JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Gibberella Ear Rot of Maize (*Zea mays*) in Nepal: Distribution of the Mycotoxins Nivalenol and Deoxynivalenol in Naturally and Experimentally Infected Maize

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The fungus *Fusarium graminearum* (sexual stage *Gibberella zeae*) causes ear rot of maize (*Zea mays*) and contamination with the 8-ketotrichothecenes nivalenol (**1**) or 4-deoxynivalenol (**2**), depending on diversity of the fungal population for the 4-oxygenase gene (*TRI13*). To determine the importance of **1** and **2** in maize ear rot, a survey of naturally contaminated maize in Nepal was combined with experiments in the field and in a plant growth room. In the survey, **1** contamination was 4-fold more frequent than **2** contamination and **1**-producers (*TRI13*) were isolated more than twice as frequently as **2**-producers (Ψ*TRI13*). In maize ear rot experiments, genetically diverse **1**-producers and **2**-producers caused ear rot and trichothecene contamination. Among strains with the same genetic background, however, **1**-producers caused less ear rot and trichothecene contamination than did **2**-producers. The high frequency of **1** contamination and the high virulence of many **1**-producers are of concern because maize is a staple food of rural populations in Nepal and because **1** has proven to be more toxic than **2** to animals.

KEYWORDS: ***Fusarium graminearum*; nivalenol; deoxynivalenol; *Gibberella* ear rot; maize; Nepal**

INTRODUCTION

Maize (*Zea mays* L.) is an important food crop in the foothills of the Himalaya Mountains of Nepal and is grown on 851,000 ha, comprising 30% of the total cultivated land (*1*). In the foothills, maize typically matures during the monsoon season, when abundant warm rains and poor storage conditions provide the ideal environment for fungal infection, ear rot, and mycotoxin production. Surveys of maize ear rotting fungal pathogens in Nepal have found a predominance of *Fusarium* species, mainly *F. verticillioides* (Saccardo) Nirenberg (sexual stage *Gibberella moniliformis* Wineland) and *F. graminearum* Schwabe [*G. zeae* Schwein (Petch)] (*2, 3*). *F. verticillioides* contaminates grain with fumonisins, which cause equine leukoencephalomalacia, swine pulmonary edema, and cancer in experimental rodents, and have been associated epidemiologically with esophageal cancer and neural tube defects in humans (*4*). *F. graminearum* contaminates grain with 8-ketotrichothecenes, mainly nivalenol (**1**) and its less oxygenated homologue 4-deoxynivalenol (**2**) (**Figure 1**). **1**, **2**, and other trichothecenes cause anemia and immunosuppression, feed refusal, emesis, and ill-thrift in animals and have been associated epidemiologically with these symptoms in humans (*4*). Production of **1** or **2** depends on diversity of the fungal population for the *TRI13* gene, which encodes the cytochrome P450 monooxygenase required for 4-oxygenation of **2** to produce **1** (*5*). Strains with a functional *TRI13* gene can produce both **1** and **2**, but usually contaminate grain with **1**, whereas strains with a nonfunctional pseudogene, Ψ*TRI13*, cannot produce **1** and can contaminate grain with **2**.

The presence of 8-ketotrichothecenes in maize naturally infected with *F. graminearum* was first reported in the United States in 1973 and in South Africa in 1977, but only **2** was detected (*6, 7*). Later surveys of maize in the Americas and Europe have often detected **2**, but have rarely detected **1** (*4, 8*). In maize in Africa and New Zealand, levels of **2** have been higher than or similar to levels of **1** and, in maize from China, higher levels of **2** than of **1** consistently have been reported (*9–14*). In Korea, **2** and **2**-producers of *F. graminearum* predominate in maize; however, **1**-producers from Korea were able to cause ear rot and **1** contamination of maize in a field test in 1995 that is, to our knowledge, the first and only study to test **1**-producers

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10.1021/jf8003702 CCC: $40.75
Published on Web 06/06/2008

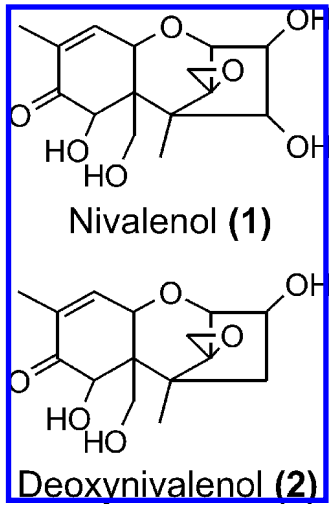

Figure 1. Structures of nivalenol (**1**) and deoxynivalenol (**2**).

of *F. graminearum* for maize ear rot and **1** production in maize ears (*15, 16*). In a survey of maize in Nepal in 1993 and 1997, 8-ketotrichothecenes were detected by quantitative fluorometry and **2** was detected by immunoassay (*3*). **1** was detected in some samples by LC-MS, but the distribution of both **1** and **2** in maize from Nepal was not determined.

Previous studies indicate that the *F. graminearum* population in Nepal is genetically highly diverse (*17–20*; unpublished results). Analyses of this population using amplified fragment length polymorphisms (AFLP) and other markers have identified four groups, designated *AFLPs1−4*, which are genetically distinct from *F. graminearum* lineage 7, the dominant phylogenetic lineage in North America. On the basis of DNA sequencing of representative strains, *AFLPs2−4*, but not *AFLP1*, have been associated with lineages that have been proposed as separate species of the *F. graminearum* complex. *AFLP2* has been associated with lineage 2 (*F. meridionale)*, *AFLP3* with lineage 6 (*F. asiaticum*), and *AFLP4* with lineage 3 (*F. boothii*) (*18, 19, 21*). Trichothecene chemotypes and *TRI13* gene alleles differ among the four AFLP groups of *F. graminearum* in Nepal: *AFLP1* has **2**-producers (Ψ*TRI13*); *AFLP2* has **1**-producers (*TRI13*); and *AFLP3* and *AFLP4* both have **1**-producers and **2**-producers.

The major aim of the present study was to investigate the importance of **1**-producers and **2**-producers of *F. graminearum* from Nepal in causing maize ear rot and contamination with **1** and **2**. We combined a survey of naturally contaminated maize in Nepal with maize ear rot experiments. Maize was surveyed by chemical analysis for distribution of **1** and **2** and by DNA analysis for distribution of *F. graminearum* strains with *TRI13* and Ψ*TRI13* alleles. *F. graminearum* strains of four AFLP groups from Nepal were tested for the ability to cause maize ear rot and to produce **1** and **2** in experimentally infected ears in the field in Nepal and in a plant growth room in the United States. This analysis provides information for risk management of **1** and **2** in grain.

