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Genetic Analysis of Fumonisin Production and Virulence of *Gibberella fujikuroi* Mating Population A (*Fusarium moniliforme*) on Maize (*Zea mays*) Seedlings†

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The phytopathogenic fungus *Gibberella fujikuroi* mating population A (anamorph, *Fusarium moniliforme*) produces fumonisins, which are toxic to a wide range of plant and animal species. Previous studies of field strains have identified a genetic locus, designated *fum1*, that can determine whether fumonisins are produced. To test the relationship between fumonisin production and virulence on maize seedlings, a cross between a *fum1*$^+$ field strain that had a high degree of virulence and a *fum1*$^-$ field strain that had a low degree of virulence was made, and ascospore progeny were scored for these traits. Although a range of virulence levels was recovered among the progeny, high levels of virulence were associated with production of fumonisins, and highly virulent, fumonisin-nonproducing progeny were not obtained. A survey of field strains did identify a rare fumonisin-nonproducing strain that was quite high in virulence. Also, the addition of purified fumonisin B_1 to virulence assays did not replicate all of the seedling blight symptoms obtained with autoclaved culture material containing fumonisin. These results support the hypothesis that fumonisin plays a role in virulence but also indicate that fumonisin production is not necessary or sufficient for virulence on maize seedlings.

Plant-pathogenic fungi produce a wide variety of phytotoxic metabolites. Classical genetic and molecular genetic analyses have demonstrated that some of these toxins are important for pathogenesis on plants (4, 11, 22, 28). It is also clear that some fungal toxins are highly specific and affect only certain genotypes of a plant species, whereas other toxins are nonspecific and affect a wide range of plants and animals as well (12, 23). Fungal metabolites toxic to animals are termed mycotoxins and are of particular interest because of their potential effects on human and animal health, as well as their potential roles in plant disease.

Gibberella fujikuroi (Sawada) Ito in Ito and K. Kimura mating population A has a *Fusarium moniliforme* Sheldon anamorph and is a major pathogen of maize worldwide, causing seedling, stalk, and ear rots (16, 18). Almost all field strains of *G. fujikuroi* mating population A tested to date produce fumonisins (14, 15, 20), which are a family of structurally closely related, water-soluble metabolites, the most prevalent of which is fumonisin B_1, a propane-1,2,3-tricarboxylic acid diester of 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane (1). Fumonisins appear to be non-host-specific toxins (13, 24) and have been associated with a variety of mycotoxicoses, including equine leukoencephalomalacia, porcine pulmonary edema, and experimental liver cancer in rats (19). The high frequency of fumonisin contamination of maize, and of fumonisin production among strains of *G. fujikuroi* mating population A from maize, raises the possibility that fumonisins play a role in virulence on maize. Further indirect evidence is provided by the structural similarity of fumonisins to AAL toxin, which is a pathogenicity factor of *Alternaria alternata* f. sp. *lycopersici* in tomato (2). In addition, purified fumonisins have been shown to cause necrosis and other symptoms in maize seedlings (13) and maize callus cultures (24) treated in vitro at a relatively low concentration (10^{-6} M). However, the role of fumonisins in maize pathogenesis has not yet been subjected to a critical genetic analysis.

Recent genetic analysis of fumonisin production in *G. fujikuroi* mating population A has shown that a single locus, designated *fum1*, or a set of closely linked loci, can determine whether fumonisins are produced (5). The purpose of this study was to test the hypothesis that a high level of virulence of *G. fujikuroi* mating population A on maize seedlings is associated with the production of fumonisins. Our main strategy was to analyze progeny of a cross between a *fum1*$^+$ field strain that is high in virulence and a *fum1*$^-$ field strain that is low in virulence. If a high level of virulence segregates independently of fumonisin production, then highly virulent, fumonisin-nonproducing progeny should be obtained.

