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Modeling effects of environment, insect damage, and *Bt* genotypes on fumonisin accumulation in maize in Argentina and the Philippines

Regina de la Campa[1], David C. Hooker[2], J. David Miller[1], Arthur W. Schaafsma[2] & Bruce G. Hammond[3]
[1]*Ottawa-Carleton Institute of Chemistry, Carleton University, Ottawa, ON, K1S 5B6, Canada;* [2]*Ridgetown College, University of Guelph, Ridgetown, ON, N0P 2C0, Canada;* [3]*Product Safety Center, Monsanto Company, 800 North Lindbergh Boulevard, St Louis, MO, 63167, USA*

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Abstract

Fumonisins are common contaminants of maize (*Zea mays* L.) grain products, especially in countries where maize is a major constituent of the diet and are harmful to human and animal health. There is a need to better define environmental conditions that favor fumonisin accumulation in the grain of maize. The impacts of biotic and abiotic factors, and hybrids containing the Cry1Ab protein from *Bacillus thuringiensis* (*Bt*), were associated with fumonisin accumulation in the grain of maize across contrasting environments in Argentina and the Philippines between 2000 and 2002. Average fumonisin concentrations in grain samples varied from 0.5 to 12 μg g^{-1} across field locations in Argentina, and from 0.3 to 1.8 μg g^{-1} across locations in the Philippines. The ratio of fumonisin B1 to fumonisin B2 was <3.0 in four of nine locations in Argentina, which proved to be due to a higher prevalence of *Fusarium proliferatum* in those locations. Most of the variability of total fumonisins among maize grain samples was explained by location or weather (47%), followed by insect damage severity in mature ears (17%), hybrid (14%), and with the use of Bt hybrids (11%). In Argentina, where conditions were more favorable for accumulation of fumonisin in the years considered, fumonisin concentrations were lower in Bt hybrids compared to their genetic isolines by an average of 40%. A model was developed to predict fumonisin concentration using insect damage to ears and weather variables as predictors in the model. Four periods of weather around silking were identified as critical for fumonisin concentrations at harvest. The model accounted for 82% of the variability of total fumonisin across all locations in 2 years of the study.

Key words: *Bt*, corn, fumonisin, hybrid, insect, maize, model, mycotoxins, Weather

Introduction

Since the discovery of fumonisin in 1988, a great deal has been learned about the effects of this toxin. Consumption of maize contaminated with fumonisin has a number of toxic effects on animals, including equine leucoencephalomalacia in horses [1], and pulmonary edema and immunosuppression in hogs [2, 3]. There is also limited evidence that dietary uptake of fumonisins is associated with esophageal cancer [4] and neural tube birth defects in humans [5]. The Joint Expert Committee on Food Additives and Contaminants set a tolerable daily intake for fumonisins that can be applied as required. For example, using national maize consumption data, the United States Food and Drug Administration (FDA) set guidelines of between 2 and 4 μg g^{-1} in grain for cleaned and dry milled products from maize depending on use [6]. A tolerance limit of

$< 5.0\ \mu g\ g^{-1}$ and $< 10\ \mu g\ g^{-1}$ of fumonisin B1 was set for horses and pigs, respectively [6]. Ultimately, guidance levels will vary around the world mainly because tolerable concentrations depend on dietary corn consumption. Results of risk assessments to humans in various countries show Canada and many European countries at a very low risk of concern because consumption of maize products is relatively low, compared to other countries like South Africa where higher risks exist because of higher consumption [1].

The most prevalent homologues of fumonisins are FB_1, FB_2 and FB_3 [6]. FB_1 is the main compound present in maize contaminated by *Fusarium verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg. Typically, FB_1 comprises approximately 70 to 80% of the total fumonisins in maize grain samples [7].

Fungal inoculum from the air is an important source of infection for many environments, although infection via the seed can also occur. For example, in one field experiment, *F. verticillioides* strains inoculated onto silks accounted for 75–95% of infection while strains from inoculated seeds accounted for 0–30% found in the kernels [8].

The incidence and severity of ear rots and associations with mycotoxins varies with environmental conditions, genotype, and location [9–14]. *F. verticillioides* has been associated with symptomless infection in maize ears that otherwise appear healthy [14–18]. In general, favorable conditions for fungal infection include high temperature [2, 13], drought stress [13, 14, 19], and insect damage stress [13]. For example, the optimal temperature for *F. graminearum* growth is a rather narrow range of 26 to 28 °C [13, 20]. In contrast, optimum temperatures for *F. verticillioides* occur at these temperatures and higher [13, 20, 21]. In one report from the United States, fumonisin concentrations were inversely proportional to rainfall in June before silking [22]. Higher concentrations of fumonisins have also been shown in Africa, Italy and Croatia, where maize hybrids were grown outside their area of adaptation; perhaps these hybrids were less tolerant of drought stress [23, 24].

Currently, agronomic management options for reducing toxins in maize are limited to choosing hybrids that are associated with lower levels of fumonisin accumulation [25]. Progress has been made recently in breeding hybrids for increased tolerance to ear moulds. Molecular markers have been used to help incorporate chromosomal regions that have a quantitative effect on resistance [25]. Studies have shown that reducing damage to maize ears from insects using hybrids transformed to produce the Cry1Ab protein from *Bacillus thuringiensis* (*Bt*) has resulted in lower recoveries of *F. verticillioides* and fumonisins [13, 26–29]. In one study for example, *Bt* hybrids lowered fumonisin concentrations to meet guidelines for human consumption [29]. Management tools that include insect control and genetic resistance to ear rots and mycotoxin accumulation are important for reducing the risk of fumonisin accumulation in maize.