MATERIALS AND METHODS

Maize Survey Sample Collection. Fifty-two samples of maize grain and two of maize flour were collected in February and March 2004 from markets (14 samples) or smallholder farms (40 samples) from 11 districts of six administrative zones of Nepal (**Figure 2**; **Tables 1** and **2**). All market samples were yellow maize; farm samples included white maize and yellow maize, introduced cultivars, and local landraces. For a high likelihood of trichothecene contamination, samples were selected for discoloration and other visible symptoms of disease. Seeds were selected from individual ears preferably or from loose seeds. Due to logistics of travel and sample collection in Nepal, we were not able to standardize sample sizes, which were a mean of 75 g and a range of 25−150 g of dry weight. All maize samples were transported from Nepal to Peoria, IL, under permit from the governments of Nepal and the U.S. Department of Agriculture and were stored at −20 °C until analysis.

Maize Ear Rot Field Experiment in Nepal. A field test was conducted at facilities of the Nepal Agricultural Research Council, Khumaltar, Nepal, using 22 previously isolated strains of *F.*

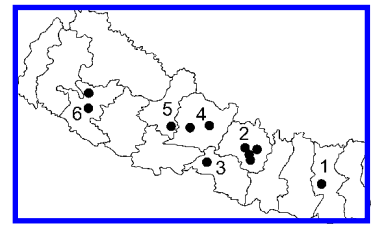

Figure 2. Map of Nepal divided into administrative zones: 1, Koshi; 2, Bagmati; 3, Narayani; 4, Gandaki; 5, Dhaulagiri; 6, Bheri. Dots indicate districts where maize survey samples were collected. Nepal is situated between the Tibet Autonomous Region of China to the north and India to the south.

Table 1. Naturally Contaminated Maize from Nepal: Distribution of Nivalenol, Deoxynivalenol, and Fumonisin B_1

	mycotoxin positives[a]		
zone	occurrence	range (ng/g)	mean (ng/g)
	Nivalenol		
Bagmati	9/14	100−34700	2600
Bheri	6/6	100−2620	1030
Dhaulagiri	4/6	100−2190	980
Gandaki	7/11	240−12800	3120
Narayani	1/1		15400
sample mean[b]	38/51	100−34700	2600
	Deoxynivalenol		
Bagmati	4/12	400−4070	1990
Bheri	1/5		4150
Dhaulagiri	3/6	740−1040	880
Gandaki	1/3		3130
sample mean	9/51	400−4150	1940
	Fumonisin B_1		
Bagmati	25/27	110−13800	3240
Bheri	3/6	430−1120	760
Dhaulagiri	3/3	3880−15700	11290
Gandaki	8/10	100−1300	530
sample mean	39/47	100−15700	3120

[a] Fourteen Bagmati samples were from markets; the Narayani sample was from the National Maize Research Program; all other samples were from smallholder farms. Mycotoxin-positive samples (≥100 ng/g) by LC-MS. [b] Sample means determined by dividing the sum of all positive samples by the number of positive samples for each toxin.

Table 2. Naturally Contaminated Maize from Nepal: Distribution of *F. graminearum TRI13* Alleles

			% of samples with strains with each allele		
zone	no. of samples	no. of strains	*TRI13* only	Ψ*TRI13* only	both alleles
Bagmati	26	31	23	4	12
Bheri	8	37	50	0	38
Dhaulagiri	6	1	0	17	0
Gandaki	11	33	18	9	45
Koshi	1	5	100	0	0
Narayani	1	11	100	0	0
all samples	53	118	26	6	21

Table 3. Maize Ear Rot Field Experiment in Nepal: Ear Rot, Nivalenol, Deoxynivalenol, and *F. graminearum TRI13* Alleles

fungal strains tested[a]			maize ear rot[b]				
strain	source zone	SCAR/AFLP	incidence	severity	toxin type[a]	ng/g in maize	*TRI13* allele
			Strains from Maize				
R-9430	Gandaki	2/nt	80	2.7	NIV	3280	nd
R-9435	Gandaki	3/nt	70	3.0	DON	2540	Ψ*TRI13*
R-9438	Bagmati	2/nt	70	4.0	NIV	150	nd
Ggm015	Gandaki	3/nt	100	4.8	NIV	380	*TRI13*
Ggm046	Koshi	2/nt	80	3.2	NIV	120	nd
Ggm070	Bagmati	3/nt	60	2.2	DON	nd	nd
Nep022	Gandaki	3/nt	60	2.8	DON	990	*TRI13*
Nep063	Gandaki	2/nt	40	2.3	NIV	1600	*TRI13*
maize strains			70 ±6	3.1±0.3			
			Strains from Wheat				
Ggm087	Gandaki	3/3	80	4.6	NIV	2,960	*TRI13*
Ggm093	Gandaki	2/2	70	2.6	NIV	nd	*TRI13*
Ggm131	Gandaki	3/3	60	1.9	DON	nd	nd
Ggm139	Gandaki	3/3	80	2.9	NIV	290	nd
Ggm154	Gandaki	3/3	100	4.1	DON	29400	Ψ*TRI13*
Ggm193	Bagmati	3/nt	90	4.5	DON	27000	Ψ*TRI13*
Ggm200	Bagmati	3/nt	33	1.3	DON	3270	Ψ*TRI13*
wheat strains			73 ±8	3.1±0.5			
			Strains from Rice				
Hkm040	Bagmati	3/nt	50	2.4	NIV	130	*TRI13*
Hkm086	Gandaki	1/nt	90	4.4	DON	nd	nd
Hkm087	Gandaki	1/1	70	2.2	DON	nd	nd
Hkm095	Gandaki	3/nt	100	4.0	DON	27300	Ψ*TRI13*
Hkm113	Bagmati	2/nt	70	2.6	NIV	940	nd
Hkm136	Bagmati	3/nt	50	2.2	NIV	120	*TRI13*
Hkm215	Bagmati	3/nt	80	4.4	NIV	6,420	*TRI13*
rice strains			73 ±7	3.2±0.4			
all strains			72 ±4	3.1±0.2			
control			20	1.3	NIV	190	*TRI13*