MATERIALS AND METHODS

Strains. Strain numbers, original hosts, and geographic origins of the field strains used in this study are given in Table 1. Most of the field strains were supplied by P. E. Nelson from the Fusarium Research Center, The Pennsylvania State University, University Park. Cultures were grown routinely on slants of V-8 juice agar under an alternating 12-h light-dark cycle of 25 and 20°C as previously described (5). Cultures were preserved for long-term storage either on lyophilized carnation leaves (21) or in 15% glycerol at −80°C

Crosses were made on carrot agar as described by Klittich and Leslie (10), but male parents were cultured on V-8 juice agar medium rather than complete medium. Random ascospores and tetrads were dissected freehand with a dissecting microscope.

VCG identifications. Vegetative compatibility was scored by pairing complementary nitrate-nonutilizing (*nit*) mutants derived from strains M-3125 and M-5500 and selected progeny of cross 57 between these two strains. *nit* mutants were generated on minimal medium containing 1.5% $KClO_3$, reisolated from single spores, and assigned to one of three classes, *nit1*, *nit3*, or NitM, on the basis of their phenotype on media containing different nitrogen sources (3). Pairings were made individually and scored for the appearance of dense aerial mycelia at the point of contact between two *mit* mutants. When two strains complemented

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TABLE 1. Fumonisin production and virulence of G. *fujikuroi* mating population A strains on seedlings of sweet maize (cultivar Golden Bantam)

Strain no.[a]		Seedling virulence[b]		Fumonisin B_1 production (ppm)[c]	Host	Origin[d]
FRC	Other	% Emerged of 30 planted	Mean shoot length (mm)			
None (control)		87	279 a			
M-5515	A-04518	63	195 b	0	Maize	Nepal
M-5538	A-04522	70	192 b	0	Maize	Nepal
M-5334	A-04521	50	182 bc	0	Maize	Nepal
M-5500	A-04516	67	172 bc	0	Maize	Nepal
M-5507	A-04517	43	168 bc	0	Maize	Nepal
M-5519	A-04519	20	159 bc	60	Maize	Nepal
M-5525	A-04520	27	154 bc	6,400	Maize	Nepal
M-5550	A-04524	37	151 bc	0	Maize	Nepal
M-3120	A-00102, PTS-F80	37	148 bc	1,230	Sorghum	United States (Calif.)
M-5542	A-04523	17	148 bc	50	Maize	Nepal
M-2657	A-02871	23	127 bc	4,420	Maize	United States (S.C.)
M-3587	A-00408, FSL-172	37	114 c	4,200–5,200	Maize	United States (Calif.)
M-1142	A-00410, FSL-174	33	98 c	320–1,900	Maize	United States (Calif.)
M-3125	A-00149, PTS-F237	30	71 c	4,830	Maize	United States (Calif.)

[a] M strain numbers, Fusarium Research Center, The Pennsylvania State University; A strains, Department of Plant Pathology, Kansas State University; PTS strains, P. T. Spieth, Department of Plant Pathology, University of California at Berkeley; FSL strains, E. G. Kuhlman, USDA Forest Service, Research Triangle Park, N.C.
[b] Results of three replicate tests of all strains, one pot of 10 seedlings per strain for each test. Seedling emergence and shoot length were scored 14 days after seed were planted in infested sand. Values followed by the same letter are not significantly different on the basis of *t* tests of least-squares means at $P < 0.05$.
[c] Fumonisin production data from reference 5. Zero indicates that ≤10-ppm fumonisin B_1 was produced on autoclaved cracked maize.
[d] Calif., California, S.C., South Carolina.

one another to form a viable heterokaryon, the two complementing strains were designated members of the same vegetative compatibility group (VCG). Strains that did not complement each other were termed vegetatively incompatible and belonged to different VCGs. All pairings were made at least twice, with one member of the pair a NitM mutant whenever possible.

Analysis of fumonisin production. Conidial suspensions prepared from strains grown on V-8 juice agar for 1 to 2 weeks were used to inoculate 50 g of autoclaved, coarsely cracked maize as previously described (20). After 28 days of incubation at (25 ± 2)°C in the dark, the cultures were extracted and analyzed by high-performance liquid chromatography (HPLC) (5). Fumonisin levels were expressed as parts per million (micrograms of fumonisin B_1 per gram of culture material). Strains that produced ≤10-ppm fumonisins were scored as nonproducers, which reflects our observation that the maize used as a substrate for this study and previous studies (5, 14, 20) contains naturally occurring fumonisin B_1, usually <10 ppm.