Forecasting systems to predict the potential for elevated fumonisin levels would be useful for harvest and marketing decisions [30]. For example, although high fumonisin concentrations are usually associated with visible symptoms of ear rot [25], predictions may be especially useful in conditions where fumonisins are synthesized in asymptomatic kernels. Little work has been published on the development of models used to predict the levels mycotoxins in field crops. One model has been developed for predicting the concentrations of deoxynivalenol in wheat [31]; the forecast during heading explained from 63 to 86% of the variability in deoxynivalenol levels at harvest [32]. Dowd [33] has reported a model predictive of fumonisin and aflatoxin in maize under US cornbelt conditions. Temperature, rainfall and insect presence were the most important variables identified.

The purposes of this study were: (1) to quantify the significance of weather, insect damage, hybrid, and the *Bt* transgene on fumonisin accumulation using actual field data from Argentina and the Philippines, (2) to identify critical weather parameters and time periods around silking that promote the occurrence and accumulation of fumonisin in various maize genotypes, including *Bt* and non-*Bt* hybrids, and (3) to develop a preliminary model for predicting concentrations of fumonisins at harvest.

Materials and methods

Field studies

Experiments were conducted in Argentina during 2000 and 2001, and in the Philippines during 2001

and 2002. In Argentina during 2000, three pairs of isolines and their *Bt* counterparts (DK615/DK615MG, DK696/DK696MG, DK752/DK752MG; Monsanto Company, St. Louis, MO) were planted at four field locations. All of the transgenic hybrids express the Cry1Ab gene (MON810 transformation). Three of the field locations (Fontezuela 60°33′ S, 33°50′ W; Salto 60°42′ S, 34°14′ W; and Bragado 60°14′ S, 35°07′ W) were in the north central Buenos Aires Province, in the area known as the Argentinean corn belt, and the fourth (Camet 57°36′ S, 37°53′ W) was located in the south eastern area of the province. The same hybrids were planted in 2001, but with the addition of the isogenic pair DK682/DK682MG, for a total of four hybrid-pairs in that year. In 2002, all of the Argentinean fields were located in the Buenos Aires Province (Ocampo 60°38′ S, 33°45′ W; Rojas 60°44′ S, 34°14′ W; Bragado 60°30′ S, 35°10′ W; and Pinto 61°56′ S, 34°46′ W).

In the Philippines, two pairs of isoline and *Bt* hybrids (C818/C818YG, and C838/C838YG, Monsanto Company, St. Louis, MO) were grown over two seasons, with the "wet season trial" between July and October 2001, and the "dry season trial" from January to May 2002. The transgenic hybrids express the *cry1Ab* gene (MON810 transformation). The hybrids were planted in two different field locations in each year: one at Cauayan, Isabela (49°82′ N, 124°59′ E), located on the northern island of Luzon, and the other at Bukidnon, Kibawe (16°49′ N, 121°59′ E), on the southern island of Mindanao.

Field plots were planted in October or November in 2000 and 2001 in Argentina, and in mid-July 2001 (for the "wet season"), and in January 2002 (for the "dry season") in the Philippines. All plots were planted in a randomized complete block design with four replications. Depending on location, plot size varied from 4 to 6 rows wide or 2.8 to 4.5 m in width, and from 7 to 10 m long, and plant population varied from 65,000 to 75,000 plants ha^{-1}. All plots were planted within larger maize fields and were surrounded by one maize guard row with fallow alleys between replicate plots. The bulk of the nitrogen requirement for the maize crops was applied as urea at between 80 and 120 kg ha^{-1} of actual N, depending on field location, and before the fourth leaf stage of maize in all fields. Weeds were controlled in most fields using recommended herbicides, with escapes controlled by hand weeding in some fields. The exception was in Ocampo, where weed escapes resulted in significant competition with the crop at silking. In 2001, the plots at Fontezuela, Bragado and Camet were planted using conventional tillage, and Salto was planted using minimum tillage. In 2002, the Ocampo and Pinto plots were planted using conventional tillage, and the Rojas and Bragado plots were planted using minimum tillage. At Bukidnon, Philippines, lime was applied before planting at the rate of 50 kg ha^{-1} kessierite to increase soil pH. Data on the initial levels of soil fertility and pH were not available. At all locations in Argentina in 2000, chlorpyrifos was applied before seedling emergence to control soil pests at the seedling stage. No insecticides or fungicides were applied in 2001. In Argentina, daily maximum and minimum temperatures, average relative humidity and total rainfall were recorded on each site by a weather station. In the Philippines, average daily relative humidity, daily rainfall accumulation, and air temperatures at 7 am and 2 pm were recorded using a weather station by Kestrel Associates Inc. at each location except the station at Isabela, where the weather data were obtained from the local weather bureau approximately 20 km from the field. Historical records from Isabela and the local bureau show similar weather between the two locations.

The date of 50% silk emergence was recorded for each hybrid at all locations. Populations of insects that cause damage to the ear of corn were observed in both countries by different methods, but attempts were made to convert the data from these observations into a single variable or index. In Argentina for example, insect damage severity per plant was determined by counting the number of borer holes in the stalks of 20 randomly selected plants per plot at harvest; these holes were caused by populations of the sugarcane borer *Diatraea saccharalis* Fabricius and corn earworm *Helicoverpa zea* Boddie. In the Philippines, the average length of tunnels in stalks, mainly caused by Asian corn borer (ACB), *Ostrinia furnacalis* Guenee, was determined at harvest from 20 plants within the center rows of each plot. An "insect damage index" was calculated from both scales so that insect severity could be compared between countries. Indices were calculated by determining the overall mean damage rating for each country

(= 1), and expressing individual plot data obtained as a proportion above or below the average for that country. For example, the average number of holes per plant in Argentina across locations was 1.2; therefore, the severity index was determined in each plot by dividing the actual number of holes per plant by 1.2. Similarly in the Philippines, the average tunnel length was 2.3 cm per plant; therefore, the severity index was determined in each plot by dividing the actual tunnel length per plant by 2.3.

