

# Comparison of Fumonisin Concentrations in Kernels of Transgenic Bt Maize Hybrids and Nontransgenic Hybrids

Gary P. Munkvold, Department of Plant Pathology, Richard L. Hellmich, USDA-ARS Corn Insects and Crop Genetics Research Unit and Department of Entomology, Iowa State University, Ames 50011, and Larry G. Rice, USDA-APHIS National Veterinary Services Laboratory, 1800 Dayton Rd., Ames, IA 50010

## ABSTRACT

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Maize hybrids genetically engineered with genes from the bacterium *Bacillus thuringiensis* (Bt maize) express CryIA(b) and other Cry proteins that are toxic to certain insects, particularly the European corn borer (*Ostrinia nubilalis*). Maize kernel feeding by *O. nubilalis* often leads to infection by fungi in the genus *Fusarium*, including the fumonisin-producing species *F. verticillioides* and *F. proliferatum*. In field experiments in 1995, 1996, and 1997, transgenic maize hybrids and near-isogenic, nontransgenic hybrids were manually infested with neonatal European corn borer larvae. Manual infestation increased *Fusarium* ear rot severity and fumonisin concentrations in kernels of nontransgenic hybrids. Transgenic hybrids with kernel expression of CryIA(b) consistently experienced less insect feeding on kernels and less *Fusarium* ear rot than their nontransgenic counterparts. In manually infested treatments, these hybrids also exhibited lower concentrations of fumonisins in kernels compared with their nontransgenic counterparts. In manually infested treatments in 1995, mean fumonisin B<sub>1</sub> concentrations were 8.8 µg/g in the nontransgenic hybrid and 6.7 or 3.0 µg/g in transgenic hybrids. In 1996, mean fumonisin B<sub>1</sub> concentrations in manually infested treatments were 4.9 µg/g (range 2.3 to 8.8) for nontransgenic and 1.2 µg/g (range 1.0 to 1.3) for transgenic hybrids with kernel expression. Mean total fumonisin concentrations (fumonisin B<sub>1</sub> + B<sub>2</sub> + B<sub>3</sub>) were 7.0 µg/g (range 3.0 to 12.2) for nontransgenic and 1.7 µg/g (range 1.5 to 1.9) for transgenic hybrids with kernel expression. In 1997, mean fumonisin B<sub>1</sub> concentrations in manually infested treatments were 11.8 µg/g (range 7.6 to 17.3) for nontransgenic and 1.3 µg/g (range 0.8 to 2.2) for transgenic hybrids with kernel expression of CryIA(b) or Cry9C. Mean total fumonisin concentrations were 16.5 µg/g (range 10.7 to 24.0) for nontransgenic and 2.1 µg/g (range 1.5 to 3.1) for transgenic hybrids with kernel expression. Transgenic hybrids that do not express CryIA(b) or Cry9C in kernels did not consistently have fumonisin concentrations different from the nontransgenic hybrids. Higher fumonisin concentrations in nontransgenic hybrids were associated with high European corn borer populations during the early reproductive stages of the maize plants. These results indicate that under some conditions, genetic engineering of maize for insect resistance may enhance its safety for animal and human consumption.

Additional keywords: corn, *Fusarium moniliforme*, mycotoxins

Fungi in the genus *Fusarium* are very common associates of maize (*Zea mays* L.) plants. These fungi can cause disease at all developmental stages of the plant, but also may infect plants without causing symptoms (24). Certain *Fusarium* species, particularly *F. verticillioides* (Sacc.) Nirenberg (synonym = *F. moniliforme* J. Sheld.) and *F. proliferatum* (T. Matsushima) Nirenberg, often infect maize plants through wounds caused by insects such as the European corn borer (*Ostrinia nubilalis*) (3,24). The same two species are capable

of producing fumonisins, a class of mycotoxins that can cause fatal leucoencephalomalacia in horses, pulmonary edema in swine, and cancer in laboratory rats (13). Esophageal cancer in humans has been associated with consumption of maize with high concentrations of the fumonisins. The compounds are now classified as Class 2B, probable human carcinogens (10,20). Fumonisin B<sub>1</sub> is typically found in the highest concentrations (16). Fumonisin-producing *Fusarium* species cause *Fusarium* ear or kernel rot, but fumonisins also can be detected in symptomless infected maize kernels (16).

The European corn borer (ECB) is a major pest of maize. The larvae of this

insect tunnel into the stalks and ears of the plants, causing yield reduction and loss of grain quality (14). In the north central United States, two generations of ECB larvae typically occur per year; the first generation attacks plants during the mid- to late-vegetative stages (V8-V10 [22]), and the second generation attacks during the reproductive stages (R1 to maturity). Adult moths lay masses of eggs on leaf surfaces, and the larvae migrate to protected sites, where they feed on leaves, stalks, and ears (14). These larvae cause physical injury to the plants, where *Fusarium* infection can occur (3), and the larvae also can carry *Fusarium* spores from the leaf surface, transmitting them to the infection court (feeding site) (25). A new strategy for the management of *O. nubilalis* is the use of transgenic maize hybrids (Bt maize). Now commercially available, these hybrids contain *cry* genes originating from the bacterium *Bacillus thuringiensis*; gene expression results in the production of insecticidal crystalline proteins in plant tissue (8). Currently available commercial hybrids express one of three Cry proteins: CryIA(b), CryIA(c), or Cry9C. Maize hybrids expressing Cry proteins have demonstrated a high level of resistance to feeding by ECB larvae (2,11). Expression of Cry proteins in specific maize tissues can be manipulated through the use of different gene promoters (11). Specific combinations of transgenes and gene promoters are often called "transformation events," and each combination must be approved separately by the U.S. Environmental Protection Agency. The use of these genetically engineered maize hybrids has been widely accepted in the United States but has met opposition elsewhere in the world because of concerns about food safety, environmental protection, and ethical issues.

Transgenic maize hybrids expressing CryIA(b) in kernels can experience less *Fusarium* infection in kernels because of the association between *Fusarium* infection and insect feeding (17). We hypothesized these hybrids also might contain lower concentrations of fumonisins than nontransgenic hybrids that are susceptible to the ECB. The objective of this study was to test this hypothesis through field experiments using near-isogenic maize hybrids with and without *cry* genes. *Fusarium* infection data from two of

Corresponding author: Gary P. Munkvold  
E-mail: munkvold@iastate.edu

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these experiments have been reported (17).

## MATERIALS AND METHODS

Field experiments were conducted in 1995, 1996, and 1997. In each experiment, transgenic hybrids were compared with near-isogenic, nontransgenic maize hybrids lacking *cry* genes. Near-isogenic hybrids were supplied by cooperating seed companies and were either identical with (except for the *cry* genes) or very closely related to their corresponding transgenic hybrids. Experiments were arranged in randomized complete block designs in 1995 and 1996; in 1997, a split-plot design was used with hybrids as main plots and infestation treatments as subplots. Plot size differed among years: in 1995, plots were four rows by 9.15 m long (approximately 40 plants per row); in 1996, they were four rows by 6.1 m long (approximately 26 plants per row); and in 1997, main plots were four rows by 3.05 m long (approximately 15 plants per row) and subplots were single rows. Row spacing was 0.76 m. Only the middle two rows of the four-row plots were treated and analyzed. There were 3.05-m or 1.5-m fallow alleyways between replicate blocks.

