Fumonisin B_1-Nonproducing Strains of *Fusarium verticillioides* Cause Maize (*Zea mays*) Ear Infection and Ear Rot

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Fumonisins are polyketide mycotoxins produced by *Fusarium verticillioides* (synonym *F. moniliforme*), a major pathogen of maize (*Zea mays*) worldwide. Most field strains produce high levels of fumonisin B_1 (FB_1) and low levels of the less-oxygenated homologues FB_2 and FB_3, but fumonisin B_1-nonproducing field strains have been obtained by natural variation. To test the role of various fumonisins in pathogenesis on maize under field conditions, one strain producing FB_1, FB_2, and FB_3, one strain producing only FB_2, one strain producing only FB_3, and one fumonisin-nonproducing strain were applied to ears via the silk channel and on seeds at planting. Disease severity on the harvested ears was evaluated by visible symptoms and by weight percent symptomatic kernels. Fumonisin levels in kernels were determined by high-performance liquid chromatography. The presence of the applied FB_1-nonproducing strains in kernels was determined by analysis of recovered strains for fumonisin production and other traits. All three FB_1-nonproducing strains were able to infect ears following either silk-channel application or seed application at planting and were as effective as the FB_1-producing strain in causing ear rot following silk-channel application. These results indicate that production of FB_1, FB_2, or FB_3 is not required for *F. verticillioides* to cause maize ear infection and ear rot.

Keywords: *Zea mays; fumonisins; Fusarium verticillioides; Fusarium moniliforme; maize ear rot*

INTRODUCTION

Fumonisins are polyketide mycotoxins produced by several *Fusarium* species, especially *Fusarium verticillioides* (synonym *F. moniliforme*, sexual stage *Gibberella fujikuroi* mating population A), a major pathogen of maize (*Zea mays*) worldwide. Consumption of fumonisins has been shown to cause a number of mycotoxicoses, including leucoencephalomalacia in horses, pulmonary edema in swine, and liver cancer in rats (Nelson et al., 1993). Consumption of fumonisin-contaminated maize also has been epidemiologically associated with human esophageal cancer in some areas of the world where maize is a dietary staple (Marasas, 1996). Fumonisins are structurally similar to sphinganine, a sphingolipid biosynthetic intermediate, and disrupt sphingolipid metabolism by inhibiting the enzyme sphinganine *N*-acyltransferase (Wang et al., 1991). Disruption of sphingolipid metabolism with the accumulation of sphingoid bases such as sphinganine is thought to be responsible for the majority of fumonisin-induced mycotoxicoses.

Fumonisins are a family of long-chain polyalcohols that vary in the position and number of hydroxyl groups. Several lines of evidence indicate that carbon atoms C-3−C-20 of the fumonisin backbone are a product of polyketide synthesis (Blackwell et al., 1996; Proctor et al., 1999). In addition, there is evidence that the oxygen atoms attached directly to the backbone are derived from molecular oxygen (Caldas et al., 1998). Fumonisin B_1 (FB_1) usually is the most abundant fumonisin in maize that is naturally infected with *F. verticillioides*, and other B-series fumonisins, FB_2, FB_3, and FB_4, usually are present at lower levels. B-series fumonisins consist of the 20-carbon backbone with an amino group at C-2, tricarballylic esters at C-14 and C-15, and methyl groups at C-12 and C-16 and differ from one another only by the extent of hydroxylation at the C-5 and C-10 positions. FB_2 lacks the C-10 hydroxyl group, FB_3 lacks the C-5 hydroxyl group, and FB_4 lacks both the C-5 and C-10 hydroxyl groups. Although structural analysis suggests that these compounds are formed along the same biosynthetic pathway, recent experimental studies indicate that the fumonisin biosynthetic pathway is branched, with FB_3 the precursor of FB_1 and FB_4 the precursor of FB_2 (Proctor et al., 2000).

There is considerable indirect evidence that fumonisins may play a role in diseases caused by *F. verticillioides* on maize. First, the overwhelming majority of strains of *F. verticillioides* isolated from maize have the ability to produce FB_1, and high levels of FB_1 and other fumonisins can accumulate in diseased kernels and in other diseased maize tissues (Desjardins and Hohn, 1997). In an extensive series of laboratory tests (Lamprecht et al., 1994), (1) fumonisins caused reductions in shoot and root length and dry mass of maize and tomato seedlings, (2) fumonisins caused necrosis in detached tomato leaves, (3) fumonisin phytotoxicity was dose-dependent, and (4) FB_1 was more phytotoxic than FB_2 or FB_3 by most parameters tested. Genetic analysis of a fumonisin-nonproducing strain of *F. verticillioides* showed an association between the production of fumonisins and high levels of virulence on maize seedlings (Desjardins et al., 1995). In addition, fumonisins are very similar in structure to AAL toxins, which are

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Table 1. Strains of *F. verticillioides* Used in This Study

strain[a]	fumonisin production	proposed genotype	source[b]
M-3125	FB_1, FB_2, FB_3, FB_4	*Fum1-1*, *Fum2-1*, *Fum3-1*	field strain from maize, California
109-R-7	FB_2, FB_4	*Fum1-1*, *Fum2-2*, *Fum3-1*	ascospore progeny of cross between field strains from maize, California and South Carolina
506-R-15	FB_3, FB_4	*Fum1-1*, *Fum2-1*, *Fum3-2*	ascospore progeny of crosses between field strains from maize, California, South Carolina, and Nepal
M-5500	none detected	*Fum1-2*, *Fum2-1*, *Fum3-1*	field strain from maize, Nepal