After physiological maturity, between 60 and 80 ears from each plot were selected at random, shelled, and dried to 14% moisture. A 2-kg subsample of the dried, shelled grain was ground with a knife mill (1 mm screen) in preparation for mycotoxin analysis. The samples from Argentina were analyzed at the Technological Laboratory of Uruguay (LATU) in Montevideo, Uruguay, and the samples from the Philippines were analyzed at the Bureau of Postharvest Research and Extension (BPHRE), Department of Agriculture, in Nueva Ecija, Philippines. All grain samples were checked for the presence or absence of the Cry1Ab protein using gene check strips (Monsanto, St. Louis, MO) to confirm the identity of the grain [34].

Mycotoxin analysis

Grain samples for trials in both countries were analyzed for FB_1, and FB_2 was also measured in the samples from Argentina. To test for consistency in the analyses, subsamples from approximately 10% of all samples were analyzed at Carleton University, Ottawa, Canada; there was no statistical evidence that indicated differences between the measurements performed in Ottawa, and those performed on similar samples at LATU or BPHRE (data not presented). In addition, reference mycotoxin standards were provided to all three laboratories to ensure comparable results.

Fumonisins were determined according to the method described by Visconti et al. [35], using double extraction with acetonitrile:methanol:water (25:25:50, v/v/v). Briefly, sample cleanup was performed with Fumonitest^{TM} immunoaffinity columns (VICAM, Watertown, MA), followed by derivatization with 50 μl of OPA solution [20 mg of *o*-phthaldialdehyde, 2.5 ml of sodium borate buffer (0.1 M), 0.5 ml methanol, 20 μl mercaptoethanol] and mixed for 30 s [35]. Derivatization was performed at room temperature. Three minutes after the addition of the OPA solution, 20 μl of the derivatized solution were injected into the HPLC system. Quantification was done using an HPLC pump system with fluorescence detection at 335 nm and 440 nm. The analytical column used for separation was a C_{18} ODS Reversed Phase HPLC column (5 μm, 250 $\times$ 4.6 mm) (Phenomenex Inc., Torrance, CA). The mobile phase was a mixture of methanol and 0.1 M solution of sodium dihydrogen phosphate (77:23, v/v); pH was adjusted to 3.35 with *o*-phosphoric acid. The flow rate was 1.0 ml min^{-1}. The lower limits of detection for FB_1 and FB_2 were 0.025 μg g^{-1} and 0.060 μg g^{-1}, respectively.

Unexpectedly, in four out of nine year 2001 samples from Argentina, the ratio of FB_1 and FB_2 was less than 3.0. As part of a separate study on the occurrence of fusaria from surface disinfested whole kernels from all samples (to be reported elsewhere), a collection of the *F. proliferatum* strains was prepared. To exclude the possibility that the maize genotype affected the production of fumonisins, production of fumonisins by these strains *in vitro* was examined. Briefly, 10 representative isolates of *F. proliferatum* were transferred into 2% malt agar slants. Distilled water (18 ml) was added to 50 g of maize kernels in 500-ml Erlenmeyer flasks. The flasks and contents were autoclaved for 30 min at 121 °C. The next day, another 18-ml water was added to each flask, and they were autoclaved again for 15 min. The cultures were inoculated with the strains, respectively, and incubated for 15 d at 25 °C with occasional gentle shaking for the first few days [36–38]. The cultures were harvested, freeze-dried, and ground into a fine meal using a coffee grinder (Black & Decker, Smartgrind). FB_1 and FB_2 were quantified using the procedure described previously. Isolates were deposited in the culture collection at Agriculture and Agri-Food Canada as DAOM 232117, DAOM 232118, DAOM 232119, DAOM 232385, DAOM 232386, DAOM 232387, DAOM 232388, DAOM 232389 and DAOM 2320390.

Statistical methods

Analysis of variance was performed using PROC MIXED (SAS). The fixed effects in PROC MIXED included location, the hybrid background, and whether or not the hybrid was a *Bt* or non-*Bt*.

Replicates were analyzed as random effects in the RANDOM statement. PROC UNIVARIATE (SAS) was used to test the plausibility of assumptions. that the data were normal and the variance was homogenous among treatments. As a result, the data for insect damage index were closer to normal and variance plots among treatments were the most homogenous with ay a ln(x + 1) transformation, and similarly, fumonisin concentrations (in μg g^{-1}) were transformed using $(x + 0.01)^{1/3}$.

The impact of insect damage, hybrid and *Bt* genotypes on fumonisin levels were obtained from simple models in SAS PROC GLM [39], which included combinations of these variables. The *R*-square (coefficient of determination) of each model analysis approximated the degree of variation attributable to variables in the model [31, 32].

The effects of weather on fumonisin concentration were modeled using regression procedures similar to those used for developing the DON-weather wheat model by Hooker and Schaafsma [31]. Daily weather data were normalized for each hybrid among all locations within both countries with the date of maize silking. Daily rainfall, daily minimum and maximum air temperatures, and hourly relative humidity (RH) were assigned to hybrids at each location from 2 weeks before silking, to 3 weeks after silking. Daily binary values were calculated for each of four weather variables with the following criteria (i.e., 0 = does not match weather criterion for the day, or 1 = matches criterion): (i) when daily rainfall >2 mm, (ii) when minimum temperature was <15 °C, and (iii) when maximum temperature was >34 °C. The 2-mm criteria for rain was speculated as sufficient amount to contribute to canopy wetness or wetness of the crop residue on the soil surface. The 15 °C threshold for a significant minimum temperature was determined by current literature as the minimum temperature for perithecial formation (e.g. Tschanz et al. 1976), and hot temperatures for inducing infections has been suggested around 34 °C [13, 14, 16, 20]. Daily binary values (i.e., 0 or 1) for each weather variable were then summed in simple, 7-day moving periods or ''windows''. Stepwise regression procedures (SAS) identified the most important weather variables and their timing for predicting fumonisin concentrations; appropriate models were selected using the same criteria as outlined by Hooker and Schaafsma [31]. Before analysis, values for concentrations of fumonisin (total, in μg g^{-1}) were transformed by ln(x + 0.01) to satisfy assumptions of normality.