The 1995 experiment, planted at the Iowa State University Woodruff Farm in Story County on 22 May, involved three maize hybrids (Table 1) and two *O. nubilalis* infestation treatments with six replicate blocks. The 1996 experiment, planted on 13 and 14 May at the ISU Johnson

Farm in Story County, included eight hybrids (Table 1) and two *O. nubilalis* infestation treatments with five replicate blocks. The 1997 experiment, planted on 20 May at the ISU Berkey Farm in Boone County, included 12 hybrids (Table 1) and four infestation treatments with 10 replicate blocks.

In 1995 and 1996, the two *O. nubilalis* infestation treatments were natural infestation and manual infestation. Laboratory-reared ECB larvae for manual infestation were obtained from the USDA Corn Insects Laboratory in Ames, Iowa. Larvae were premixed in ground corn and applied to the plants using a volumetric dispenser that dispenses 1.0-g aliquots of mixture containing 50 neonatal larvae. Manual infestation was conducted by dispensing 50 larvae into the whorl of each plant at growth stage V8-V10 (22) (first generation) and into the axil of the ear leaf at growth stage R1 (second generation). Manual infestation was conducted to reduce plant-to-plant variability in insect feeding and to simulate a uniform infestation throughout the plots. This method and intensity for manual infestation are standard for European corn borer research (7). In 1997, the four infestation treatments were: natural infestation, manual infestation, *F. verticillioides*-contaminated first-generation manual infestation (Manual-F1); and *F. verticillioides*-contaminated second-generation manual infestation (Manual-F2). Larvae for the Manual-F1 treatment were prepared by growing *F.*

*verticillioides* strain M-8114 (Pennsylvania State University *Fusarium* Research Center, originally isolated in Iowa) on carnation-leaf agar (19) for 7 days, then scraping the contents of four CLA cultures into 500-ml bottles containing approximately 33,000 larvae. The contents were thoroughly mixed by hand before applying to the plants as already described. For the Manual-F2 treatment, five third-instar larvae were placed in 10-ml plastic beakers and lightly misted with a suspension of strain M-8114 conidia (approximately  $10^5$ /ml) in sterile water. One beaker was immediately placed into the axil of the ear shank of each of five plants per subplot at growth stage R1. Surface contamination of the larvae in the F1 and F2 treatments with *F. verticillioides* was confirmed by placing 50 neonatal larvae or five third-instar larvae on Nash-Snyder Medium (19). *F. verticillioides* was isolated from 60 to 75% of neonatal and 100% of third-instar larvae, compared with 5 to 10% of the larvae that were not intentionally contaminated.

After the plants reached maturity (kernel moisture of approximately 20%) in 1995 and 1996, 10 arbitrarily selected ears per plot were visually evaluated in the field for Fusarium ear rot and insect feeding on kernels. In 1997, five ears per subplot were hand-harvested and evaluated in the laboratory. For each ear, the numbers of kernels with symptoms of Fusarium ear rot and insect feeding were recorded. Incidences of Fusarium ear rot and insect feeding were calculated as the percentages of plants per plot with symptoms. Severity of Fusarium ear rot and insect feeding were calculated as the mean number of kernels per ear with symptoms. In 1995, insect feeding was recorded in a different way; a 0 to 3 scale was used in which 0 = no damage, 1 =  $\leq 5$  cm<sup>2</sup> of damage per ear, 2 =  $>5 \leq 10$  cm<sup>2</sup> of damage per ear, and 3 =  $>10$  cm<sup>2</sup> of damage per ear. Incidence and severity calculations were done as in the other years. The number of ECB larvae found on each plant also was recorded after splitting the stalks

**Table 1.** Transformation events, Cry proteins, and maize hybrids represented in field experiments examining the effects of Cry protein expression on fumonisin concentrations in kernels

Year	Transformation event	Cry protein	Cry protein expression in all plant tissues <sup>†</sup>	Hybrid
1995	MON810	CryIA(b)	Yes	Monsanto <sup>u</sup> 802
	MON810	CryIA(b)	Yes	Monsanto <sup>u</sup> 810
	None	None	...	B73 × Mo17
1996	MON810	CryIA(b)	Yes	Monsanto <sup>u</sup> 810
	None	None	...	B73 × Mo17
	BT11	CryIA(b)	Yes	Northrup King <sup>v</sup> X6534CBR
	None	None	...	Northrup King <sup>v</sup> N6800
	176	CryIA(b)	No	Mycogen <sup>w</sup> NG7059BT
	None	None	...	Mycogen <sup>w</sup> 7050cb <sup>x</sup>
	176	CryIA(b)	No	Ciba <sup>v</sup> MAX454
	None	None	...	Ciba <sup>v</sup> 4494
	1997	MON810	CryIA(b)	Yes
None		None	...	Pioneer <sup>s</sup> hybrid 3489
Bt11		CryIA(b)	Yes	Novartis <sup>v</sup> NK 7070BT
None		None	...	Novartis <sup>v</sup> NK 7070
176		CryIA(b)	No	Mycogen <sup>w</sup> NG 7059
None		None	...	Mycogen <sup>w</sup> 7059cb <sup>x</sup>
176		CryIA(b)	No	Ciba <sup>v</sup> Max 454
None		None	...	Ciba <sup>v</sup> 4494
DBT418		CryIA(c)	Yes	DeKalb <sup>y</sup> 580BT
None		None	...	DeKalb <sup>y</sup> 580
CBH351		Cry9C	Yes	AgrEvo <sup>z</sup> Bt
None	None	...	AgrEvo <sup>z</sup> non-Bt	

<sup>†</sup> As indicated by supplier.

<sup>u</sup> Monsanto Co., St. Louis, MO.

<sup>v</sup> Novartis Seeds, Greensboro, NC.

<sup>w</sup> Mycogen Corp., San Diego, CA.

<sup>x</sup> Hybrid with naturally occurring resistance to European corn borer.

<sup>y</sup> DeKalb Plant Genetics, DeKalb, IL.

<sup>z</sup> Agrevo USA Co., Apple Valley, MN.

**Table 2.** *P* values for the effects of hybrid brand, presence of *cry* gene, and European corn borer (ECB) infestation on fumonisin concentrations in kernels for field experiment near Ames, Iowa, in 1996

Effect	Fumonisin B <sub>1</sub>	Total fumonisins
Brand	0.0001	0.0001
<i>cry</i> gene <sup>y</sup>	0.0002	0.0003
ECB infestation <sup>z</sup>	0.0001	0.0001
Block	0.0026	0.0014
Brand × <i>cry</i> gene	0.0065	0.0061
Brand × ECB	0.0798	0.0606
<i>cry</i> gene × ECB	0.0008	0.0003
Brand × <i>cry</i> gene × ECB	0.0141	0.0112

<sup>y</sup> Effect of presence of *cry* gene.