[a] Strains R-9430, R-9435, R-9438, Nep022, and Nep063 were collected in 1993, all others in 1997. nt, AFLP not tested. [b] NIV, nivalenol; DON, deoxynivalenol. nd, <100 ng/g or *F. graminearum* strains not recovered from field test maize samples. *TRI13* and Ψ*TRI13*, alleles detected by PCR in recovered strains.

graminearum (*3, 17*) (**Table 3**). Trichothecene chemotypes of the strains were determined by at least two of the following methods: in culture on rice or maize grain substrate (*17*), in infected wheat heads (*20*), or by *TRI13*-specific PCR as described below. The field test consisted of a plot of the open-pollinated maize variety Arun-4, which is susceptible to *Gibberella* ear rot. Arun-4 was planted in May 2003 as the main plot, and fungal strains were inoculated as subplots without replication. Chemical fertilizer was applied at a rate of 120:60:40 NPK at 1 kg/ha. The plot size for each strain was 22 plants in a single row 5.5 m long with 1 m between rows. Ten ears per treatment were inoculated 5 days after silking by injection of 1−2 mL at 1×10^5 spores/mL into the silk channel (*22*), except that fungi were grown in mung bean liquid medium (*23*) with shaking at 90 rpm for 4−5 days. Spore suspensions were prepared and control plants were injected with mung bean medium. After physiological maturity in September, ears were harvested, husked, air-dried, and scored for disease. Disease incidence was the percentage of 10 ears with ear rot symptoms by visual estimation. Disease severity ratings were based on the percentage of visibly damaged seeds on an ear: 1 = 0%, 2 = 1−3%, 3 = 4−10%, 4 = 11−25%, 5 = 26−50%, 6 = 51−75%, 7 = 76−100% (*22*). Each ear was hand-shelled, and all seeds from all replicate ears of each treatment were combined to produce one sample per treatment. Seed samples were dried and stored at room temperature until March 2004, when representative 400 g subsamples of each sample were shipped to the U.S. Department of Agriculture, where they were stored at −20 °C until analysis. For trichothecene analysis, half of each sample was ground.

Maize Ear Rot Field Experiments in the United States. Field tests were conducted in 1999 and 2000 at a site near Peoria, IL. Both tests included *F. graminearum* strain GZ3639, a **2**-producer isolated in Kansas; the 1999 test also included strain GZ3639P5, a laboratory-derived, **2**-producing mutant of GZ3639 with reduced virulence (*24*). Maize hybrid 3394 (Pioneer Hi-Bred International, Inc.) was inoculated at silking by injection of 2 mL at 10^6 spores/mL of Bilay's medium into the silk channel (*22*). Control ears were injected with Bilay's medium. The number of ears inoculated per treatment was 25 in 1999 and three replicates of ten ears in 2000. Ears were harvested at maturity, scored for disease severity as described above (*22*), and hand-shelled. For trichothecene analysis for the 1999 field test, ears from each treatment were randomly pooled into five seed samples with five ears per sample, and data from the five pools were averaged for each treatment. For the 2000 field test, ears from each replicate of 10 ears were pooled, and data from the three pools were averaged for each treatment. Each seed sample was ground and subsampled for trichothecene analysis.

Maize Ear Rot Experiments in the Plant Growth Room. Thirty *F. graminearum* strains that were previously isolated from smallholder farms in Lamjung district, Gandaki zone (*20*), were used for plant growth room tests (**Table 4**). Trichothecene chemotypes of the strains were determined as described above. To accommodate sufficient plant replications in limited growth room space, seven tests were conducted over two years. Each fungal strain was included in two independent tests, with 18 ± 0.3 (mean ± SE) ears per strain for each test. Strain GZ3639 was included in six tests as a standard with high virulence, and mutant GZ3639P5 was included in three tests as a standard with low virulence. **1**-producers and **2**-producers with similar genetic backgrounds were tested concurrently. An initial analysis of the data from strain GZ3639 confirmed that virulence did not change across tests, allowing data to be combined into a single statistical analysis.

Seeds of maize landrace Gaspe Flint (accession no. PI 401757) were obtained from M. Millard, North Central Regional Plant Introduction Station, Ames, IA, and propagated at the U.S. Department of Agriculture with selection for uniform height and maturation. Gaspe Flint is a very early flowering, short, yellow-seeded landrace of the Northern Flint race, originally collected in Quebec, Canada. In the greenhouse (tests 1 and 2) or plant growth room (tests 3−7), Gaspe Flint grew to a height of 0.6−0.9 m and matured at 65 days after seeding, producing ears up to 10 cm long with up to 8 rows of kernels (**Figure 3**). Conditions were 14 h light/23 °C and 10 h dark/17 °C. Seeds were planted in plastic pots, either at one plant per square pot

Table 4. Maize Ear Rot Experiments in the Plant Growth Room: Ear Rot, Nivalenol, 4-Acetylnivalenol, and Deoxynivalenol

AFLP genotype	strain[a]	toxin[a]	ear rot rating (%)[b]	NIV	AcNIV	DON
1-1	Nep223	DON	43	nd	nd	8620
1-1	Nep228	DON	36	nd	nd	4730
2-1	Ggm093	NIV	20	nd	nd	nd
2-1	Ggm096	NIV	31	nd	nd	nd
4-1	Nep241	NIV	69	5070	1640	nd
4-2	Nep306	DON	75	nd	nd	44000
3-1	Ggm087	NIV	75	2430	430	nd
3-1	Ggm291	NIV	32	nd	nd	nd
3-1	Nep181	NIV	15	nd	nd	nd
3-1	Ggm235	DON	12	nd	nd	nd
3-2	Ggm259	NIV	27	390	270	nd
3-3	Nep094	DON	76	nd	nd	45300
3-4	Ggm240	NIV	65	3100	950	nd
3-6	Ggm244	DON	31	nd	nd	9200
3-5	Ggm231	NIV	35	950	200	nd
3-5	Nep168	NIV	48	980	110	nd
3-7	Ggm248	NIV	24	540	540	nd
3-7	Ggm247	DON	55	nd	nd	22200
3-8	Ggm237	NIV	26	560	770	nd
3-8	Ggm168	DON	62	nd	nd	25700
3-9	Ggm288	NIV	36	1000	710	nd
3-9	Ggm154	DON	56	nd	nd	14600
3-9	Nep218	NIV	53	1860	540	nd
3-9	Nep147	DON	28	nd	nd	1090
3-10	Nep249	NIV	32	180	160	nd
3-10	Ggm282	DON	25	nd	nd	29500
3-11	Ggm167	NIV	64	1820	560	nd
3-11	Ggm232	NIV	23	100	nd	nd
3-13	Nep281	NIV	37	1940	260	nd
3-12	Nep251	DON	60	nd	nd	30200
control			4	nd	nd	100

[a] Nep094 and Nep306 were from maize; Nep147, Nep168, and Nep181 were from rice; Nep249, Nep251, and Nep281 were from debris; and Nep218, Nep223, Nep228, and Nep241 were from weeds; all were collected in 2000. All others were from wheat collected in 1997. [b] NIV, nivalenol; AcNIV, 4-acetylnivalenol; DON, deoxynivalenol; nd, <100 ng/g. Each toxin level is the mean of two replicate maize samples, each of which was sampled from a pool of all ears from each replicate sample.