[a] The prefix M refers to the Fusarium Research Center, The Pennsylvania State University, University Park, PA. For strains derived from laboratory crosses, the first number indicates the cross number, R indicates that the strain was derived from a random ascospore, and the last number indicates the ascospore number. [b] The sources of the field strains and the laboratory crosses used to derive the two ascospore strains are described in Desjardins et al. (1996).

required for pathogenicity of strains of *Alternaria alternata* on tomato cultivars that are homozygous for the *asc* allele (Gilchrist, 1998). AAL toxin-nonproducing strains of this pathogen obtained by mutagenesis or by natural variation lost the ability to cause leaf necrosis on susceptible tomato cultivars (Akamatsu et al., 1997). Furthermore, treatment of tomato, tobacco, and duckweed with either FB_1 or AAL toxin led to accumulation of sphinganine and other sphingoid bases (Abbas et al., 1994). On the other hand, a fumonisin-nonproducing strain of *F. verticillioides* was as aggressive as fumonisin-producing strains in causing stalk rot of mature maize plants in greenhouse tests (Jardine and Leslie, 1999). Thus, the importance of fumonisins in pathogenesis on maize is not clear.

Toxin-nonproducing strains obtained by mutagenesis or natural variation are powerful tools to more directly test the role of toxins in plant pathogenesis. In previous surveys of field strains, we identified rare natural variants that produce no FB_1, including a strain that produces only FB_2 and FB_4, a strain that produces only FB_3 and FB_4, and a strain that produces no fumonisins, in liquid culture media and on autoclaved maize meal. Meiotic analysis of these strains identified two fumonisin biosynthetic loci (*Fum2* and *Fum3*) responsible for hydroxylation at C-10 and C-5, respectively. Strains with a defective *Fum2* allele are unable to hydroxylate the C-10 position and, as a result, produce only FB_2 and FB_4, whereas strains with a defective *Fum3* allele are unable to hydroxylate the C-5 position, and, as a result, produce only FB_3 and FB_4. Further meiotic analysis of strains with these defective alleles indicated that *Fum2* and *Fum3* are linked to one another and to a third locus, *Fum1*, which governs whether fumonisins are produced (Desjardins et al., 1996).

Our objective in this study was to examine the importance of fumonisins in the ability of *F. verticillioides* to cause maize ear infection and ear rot under field conditions. In field tests, we compared a strain that produces FB_1, FB_2, and FB_3 with three natural variants—an FB_2-producing strain, an FB_3-producing strain, and a fumonisin-nonproducing strain—for the ability to cause maize ear infection and ear rot following silk-channel application and seed application at planting. Fumonisin levels in kernels were determined by high-performance liquid chromatography (HPLC). The presence of the applied FB_1-nonproducing strains in kernels was determined by analysis of recovered strains for fumonisin production and other traits.

MATERIALS AND METHODS

Fungal Strains. Strains of *F. verticillioides* used in this study, their fumonisin production phenotypes, and their genotypes with respect to *Fum* loci are shown in Table 1. Genotype designations for these strains are based on the proposed genetic nomenclature for plant pathogenic fungi (Yoder et al., 1986). For application to the silk channel, spore suspensions were prepared from strains grown on V-8 juice agar plates and diluted to a final concentration of 5×10^6 spores/mL in half-strength, modified, Bilay's medium (Harris et al., 1999). For application to seed at planting, fungal-infested toothpicks were used following the method of A. Tomas (personal communication). Wooden toothpicks were first boiled in three changes of distilled water, placed in groups of 36 into 50 mL Erlenmyer flasks containing 25 mL of modified Bilay's medium, and autoclaved. The flasks with toothpicks were then inoculated with fungal strains and incubated with shaking at 200 rpm in the dark at 28 °C for 2 weeks. Flasks with infested toothpicks were held at 4 °C for up to 2 weeks until the maize planting dates. Fungal infestation was monitored by placing toothpicks on a selective medium containing pentachloronitrobenzene (Nelson et al., 1983) and testing recovered *Fusarium* strains for fumonisin production in liquid culture as described below.

Field Plots. Field tests were conducted under permit 960383 from Plant Protection and Quarantine, Animal and Plant Health Inspection Service, U.S. Department of Agriculture. Field tests were conducted in 1996 and 1997 at a site near Peoria, IL. Field test size was limited to minimize the potential for environmental release of fungal strains that are not endemic to Illinois. In the 1996 field test, experiments A–D compared the ability of fungal strains M-3125 and 109-R-7 to cause ear infection and ear rot on commercial hybrids 3394 and 3489 (Pioneer Hi-Bred International, Inc.), which are highly susceptible to *F. verticillioides*. In the 1997 field tests, experiments E and F compared the ability of fungal strains M-3125, 109-R-7, 506-R-15, and M-5500 to cause ear infection and ear rot on hybrid 3394. For application to the silk channel, 2 mL of spore suspension was injected into the silk channel of the primary ear 4 days after silk emergence (Reid et al., 1996). Control ears were injected with sterile half-strength Bilay's medium. For application to seed at planting, a fungal-infested toothpick was placed in the soil next to each seed at planting. Control seeds were planted next to a toothpick soaked in sterile Bilay's medium.