<sup>z</sup> Plants were either manually infested with 50 neonatal ECB larvae (growth stages V8 to V10 and R1) or naturally infested.

and ear shanks longitudinally and examining these tissues for larvae.

In 1995 and 1996, 30 ears per plot were hand-harvested (including the ears evaluated for disease and insect feeding), and the kernels were removed by hand or by an electric sheller. Symptomless infection was determined by culturing 50 arbitrarily selected symptomless kernels (surface-disinfested for 3 min in a 0.5% solution of sodium hypochlorite) from each plot on Nash-Snyder medium (NSM) (19). In 1995 and 1996, *Fusarium* colonies were identified after 7 to 10 days by colony and conidial morphology (19). Colonies were not identified to species in 1995 and 1996 but were classified into groups of *Fusarium* spp., and the percentage of kernels (incidence) infected with each group was recorded. In 1997, after the five ears harvested from each subplot had been evaluated for ear rot and insect feeding severity, they were shelled with an electric sheller, and 25 symptomless kernels per subplot were cultured on NSM. From five infected kernels per subplot, a single colony was transferred from NSM to carnation leaf agar (CLA) and identified to species according to the keys and descriptions of Nelson et al. (19). Incidence of infection by each species was recorded.

In 1995 and 1996, a 0.6-kg sample was collected arbitrarily from the shelled kernels from each plot and ground in a Wiley mill with a 2-mm mesh screen. In 1997, after the 25-kernel sample was removed and cultured, the remainder of the kernels was ground for fumonisin analysis. The ground maize was thoroughly mixed, and 10-g samples were taken for analysis of fumonisin B<sub>1</sub> in 1995 by the procedure of Hopmans and Murphy (9) and of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> (total fumonisins) in 1996 and 1997 by the procedure of Rice et al. (21). Briefly, 10-g samples were extracted with 50 ml of 50% acetonitrile (ACN) for at least 30 min. The supernatant was filtered through filter paper, and 2 ml

of the filtered extract was diluted with 6 ml of 1% potassium chloride (KCl). The diluted sample was then cleaned up using a 500-mg C<sub>18</sub> solid-phase extraction (SPE) column (Waters/Millipore, Milford, MA) by rinsing with 1 ml of 1% KCl and 3 ml of 15% ACN. The fumonisins were then eluted using 2 ml of 70% ACN. Fluorescent derivatives of the fumonisins were formed using *o*-phthalaldehyde (OPA) and detected using a fluorescence detector (LC-240, Perkin-Elmer, Norwalk, CT) with an excitation wavelength of 335 nm and an emission wavelength of 440 nm. Derivatization was accomplished at room temperature using a 0.25-ml sample, 0.25 ml of borate buffer, (pH 8.3), 0.25 ml of OPA solution (15 mg of OPA and 20 µl of 2-mercaptoethanol in 10 ml of ACN), and 0.25 ml water. Reaction time was 10 min.

Ten microliters of derivatized sample (0.5 mg equivalent) was injected into a 3 cm × 4.6 mm high speed, 3 µm packing material, C<sub>18</sub> column (Perkin-Elmer) with quantitation made using external standards and peak height measurements. Fumonisin B<sub>1</sub> and B<sub>2</sub> standards were purchased from Sigma Chemical Co. (St. Louis, MO), and fumonisin B<sub>3</sub> standard was obtained from Ron Plattner (USDA-ARS, NCAUR, Peoria, IL). Detection limits were 0.1 µg/g for fumonisin B<sub>1</sub> and 0.5 µg/g for fumonisins B<sub>2</sub> and B<sub>3</sub>.

Analysis of variance (SAS, Cary, NC, or SigmaStat, Jandel Scientific, San Diego, CA) was utilized to evaluate insect feeding severity, numbers of ECB larvae, *Fusarium* ear rot severity, incidence of symptomless infection, fumonisin B<sub>1</sub> concentration, and total fumonisin concentration. A factorial

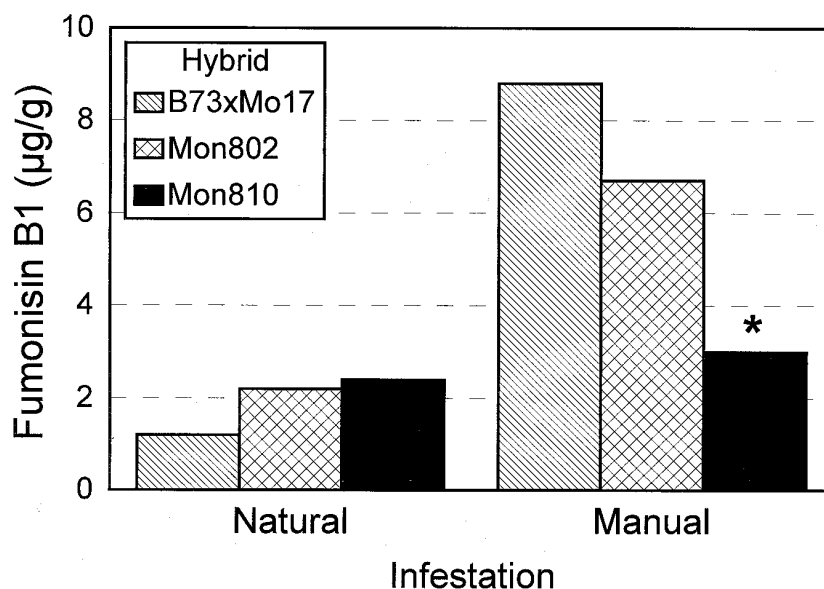


Fig. 1. Fumonisin B<sub>1</sub> concentrations in kernels of transgenic *Bt* maize hybrids (Mon802 and Mon810) and a near-isogenic standard hybrid (B73×Mo17) in 1995. Manually infested plants were infested with 50 neonatal European corn borer (*Ostrinia nubilalis*) larvae at growth stages V8 to V10 and R1. \* indicates a significant difference ( $P \leq 0.05$ ) between standard and transgenic hybrid.