(12.5 cm × 15 cm deep) or at two plants per round pot (19 cm × 15 cm deep). Potting soil was Sun Gro Sunshine SB300 Professional Growing Mix, a medium-textured, well-drained mix (Sun Gro Horticulture Canada, Ltd., Vancouver, BC, Canada). Plants were treated with a mild nitrogen fertilizer weekly from seedling emergence through anthesis. To prevent root rot, to which Gaspe Flint is susceptible, plants were watered from the bottom of the pot, allowing soil to become slightly dry between waterings. Tillers were removed from the plants. Approximately 30 days after planting, silks were trimmed to 2−3 cm and pollinated by hand the two following days. After the second pollination, silk channels were injected with 1 mL at 10^6 spores/mL in mung bean medium (*23*). Ears were harvested at maturity, dried at room temperature, and weighed. A single individual scored all samples for visible symptoms of rot (discolored, moldy, or shriveled seeds or cob) as a percentage of the ear, rounded up to the next 10%: 0 = no disease; 10% = 1−10% disease, 20% = 11−20% diseased, etc. Ratings were made without knowledge of the type of strain treatment. Ears from each strain treatment were pooled and stored at −20 °C. After all tests were completed, each bag of ears (including cobs) was ground and sampled for trichothecene analysis. Disease rating data from individual ears from both replicate tests were combined for statistical analysis using the general linear model procedure of SAS.

Chemical Analysis of Mycotoxins. 1, **2**, 4-acetylnivalenol, 4,15-diacetoxyscirpenol, and HPLC-grade solvents were from Sigma Chemical Co. (St. Louis, MO). 3-Acetyldeoxynivalenol and 15-acetyldeoxynivalenol were from Biopure Referenzsubstanzen GmbH (Tulln, Austria). Fumonisin B_1 (FB_1) was purified from cultures of *F. verticillioides* (*25*). Production of trichothecenes was analyzed in fungal cultures grown for 4 weeks on 30 g of either cracked maize grain or rice grain in 150 mL Erlenmeyer flasks as previously described (*17*).

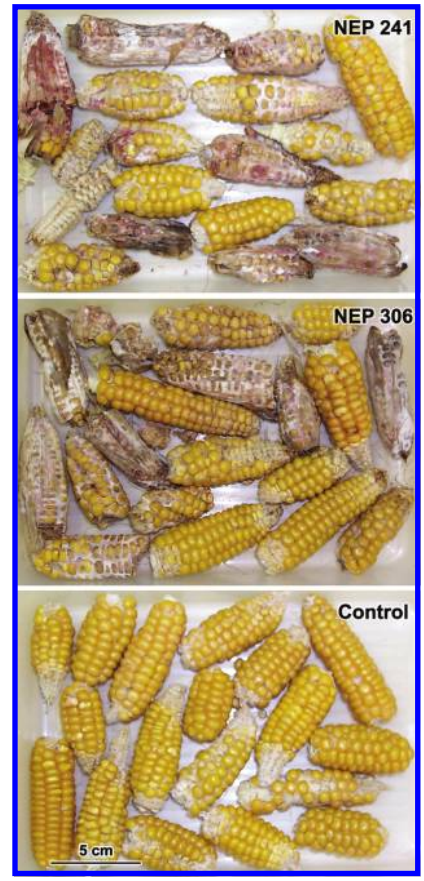

Figure 3. *Gibberella* ear rot of Gaspe Flint maize. Control ears and ears treated with *F. graminearum* strains with genotype *AFLP4-1*: **1**-producer Nep 241 with an average of 69% ear rot and 5620 ng/g total **1** and **2**-producer Nep306 with an average of 76% ear rot and 21240 ng/g **2**, experiment 6.

LC-MS analysis of mycotoxins utilized slight modifications of described methods (*25*). For trichothecene analysis, 10 g of dried, ground sample from fungal cultures, the maize survey, field tests, or growth room tests was extracted with 40 mL of acetonitrile/water (84:16, v/v). Extracts were filtered through Whatman no. 1 paper and stored at 4 °C until analysis by LC-MS. For FB_1 analysis, 10 g of ground sample from the maize survey was extracted with 50 mL of acetonitrile/water (1:1, v/v). Analyses were conducted utilizing an LC-MS system consisting of a ThermoSpectraphysics P4000 gradient pump and an AS3000 autoinjector coupled to a ThermoFinnigan LCQ-DECA ion trap mass spectrometer operating in electrospray ionization mode. Chromatographic separations were achieved on a 150 mm × 3 mm i.d. MetaChem C18 column utilizing a 0.3 mL/min gradient flow. For trichothecene analysis, a gradient of 0−40% aqueous methanol (with 0.1% acetic acid) over 15 min was used. MS/MS detection of **2** and **1** was done by monitoring distinctive *m/z* 265 (**2**) and 281 (**1**) fragment ions from the parent $[M + \text{acetic acid} - H]^-$ ions at *m/z* 355 and 371, respectively. MS/MS detection of 4-acetylnivalenol was done by monitoring the *m/z* 247 fragment ion from the parent $[M + H]^+$ ion at *m/z* 355. MS detection of diacetoxyscirpenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol was done by monitoring $[M + H]^+$ ions at *m/z* 367 and 339. MS detection of FB_1 was done by monitoring the *m/z* 722 $[M + H]^+$ ion eluting from a gradient of 35−95% aqueous methanol (with 0.3% acetic acid) over 25 min. External standards were used for quantitation. Calibration curves were generated for each toxin just prior to sample analysis and were based on responses from diluted concentration standards. Quantitations were done by comparison of integrated chromatographic intensities with those obtained from a "single-point" calibration utilizing a 10 μg/mL concentration standard for each of the toxins.