The 1996 field test consisted of one block of hybrid 3394 for experiment A, one block of hybrid 3394 for experiments C and D, and one block of hybrid 3489 for experiment B. Each block contained four randomly assigned replicate plots of each of the two fungal strains being tested and four control plots. Each plot was 4 m long and contained two rows of ~15 plants each. In experiment B, fungal-infested toothpicks were placed near seeds of hybrid 3489 at planting for 10 plants in one row of each plot, and plants in the second row of each plot were left untreated. In experiment A, fungal strains were applied to the silk channel of 10 plants of hybrid 3394 in one row of each plot, and plants in the second row of each plot were left untreated. For experiments C and D, the hybrid 3394 block had a split-plot design. Fungal-infested toothpicks were placed near seeds at planting for 10 plants in both rows of each plot. For experiment C, the same fungal strains were reapplied to the silk channel of 10 plants in one row of each plot and, for experiment D, plants in the second row of each plot were not re-treated.

The 1997 field test consisted of one block of hybrid 3394 for experiment E and one block of hybrid 3394 for experiment F. Each block contained four randomly assigned replicate plots of each of the four fungal strains being tested and four control plots. Each plot was 4 m long and contained two rows of ~15 plants each. In experiment E, fungal strains were applied to the silk channel of 10 plants in one row of each plot, and plants in the second row of each plot were left untreated. In experiment F, fungal strains were applied near seeds at planting for 10 plants in one row of each plot, and the other row of each plot was left untreated.

Disease Ratings. At physiological maturity, all ears from treated plants were hand-picked, husked, and air-dried in the laboratory for ≥2 weeks. Ears from untreated plants were not harvested. Each ear was then individually evaluated using a disease severity rating scale based on visual estimation of the percentage of visibly infected kernels on an ear as follows: 1 = 0%; 2 = 1−3%; 3 = 4−10%; 4 = 11−25%; 5 = 26−50%; 6 = 51−75%; and 7 = 76−100% (Reid et al., 1996). Each ear was sampled by removing five to seven kernels for microbiological analysis. The remaining kernels were hand-shelled and separated into a nonsymptomatic fraction and a symptomatic fraction that contained all kernels that were visibly moldy, darkened, streaked, or chalky in appearance. The number of ears sampled for percentage ear rot by weight and fumonisin analysis differed between the tests. For the control treatments in the 1996 test, and for all treatments in the 1997 test, the symptomatic fraction and the nonsymptomatic fraction from all 10 ears of each replicate plot were each pooled, weighed, ground, and analyzed for fumonisins. For the fungal treatments in the 1996 tests, two representative ears were selected by disease severity visual rating from each replicate plot of 10 ears. The symptomatic fraction and the nonsymptomatic fraction of the two representative ears from each replicate plot were each pooled, weighed, ground, and analyzed for fumonisins. To simplify data presentation, fumonisin levels and weights of symptomatic and nonsymptomatic kernels were used to determine the fumonisin level in the whole ear. This was appropriate because the main objective of the study was to compare overall fumonisin levels in whole ears, not the distribution of fumonisins within ears.

Fumonisin Analysis. Fumonisins were analyzed by HPLC of their orthophthalaldehyde (OPA) derivatives as described (Sydenham et al., 1992). The detection limits for FB_1, FB_2, and FB_3 were 0.05, 0.1, and 0.1 μg/g, respectively, of the maize dry weight. To ensure reliability and reproducibility of the analytical procedures, a reference maize meal containing ~1 μg/g fumonisins was run as a control with every set of samples. In addition, replicate extractions and HPLC analyses were done on randomly chosen samples. Fumonisin levels in samples generally were reproducible across the quantitation range, with a coefficient of variation of <20%. Fumonisin B_4 was not quantitated in this study but was present at low levels in samples that contained FB_1, FB_2, or FB_3.

The production of FB_1, FB_2, and FB_3 by fungal strains was assessed in 25 mL liquid cultures in 50 mL Erlenmyer flasks as described (Plattner and Shackelford, 1992). After 14 days of incubation with shaking at 200 rpm at 28 °C in the dark, the cultures were filtered and analyzed by HPLC of OPA-derivatized samples as described (Sydenham et al., 1992). Detection limits for FB_1, FB_2, and FB_3 were 1 μg/mL in liquid culture filtrates.

Identification of Fungal Strains Recovered from Maize Kernels. Five to seven kernels, selected to be representative of symptomatic and nonsymptomatic kernels on each ear, were removed from each harvested ear before it was hand-shelled. Kernels obtained from ears from randomly selected plots from various experiments and treatment groups were tested for the presence of *Fusarium* species. All kernels sampled from each group of 10 ears from a replicate plot were surface-disinfested in 0.5% sodium hypochlorite and placed on a selective medium as previously described (Nelson et al., 1983). One fungal colony per kernel was reisolated from a single spore and identified as *F. verticillioides* by production of microconidia in chains. This trait is not sufficient to distinguish *F. verticillioides* from the closely related species *F. proliferatum* and *F. thapsinum,* which have been found rarely in maize in the Midwest U.S. region (Munkvold and Desjardins, 1997). However, for the objectives of this study it was not necessary to identify all strains to the species level because the fumonisin production profile and other traits were used to determine which strains were similar to the applied FB_1-nonproducing strains.

To obtain additional information for the identification of selected strains that were recovered from the 1996 field test, genomic DNA was isolated from strains using a previously described method (Desjardins et al., 1996). PCR amplification using randomly amplified polymorphic DNA (RAPD-PCR) was carried out using Operon oligonucleotides OPA-01 (CAGGC-CCTTC) and OPB-01 (GTTTCGCTCC) (Operon Technologies, Inc., Alameda, CA). OPA-01 amplified RAPD markers unique to strain 109-R-7, and OPB-01 amplified RAPD markers unique to strain M-3125. RAPD-PCR was carried out in a Perkin-Elmer 480 thermocycler essentially as described (Williams et al., 1990) except that 25−75 ng of genomic DNA and 0.225 mM of each dNTP were used. Thermocycler settings were as follows: 45 cycles of 94 °C for 1 min, 35 °C for 1 min, 72 °C for 2 min. PCR products were analyzed by agarose gel electrophoresis in 1% agarose gels following standard protocols (Sambrook et al., 1989).