Table 3. *P* values for the effects of hybrid brand, presence of *cry* gene, and European corn borer (ECB) infestation on *Fusarium* kernel rot incidence, *Fusarium* ear rot severity, and incidence of symptomless kernel infection by *Fusarium* spp. in a field experiment in Boone County, Iowa, in 1997

Effect	Insect feeding		Fusarium ear rot		Symptomless <i>Fusarium</i> infection <sup>v</sup>			Fumonisin concentration		
	Incidence <sup>w</sup>	Severity <sup>x</sup>	Incidence <sup>w</sup>	Severity <sup>x</sup>	Total	<i>F. vert.</i>	<i>F. prol.</i>	<i>F. subg.</i>	FB <sub>1</sub>	Total
Brand	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0003	0.0189	0.5145	0.5684
<i>cry</i> gene <sup>y</sup>	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.1188	0.2815	0.0001	0.0001
ECB infestation <sup>z</sup>	0.1300	0.0989	0.0040	0.0019	0.0001	0.0001	0.8722	0.0001	0.0001	0.0001
Block	0.1149	0.0016	0.2693	0.0079	0.0003	0.0001	0.7183	0.1943	0.0152	0.0128
Brand × <i>cry</i> gene	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0120	0.0908	0.0001	0.0001
Brand × ECB	0.8711	0.2805	0.1352	0.3543	0.0051	0.0077	0.1536	0.3557	0.9550	0.9419
<i>cry</i> gene × ECB	0.3668	0.2739	0.0322	0.0482	0.4020	0.5560	0.4382	0.2786	0.0338	0.0345
Brand × <i>cry</i> gene × ECB	0.8586	0.2812	0.4718	0.2728	0.4261	0.4578	0.1545	0.5433	0.7115	0.6732

<sup>v</sup> Measured as the percentage of infected kernels, based on isolation from 10 replications of 25 kernels each. *F. verticilloides*, *F. proliferatum*, and *F. subglutinans*.

<sup>w</sup> Measured as the percentage of plants with symptoms, based on 10 replications of five plants each.

<sup>x</sup> Measured as the mean number of kernels with symptoms per ear, based on 10 replications of five plants each.

<sup>y</sup> Effect of presence of *cry* gene.

<sup>z</sup> There were four infestation treatments: natural ECB infestation, manual infestation, or manual infestation with *F. verticilloides* contamination at growth stage V8 to V10 or R1.

analysis was used to evaluate the main effects and interactions of hybrid brand, presence of *cry* genes, and ECB infestation treatment. Multiple comparison tests were performed according to the Student-Newman-Keuls test on treatment means if there were interactions. A square-root-square-root transformation was used to obtain a normal distribution for the fumonisin data. Linear correlation coefficients were calculated for the relationships among severity of Fusarium ear rot, insect feeding severity, and fumonisin concentration (SigmaStat), using data from individual experimental units.

## RESULTS

Hybrid brand, presence of *cry* genes, and insect infestation treatments had effects on fumonisin B<sub>1</sub> and total fumonisin concentrations, and the significance of these effects differed among years. In 1995, when only one brand was included, the main effect of hybrid brand on fumonisin B<sub>1</sub> concentration was not significant ( $P = 0.240$ ), but infestation had a significant effect ( $P = 0.032$ ) and there was a significant interaction ( $P = 0.044$ ). In 1996, effects of hybrid brand, presence of *cry* genes, and infestation treatment were significant, but there were significant two-way and three-way interactions (Table 2). In 1997, the significant main effects were those of *cry* gene presence and infestation treatment, and there were significant two-way interactions (Table 3). Fumonisin concentrations were significantly lower in some transgenic hybrids compared with their nontransgenic counterparts, and fumonisin concentrations were enhanced when maize plants were manually infested with ECB larvae (Figs. 1 to 3). Significant differences among hybrids were more evident under manual infestation. In 1997, the fumonisin concentrations were higher than in the other 2 years, and the differences between transgenic and nontransgenic hybrids were most dramatic. Transgenic hybrids in some cases had concentrations less than 10% of their nontransgenic counterparts. The Manual-F1 treatment resulted in the highest fumonisin concentrations in 1997, with a mean of 17.3  $\mu\text{g/g}$  (total fumonisins), compared with 12.1  $\mu\text{g/g}$  in the Manual-F2 treatment, 11.3  $\mu\text{g/g}$  in the manually infested treatment, and 7.8  $\mu\text{g/g}$  in the naturally infested treatment. Fumonisin B<sub>1</sub> accounted for approximately 70% of the total fumonisins, and ANOVA on total fumonisins yielded results almost identical to those for fumonisin B<sub>1</sub>.

In all 3 years, presence of *cry* genes had a significant ( $P \leq 0.0001$ ) effect on insect feeding severity on ears, with lower severity in transgenic hybrids than in nontransgenic hybrids (Tables 4 and 5). In 1996, the effects of hybrid brand and the presence of *cry* genes were significant ( $P \leq 0.0001$ ), but there also were significant two-way and three-way interactions ( $P \leq 0.05$ ).

Insect infestation had a significant effect on feeding severity in 1996 ( $P \leq 0.0001$ ) but not in 1995 and 1997 ( $P = 0.8235$  in 1995 and  $P = 0.0989$  in 1997). Significantly more larvae were found in naturally infested plants than in manually infested plants ( $P = 0.0043$  in 1995 and  $P = 0.0277$  in 1996) (Tables 4 and 5). Numbers of larvae were affected significantly by hybrid brand and presence of *cry* genes (transgenic hybrids had fewer larvae than nontransgenic hybrids) (Tables 4 and 5), and there was a significant ( $P = 0.001$ ) interaction between these two factors.

Fusarium ear rot severity was affected by hybrid brand, presence of *cry* genes, and ECB infestation ( $P \leq 0.0001$ ), but there were significant interactions among these factors (17; Table 6). Generally, symptoms were less for transgenic hybrids

than for nontransgenic, and less for natural infestation than for manual infestation, but these effects depended on the hybrid brand. In 1997, the second-generation manual infestation treatment that included *F. verticillioides* contamination (Manual-F2) had the highest ear rot severity (Table 6).

Incidence of symptomless kernel infection by *Fusarium* spp. was significantly affected by hybrid brand, presence of *cry* genes, and infestation treatment; there also were significant two-way interactions (17; Table 6). Transgenic hybrids had lower incidence of symptomless infection (43.4% in 1997) than nontransgenic hybrids (53.5% in 1997), but the difference depended on hybrid brand and infestation treatment. In 1997, the Manual-F1 treatment resulted in the highest incidence of symptomless infection in 11 of 12 hybrids