Fungal Identification and Genetic Analysis. All strains used for maize ear rot tests were identified as *F. graminearum* by morphology

(*26*) and by species-specific, monomorphic, and polymorphic sequence-characterized amplified region (SCAR) markers (*17*). *Fusarium* contamination was analyzed in maize seed samples from the survey and field test in Nepal. Numbers of seeds tested per sample were 75−200 seeds for the 33 larger samples from the survey, 5−24 seeds for the 19 smaller samples from the survey, and 22 seeds for the 23 samples from the field test. Seeds were surface disinfested with 0.5% sodium hypochlorite and then placed on a *Fusarium*-selective medium (*26*). No more than one strain per seed was purified by single-spore isolation and identified by morphology as *F. graminearum*. More than half of the strains from the maize survey were identified further using species-specific SCAR markers (*17*), and all were confirmed as *F. graminearum*. DNA for PCR amplification was extracted using an Ultraclean DNA Purification Kit (Mo Bio Laboratories, Inc., Solana Beach, CA) from the lyophilized mycelium of 7−10-day cultures grown on V-8 juice (Campbell Soup Co., Camden, NJ) agar. PCR amplification was used to distinguish *TRI13* in **1**-producers and Ψ*TRI13* in **2**-producers, using primers GzTri13/p1 (5′-ATACTAMAAGYCTAGKACGACGC-3′) and GzTri13/p2 (5′-GTGRTRTCCCAGGATCTGCGTGTC-3′), which amplify 760 and 470 bp products from **1**-producers and **2**-producers, respectively (*27*). DNA from some strains was also examined with primers Tri13F (5′-CATCATGAGACTTGTKCRAGTTTGGG-3′) and Tri13R (5′- TTGAAAGCTCCAATGTCGTG-3′), which amplify 1075 and 799 bp products from **1**-producers and **2**-producers, respectively (*18*). In the primer sequences above, K indicates G or T; M indicates A or C; R indicates A or G; and Y indicates C or T. Thermal cycler conditions consisted of 94 °C for 2 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 52 or 48 °C for 45 s, and extension at 72 °C for 60 s. The conditions were completed with a final extension at 72 °C for 5 min. The 52 and 48 °C annealing temperatures were used with primers GzTri13/p1-GzTri13/p2 and Tri13F-Tri13R, respectively.

DNA isolation and amplification procedures for AFLP were the same as in ref *28*, except that a fluorescent dye label was used instead of a radioactive label and the final amplification products were separated at the Michigan State University Gene Sequencing Facility (East Lansing, MI). Initial digests were performed using the restriction enzymes *Eco*R1 and *Mse*1 at 37 °C for 2 h. Selective amplification was done using the *Mse*1-CA specific primer and the fluorescently labeled *Eco*R1-AA primer (Applied Biosystems, Foster City CA). The presence or absence of 64 bands, ranging from 50 to 490 bp in length, was scored. Forty-nine variable AFLP bands were used to construct a tree based on neighbor-joining (**Figure 4**). An optimal tree of length 3.29049 was obtained, and 1000 heuristic bootstrap replicates were performed in PAUP*4.10b (*29*), with optimality criterion set to distance.

Statistical Analyses. Statistical analyses were carried out using the SAS statistical package (*30*). Analyses involving nonparametric disease assessments, that is, maize field disease ratings, were analyzed using the Spearman rank correlation option of the PROC CORR procedure. All other analyses were performed using the PROC GLM procedure with strain and toxin type and toxin concentration treated as random variables. Samples that contained less than the mycotoxin detection limit of 100 ng/g were not utilized in regression analysis.

RESULTS AND DISCUSSION

Survey of Naturally Contaminated Maize. The distribution of **1** and **2** was determined in 49 seed and 2 flour samples collected from hill regions of five administrative zones across Nepal (**Table 1**; **Figure 2**). Seventy-five percent of samples were positive for **1**; 18% were positive for **2**; all samples that were positive for **2** also were positive for **1**. **1** was ≥1000 ng/g, a level of concern for food safety (*31, 32*), in 37% of samples; **2** was ≥1000 ng/g in 8% of samples; **1** and **2** combined were ≥1000 ng/g in 40% of samples. The highest level of **1** (34700 ng/g) and the second highest level of **2** (4070 ng/g) were detected in samples from Kakani village at an altitude of 1800 m in the Bagmati zone in central Nepal. The highest level of **2** (4150 ng/g) was detected in a sample from Kimu village at an altitude of 1200 m in the Bheri zone in midwestern Nepal. For positive samples, mean levels of each toxin were similar, at 2600 ng/g for **1** and 1940 ng/g for **2**; however, frequency distributions for positive samples were different for the two toxins due to higher frequencies of samples with lower levels of **1** and higher frequencies of samples with higher levels of **2** (**Figure 5A**).

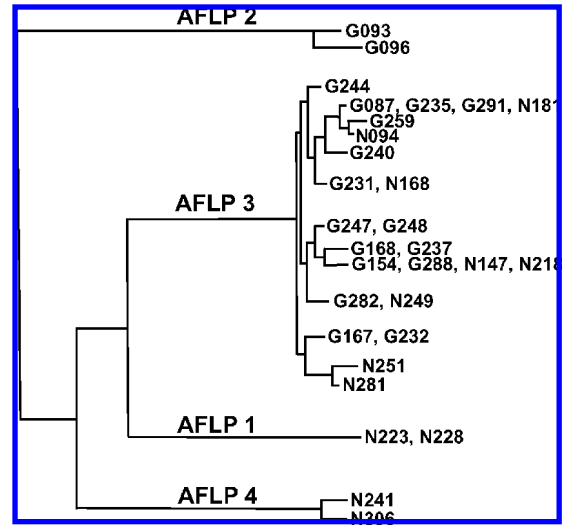

Figure 4. Phylogenetic tree of the relationships among 30 strains of *F. graminearum* from Nepal based on amplified fragment length polymorphism (AFLP) analysis as described under Materials and Methods. Numbers prefixed by G indicate Ggm series strains; numbers prefixed by N indicate Nep series strains. A horizontal bar length of 20 mm represents 0.1 changes per AFLP locus, that is, 1 change in band presence/absence for every 10 loci scored.