To obtain additional information to determine whether fumonisin-nonproducing strains were identical to the applied strain M-5500, all fumonisin-nonproducing strains that were recovered from the 1997 field test were tested for mating population and mating type. Strains were tested on carrot agar (Klittich and Leslie, 1988) for fertility with strain M-3125, a *MAT1-2* female fertile tester strain of *G. fujikuroi* mating population A. Fertility with strain M-3125 indicated that the tested strains were of mating population A, mating type *MAT1-1*, which is the mating type of strain M-5500. Strains that were of the same mating type as strain M-5500 were then tested for vegetative compatibility with strain M-5500. Nitrate nonutilizing mutants were generated and used in vegetative compatibility assays as previously described (Klittich and Leslie, 1988; Desjardins et al., 1998).

Statistical Analyses. For each field test, analyses of variance were conducted on the disease severity visual ratings and on the percentage ear rot by weight and, for seed application experiments, on the relative proportions of fumonisin homologues in the harvested kernels (Statistical Analysis Systems, Cary, NC). The major objectives of these analyses were to test whether the levels of disease and the relative proportions of fumonisin homologues produced by the applied strains were different. Additional analyses were conducted to test whether the incidence of kernel infection by applied strains was increased compared to controls. Differences in recovery of applied strains were analyzed by breaking the data into a 2 × 2 table that consisted of strain (present or absent) and treatment (applied or not) and then using the Chi-square test of independence.

RESULTS

Silk-Channel Application−1996 Field Test. In the 1996 field test, the $FB_1FB_2FB_3$-producing strain M-3125 was compared to the FB_2-producing strain 109-R-7 for their ability to cause ear infection and ear rot following silk-channel application in experiments A and B and following combined silk-channel and seed application in experiment C. Silk-channel application of either strain, with or without additional seed application, was highly effective in producing ear rot. More than 45% of the ears treated with either fungal strain exhibited disease symptoms over >25% of the ear (disease rating >4). Figure 1 illustrates the frequency distribution of the disease severity visual ratings for a total of 360 individual ears from experiments A−C. Mean disease severity visual ratings for strains M-3125 and 109-R-7 ranged from 4.0 to 5.1 and were not significantly ($P > 0.05$)

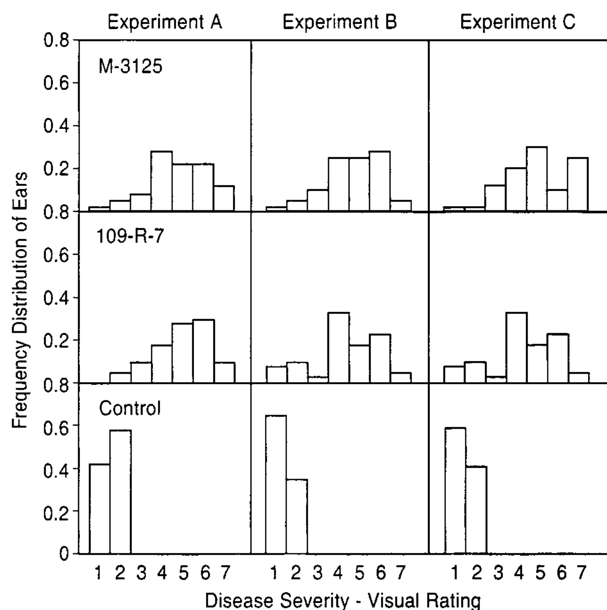

Figure 1. Frequency distribution of disease severity ratings for 360 individual maize ears from plants treated by the silk-channel application method with fungal strain M-3125 or 109-R-7 or controls in experiments A–C of the 1996 field test. Fungal strains and treatment applications are described in Tables 1 and 2. The silk-channel application method and the field tests are described under Materials and Methods.

different from each other. Both fungal strains caused more disease ($P < 0.05$) than the control ears, which had mean disease severity ratings ranging from 1.4 to 1.6 (Table 2).

Both strains M-3125 and 109-R-7 also caused high levels of disease as measured by percentage ear rot by weight, and both produced high levels of fumonisins in kernels following silk-channel application. Figure 2 (top) illustrates the percentage ear rot by weight versus disease severity visual ratings for a total of 168 representative ears from experiments A–C. The mean percentage of ear rot caused by strains M-3125 and 109-R-7 ranged from 68 to 86%, and the strains were not significantly different ($P > 0.05$) from each other. Both fungal strains caused more ear rot ($P < 0.05$) than the control ears, which had mean ear rot ranging from 7 to 8% (Table 2). Figure 2 (bottom) also illustrates the percentage of ear rot by weight versus fumonisin levels in kernels from experiments A–C. Because the hybrids tested were highly susceptible to *F. verticillioides*, even the control ears contained fumonisins at mean levels ranging from 11 to 18 μg/g due to background infection and insect damage (Table 2). Mean fumonisin levels following fungal treatments were much higher, ranging from 130 to 1160 μg/g. Although kernels from ears treated with strain M-3125 contained FB_1, FB_2, and FB_3, kernels from ears treated with strain 109-R-7 contained only FB_2 (Table 2).