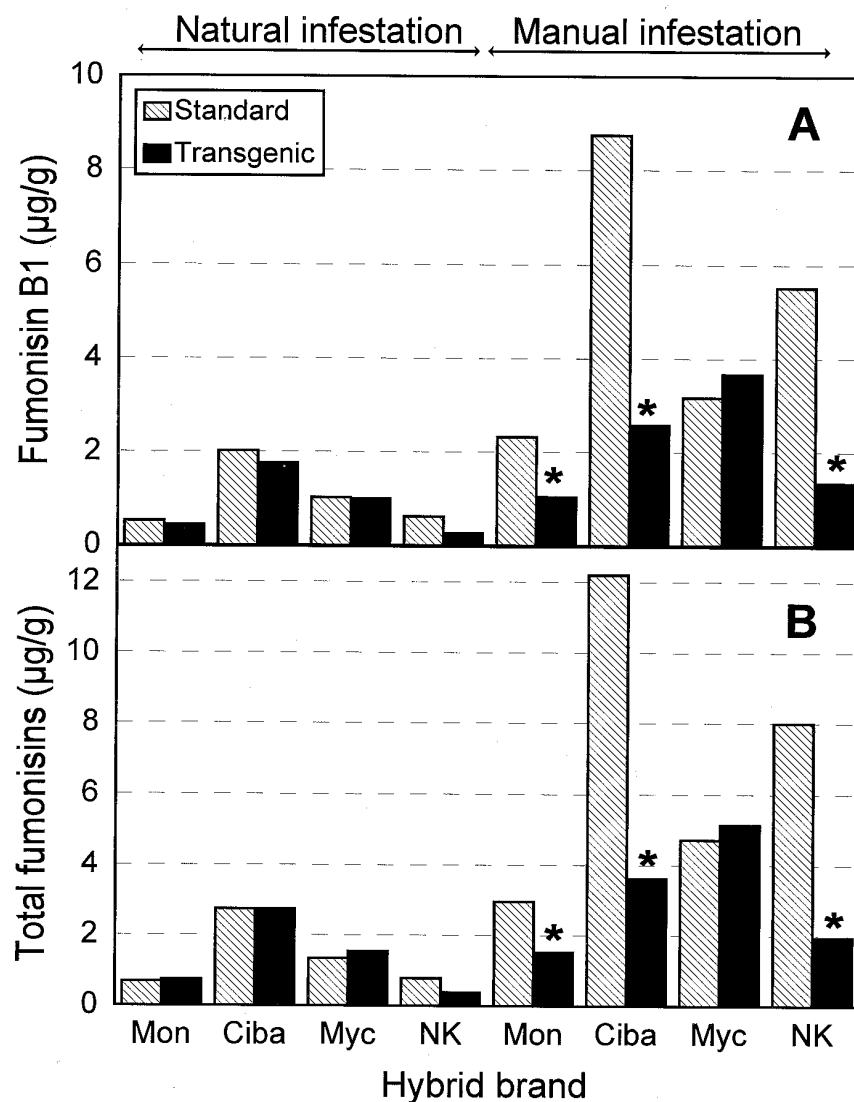


Fig. 2. Fumonisin B<sub>1</sub> (A) and total fumonisin (B) concentrations in kernels of transgenic *Bt* maize hybrids and near-isogenic standard hybrids in 1996. Transgenic hybrids are paired with their near-isogenic counterparts. Manually infested plants were infested with 50 neonatal European corn borer (*Ostrinia nubilalis*) larvae at growth stages V8 to V10 and R1. Hybrids are identified by brand: Mon (Monsanto), Ciba (Novartis), Myc (Mycogen), NK (Novartis). \* indicates a significant difference ( $P \leq 0.05$ ) between standard and transgenic hybrids.

(Table 6). Three species, *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*, accounted for 99.0% of the *Fusarium* colonies identified from symptomless infected kernels. *F. verticillioides* comprised the majority (81.4%) of the *Fusarium* colonies identified from all treatments. Analysis of variance on individual species indicated that incidence of infection by *F. verticillioides* was higher in the manual ECB infestation treatments than in the natural infestation treatment, but incidence of the other two species was either the same or lower in the manual infestations than in the natural infestation.

In 1996 and 1997, insect feeding severity, *Fusarium* ear rot severity, and incidence of symptomless infection were significantly correlated with fumonisin B<sub>1</sub> concentration (Fig. 4). In 1995, fumonisin B<sub>1</sub> concentrations were significantly correlated only with incidence of symptomless infection.

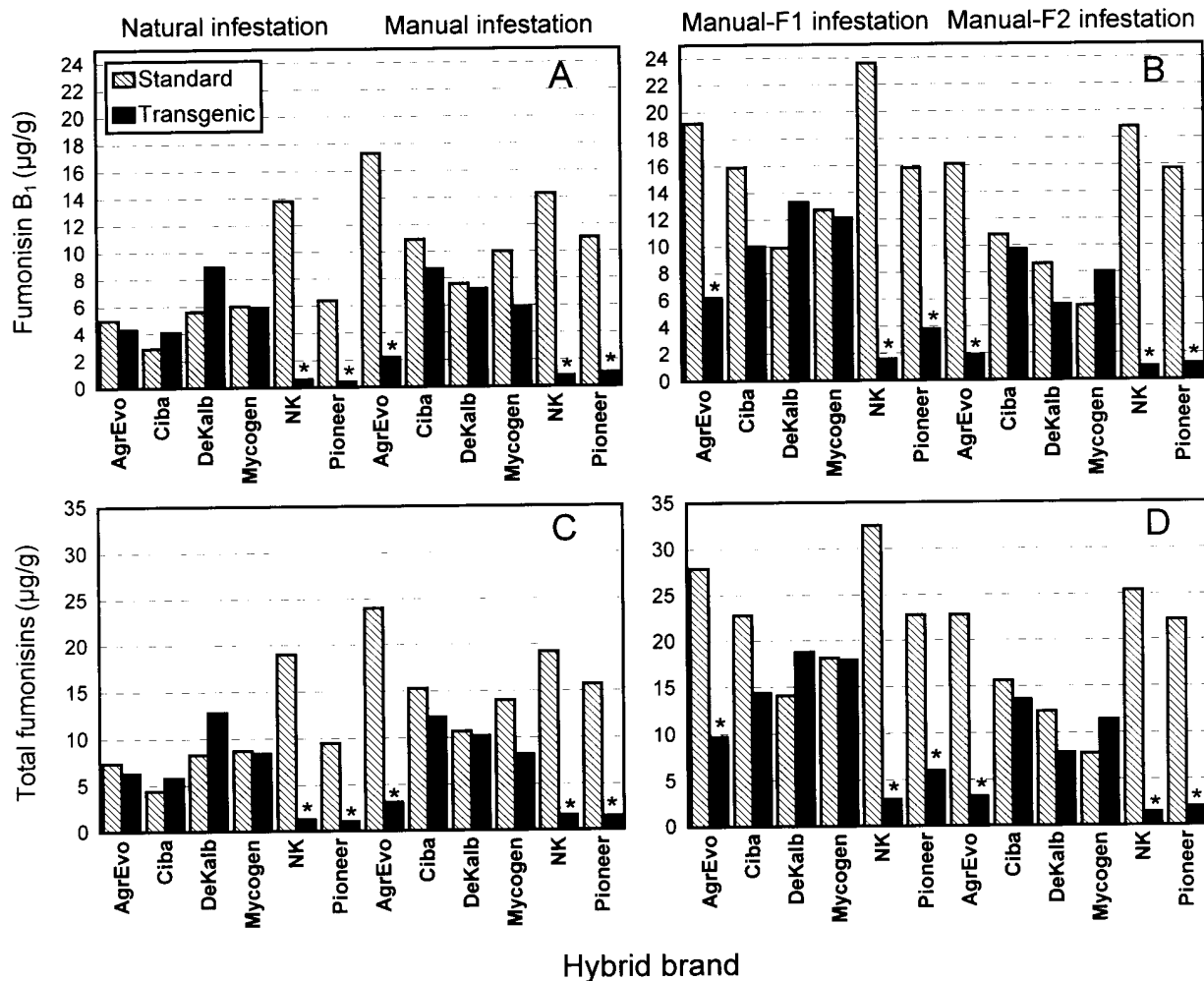
### DISCUSSION

Under some conditions, transgenic maize hybrids expressing *cry* genes from

*B. thuringiensis* can contain lower concentrations of fumonisins than do nontransgenic hybrids. These lower concentrations were associated with earlier-reported reductions in *Fusarium* ear rot and/or symptomless infection (17) that, in turn, can be attributed to reduced insect feeding on kernels of transgenic hybrids. This finding indicates that the safety of maize for human and animal consumption may be enhanced by genetic engineering for insect resistance. The magnitude of the differences in fumonisin concentrations between transgenic and nontransgenic hybrids was sufficient to affect the toxicity of these maize kernels to horses (15) and to human cell cultures (1,23,26). The fumonisin concentrations that occurred in some nontransgenic hybrids were high enough to be considered unsafe for some animal species and inadvisable for human consumption. The American Association of Veterinary Laboratory Diagnosticians (AAVLD) has recommended that feeds for horses contain less than 5 µg of fumonisins per g and that feeds for swine contain less than 10 µg of