Forty-seven maize samples that had been analyzed for 8-ketotrichothecenes also were analyzed for FB_1 (**Table 1**). Eighty-three percent of samples were positive for FB_1, and 38% contained levels of FB_1 ≥1000 ng/g. Levels of FB_1 >10000 ng/g were detected in one sample from a Kathmandu market and in four samples from farms: in Kakani village, in Durlung village in the Dhaulagiri zone, and in Khokana village in the Bagmati zone. For all positive samples, the mean level of FB_1 was 3120 ng/g. The cumulative frequency distribution for FB_1 was similar to the distribution for **1** due to higher frequencies of positive samples with lower levels of FB_1 (**Figure 5A**). Ninety-four percent of samples were contaminated with at least one of the three toxins tested, and 61% were cocontaminated with FB_1 and either **1** or **2**. One sample from Kakani village contained significant levels of all three mycotoxins: **1** at 3670 μg/g, **2** at 2110 ng/g, and FB_1 at 8720 ng/g.

The distribution of *TRI13* alleles was determined among *F. graminearum* isolated from 53 seed samples collected from hill regions of six administrative zones across Nepal (**Table 2**). *F. graminearum* strains were isolated from 53% of samples, including 55% of samples from smallholder farms and 39% of samples from markets. A total of 118 strains, representing a mean of 4.4 strains per seed sample, were scored by PCR for the presence of *TRI13* alleles. Eighty-one percent of strains contained a PCR band consistent with the size of a functional *TRI13* allele and thus were putative **1**-producers. Nineteen percent of strains contained a PCR band consistent with the size of a Ψ*TRI13* allele and thus were putative **2**-producers. Among the seed samples tested, 26% were infected with strains with *TRI13*, 6% with strains with Ψ*TRI13*, and 21% with both genotypes of strains. The presence of culturable *F. graminearum* was not a consistent indicator of trichothecene contamination

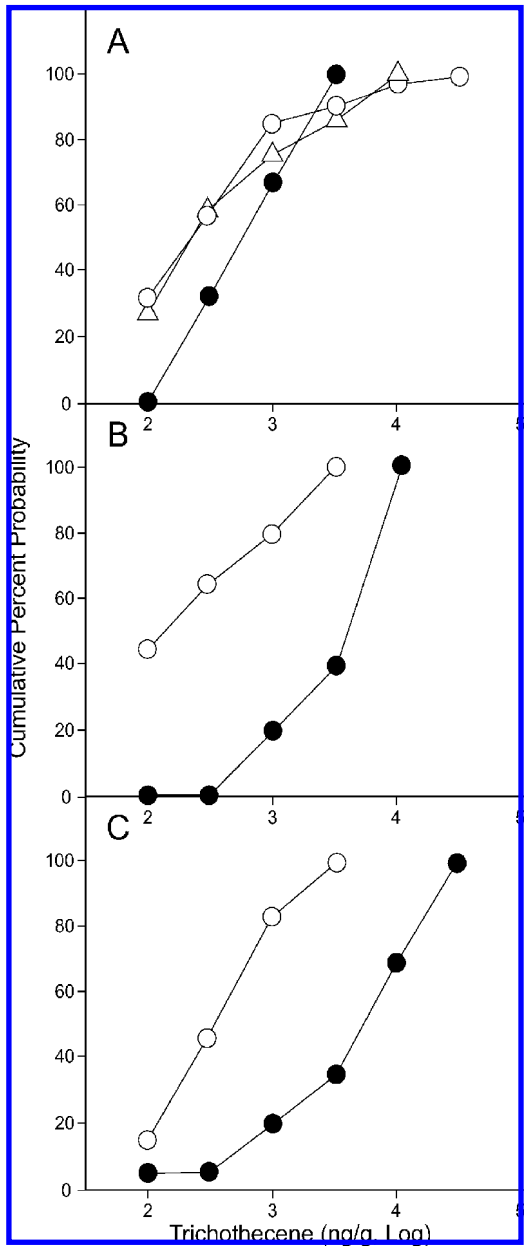

Figure 5. Cumulative frequency distributions for **1** (○), **2** (●), and FB_1 (△) levels in maize: (**A**) naturally infected samples; (**B**) samples from Nepal field test; (**C**) samples from Gaspe Flint tests. Only mycotoxin-positive samples (≥100 ng/g) were included in the analysis. Mycotoxins were analyzed by LC-MS as described under Materials and Methods. Data for **1** in (**C**) are "total **1**", which includes 4-acetylnivalenol. Numbers of samples included for each test and mycotoxin were (**A**) 36 for **1**, 9 for **2**, and 39 for FB_1; (**B**) 11 for **1** and 5 for **2**; and (**C**) 20 for **1** and 26 for **2**.

of seed samples and, conversely, trichothecenes were sometimes detected in samples from which *F. graminearum* was not culturable. For example, **1** was not detected in 26% of 23 samples that yielded strains with a *TRI13* allele, and **2** was not detected in 70% of 13 samples that yielded strains with a Ψ*TRI13* allele.

Mycotoxin contamination was higher in the present survey than in a previous survey of maize in Nepal in 1997, in which 8-ketotrichothecenes and FB_1 were ≥1000 in only 16 and 22% of samples, respectively (*3*). Mycotoxin levels likely were higher because many samples in the present survey were selected for visible symptoms of disease, but samples in the previous survey were not. In the present survey, use of LC-MS for 8-ketotrichothecene analysis has provided the first definitive evidence for the widespread occurrence of **1** in maize in Nepal. As far as we are aware, this is the first report of **1** as the dominant trichothecene in maize in Asia and the only report worldwide, other than a 1985 survey of moldy maize in South Africa (*9*), of **1** as the dominant trichothecene in maize being used for human consumption.

Maize Ear Rot Field Experiment in Nepal. The field experiment in Nepal was conducted with strains selected to represent **1**-producers and **2**-producers, a range of geographic locations, and three hosts of origin (maize, wheat, and rice) (**Table 3**). The majority of strains produced symptoms of ear rot, with an incidence (the presence or absence of visible symptoms of ear rot) of 72 ± 4% (mean ± SE) and a severity (the extent of ear rot) of 3.1 ± 0.2 for all strains. Disease incidence and severity did not differ between strains isolated originally from maize, wheat, or rice or between **1**-producers and **2**-producers ($p > 0.05$, Student's paired t test) (**Table 3**).

Ninety-two percent of **1**-producers produced **1** in planta and 60% of **2**-producers produced **2** in planta (**Table 3**). Across all positive samples, the mean ± SE for **1** was 1490 ± 610 ng/g with a cumulative frequency distribution skewed to the left, and the mean ± SE for **2** was 15080 ± 5870 ng/g with a cumulative frequency distribution skewed to the right (**Figure 5B**). Maize ear rot disease intensity (incidence/100 × severity) was not correlated with levels of **1**, but showed a nonsignificant tendency to be related to levels of **2** ($R = 0.55$, $p = 0.094$), using the Spearman rank correlation.