High levels of exclusively FB_2 in ears treated with strain 109-R-7 indicated successful infection of the ears following silk-channel application with the FB_2-producing strain. To confirm this result in the 1996 field test, *F. verticillioides* strains were recovered from kernels from 10 ears from each of two replicate plots treated with strain 109-R-7 and from 10 control ears from one replicate plot. The mean percentage of infected kernels ranged from 88 to 90% for the treatment groups (Table 3). One hundred and twenty-five of the recovered strains were tested for fumonisin production in liquid culture.

Table 2. Mean Values of Disease Severity Visual Rating, Percentage Ear Rot by Weight, and Fumonisin Levels in Maize Kernels Following Application of *F. verticillioides*

year and expt[a]	treatment[a]	disease severity visual rating[b]	percentage ear rot by wt[b]	fumonisins in kernels[c] (μg/g)		
				FB_1	FB_2	FB_3
		Silk-Channel Application				
1996, A	M-3125	4.9 a	68 a	450	140	42
	109-R-7	5.0 a	76 a	0	400	0
	control	1.6 b	7 b	8.2	2.6	1.2
1996, B	M-3125	4.7 a	86 a	760	360	40
	109-R-7	4.0 a	77 a	0	180	0
	control	1.4 b	8 b	7.9	2.6	0
1996, C	M-3125	5.1 a	78 a	700	240	72
	109-R-7	4.3 a	72 a	0	130	0
	control	1.5 b	8 b	12	5.0	1.2
1997, E	M-3125	4.6 a	50 a	200	100	21
	109-R-7	4.6 a	48 a	0	220	0
	506-R-15	5.1 a	56 a	0	0	450
	M-5500	5.0 a	56 a	0	0	0
	control	2.3 b	10 b	4.7	1.3	0.3
		Seed Application				
1996, D	M-3125	1.6 a	7 a	6.2	1.9	0.2
	109-R-7	1.8 a	5 a	1.1	10	0.2
	control	1.4 a	4 a	4.7	2.5	0.3
1997, F	M-3125	2.0 a	10 a	2.8	1.0	0.1
	109-R-7	2.1 a	10 a	1.7	1.9	0
	506-R-15	2.0 a	8 a	6.3	1.4	2.2
	M-5500	1.9 a	9 a	2.9	0.5	0.2
	control	2.0 a	10 a	3.3	1.6	0.1

[a] Strains applied as treatments are described in Table 1. Control treatments were half-strength Bilay's medium. Application methods and field tests are described under Materials and Methods. [b] A disease severity rating scale from 1 to 7 was based on visual estimation of infected kernels on each ear (Reid et al., 1996), and percentage ear rot was determined by weighing symptomatic and nonsymptomatic kernels from each ear. For each experiment, A, B, C, D, E, or F, numbers in each column followed by the same letter are not significantly different by ANOVA ($P < 0.05$). [c] Fumonisin levels in kernels were determined by HPLC by the method of Sydenham et al. (1992) as described under Materials and Methods.

Eighty-six percent of the strains recovered from control ears produced FB_1, FB_2, and FB_3. RAPD-PCR marker analysis of 29 of these FB_1-producing strains indicated a diverse population of endemic FB_1-producing strains at the field test site. By RAPD-PCR marker analysis, none of the 29 FB_1-producing strains from the control ears were identical to strain M-3125, indicating that the applied strain M-3125 had not moved from a plot where it was applied into the tested kernels from the control plot. Only one strain that produced exclusively FB_2 was recovered from control ears, and this stain was identical to strain 109-R-7 by RAPD-PCR marker analysis, indicating that strain 109-R-7 had moved from a plot where it was applied into the control plot. Ninety-three percent of the strains recovered from ears treated with strain 109-R-7 produced only FB_2, a significant ($P < 0.01$) increase in frequency compared to plants to which strain 109-R-7 had not been applied. Thus, microbiological analysis confirmed the ability of strain 109-R-7 to infect ears following silk-channel application (Table 3).

Silk-Channel Application—1997 Field Test. In the 1997 field test, experiment E, the $FB_1FB_2FB_3$-producing strain M-3125 was compared to the FB_2-producing strain 109-R-7, the FB_3-producing strain 506-R-15, and the fumonisin-nonproducing strain M-5500 for their ability to cause ear infection and ear rot following silk-channel application. Silk-channel application of all four strains was highly effective in producing ear rot. More

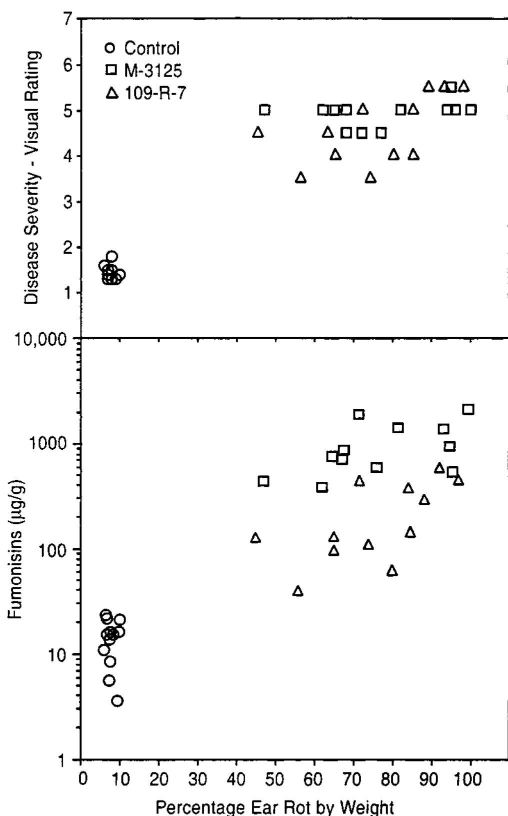

Figure 2. Relationships between percentage ear rot by weight and disease severity visual ratings (top) and fumonisin levels ($FB_1 + FB_2 + FB_3$) in kernels (bottom) for combined experiments A–C of the 1996 field test. Fungal strains and treatment applications are described in Tables 1 and 2. The silk-channel application method and the field tests are described under Materials and Methods. Each point represents the mean of sampled ears in one replicate plot: 12 plots treated with strain M-3125 (squares), 12 plots treated with strain 109-R-7 (triangles), and 12 control plots (circles).