Results

FB_1 versus FB_2

When strains of *F. proliferatum* from the 2002 crop in Argentina were cultured on maize, four out of nine produced FB_1:FB_2 in ratios of less than 3.0. The FB_1:FB_2 ratios were: Rojas (DAOM 232385), **5.0:1**; Rojas (DAOM 232386), **4.6:1**; Rojas (DAOM 232387): **4.2:1**, Bragado (DAOM 232388), **4.5:1**; Bragado (DAOM 232389), **4.8:1**; Bragado (DAOM 232117), **2.6:1**; Bragado (DAOM 232390), **1.36: 1**; Ocampo (DAOM 232119): **1.8:1** and Ocampo (DAOM 232118): **2.5:1**. The ratios of FB_1 and FB_2 in maize harvested in Argentina were not affected by the *Bt* genotype (data not shown).

Effects of location on total fumonisin

Average fumonisin concentrations across hybrids varied with field location. Average concentrations varied from 0.5 to 12 μg g^{-1} across field locations in Argentina, and from 0.3 to 1.8 μg g^{-1} across locations in the Philippines (Table 1). Fumonisin was detected in more than 50% of grain samples in 7 of 12 field locations in both years, indicating that environmental conditions were favorable for fumonisin accumulation in most field locations of this study.

Briefly, at the locations where the highest average concentrations of fumonisin were detected (Fontezuela and Salto, Argentina, 2000; Table 1), relatively moist (>25 mm $week^{-1}$) and warm (30 to 32 °C daily maximum) conditions occurred during 2 weeks before the average silking date, and relatively dry conditions occurred 2 weeks after silking. In contrast, the lowest fumonisin average concentrations in Argentina occurred at Pinto in 2002, where relatively moist conditions and cool–warm daily maximum temperatures (26 to 30 °C) occurred during a 4-week period around the average silking date across hybrids. In the Philippines in 2001, relatively hot (33 to 34 °C daily maximum) and dry conditions occurred around silking at Cauayan, which coincided with the highest average fumonisin concentrations

Table 1. Statistics of fumonisin concentrations in grain samples among locations in Argentina and the Philippines during 2001 and 2002

Country, Year/Location	Proportion of samples >1.0 $\mu g\ g^{-1}$ (%)	Concentrations of total fumonisin ($\mu g\ g^{-1}$)			
		Minimum	Maximum	Average	SD
Argentina, 2000					
Fontezuela	100	1.8	27.0	12.0	6.99
Salto	96	0.8	21.4	8.3	5.69
Bragado	63	0.1	14.0	3.1	3.84
Camet	46	<0.1	11.4	2.5	3.36
Argentina, 2001					
Bragado	81	0.4	17.1	5.1	5.53
Pinto	13	<0.1	4.8	0.5	0.89
Rojas	66	0.1	23.5	4.0	5.43
Ocampo	50	0.1	11.9	2.5	2.96
Philippines, 2001					
Cauayan, Isabela	67	0.5	6.0	1.8	1.51
Bukidnon, Kibawe	17	0.2	1.8	0.5	0.50
Philippines, 2002					
Cauayan, Isabela	17	0.2	2.1	0.6	0.53
Bukidnon, Kibawe	17	0.1	1.6	0.3	0.51

among locations in the Philippines in this study (Table 1; $p = 0.002$, see LOC effect in Table 2). More rainfall and slightly cooler temperatures (28 to 32 °C) occurred around silking at most other locations in the Philippines in both years, with the exception of Cauayan in 2002, where average daily maximum temperatures varied between 34 and 36 °C in a four week period centered on silking.

Effects of Bt *and insect damage on level of total fumonisin*

In general, the main effect of location on fumonisin was significant in every year across both countries ($0.04 < p < 0.0001$), and the effects of *Bt* and hybrid were significant ($p < 0.0008$) for both years in Argentina. More importantly, the effects of *Bt* and hybrid on fumonisin in Argentina interacted with the location in 2001, indicating that the effects of *Bt* and hybrid were not consistent with location during that year (Table 2). The effect of *Bt* on insect damage severity was also dependent on location in both years and countries (Table 3; $p < 0.0001$). Insect damage severity also depended on the hybrid in Argentina in 2000 (Table 3; $p = 0.04$) and in the Philippines in 2002 (Table 3; $p = 0.0009$); indicating that some hybrids were more susceptible to insect damage than others.

Table 2. Analysis of variance results for total fumonisin concentrations of field locations, *Bt* or isoline, and maize hybrid from Argentina and Philippines during 2000 and 2002

Source	Argentina						Philippines					
	2000			2001			2001			2002		
	df	*F* value	$p > F$	df	*F* value	$p > F$	df	*F* value	$p > F$	df	*F* value	$p > F$
Location (LOC)	3	38.21	<0.0001	3	26.90	<0.0001	1	13.29	0.002	1	4.95	0.041
BT	1	32.49	<0.0001	1	64.48	<0.0001	1	0.30	0.591	1	2.40	0.141
Hybrid (HYB)	2	10.05	0.0001	3	6.18	0.0008	1	0.00	0.999	1	0.72	0.408
LOC*BT	3	1.28	0.289	3	4.47	0.006	1	0.79	0.388	1	2.46	0.136
LOC*HYB	6	3.22	0.007	9	2.45	0.016	1	0.01	0.926	1	3.72	0.072
HYB*BT	2	2.59	0.082	3	0.58	0.629	1	0.15	0.704	1	0.24	0.630
LOC*HYB*BT	6	0.47	0.826	9	0.68	0.727	1	3.08	0.099	1	0.37	0.554

Table 3. Analysis of variance results for insect damage levels[a] of field locations, *Bt* or isoline, and maize hybrid from Argentina and Philippines between 2000 and 2002