Strains identified by morphology as *F. graminearum* were isolated from 61% of the 23 maize samples from the field experiment (**Table 3**). Each isolated strain had a *TRI13* band consistent with the strain with which the ears were injected, except for ears injected with the **2**-producer Nep022 and for control ears. Both of these samples were contaminated with strains with the *TRI13* allele and, along with the sample from **2**-producer Ggm200, contained low levels of **1** (250 ± 90 ng/g, mean ± SE), probably due to background contamination with **1**-producers during the field test.

All strains in the maize ear rot field test had been included in a previous study of the ability of *F. graminearum* to cause wheat head blight (*20*). Maize ear rot disease intensity was compared to wheat head blight (percentage of spikelets blighted at 20−21 days after inoculation), using the Spearman rank correlation. The correlation between maize ear rot disease intensity and wheat head blight was significant ($R = 0.47$, $p = 0.026$), but was driven predominately by the strong correlation for **2**-producers.

Maize Ear Rot Experiments in the Plant Growth Room in the United States. Additional maize ear rot experiments on **1**-producers and **2**-producers of *F. graminearum* from Nepal were conducted on cultivar Gaspe Flint. One disadvantage of Gaspe Flint was that the size and weight of uninfected ears were too variable for ear weight to be suitable as a disease index; thus, each ear received a disease rating based on visible symptoms of rot (discolored, moldy, or shriveled seeds or cob) as a percentage of the ear (**Figure 3**). To validate the assay, the virulence of two standard *F. graminearum* strains, GZ3639 and GZ3639P5, was compared on hybrid maize in the field and on Gaspe Flint in the growth room. The virulent strain GZ3639 caused ear rot with a severity rating of 4.1 (equivalent to 11−25% ear rot) and **2** contamination of 175000 ng/g in the field, and ear rot of 42 ± 2% (mean ± SE) and **2** contamination of 10000 ± 4170 ng/g on Gaspe Flint. The reduced virulence

mutant GZ3639P5 caused <3% ear rot and **2** contamination of 55000 ng/g in the field and 14 ± 2% ear rot with no toxin on Gaspe Flint. Controls had <3% ear rot and **2** contamination of 10000 ng/g in the field and 4 ± 2% ear rot with no toxin on Gaspe Flint. Thus, under growth room conditions, Gaspe Flint was susceptible to *Gibberella* ear rot and **2** contamination, but strain GZ3639 produced less toxin on Gaspe Flint than in the field.

For Gaspe Flint ear rot tests, *F. graminearum* strains were selected to represent all four AFLP groups and, when possible, **1**-producers and **2**-producers with identical, or closely related, AFLP genotypes (**Figure 4**; **Table 4**). *AFLP1* and *AFLP2* were each represented by two strains with identical or closely related AFLP genotypes. *AFLP4* was represented by two closely related strains that differ in toxin chemotype. *AFLP3* was represented by 15 **1**-producers and 9 **2**-producers, including 8 pairs of strains with identical or closely related AFLP genotypes that each included at least one strain of each chemotype. The majority of strains tested produced symptoms of ear rot on Gaspe Flint, with a disease rating of 42 ± 3% (ear rot mean ± SE) for all 30 strains, compared to a disease rating of 42 ± 2% for the concurrently tested U.S. strain GZ3639 (**Table 4**). On the basis of the small number of strains tested for *AFLP1*, *AFLP2*, and *AFLP4*, *AFLP1* (39 ± 4% rot) was more virulent that *AFLP2* (26 ± 4% ear rot, $p < 0.0001$), but less virulent than *AFLP4* (71 ± 6% rot; $F_{5,1066} = 12.84$, $p < 0.0001$). *AFLP4* was more virulent than *AFLP3* (42 ± 2% rot). The disease rating was not significantly different between **1**-producers and **2**-producers for all 30 strains tested ($p = 0.19$) or between the **1**-producer and **2**-producer of *AFLP4*. In *AFLP3*, however, **1**-producers (40 ± 2% rot) were less virulent than **2**-producers (48 ± 2% rot; $F_{1,851} = 8.45$; $p = 0.0037$). Between sets of *AFLP3* strains with identical or closely related genotypes, **1**-producers (41 ± 2%) were less virulent than **2**-producers (50 ± 2% rot; $F_{1,780} = 10.48$; $p = 0$ 0.0013).

Seventy-eight percent of **1**-producers produced **1** in planta, and 92% of **2**-producers produced **2** in planta (**Table 4**). All **1**-producers, except one, also produced 4-acetylnivalenol, averaging 32 ± 5% by weight of the combined **1** and 4-acetylnivalenol. To simplify comparisons and adjust for the different molecular weights of **1** and 4-acetylnivalenol, the sum of **1** and (acetylnivalenol × [**1** mwt/acetylnivalenol mwt]) was expressed as "total **1**". Across all positive samples, the mean ± SE for total **1** was 2230 ± 440 ng/g with a cumulative frequency distribution skewed to the left, and the mean ± SE for **2** was 23500 ± 4490 ng/g with a cumulative frequency distribution skewed to the right (**Figure 5C**). The correlation between disease severity and toxin level was significant for **1**-producers ($R^2 = 0.44$, $p < 0.0001$) and **2**-producers ($R^2 = 0.51$, $p < 0.0001$). However, the slope of the correlation was steeper for **1**-producers compared to that for **2**-producers (**Figure 6**).

Three *AFLP3* strains selected for the Gaspe Flint tests also had been included in the field test in Nepal. Results were consistent under both conditions: strain Ggm087 was virulent and produced **1** in both tests; strain Ggm154 was virulent and produced **2** in both tests; and strain Ggm093 was low in virulence and produced no detectable toxin in either test (**Tables 3** and **4**). All 30 strains selected for the Gaspe Flint tests had been included in a previous study of the ability of *F. graminearum* to cause wheat head blight (*20*). The correlation between Gaspe Flint ear rot (percent) and wheat head blight (percentage of spikelets bleached, area under disease progress curve) was not significant ($F_{1,28} = 2.30$; $p = 0.1408$).