fumonisin per g (15). One or both of these criteria were exceeded by nontransgenic hybrids in all 3 years. There are no widely accepted standards regarding safe fumonisin concentrations for human consumption.

The hybrid brand × *cry* gene interaction is important in the analysis of our results because it indicates whether Bt transformation events differ in their effects on the dependent variables that we measured. The transgenic hybrids included in the 1996 and 1997 experiments are the products of different transformation events, so they differ either in the gene promoter or the Cry protein. Directly comparing data from different transgenic hybrids is problematic because the background *Fusarium* susceptibility of the hybrids differs and the effects of background genetics cannot be separated from the effects of the transgenes. By examining the hybrid brand × *cry* gene interactions, we can assess how different transformation events affected the *Fusarium* ear rot, *Fusarium* infection, and fumonisin concentrations of their respective background hybrids. These interactions were



**Fig. 3.** Fumonisin B<sub>1</sub> (A and B) and total fumonisin (C and D) concentrations in kernels of transgenic *Bt* maize hybrids and near-isogenic standard hybrids in 1997. Transgenic hybrids are paired with their near-isogenic counterparts. Manually infested plants were infested with 50 neonatal European corn borer (*Ostrinia nubilalis*) larvae at growth stages V8 to V10 and R1; plants in the manual-F1 treatment were infested with 50 *Fusarium verticillioides*-contaminated neonatal larvae at stages V8 to V10; and plants in the manual-F2 treatment were infested with five *F. verticillioides*-contaminated third-instar larvae at stage R1. Hybrids are identified by brand. \* indicates a significant difference ( $P \leq 0.05$ ) between standard and transgenic hybrids.

often significant, indicating that the MON810 and BT11 transformation events led to significantly greater reductions in *Fusarium* ear rot severity, incidence of symptomless infection, and fumonisin concentrations than did the 176 event.

The differential effects of the transformation events can be attributed to differences in *cry* gene expression. Maize hybrids with the MON810 or BT11 transformation types utilize a CaMV 35S gene promoter (2) that results in season-long expression of CryIA(b) in all plant tissues; whereas transformation 176 utilizes a combination of two maize-derived, tissue-specific promoters: a phosphoenolpyruvate carboxylase (PEPC) promoter that results in gene expression only in green plant tissues, and a pollen-specific promoter (11). Hybrids expressing

CryIA(b) in kernels were better protected against *Fusarium* infection than those expressing the protein only in green tissues and pollen. This trend was evident but not universal. Hybrids with the MON810 and BT11 transformation events consistently demonstrated significant reductions in *Fusarium* infection and fumonisin concentration in the manual infestation treatments. Although hybrid Mon802 also expresses CryIA(b) in kernels, it did not exhibit reduced fumonisin concentration. This hybrid expresses lower concentrations of CryIA(b) than Mon810 (5), and the lower expression in this hybrid may not have controlled insect feeding on kernels sufficiently to affect fumonisin concentrations. Insect feeding severity for this hybrid was higher than, but not significantly different from, insect feeding severity for

Mon810. Therefore, the difference in fumonisin B<sub>1</sub> concentration between these two hybrids cannot be explained completely with the available data. Hybrids with the 176 transformation event do not have kernel expression of CryIA(b) and did not consistently demonstrate effects on *Fusarium* ear rot or fumonisins. Ciba hybrid Max454 demonstrated significantly less *Fusarium* ear rot in 1996 and 1997 and lower fumonisin concentrations in 1996 than its nontransgenic counterpart, 4494, but the Mycogen transgenic hybrid did not exhibit any significant reductions in any of the variables in the manual infestation treatment. This result might have been due to differences in the genetic backgrounds of the hybrids; the nontransgenic Mycogen hybrid is known to have native resistance to *O. nubilalis*. The DBT418 transforma-

**Table 4.** Insect feeding severity on maize ears and European corn borer (ECB) larvae per plant in transgenic (Bt) and nontransgenic hybrids in field experiments conducted in 1995 and 1996<sup>v</sup>

Year	Hybrid	Insect feeding severity <sup>w</sup>			ECB larvae /plant <sup>x</sup>		
		Natural	Manual <sup>y</sup>	Mean	Natural	Manual <sup>y</sup>	Mean
1995	B73 × Mo17	0.7 a	0.7 a	0.7	1.17 a	0.42 b	0.79
	Mon 802* <sup>z</sup>	0.2 b	0.3 b	0.2	0.02 c	0.00 c	0.01
	Mon 810*	0.1 b	0.1 b	0.1	0.00 c	0.00 c	0.00
	Means	0.3	0.4	0.3	0.40	0.14	0.27
1996	Mon 810*	0.5 ef	0.2 f	0.3	0.00 f	0.00 f	0.00
	B73 × Mo17	2.2 def	2.3 def	2.2	1.02 a	0.86 abc	0.96
	Ciba MAX454*	3.0 cde	2.3 def	2.6	0.16 ef	0.10 f	0.13
	Ciba 4494	5.1 bc	12.3 a	8.7	0.94 ab	0.80 abc	0.87
	Mycogen NG7059BT*	3.3 cd	7.1 b	5.2	0.52 cde	0.20 def	0.36
	Mycogen 7050cb	6.3 b	7.6 b	6.9	0.78 abc	0.52 cde	0.66
	Northrup King X6534CBR*	0.8 def	0.4 ef	0.6	0.00 f	0.00 f	0.00
	Northrup King N6800	2.0 def	7.0 b	4.5	0.58 bc	0.54 cd	0.56
	Means	2.9	4.9	3.9	0.50	0.38	0.44

<sup>v</sup> Near-isogenic hybrids are paired in the table. Values are means for six replications of 10 ears in 1995 and five replications of 10 ears in 1996. Values within a year followed by the same letter are not significantly different (Student-Newman-Keuls test,  $\alpha = 0.05$ ).

<sup>w</sup> Insect feeding severity was assessed on a 0 to 3 scale in 1995 and as the mean number of damaged kernels per plant in 1996 (10 plants per plot).