than 47% of the ears treated with a fungal strain exhibited disease symptoms over >25% of the ear (disease rating >4). Figure 3 illustrates the frequency distribution of the disease severity visual ratings for a total of 200 individual ears from experiment E. Mean disease severity ratings for the four fungal strains ranged from 4.6 to 5.1 and were not significantly different ($P > 0.05$) from each other. All fungal strains caused more disease ($P < 0.05$) than the control ears, which had a mean disease severity rating of 2.3 (Table 2).

All four fungal strains also caused high levels of disease as measured by percentage ear rot, and all strains, except the fumonisin-nonproducing strain M-5500, produced high levels of fumonisins in kernels following silk-channel application. Figure 4 (top) illustrates the percentage ear rot by weight versus disease severity visual ratings for all 360 ears from experiment E. The mean percentage of ear rot caused by the four fungal strains ranged from 48 to 56%, and the strains were not significantly different ($P > 0.05$) from each other. All fungal strains caused more ear rot ($P < 0.05$) than the control ears, which had a mean of 10% ear rot (Table 2). Figure 4 (bottom) also illustrates the percentage of ear rot by weight versus fumonisin levels in kernels from experiment E. Mean fumonisin levels following application of fumonisin-producing strains were high, ranging from 220 to 450 μg/g. Kernels from control ears and from ears treated with strain M-3125 contained FB_1, FB_2, and FB_3, but kernels from ears treated with strain 109-R-7 contained only FB_2, and kernels from ears treated with strain 506-R-15 contained only FB_3. Kernels from control ears contained a mean of 6.3 μg/g fumonisins, but kernels from ears treated with the fumonisin-nonproducing strain M-5500 contained no detectable fumonisins (Table 2). Thus, the FB_2-producing strain, the FB_3-producing strain, and the fumonisin-nonproducing strain were able to infect ears and cause ear rot following silk-channel application.

Seed Application at Planting—1996 Field Test. In the 1996 field test, the $FB_1FB_2FB_3$-producing strain M-3125 was compared to the FB_2-producing strain 109-R-7 for their ability to cause ear infection and ear rot following seed application at planting. Seed application of fungal strains caused no significant increase ($P > 0.05$) in disease severity visual ratings, percentage ear rot by weight, or fumonisin levels in kernels compared to control ears (Table 2). Mean proportions of fumonisin homologues in the kernels were determined by dividing the level of each fumonisin by the combined level of $FB_1 + FB_2 + FB_3$ (μg/g). Kernels from control plants and from plants treated with strain M-3125 contained mean proportions of FB_2 of 33 and 21%, respectively. Kernels from plants treated with strain 109-R-7 contained a mean proportion of 88% FB_2, but these proportions were not significantly ($P > 0.05$) different from the control treatments due to the low levels of fumonisins and the high coefficients of variation between replicate plots.

To investigate further whether the frequency of strain109-R-7 in kernels was increased by seed application at planting, *F. verticillioides* strains were recovered from kernels from plants to which the strain had been applied and from control plants. Eighty-six percent of the strains recovered from control ears produced FB_1, FB_2, and FB_3, and only one strain produced FB_2 (Table 3). In contrast, the frequency of FB_2-producing strains increased significantly ($P < 0.01$) to 47% following seed application with strain 109-R-7 (Table 3).

Seed Application at Planting—1997 Field Test. In the 1997 field test, the $FB_1FB_2FB_3$-producing strain M-3125 was compared to the FB_2-producing strain 109-R-7, the FB_3-producing strain 506-R-15, and the fumonisin-nonproducing strain M-5500 for their ability to cause ear infection and ear rot following seed application at planting. Seed application of fungal strains caused no significant increase ($P > 0.05$) in disease severity visual ratings, percentage ear rot by weight, or fumonisin levels in kernels compared to control ears (Table 2). Kernels from control plants and from plants treated with strain M-3125 contained mean proportions of FB_2 of 32 and 26%, respectively, and contained mean proportions of FB_3 of 2 and 3%, respectively. Kernels from ears treated with strain 109-R-7 contained a mean proportion of 53% FB_2, and kernels from ears treated with strain 506-R-15 contained a mean proportion of 22% FB_3. These proportions, however, were not significantly ($P > 0.05$) different from the control treatments due to the low levels of fumonisins and the high coefficients of variation between replicate plots.