Source	Argentina						Philippines					
	2000[b]			2001			2001			2002		
	df	*F* value	$p > F$	df	*F* value	$p > F$	df	*F* value	$p > F$	df	*F* value	$p > F$
Location (LOC)	–	–	–	3	77.37	< 0.0001	1	28.22	< 0.0001	1	83.93	< 0.0001
BT	1	1262.22	< 0.0001	1	1920.72	< 0.0001	1	132.08	< 0.0001	1	2361.35	< 0.0001
Hybrid (HYB)	2	1.70	0.188	3	0.82	0.487	1	0.11	0.743	1	16.60	0.0009
LOC*BT	–	–	–	3	67.99	< 0.0001	1	28.22	< 0.0001	1	83.93	< 0.0001
LOC*HYB	–	–	–	9	0.97	0.472	1	0.23	0.637	1	0.23	0.641
HYB*BT	2	3.24	0.044	3	0.12	0.949	1	0.11	0.743	1	16.60	0.0009
LOC*HYB*BT	–	–	–	9	1.08	0.386	1	0.23	0.637	1	0.23	0.641

[a] Insect damage levels are indices relative to the average number of holes or tunnel lengths from boring insects in the stalks of maize across locations in Argentina and Philippines at harvest.
[b] Measurements were averaged across replications in Argentina in 2001, therefore, variance of other sources was determined across locations.

Across all locations in Argentina, *Bt* hybrids reduced fumonisin concentrations from 6.29 to 2.46 $\mu g\ g^{-1}$ in 2000 (Table 4; $p < 0.0001$), and from 3.06 to 0.56 $\mu g\ g^{-1}$ in 2001 (Table 4; $p < 0.0001$). The effect of *Bt* depended on location, however, as *Bt* hybrids did not reduce fumonisin in two out of the eight location-years in Argentina. The effect of *Bt* on reducing fumonisin at Fontezuela in 2000 was weak ($p = 0.187$), even though average concentration (10.7 $\mu g\ g^{-1}$) at this

Table 4. Effects of the Bt transgene on mean concentrations of fumonisin and insect damage levels in locations from Argentina and Philippines between 2000 and 2002

Country, Year Location	Fumonisin ($\mu g\ g^{-1}$)			Insect Damage Level [a]		
	Isoline	*Bt*	*p*	Isoline	*Bt*	*p*
Argentina, 2000						
Fontezuela	12.42	9.06	0.187	0.87	0.00	–
Salto	9.79	4.42	0.006	3.64	0.02	–
Bragado	4.15	0.74	0.0001	3.25	0.00	–
Camet	2.32	0.46	0.002	2.06	0.01	–
Average	6.29	2.46	< 0.0001	2.17	0.01	< 0.0001
Argentina, 2001						
Bragado	7.36	1.24	< 0.0001	4.12	0.01	< 0.0001
Pinto	0.30	0.13	0.237	0.52	0.01	< 0.0001
Rojas	4.81	0.84	< 0.0001	2.02	0.01	< 0.0001
Ocampo	3.39	0.47	< 0.0001	0.40	0.00	< 0.0001
Average	3.06	0.56	< 0.0001	1.20	0.01	< 0.0001
Philippines, 2001						
Cauayan, Isabela	1.85	1.25	0.325	2.29	0.00	< 0.0001
Bukidnon, Kibawe	0.43	0.49	0.814	0.22	0.00	0.0005
Average	0.97	0.81	0.591	0.78	0.00	< 0.0001
Philippines, 2002						
Cauayan, Isabela	0.82	0.28	0.043	2.38	0.00	< 0.0001
Bukidnon, Kibawe	0.21	0.21	0.988	0.80	0.00	< 0.0001
Average	0.45	0.25	0.141	1.39	0.00	< 0.0001

[a] Insect damage levels are indices proportionate to the number of holes per plant (i.e., 1.0 = 1.3 holes per plant, on average, in Argentina) or tunnel lengths (i.e., 1.0 = 2.3 cm tunnel length, on average, in the Philippines) from boring insects in the stalks of maize at harvest.

field was the highest of any other location in this study (Table 4).

Bt hybrids reduced insect damage to near zero across all location-years; however, insect damage across non-*Bt* hybrids varied depending on the location within each country and year (Table 4). This resulted in the previous significant interaction between location and *Bt* hybrids within each country and year (Table 3; $p < 0.0001$).

Average fumonisin concentrations were different among hybrids in 6 of 8 locations across Argentina in 2000 and 2001 (Table 5), but concentrations were not different between two different hybrids planted in the Philippines in both years (data not shown). On average, both *Bt* and non-*Bt* isolines of DK752 accumulated significantly lower fumonisin in most comparisons ($p < 0.05$), with 2.65 μg g^{-1} compared with 3.72 and 6.39 μg g^{-1} for DK615 and DK696 in 2000, respectively, and 0.76 μg g^{-1} compared with concentrations between 1.13 and 2.40 μg g^{-1} in the other three hybrids in 2000. The *Bt* versions of DK615, DK682, and DK752 reduced fumonisin concentrations by 40% compared to their non-*Bt* counterparts ($p < 0.008$) when averaged across all locations in Argentina (data not shown); however, concentrations in *Bt* and non-*Bt* hybrids of DK696 were not different in six of eight locations or when averaged across locations (contrast not shown; $p = 0.15$).

Differences between fumonisin and relative insect damage among hybrids were not consistent at each location. For example, concentrations of fumonisin were higher in DK696 compared to DK752 across locations in Argentina in both 2000 and 2001 (Table 5; $p < 0.05$). Insect damage was higher in DK696 compared to DK752 in 2000, but there were no differences in insect damage at any of those locations in 2001 ($p > 0.05$).