<sup>x</sup> Numbers of live and dead ECB larvae in the ears, stalks, and shanks were recorded at harvest maturity (10 plants per plot).

<sup>y</sup> Manual infestation consisted of 50 neonatal ECB larvae placed in the whorl at stage V8 to V10 and in the axil of the ear leaf at stage R1.

<sup>z</sup> \* = Bt hybrids.

**Table 5.** Insect feeding severity on maize ears in transgenic (Bt) and nontransgenic hybrids in a field experiment conducted in 1997<sup>u</sup>

Year	Hybrid	Insect feeding severity <sup>v</sup>				Mean
		Natural	Manual <sup>w</sup>	Manual-F1 <sup>x</sup>	Manual-F2 <sup>y</sup>	
1997	Pioneer hybrid 34R06* <sup>z</sup>	0.5	0.7	1.7	1.3	1.0 e
	Pioneer hybrid 3489	12.8	15.7	16.0	22.6	16.8 bc
	Novartis NK 7070BT*	0.7	0.3	0.7	1.3	0.7 e
	Novartis NK 7070	17.1	20.8	19.9	20.6	19.6 b
	Mycogen NG 7059*	11.7	13.6	13.4	12.6	12.8 cd
	Mycogen 7059cb	14.9	15.1	16.3	11.6	14.5 cd
	Ciba Max 454*	9.9	11.4	8.7	13.4	10.8 d
	Ciba 4494	15.3	22.1	17.6	20.0	18.7 b
	DeKalb 580BT*	9.7	11.1	13.2	9.3	10.8 d
	DeKalb 580	18.9	15.8	13.1	14.5	15.6 bc
	AgrEvo Bt*	4.4	3.1	1.9	2.3	2.9 e
	AgrEvo	28.3	34.2	26.1	36.7	31.3 a
	Means	12.0	13.7	12.4	13.9	13.0

<sup>u</sup> Near-isogenic hybrids are paired in the table. Values are means for 10 replications of five ears. Values followed by the same letter are not significantly different (Student-Newman-Keuls test,  $\alpha = 0.05$ ).

<sup>v</sup> Insect feeding severity was assessed as the mean number of damaged kernels per plant in 1997 (five ears per plot).

<sup>w</sup> Manual infestation consisted of 50 neonatal European corn borer (ECB) larvae placed in the whorl at stage V8 to V10 and in the axil of the ear leaf at stage R1.

<sup>x</sup> Manual-F1 treatment consisted of 50 neonatal ECB larvae contaminated with *Fusarium verticillioides* conidia and placed in the whorl at stage V8 to V10.

<sup>y</sup> Manual-F2 treatment consisted of five third-instar ECB larvae contaminated with *F. verticillioides* conidia and placed in the ear shank axil at stage R1.

<sup>z</sup> \* = Bt hybrids.

tion event demonstrated results similar to the 176 event, and the CBH351 event demonstrated results similar to the MON810 event, but we have only 1 year of data for DBT418 and CBH351.

Fumonisin concentrations were significantly correlated with insect feeding severity, Fusarium ear rot, and symptomless *Fusarium* infection in 1996 and 1997, but only with symptomless infection in 1995.

Symptomless infection occurs by a number of pathways that may or may not be related to insect feeding (18). Typically, fumonisin concentrations are higher in symptomatic kernels (6), and Fusarium ear rot severity

**Table 6.** Fusarium ear rot severity and symptomless kernel infection in Bt and non-Bt hybrids subjected to manual and natural infestations of European corn borer (ECB) in Boone County, Iowa, in 1997<sup>a</sup>

Brand	Hybrid	Fusarium ear rot severity (kernels/ear)					Symptomless infection (% of kernels)				
		Manual <sup>v</sup>	Manual-F1 <sup>w</sup>	Manual-F2 <sup>x</sup>	Natural <sup>y</sup>	Mean	Manual <sup>v</sup>	Manual-F1 <sup>w</sup>	Manual-F2 <sup>x</sup>	Natural <sup>y</sup>	Mean
AgrEvo	Bt <sup>z</sup>	2.8 c	2.5 d	3.2 c	4.8 c	3.3	25.9 d	56.4 a	30.4 c	19.6 d	33.1
AgrEvo	non-Bt	38.4 a	23.1 ab	31.9 a	21.5 a	28.7	43.5 bcd	64.4 a	48.4 ab	30.2 b-e	46.6
Ciba	Max454*	10.1 bc	10.6 c	13.9 b	9.2 bc	11.0	67.8 ab	64.4 a	60.0 ab	46.7 ab	59.7
Ciba	4454	25.7 b	17.6 bc	26.6 ab	14.1 ab	21.0	71.6 a	72.8 a	66.0 a	49.6 a	65.0
DeKalb	DK580BT*	17.9 b	19.2 abc	19.1 ab	15.7 ab	18.0	58.0 abc	70.0 a	58.4 ab	44.8 ab	57.8
DeKalb	DK580	19.8 b	18.5 abc	18.9 ab	21.5 a	19.7	59.1 abc	73.0 a	61.1 ab	39.2 abc	58.1
Mycogen	NG7059*	13.7 bc	15.9 bc	15.4 b	13.6 ab	14.7	54.8 abc	65.3 a	54.9 ab	37.8 a-d	53.2
Mycogen	7050cb	17.1 b	18.9 abc	16.2 b	16.2 ab	17.1	40.4 cd	60.4 a	41.2 bc	28.7 bcd	42.7
NK	NK7070BT*	2.1 c	2.1 d	2.6 c	1.3 c	2.0	26.4 d	39.6 b	29.6 c	19.2 d	28.7
NK	NK7070	25.1 b	26.6 a	27.2 ab	20.3 a	24.8	59.6 abc	67.6 a	57.2 ab	53.2 a	59.4
Pioneer	34R06*	1.7 c	2.3 d	2.5 c	0.5 c	1.8	28.4 d	57.2 a	12.0 d	13.2 e	27.7
Pioneer	3489	17.4 b	15.5 bc	26.5 ab	15.6 ab	18.8	56.9 abc	66.2 a	52.0 ab	24.0 cd	49.8
Means		16.0	14.4	17.0	12.9	15.1	49.3	63.1	47.6	33.7	48.4

<sup>a</sup> Values in a column followed by the same letter are not significantly different (Student-Newman-Keuls test,  $\alpha = 0.05$ ).