To determine whether the frequency of FB_1-nonproducing strains in kernels was increased by seed application at planting, *F. verticillioides* strains were recovered from 10 ears of one replicate plot of each of the five treatment groups. The mean percentage of infected kernels ranged from 63 to 93% for the treatment groups (Table 3). One hundred and eighty-four of the recovered

Table 3. Infection of Maize Kernels with *F. verticillioides* and Recovery of Applied Strains

year and expt[a]	treatment[a]	no. of kernels tested	no. of kernels infected	no. of strains tested	no. of recovered strains with each fumonisin phenotype[b] $B_1B_2B_3$	B_2	B_3	none	other
			Silk-Channel Application						
1996, A	109-R-7	51	46	35	0 b	30 a	0 a	5 a	nt
1996, C	109-R-7	59	55	46	1 b	45 a	0 a	0 a	nt
1996, C	control	60	53	44	38 a	1 b	5 a	0 a	nt
			Seed Application						
1996, D	109-R-7	56	47	32	14 b	15 a	1 a	2 a	nt
1996, C	control	60	53	44	38 a	1 b	5 a	0 a	nt
1997, F	M-3125	62	39	39	36 a	1 b	0 b	0 b	2
1997, F	109-R-7	59	39	36	7 b	28 a	0 b	0 b	1
1997, F	506-R-15	60	40	37	12 b	6 a	18 a	1 b	0
1997, F	M-5500	60	56	35	15 b	5 a	0 b	12 a	3
1997, F	control	59	39	37	24 a	0 a	0 b	0 b	13

[a] Strains applied as treatments are described in Table 1. Control treatments were half-strength Bilay's medium. Application methods and field tests are described under Materials and Methods. [b] The presence of strains was determined by isolation on selective medium followed by HPLC analysis of fumonisin production in liquid culture. For the 1996 field test, "none" designates fumonisin-nonproducing strains, and "nt" indicates not tested. For the 1997 field test, "none" designates fumonisin-nonproducing strains that were identical to the applied strain M-5500 by mating tests and vegetative compatibility tests, and "other" indicates fumonisin-nonproducing strains that were not identical. For each year, numbers in each column followed by the same letter are not significantly different by Chi-square test ($P < 0.05$).

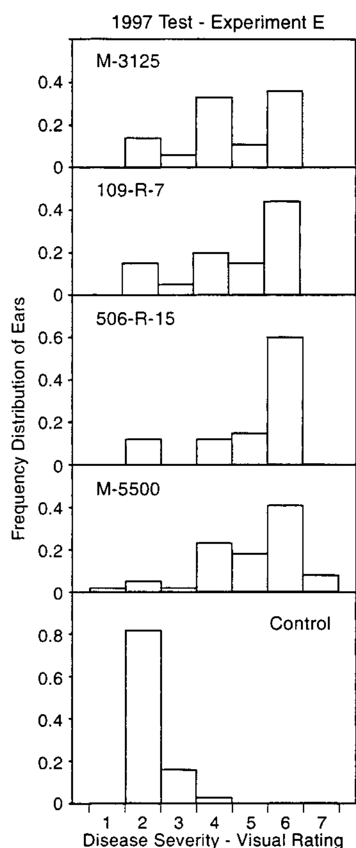

Figure 3. Frequency distribution of disease severity ratings for 200 individual maize ears from plants treated by the silk-channel application method with fungal strain M-3125, 109-R-7, 506-R-15, or M-5500 or controls in experiment E of the 1997 field test. Fungal strains and treatment applications are described in Tables 1 and 2. The silk-channel application method and the field test are described under Materials and Methods.

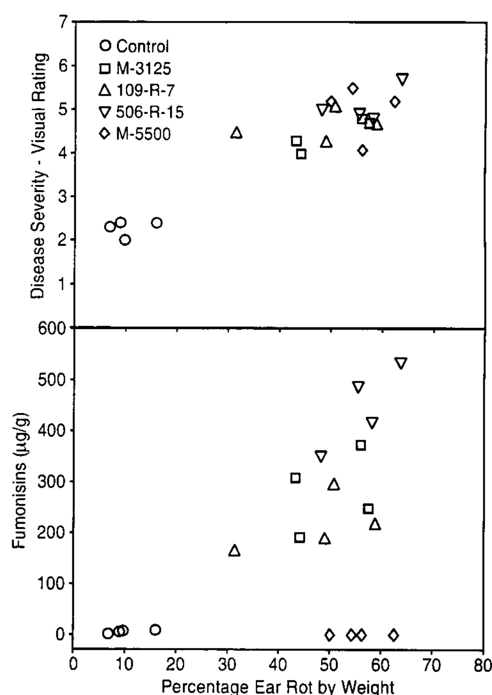

Figure 4. Relationships between percentage ear rot by weight and disease severity visual ratings (top) and fumonisin levels ($FB_1 + FB_2 + FB_3$) in kernels (bottom) from experiment E of the 1997 field test. Fungal strains and treatment applications are described in Tables 1 and 2. The silk-channel application method and the field test are described under Materials and Methods. Each point represents the mean of sampled ears in one replicate plot: four plots treated with strain M-3125 (squares), four plots treated with strain 109-R-7 (triangles), four plots treated with strain 506-R-15 (inverted triangles), four plots treated with strain M-5500 (diamonds), and four control plots (circles).

strains were tested for fumonisin production in liquid culture. Ninety-two percent of the strains recovered after application of strain M-3125 produced FB_1, FB_2, and FB_3; 78% recovered after application of strain 109-R-7 produced only FB_2; 49% recovered after application of strain 506-R-15 produced only FB_3; and 43% recovered after application of strain M-5500 produced no fumonisins (Table 3). Strains that produced only FB_2 or FB_3 were recovered at frequencies of only 8 and 0.5%, respectively, among strains from plants to which they had not been applied. Thus, the frequency of FB_2-producing strains and FB_3-producing strains increased significantly ($P < 0.01$) following seed application of strains 109-R-7 and 506-R-15, respectively.