Table 5. Effects of hybrid on mean concentrations of fumonisin and insect damage levels among locations in Argentina during 2000 and 2001

Country, Year Field Location	Fumonisin[A, C] (μg g^{-1})					Insect Damage Level[B, C]				
	DK615	DK696	DK752	DK682	Average	DK615	DK696	DK752	DK682	Average
Argentina, 2000										
Fontezuela	8.18 b	19.56 a	6.73 b	–[D]	10.64	0.66 –	1.15 -	0.85 -	–[D]	0.87
Salto	8.16 a	10.11 a	3.32 b	–	6.74	3.32 -	4.71 -	3.09 -	–	3.64
Bragado	2.97 a	1.59 a	1.53 a	–	1.97	2.41 -	4.21 -	3.39 -	–	3.25
Camet	0.39 b	2.73 a	1.00 a	–	1.14	2.84 -	2.76 -	1.09 -	–	2.06
Average	3.72 b	6.39 a	2.65 b	–	4.07	2.00 b	2.84 a	1.78 b	–	2.17
Argentina, 2001										
Bragado	6.85 a	4.83 a	1.82 b	1.80 b	3.43	3.76 a	4.34 a	4.80 a	3.72 a	4.12
Pinto	1.02 a	0.04 b	0.09 b	0.13 b	0.20	0.72 a	0.44 b	0.51 ab	0.45 b	0.52
Rojas	2.95 a	4.02 a	0.78 b	2.25 ab	2.27	1.65 b	2.19 ab	1.65 b	2.76 a	2.02
Ocampo	1.02 a	2.56 a	1.15 a	1.44 a	1.47	0.33 b	0.41 ab	0.33 b	0.57 a	0.40
Average	2.40 a	1.97 a	0.76 b	1.13 b	1.47	1.13 a	1.12 a	1.32 a	1.20 a	1.20

[A] Fumonisin concentrations averaged across Bt and Isoline within each base hybrid.
[B] Insect damage levels are indices proportionate to the number of holes per plant (i.e., 1.0 = 1.3 holes per plant, on average, in Argentina) from boring insects in the stalks of maize at harvest.
[C] Means with the same letter within each location are not different according to the Protected LSD test at $p = 0.05$.
[D] Hybrid not tested in 2001.

Factors associated with fumonisin

When location, hybrid, *Bt* or non-*Bt*, and insect damage severity levels were analyzed individually against total fumonisin accumulation, location effects explained the most variability in fumonisin (47%), followed by insect damage severity (17%), hybrid (14%), and Bt (11%)(Table 6). In addition, location effects interacted significantly with all the terms in the simple linear models (Table 6), indicating that environmental factors at each location

Table 6. The amount of variation in total fumonisin associated with simple effects and interactions of location, hybrid, *Bt*, and insect damage using simple additive models in PROC GLM across all data from Argentina and Philippines

Highest Order Effect in Linear Model [a]	Variation Explained by Effect [b] (%)
Location	47
Hybrid	14
Bt	11
Insect Damage Level (INSECT)	17
Location × Hybrid	51
Location × *Bt*	58
Location × INSECT	58
Location × Hybrid × *Bt*s	63
Location × Hybrid × *Bt* × INSECT	63

[a] Components of interactions were included in the models as simple effects.
[b] R^2 of each model. All models were significant at $p < 0.0001$.

(e.g. weather) are the primary factors responsible for the ultimate fumonisin concentrations in the grain at harvest. Overall, a maximum of 63% of the variability in fumonisin was explained when all four effects and their interactions were included in the model (Table 6).

Development of a preliminary model to predict total fumonisin

Using data from all locations, multiple regression analysis revealed that weather conditions were most influential in four, 7-day critical periods relative to silking: (1) 4 to 10 d before silking, (2) from 4 days before silking to 2 days after silking, (3) 2 to 8 d after silking, and (4) 8 to 14 days after silking (Figure 1). Partial regression analysis showed that the first period of weather, 4 to 10 d before silking, explained 18.3% of the variation in fumonisin, the second period explained 2.9%, the third period of weather explained 42.9%, and the fourth period of weather explained 10.9% of the variability of fumonisin. An empirical equation was developed using insect damage severity and the weather information from 10 d before silking to 14 d after silking. Fumonisin was predicted best when data from both ear damage from insects and weather were included. An equation was developed to predict fumonisin accumulation using insect, rainfall and temperature data:

$$\begin{aligned}&\text{Total Fumonisin}(\mu\text{g g}^{-1})\\&= \exp\{1.14 + 0.46\text{INSECT} - 0.64\,\text{TMIN1}\\&\quad - 0.22\,\text{TMAX1} + 0.32\text{RAIN1}\\&\quad - 0.33(\text{RAIN1} * \text{TMX1}) + 0.54\,\text{TMAX2}\\&\quad - 0.94(\text{RAIN3}) + 1.26(\text{TMAX3})^2\\&\quad - 0.25(\text{TMAX3})^2 + 0.04(\,\text{RAIN3} * \text{TMX3})\\&\quad - 1.00\,\text{TMIN3} + 0.50\,\text{RAIN4}\\&\quad - 0.11(\,\text{RAIN4})^2\} - 0.01,\end{aligned}$$

where INSECT is the insect damage severity, TMIN1 is the number of days daily minimum temperature < 15 °C in the 7-day period from 4 to10 days before silking, TMAX1 is the no. of days daily maximum temperature > 34 ° C in the 7-day period from 4 to 10 days before silking, RAIN1 is the no. of days of rain > 2 mm in the 7-day period from 4 to 10 days before silking, RAIN1*TMX1 is the interaction term between RAIN1 and TMX1, TMAX2 is the no. days of daily maximum temperatures > 34 °C in the 7-day period from 4 days before silking to 2 days after

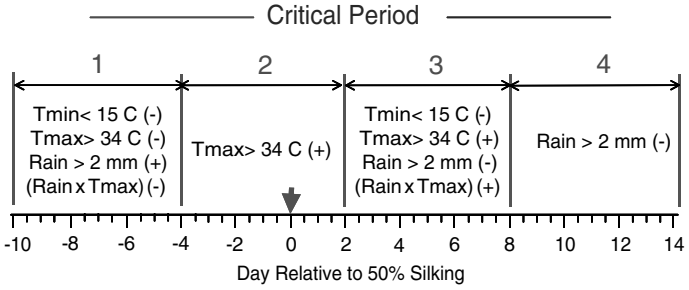

Figure 1. Four critical periods of weather variables and thresholds around corn silking and their general effect on total fumonisin concentrations in grain corn at maturity. The effect of (−) or (+) beside each weather variable indicates a general decrease or increase of fumonisin, respectively, with an increase in the number of days that exceed the threshold within each 7-d critical period.

silking, RAIN3 is the no. of days of rain >2 mm in the 7-day period from 2 to 8 days after silking, TMAX3 is the no. of days daily maximum temperature >34 °C in the 7-day period from 2 to 8 days after silking, RAIN3*TMX3 is the interaction term between RAIN3 and TMX3, TMIN3 is the no. of days when daily maximum temperature <15 °C in the 7-day period from 2 to 8 days after silking, and RAIN4 is the no. days of rain >2 mm in the 7-day period from 8 to 14 days after silking. Statistics of the predictor variables are presented in Table 7.