Virulence tests with maize seedlings. One milliliter of a conidial suspension (10^7 conidia per ml) prepared from strains grown on V-8 juice agar for 1 to 2 weeks was used to inoculate 15 g of maize meal autoclaved with 6.6 ml of water in a 125-ml Erlenmeyer flask with a Morton closure (20). After 5 to 14 days of incubation at (25 ± 2)°C in the dark, the maize meal cultures were used as inocula. All inocula used in each test were grown at the same time and were the same age. For one series of experiments, fungal inocula were autoclaved for 30 min prior to virulence assays. Plating tests showed that viable propagules were not recovered after autoclaving. For some tests, purified fumonisin B_1 was added to the maize meal controls and to inocula prepared from strain M-5500 at a concentration of 2.0 mg of fumonisin B_1 in 1 ml of sterile water added to 1 g of maize meal.

Maize seeds were obtained from Johnny's Selected Seeds, Albion, Maine, and were surface disinfected with 0.5% NaOCl for 1 min, rinsed in sterile water, and blotted dry on paper toweling. To avoid potential problems with fungicide residues, we used maize seed without fungicide treatments. Maize cultivar Silver Queen was used for most tests, but cultivar Golden Bantam was substituted when fungicide-untreated seed of Silver Queen was unavailable. Seedlings were grown in 10.5-cm-diameter clay pots (10 seed per pot) containing moist, autoclaved, coarse sand. A 15-30-15 (nitrogen-phosphorus-potassium) soluble fertilizer was applied before planting and twice a week thereafter. For inoculations, 1.0 g of *Fusarium*-infested maize meal was thoroughly mixed with the top 5 cm of sand in each pot, and seeds were immediately planted at a depth of 3 to 5 cm. Control pots received 1.0 g of autoclaved maize meal. Planted pots were arranged on two shelves of a controlled-environment chamber under a 12-h light-dark cycle of 26 and 21°C. Each shelf was illuminated with two General Electric cool white fluorescent tubes 50 cm above the bench. Pots were autoclaved in individual bags, which were retained during the virulence assay to help control spread of *G. fujikuroi* between pots.

For this study, virulence was defined as the ability to cause two types of disease symptoms on maize seedlings: inhibition of seedling emergence and inhibition of shoot elongation. Fourteen days after being planted, the seedlings were harvested and seedling emergence and shoot length were determined. Seeds with no visible shoot growth were scored as not emerged. This group included both seeds that had never germinated and germinated seeds whose shoots had completely rotted away. The length of each seedling from the seed attachment site to the tip of the longest leaf was measured.

Shoot length data were compared by analysis of variance. Means were compared by *t* tests of differences in least-squares means. Emergence data were compared as percentages by *z* tests.

Recovery of *nit* mutants from infected seedlings. For selected virulence tests of cross 57 parents and progeny, *nit* mutants were used for inoculum production as described above. At the end of the 2-week virulence assay, 1- to 2-cm-long leaf tissue samples were taken from three seedlings in each treatment or control pot. Leaf pieces were surface disinfected with 0.5% NaOCl and 1 drop of 1% Triton X-100 for 1 min, rinsed in sterile water, and blotted dry on paper towels. Three leaf pieces from each pot were then placed on a petri plate containing a modified peptone-PCNB medium (21) and incubated for 3 to 5 days at 25°C. A 2- to 3-mm^3 block was transferred from each fungal colony to minimal medium (3) and paired with a complementary *nit* mutant of the strain used for seedling inoculation.