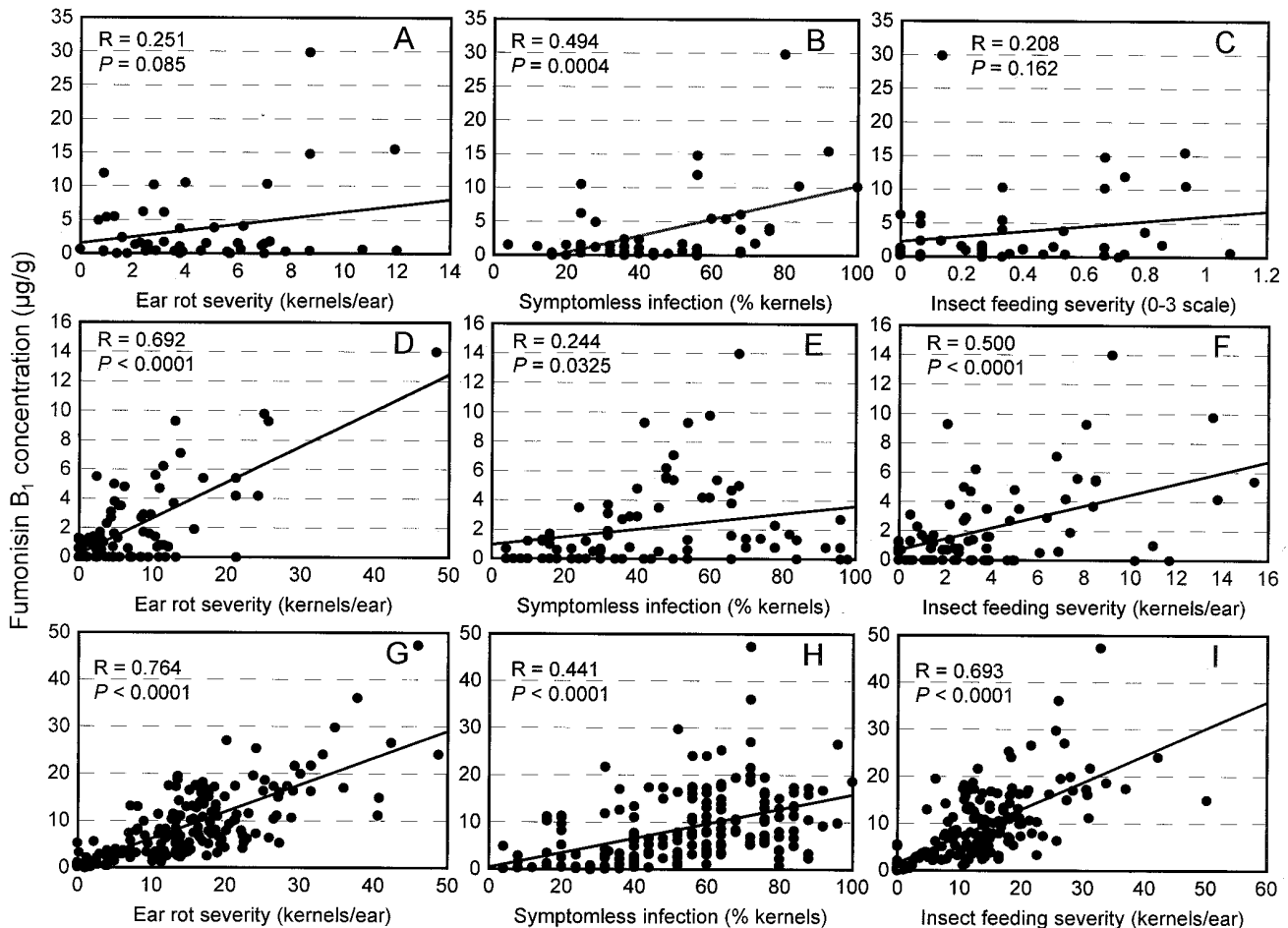
<sup>v</sup> Manual infestation treatment consisted of 50 neonatal ECB larvae placed in the whorl at stage V8 to V10 and in the axil of the ear leaf at stage R1.

<sup>w</sup> Manual-F1 treatment consisted of 50 neonatal ECB larvae contaminated with *Fusarium verticillioides* conidia and placed in the whorl at stage V8 to V10.

<sup>x</sup> Manual-F2 treatment consisted of five third-instar ECB larvae contaminated with *F. verticillioides* conidia and placed in the ear shank axil at stage R1.

<sup>y</sup> The natural infestation treatment consisted of the endemic ECB population.

<sup>z</sup> \* = Bt hybrids.



**Fig. 4.** Linear correlations of Fusarium ear rot severity, symptomless *Fusarium* infection, and insect feeding severity with fumonisin B<sub>1</sub> concentrations in 1995 (A to C), 1996 (D to F), and 1997 (G to I). Correlations were calculated on individual experimental units.

is enhanced by insect feeding (18). The highest fumonisin concentrations occurred in 1997, the year in which insect feeding severity and Fusarium ear rot severity were highest. The 1996 and 1997 correlations are consistent with these relationships, but the 1995 correlations are less so. The lack of correlation between fumonisin concentrations and ear rot severity in 1995 is a result of the low fumonisin concentration in hybrid B73×Mo17 in the naturally infested compared with the manually infested treatment. Although manual infestation did not affect ear rot severity, it strongly affected fumonisin concentration. Sampling may also have affected correlation coefficients. In 1995 and 1996, Fusarium ear rot and insect feeding severity were assessed in the field on 10 ears per plot, but symptomless infection and fumonisin concentrations were analyzed on a subsample of grain harvested from each entire plot. In 1997, all assessments and analyses were performed on the same five ears per plot. As a result, correlation coefficients tended to be higher in 1997 than in 1996.

Our results also indicate that fumonisin concentrations in maize kernels can be affected by ECB infestation. Differences in Fusarium ear rot and fumonisin concentrations between transgenic and nontransgenic hybrids were evident primarily in manually infested treatments. The effect may be related to larval numbers, placement, and/or timing of infestation. Manual infestation with ECB larvae was performed in order to reduce variability in insect feeding among the plants and ensure that some viable larvae occurred on each plant. The number of larvae used (50) is a standard and realistic population for manual infestation (6,14). Natural mortality of ECB larvae hatching on maize leaf surfaces is about 70% in the first instar, and 50 to 80% of the surviving larvae die before tunneling into the plant (14). The number of larvae likely to survive in the manual infestation was therefore similar to that which would occur if one egg mass were laid per plant. Manual infestation did not increase the total number of larvae recovered from plants at maturity in 1995 or 1996, nor did it affect insect feeding severity on kernels in 1995 or 1997, indicating that the number of larvae per plant was not unrealistic in the manual infestation treatment. Nevertheless, this treatment did significantly affect Fusarium ear rot and fumonisin concentrations in some hybrids. This is probably more related to the timing and placement of the infestation than to the total number of larvae. Manual second-generation infestation occurred at stage R1, and larvae were placed in the ear-leaf axil; both timing and placement were optimal for the larvae to damage the kernels at an early plant reproductive stage, allowing the maximum time for fumonisin accumulation before harvest. In 1997, the

manual infestation treatments that included *F. verticillioides* inoculum resulted in the highest Fusarium ear rot severity and fumonisin concentrations, as expected.

Other mycotoxins in maize also may be affected by insect activity. Most important among these are the aflatoxins, produced primarily by *Aspergillus flavus* (12). Seed of transgenic *Bt* cotton sometimes have lower aflatoxin concentrations than seed of nontransgenic cotton (4), but there are no reports demonstrating a similar effect in corn kernels. This possibility warrants further study.

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