Partial regression analysis showed that the insect damage index explained approximately 6% of the variation in fumonisin, while weather variables accounted for approximately 76% of the remaining variation explained by the model (Table 7). Overall, the model accounted for 82.3% of the variation in total fumonisin (Figure 2, $p < 0.0001$).

Discussion

The varied levels of fumonisin across field environments and two countries in the study were conducive to investigating the effects weather, insect damage, and hybrid on fumonisin accumulation. Simple linear models revealed that location alone explained most of the variability (47%) in fumonisin in both countries and years, while insect damage severity, hybrid, or *Bt*, each explained from 11 to 17% of the variability in fumonisin, suggesting that effects due to differences in weather among locations were much more influential on fumonisin accumulation than level of insect damage or hybrid or *Bt*. Therefore, controlling insects that feed in ears does not preclude the risk of high levels fumonisin. However, the effects of weather in each location likely influenced populations of the two lepidopteran species native to Argentina and Philippines:

Table 7. Statistics of equations for predicting total fumonisin[a]

Variable[b]	Parameter estimate	SE	Parameter estimate $p > \|T\|$	Partial[c] R^2
Constant	1.14	0.629	0.077	–
INSECT	0.46	0.095	<0.0001	0.062
TMIN1	−0.64	0.119	<0.0001	0.094
TMAX1	−0.22	0.134	0.102	0.039
RAIN1	0.32	0.182	0.085	0.023
RAIN1 × TMX1	−0.33	0.092	0.001	0.037
TMAX2	0.54	0.203	0.011	0.029
RAIN3	−0.94	0.120	<0.0001	0.196
TMAX3	1.26	0.279	<0.0001	0.092
$(TMAX3)^2$	−0.25	0.042	<0.0001	0.119
RAIN3 × TMX3	0.04	0.044	0.320	0.004
TMIN3	−1.00	0.307	0.002	0.018
RAIN4	0.50	0.443	0.269	0.004
$(RAIN4)^2$	−0.11	0.087	0.200	0.105
Total R^2 of Model [d]				0.823

[a] ln (fumonisin + 0.01) predicted in $\mu g\ g^{-1}$.
[b] INSECT = insect damage level, where the level is proportionate to the average number of holes per plant in Argentina (i.e., 1.0 = 1.3 holes per plant, on average) or tunnel lengths in the Philippines (i.e., 1.0 = 2.3 cm tunnel length, on average) from boring insects in the stalks of maize at harvest.
TMIN1 = no. days daily minimum temperature <15 °C in the 7-day period from 4 to10 days before silking.
TMAX1 = no. days daily maximum temperature >34 °C in the 7-day period from 4 to 10 days before silking.
RAIN1 = no. days of rain >2 mm in the 7-day period from 4 to 10 days before silking.
RAIN1*TMX1 = interaction term between RAIN1 and TMAX1.
TMAX2 = no. days daily maximum temperature >34 °C in the 7-day period from 4 days before silking to 2 days after silking.
RAIN3 = no. days of rain >2 mm in the 7-day period from 2 to 8 days after silking.
TMAX3 = no. days daily maximum temperature >34 °C in the 7-day period from 2 to 8 days after silking.
RAINTMX3 = interaction term between RAIN3 and TMAX3.
TMIN3 = no. days daily minimum temperature <15 °C in the 7-day period from 2 to 8 days after silking.
RAIN4 = no. days of rain >2 mm in the 7-day period from 8 to 14 days after silking.
[c] Partial coefficient of determination (R^2) values of each variable.
[d] R^2 = overall coefficient of determination of the model. Overall model is significant at $p < 0.0001$ with 41 df.

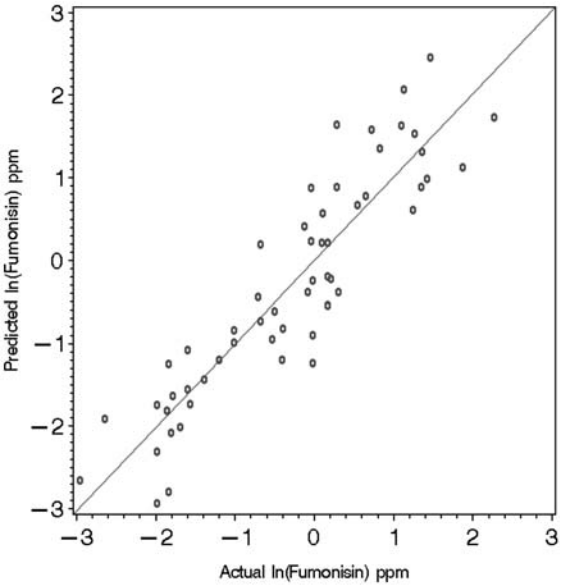

Figure 2. Actual versus predicted total fumonisin concentrations (FB_1 + FB_2) in all field locations across Argentina and Philippines between 2000 and 2002. $R^2 = 0.82$, $n = 55$.

Diatraea saccharalis (maize borer) and *Helicoverpa zea* (ear caterpillar), and therefore, the effects of weather and insect damage may be slightly confounded and not truly independent from each other. If both insect damage and location were independent from each other, then a total of 64% of variability may be explained by the two effects (17 plus 47%). However, because a total of 58% of the variability in fumonisin was explained by the two effects, both effects are likely important for predicting fumonisin despite some confounding.