RESULTS

Fumonisin production and virulence of field strains. Field strains of *G. fujikuroi* mating population A were analyzed to determine if there was a clear correlation between fumonisin production and virulence on maize seedlings. Fourteen field strains isolated from maize and sorghum were previously analyzed for fumonisin production on autoclaved cracked maize (5). Eight of these strains, including three strains from Nepal, produced fumonisin B_1 in culture, whereas six strains, all from Nepal, produced no fumonisin or only trace amounts of fumonisins (<10-ppm). In the present study, these 14 strains were assayed for their virulence on maize seedlings. Virulence was assayed by inhibition of seedling emergence and of seedling shoot elongation in three replicate tests of maize cultivar Golden Bantam planted in *Fusarium*-infested sand. All strains produced some blight symptoms on maize seedlings, but the overall mean emergence (28%) and mean shoot length (124 mm) of seedlings infected with the group of eight fumonisin-producing (>10-ppm) strains were significantly ($P < 0.05$) different from the overall mean emergence (55%) and mean shoot length (179 mm) of seedlings infected by the group of six fumonisin-nonproducing strains (Table 1). When individual

TABLE 2. Virulence tests of strain M-3125, a high-level fumonisin producer, and strain M-5500, a nonproducer, on various maize cultivars

Cultivar	Type	Seedling treatment group[a]					
		Control		M-3125		M-5500	
		Emergence (%)	Mean shoot (mm)	Emergence (%)	Mean shoot (mm)	Emergence (%)	Mean shoot (mm)
Alpine	White sweet	100	238 a	80	140 b	90	249 a
Golden Bantam	Yellow sweet	90	429 a	100	156 c	100	334 b
Jubilee	Yellow sweet	90	350 a	100	159 b	100	301 a
Longfellow Flint	Yellow flint	90	384 a	80	150 b	90	374 a
Mandan Bride	Flour	100	356 a	80	209 b	100	376 a
Matinee	Popping	100	286 a	60	150 b	70	294 a
Northstine Dent	Yellow dent	100	403 a	90	267 b	90	386 a
Silver Queen	White sweet	100	311 a	50	104 b	100	270 a
Tom Thumb	Popping	100	358 a	40	132 b	100	310 a

[a] Results of one simultaneous test of all cultivars, one pot of 10 seedlings per cultivar per treatment. The assay was as described in Table 1, footnote *b*. Means in each row that are followed by the same letter are not significantly different on the basis of *t* tests of least-squares means at $P \leq 0.05$.

strains were compared, however, the results of the correlation analysis were inconclusive because one fumonisin-nonproducing strain, M-5550, was quite virulent. Therefore, the causal relationship between fumonisin production and virulence was tested further by a genetic analysis.

Strains M-3125 and M-5500 were used as parents for the genetic analysis of virulence because they were highly interfertile and differed at the *fum1* locus (5). The relative virulence of strains M-3125 and M-5500 was demonstrated in a simultaneous test of eight maize cultivars chosen to represent a range of maize types, including hybrid sweet maize, yellow dent maize, yellow flint maize, and popping maize (Table 2). In this single, unreplicated test, neither M-3125 nor M-5500 inhibited seedling emergence of most cultivars, but strain M-3125 was more inhibitory than M-5500 to shoot elongation of all cultivars tested. Cultivar Silver Queen was selected as a typical cultivar for further tests. When fungicide-untreated seed of cultivar Silver Queen was not available, cultivar Golden Bantam was used; both cultivars are hybrid sweet maize and highly susceptible to *G. fujikuroi*.

Seedling blight symptoms produced by fumonisin-producing and -nonproducing strains were similar and included seed rotting, preemergent damping-off, root rot and browning, wilting, and death of the seedling leaves. At the conclusion of one virulence experiment, maize leaves were harvested from one pot each of uninoculated controls, strain M-3125-infected seedlings, and strain M-5500 infected seedlings. The leaves were dried, extracted, and analyzed for fumonisins by combined gas chromatography-mass spectroscopy (5). Fumonisin B_1 was detected at approximately 50 ppm in leaves of seedlings infected with strain M-3125 but was not detected in leaves from control seedlings or from strain M-5500-infected seedlings.

Segregation of fumonisin production and virulence. Previous genetic analysis indicated that the ability to produce fumonisins segregated as a single gene, designated *fum1*, or a group closely linked genes, among random ascospore progeny and tetrad progeny of cross 57 between strains M-3125 and M-5500 (5). Tetrad 57-3 was selected for a trial experiment because the eight progeny had previously been scored for fumonisin production and mating type, and all four meiotic products could be distinguished by using these markers. In *G. fujikuroi*, meiosis in the ascus is followed by a mitotic division, so there are four sets of identical twins in each complete ascus.