Fumonisin-nonproducing strains were recovered at a frequency of 11% from plants to which strain M-5500 had not been applied (Table 3). Due to this relatively high frequency, additional tests were conducted to

determine whether the 32 fumonisin-nonproducing strains recovered from the 1997 field test were identical to strain M-5500. Mating tests and/or vegetative compatibility tests confirmed that 12 of 15 fumonisin-nonproducing strains from plants treated with strain M-5500 were identical to this strain. However, only 1 of the 17 fumonisin-nonproducing strains from plants not treated with stain M-5500 were identical to this strain. The remaining 16 fumonisin-nonproducing strains apparently are endemic fumonisin-nonproducing strains of *F. verticillioides* or of a morphologically similar species. Thus, the frequency of strain M-5500 increased significantly ($P < 0.01$) following seed application.

DISCUSSION

The first goal of the present study was to test whether FB_1-nonproducing strains of *F. verticillioides* could infect maize ears and cause ear rot following silk-channel application. In field tests in 1996 and 1997, natural variants that produced only FB_2 or FB_3 or produced no detectable fumonisins, in liquid culture media or on autoclaved maize meal, were able to cause ear rot and appeared to be as virulent as a strain that produced FB_1, FB_2, and FB_3. Analysis of kernels from severely rotted ears confirmed that neither the FB_2-producing strain nor the FB_3-producing strain produced any FB_1 in planta and that the fumonisin-nonproducing strain produced no detectable fumonisins in planta. Further analysis of *F. verticillioides* isolated from individual kernels in the 1996 field test confirmed that the FB_2-producing strain infected a mean of 68% of tested kernels and a mean of 93% *F. verticillioides*-infected kernels from ears following silk-channel application. These results argue that production of FB_1, FB_2, or FB_3 is not required for *F. verticillioides* to infect maize ears and cause ear rot following silk-channel application to a highly susceptible maize cultivar. This result is consistent with the previous report that production of fumonisins also is not required for *F. verticillioides* to cause maize stalk rot under greenhouse conditions (Jardine and Leslie, 1999).

In contrast, it is well-established that AAL toxins, which are closely related in structure to fumonisins, are required for strains of *A. alternata* to cause disease on tomato cultivars that are homozygous for the *asc* allele (Gilchrist, 1998). This discrepancy is not unprecedented in the field of mycotoxin research. Previous results from our laboratory have shown that the importance of trichothecene mycotoxins in plant disease varies among plant−fungal systems. Strains altered in trichothecene biosynthesis have been obtained by mutagenesis or specific gene disruption in *F. graminearum*, *F. sambucinum*, and *F. sporotrichioides*. Tests of these mutants under laboratory and field conditions have shown that trichothecenes are important for virulence of *F. graminearum* on maize and wheat ears and of *F. sambucinum* and *F. sporotrichioides* on parsnip root (Desjardins and Hohn, 1997; Harris et al., 1999). Trichothecenes, however, are not required for *F. sambucinum* to cause potato tuber rot (Desjardins and Hohn, 1997). These results indicate that one should be cautious in generalizing results from one plant−fungal system to another when assessing the role of any class of mycotoxins in plant disease.

The second goal of the present study was to test whether FB_1-nonproducing strains of *F. verticillioides* could infect maize ears following seed application at planting. Numerous previous studies have shown that *F. verticillioides* can not only cause severe ear rot but also infect many parts of the maize plant without causing obvious symptoms (Munkvold and Desjardins, 1997). Because *F. verticillioides* is endemic in maize fields and individual maize plants can be infected with multiple fungal strains, methods are necessary to distinguish applied strains from endemic strains (Kedera et al., 1994; Munkvold et al., 1997b). In several recent studies, vegetative compatibility group analysis has been used to distinguish applied strains from endemic strains. These studies have shown that strains applied to maize seed at planting can be recovered from seedlings and stalks and can be recovered from kernels from mature ears (Desjardins et al., 1998; Kedera et al., 1994; Munkvold and Carlton, 1997; Munkvold et al., 1997). The present study showed that FB_2-producing, FB_3-producing, and fumonisin-nonproducing strains occurred at low frequencies among the *F. verticillioides* population endemic to our field test sites in central Illinois in 1996 and 1997. Thus, we were able to distinguish applied strains from endemic strains by fumonisin production profiles, with some additional analyses by mating type, vegetative compatibility group, and RAPD-PCR. In field tests in 1996 and 1997, analysis of *F. verticillioides* isolated from individual kernels showed that strains that produced only FB_2 or FB_3, or produced no detectable fumonisins, were able to infect a high proportion of the kernels in ears following seed application at planting. These results argue that production of FB_1, FB_2, or FB_3 is not required for *F. verticillioides* to infect maize ears following seed application at planting.

Substantial levels of fumonisins occur in maize crops worldwide (Marasas, 1996). As concerns about the toxicity and carcinogenicity of fumonisins increase, new approaches are needed to reduce the levels of fumonisins in maize. The prevalence of endemic FB_1FB_2 FB_3-producing strains of *F. verticillioides* in maize is a formidable challenge (Munkvold and Desjardins, 1997). Our study has shown that strains that produce only the less phytotoxic homologues FB_2 or FB_3 or produce no detectable fumonisins can infect maize kernels and can change the profile of fumonisins produced in the kernels of infected ears. Thus, it may be possible to reduce infection by endemic fumonisin-producing strains through competition with fumonisin-nonproducing strains and thus decrease the potential for contamination with fumonisins. In the present study, application of FB_1-nonproducing strains to seeds at planting increased the frequency of these strains in kernels but did not increase levels of ear rot or prevent the production of FB_1 in kernels. In contrast, silk-channel application of FB_1-nonproducing strains prevented the production of FB_1 in the kernels but also caused high levels of ear rot. Thus, the success of fumonisin-nonproducing strains as biological control agents will require further development of fungal strains and application methods to produce a low level of ear rot but a higher level of nonsymptomatic kernel infection than was observed in the present study.

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