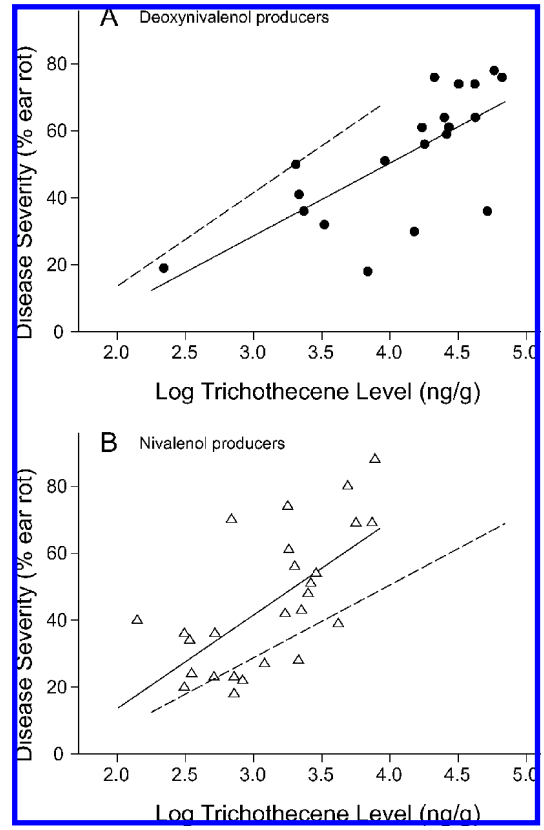

Figure 6. Relationship between maize ear rot and trichothecenes (**A**) deoxynivalenol and (**B**) nivalenol in Gaspe Flint ears infected with 30 strains of *F. graminearum*. Each symbol represents one maize treatment replicate; only trichothecene-positive samples (≥100 ng/g) are included in the regression analysis. For comparison, the regression line from **A** is shown as a dashed line in **B** and the regression line from **B** is shown as a dashed line in **A**.

In our experiments in the field in Nepal and in a plant growth room in the United States, the majority of **1**-producers and **2**-producers of *F. graminearum* from Nepal caused maize ear rot and trichothecene contamination. A previous study of maize ear rot in Korea also found that most **1**-producers and **2**-producers of *F. graminearum* caused ear rot and trichothecene contamination (*16*). In tests in Korea, Nepal, and the United States, visible symptoms of ear rot were severe, and trichothecene levels were correlated with ear rot severity. However, levels of **1** and **2** were generally higher in the maize field test in Korea than in our tests in Nepal and the United States; the reasons for these differences in trichothecene levels are unknown. In our tests of *F. graminearum* in the field in Nepal and in a plant growth room in the United States, levels of total **1** (including 4-acetylnivalenol) often were several-fold lower than levels of **2** in maize ears with similar levels of visible disease symptoms. Biological explanations for this difference are not obvious because **1** and **2** are intermediates in the same biosynthetic pathway, and **1**-producers and **2**-producers often were genetically closely related. In maize infected with **1**-producers, LC-MS analysis found no evidence for the presence of significant levels of other trichothecenes, either 4,15-diacetoxyscirpenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, or **2**. Efforts are now underway in our laboratories to investigate reasons for the apparent differences in levels of **1** and **2** in planta, including reported differences in the stabilities of **1** and **2** in some matrices (*33*).

Risk Assessment. The high frequency of **1** contamination and the high virulence of many **1**-producing strains of *F.*

graminearum are of concern because maize is a staple food of rural populations in Nepal and because **1** has proven to be more toxic than **2** in most animal systems (*3*). The recommended total daily intakes are <700 ng/kg of body weight/day for **1** and 1000 ng/kg of body weight/day for **2** (*31, 32*). In rural Nepal, a 60 kg individual who each day consumes 500 g of maize with a **1** level of 100 ng/g would consume 830 ng/kg of body weight, which is higher than the acceptable daily intake of NIV. Cocontamination of maize with **1**, **2**, and FB_1 would create an even greater health risk.

Although **2**-producer populations of *F. graminearum* lineage 7 are well-established in North America and Europe, **1**-producers have been observed only rarely (*4, 34*). Due to the higher mycotoxicity of **1**, there is a strong rationale for preventing **1**-producers of the *F. graminearum* complex from becoming more prevalent in North America or Europe. We suggest that **1**-producers are unlikely to evolve by mutation from **2**-producers, because the Ψ*TRI13* gene has multiple insertions and large deletions. Thus, migration of a **1**-producer is the only manner of introducing the **1**-chemotype into North America and other regions where it is rare. To have a significant impact in North America, the **1**-producer migrant must invade a resident **2**-producer population of *F. graminearum* or introgress with extant **2**-producers to yield **1**-producer offspring that are favored by natural selection. Introgression may occur among strains from the same lineage or different lineages, because hybrids between lineages have been produced in the laboratory and found in nature (*21, 35*; unpublished results).

A migrant that is better adapted to a host plant might be able to displace the extant population. However, our comparison of strains from the United States and Nepal indicates that, on heads of wheat cultivar Wheaton and ears of Gaspe Flint maize at least, **1**-producers are of similar or lower virulence than **2**-producers and thus would not easily displace extant **2**-producers. In our previous study of wheat head blight, **1**-producers were, on average, 40% less virulent than **2**-producers across all AFLP groups and 30% less virulent within the *AFLP3* genetic background (*20*; unpublished results). In the present study of ear rot of Gaspe Flint maize, **1**-producers were, on average, as virulent as **2**-producers across all AFLP groups, but 20% less virulent within the *AFLP3* genetic background.

Our current understanding of the chemistry, genetics, and biology of 8-ketotrichothecenes indicates that the potential may be relatively low for **1**-producers of the *F. graminearum* complex to have a significant toxicological impact in North America. Even if the risk is low, the consequences could be so serious that the prudent course is development and application of improved methods to monitor **1** in cereal grains in North America and worldwide.

SAFETY

Trichothecene-contaminated plant materials should be handled so as to minimize exposure via the skin and respiratory system.

ABBREVIATIONS USED

SCAR, sequence-characterized amplified region; AFLP, amplified fragment length polymorphism.

ACKNOWLEDGMENT

We thank A. Tomas (Pioneer Hi-Bred International, Wilmington, DE) and R. Stessman for their collaboration in development of the Gaspe Flint system; S. Folmar, N. Deppe, M. Moore, D. Shane, N. Puent, and D. Engel for technical assistance; the W. Christ family for assistance with maize field tests; and S. Prather for scientific illustration.

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Received for review February 5, 2008. Revised manuscript received March 31, 2008. Accepted March 31, 2008. Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

JF8003702