The first objective of this experiment was to determine if virulence was heritable and if it correlated with fumonisin production. The second objective was to investigate environmental effects on virulence, by comparing three replicate pots of seedlings treated with the same maize inoculum and comparing pots of seedlings treated with strains that were genetically identical twins. As determined by shoot length assay, the four fumonisin-producing progeny (progeny 1 twins A and B and progeny 2 twins A and B) of tetrad 57-3 were virulent, and each of these progeny demonstrated a higher average level of virulence than each of the four fumonisin-nonproducing progeny (progeny 3 twins A and B and progeny 4 twins A and B) (Table 3). The mean shoot length (124 mm) of the group of four fumonisin-producing progeny was also significantly different from the mean shoot length (311 mm) of the group of four fumonisin-nonproducing progeny on the basis of *t* tests of differences of least-squares means at $P < 0.05$. In this test, none of the progeny strains were as virulent as the fumonisin-producing parent M-3125, which alone consistently inhibited seedling emergence. To compare the variation among three replicate pots of a treatment group, coefficients of variation were calculated for all parent and progeny strains (Table 3). Coefficients of variation varied from 5 to 14% for seedling emergence and shoot length assays of three replicate pots of each of the fumonisin-nonproducing strains and from 5 to 33% for each of the fumonisin-producing strains. The environmental variation of virulence for each pair of twins was comparable to that for replicate pots of each individual twin, indicating that pot-to-pot variation, not the origin of the inoculum, was the major source of variation in the virulence assay. Efforts to decrease the amount of variation in seedling assay data by using two inbred lines of maize were unsuccessful because seedlings of the inbred lines grew very poorly in the growth chamber.

Potential internal contamination of seed and cross contamination of seedlings by other *G. fujikuroi* strains during virulence assays have been observed (7, 9) and led to development of a modified virulence assay. Plating tests indicated that approximately 10% of the seed of cultivars Golden Bantam and Silver Queen planted were internally infected with *G. fujikuroi*, which could produce anomalous virulence test results because almost all field strains produce high levels of fumonisins. This problem and other potential contamination problems were addressed by using marked (nitrate-nonutilizing mutant) strains and monitoring their recovery from infected plants to determine the extent of contamination.

To determine whether nitrate-nonutilizing (*nit*) mutants retained virulence, strain M-3125 and four independently de-

TABLE 3. Segregation of fumonisin production and virulence among eight ascospore progeny of tetrad 57-3

Organism(s)	Mean fumonisin B_1 ppm ± SD[a]	Seedling virulence[b]			
		Emergence		Shoot length	
		% (mean ± SD)	Coefficient of variation (%)	mm (mean ± SD)	Coefficient of variation (%)
None (control)	None	85 ± 5	6	295 ± 0	0
Parent M-3125	3,184 ± 2,224	53 ± 12	23	90 ± 20	23
Parent M-5500	6 ± 2	96 ± 5	5	296 ± 28	10
Progeny 1 twin(s)					
A	1,402	97 ± 5	5	142 ± 40	29
B	2,398	73 ± 9	12	88 ± 11	13
A + B	1,900 ± 498	85 ± 14	16	115 ± 44	38
Progeny 2 twin(s)					
A	1,301	57 ± 19	33	137 ± 27	20
B	167	90 ± 8	9	146 ± 20	14
A + B	734 ± 567	76 ± 21	28	142 ± 24	17
Progeny 3 twin(s)	10	87 ± 12	14	329 ± 19	6
A					
B	7	93 ± 4	5	320 ± 15	5
A + B					
Progeny 4 twin(s)	8 ± 1	90 ± 10	11	324 ± 18	5
A	10	90 ± 8	9	270 ± 28	10
B	10	87 ± 12	14	329 ± 19	6
A + B	10 ± 0	88 ± 11	12	300 ± 38	13