As noted, the ratio of FB_1 and FB_2 was less than 3.0 in 4 of 9 samples collected in Argentina in 2001. This was unexpected because FB_1 is usually 80% of total fumonisin (FB_1 + FB_2) [40, 41]. Our results confirmed those of Chulze et al. [42] who reported that some Argentinean strains of *F. proliferatum* produce more FB_2 than FB_1. Although it *F. proliferatum* is commonly isolated, little is known about the factors that govern it becoming the dominant species in corn with kernel rot. The present data suggest that the climate conditions that favor its growth are not identical to those for *F. verticillioides*.

Most of the *Bt* hybrids in Argentina reduced total fumonisin concentrations by an average of 40% to safer levels of fumonisin in both years of the study. One hybrid however, DK696, showed no statistical differences between the non-*Bt* and transgenic in six out of eight locations across Argentina, even though the transgenic had near zero visible insect damage to the ear, and the non-transgenic tended to have greater insect damage than other non-transgenic hybrids. While most *Bt* hybrids that express *Cry* proteins in the kernel may reduce fumonisin under conditions where insect damage to kernels occurs [28], the effect doesn't preclude the genetic susceptibility of the base hybrid to ear rots associated with *Fusarium* or to accumulate fumonisin. Therefore, genetic coefficients for specific hybrids would be useful in models to predict fumonisin.

An empirical relationship was developed to predict concentrations of fumonisin from weather variables in four periods around silking and insect damage. The results support the hypothesis that concentrations of fumonisin are associated with environmental conditions around silking. Although others have correlated general weather conditions with elevated concentrations of fumonisin [2, 16], our analysis has quantified weather effects in four relatively narrow time periods around silking.

Regression analysis identified insect damage as a significant predictor in the model, with higher concentrations of fumonisin predicted in insect-damaged maize ears. This relationship is supported with analysis of variance procedures in this study and others [21, 39] that show decreased levels of fumonisin by controlling insect damage with *Bt* hybrids expressing *Cry* protein in the kernels.

Weather in 4 critical periods relative to silking explained 76% of the variability of fumonisin. In the first critical period 4 to 10 days before silking, the data suggest that temperatures < 15 °C and > 34 °C reduce fumonisin (TMIN1 and TMAX1, respectively), and rain (RAIN1) increases fumonisin; however, the effects of rain were negated by temperatures > 34 °C, as indicated by a negative interaction between the two variables. Extreme temperatures and dry weather before silking likely delayed or reduced sources of inoculum during this period before silking. Inoculum sources are important factors for all fusaria affecting corn [43]. Low levels of inoculum from *Gibberella*, for example, are associated with low temperature or dry conditions [44], but

little is known about the effects of inoculum sources and timing for fumonisin-producing *Fusarium* spp.

In the second, third, and fourth critical periods, the data indicate that hot (TMAX2 and TMAX3) and dry conditions (RAIN3, RAIN4) from 4 days before silking to 14 days after silking increases fumonisin, and cool temperatures < 15 °C (TMIN3) reduced fumonisin. As reported earlier, the highest levels of fumonisin in this study were measured at locations with dry, warm weather around silking (e.g. Fontezuela and Salto locations in 2001) and the lowest levels were measured at locations with rainfall or adequate moisture during this period (e.g. the Pinto location in 2001). The frequency of rain in the third and fourth critical periods between 2 and 14 days after silking were the most important predictors in the model (RAIN3 and RAIN4), which explained a total of 31% of the variability of fumonisin, according to a ranking of partial coefficients of determination (Table 6). Hot and dry conditions around silking have been observed by others as conditions favorable for fumonisin accumulation [13, 14, 16, 19]. Similar critical periods but different weather parameters were established in predicting DON in wheat [31] using the same approach for model development. In the wheat model and with this model for maize, predictions of mycotoxin levels at harvest were developed relative to periods of the highest susceptibility of infection around anthesis or silking [45].

The model was developed using 57 data points of fumonisin concentrations; each point was an average of concentrations across replications. By comparison, the model reported previously for predicting deoxynivalenol in wheat [31] used 399 data points of actual toxin concentrations and different environments. It is apparent that the robustness of this fumonisin model across different environments may be improved with data points from more locations with more extremes in weather conditions and with varying levels of insect damage. No attempts were made to develop models for each country because models are generally more robust when developed using data from different environments. In other words, we were not interested in country-specific weather or insect effects; any country-specific differences or interactions with weather or insect would reduce the predictability or significance of the predictors in the model. Incorporating data from both countries into a predictive model indicates that the model is robust under varied environments.

There was evidence in this study, and suggestions from others [25, 28, 45], that a significant interaction exists between hybrid susceptibility to fumonisin accumulation and environment; genetic coefficients of hybrids were not implemented here because data were only available from six hybrids of different genetic backgrounds. Further studies are needed to investigate genetic differences and stability of hybrids for susceptibility or resistance to accumulate fumonisin, and to quantify these differences as genetic coefficients for predictive models.

In summary, specific environmental conditions around silking were found to be most important for fumonisin accumulation, followed by insect damage, hybrid, and the effect of the *Bt* transgene. Weather and insect damage severity accounted for approximately 82% of the variability in fumonisin from locations across Argentina and Philippines. Further studies across a range of environments are needed for sensitivity analysis and model validation. Management decisions to use *Bt* maize genotypes are more likely to produce grain that meet tolerable fumonisin concentrations, which agrees with similar research in United States [28]. At some locations in Argentina, FB_1:FB_2 ratios of < 3.0 indicated higher than expected prevalence of *F. proliferatum*, as similar observations in Argentina were reported earlier by Chulze et al. [42].

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Address for correspondence: J. David Miller, Ottawa-Carleton Institute of Chemistry, Carleton University, Ottawa, ON K1S 5B6, Canada
Phone: +1-613-520-2600, ext: 1053; Fax: +1-613-520-3749
E-mail: david_miller@carleton.ca