[a] Data from reference 5. Two replicate tests were done for parent strains.
[b] Results of one simultaneous test of all strains, with three pots of 10 seedlings each of cultivar Silver Queen for each treatment, except that there were only two control pots. The assay was as described in Table 1, footnote *b*.

rived NitM mutants were each tested for virulence on two replicate pots of 10 maize seedlings. All four *nit* mutants retained a high level of virulence that was not significantly different ($P < 0.05$) from that of the wild-type parent (data not shown). Mutant A-00707 (10) was selected for further virulence tests. To determine whether *nit* mutants could be recovered from infected plants, *Fusarium* spp. were reisolated from maize leaf tissue from each of the eight pots of 2-week-old seedlings infected with the four NitM mutants. Strain M-3125 NitM mutants were recovered from leaf tissue from seven pots; leaf tissue from one pot infected with A-00707 yielded a wild-type strain. It was not determined if this strain was a revertant or a contaminant. To determine whether *nit* mutants retained the ability to produce fumonisins, strain M-3125 NitM A-00707 was grown for 4 weeks on autoclaved cracked maize. This NitM mutant retained the fumonisin production phenotype of the parent strain M-3125 (data not shown).

As a result of the previous experiments, cross 57 was used for a larger-scale analysis of the relationship between fumonisin production and virulence on maize seedlings. Twenty random ascospore progeny of cross 57 had previously been analyzed for fumonisin production (5), and for this study an additional 36 progeny were analyzed. The results for these additional progeny were consistent with our previous findings that a single locus, or set of closely linked loci, can determine whether fumonisins are produced. Twenty-five of the 56 progeny produced ≤10-ppm fumonisin B_1 and were classified as fumonisin-nonproducing strains (Fig. 1). As noted above, the maize kernels used as a growth substrate are often naturally contaminated with low levels of fumonisins, i.e., up to 10 ppm (5). The 25 progeny that produced more than 1,000-ppm fumonisin B_1 and the 6 progeny that produced intermediate levels (from 43 to 937 ppm) were all classified as fumonisin-producing strains (Fig. 1). Previous studies have shown that coefficients of variation for fumonisin production on autoclaved maize kernels are high, that this variation is due largely to environmental rather than genetic or analytical variables, and that even strains that usually produce high levels of fumonisins will occasionally produce intermediate levels (5).

The virulence of *nit* mutants of the parent strains M-3125 and M-5500, 18 fumonisin-producing progeny and 14 fumonisin-nonproducing progeny, was tested on seedlings of cultivar Silver Queen. An additional seven fumonisin-producing progeny and seven fumonisin-nonproducing progeny were tested for virulence as prototrophs. Because of limited space in the growth chambers, all 46 progeny could not be tested simultaneously in the same growth chamber and were therefore analyzed in a series of tests. Each test included pots of control

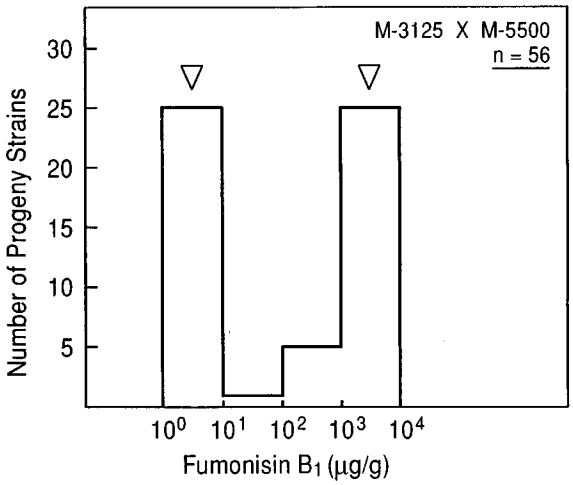

FIG. 1. Segregation of fumonisin production among 56 random ascospore progeny of cross 57. Maize cultures of the strains were incubated for 28 days and analyzed for fumonisins by HPLC. Data for 20 strains are from reference 5. Triangles indicate fumonisin production of parent strains M-3125 and M-5500.

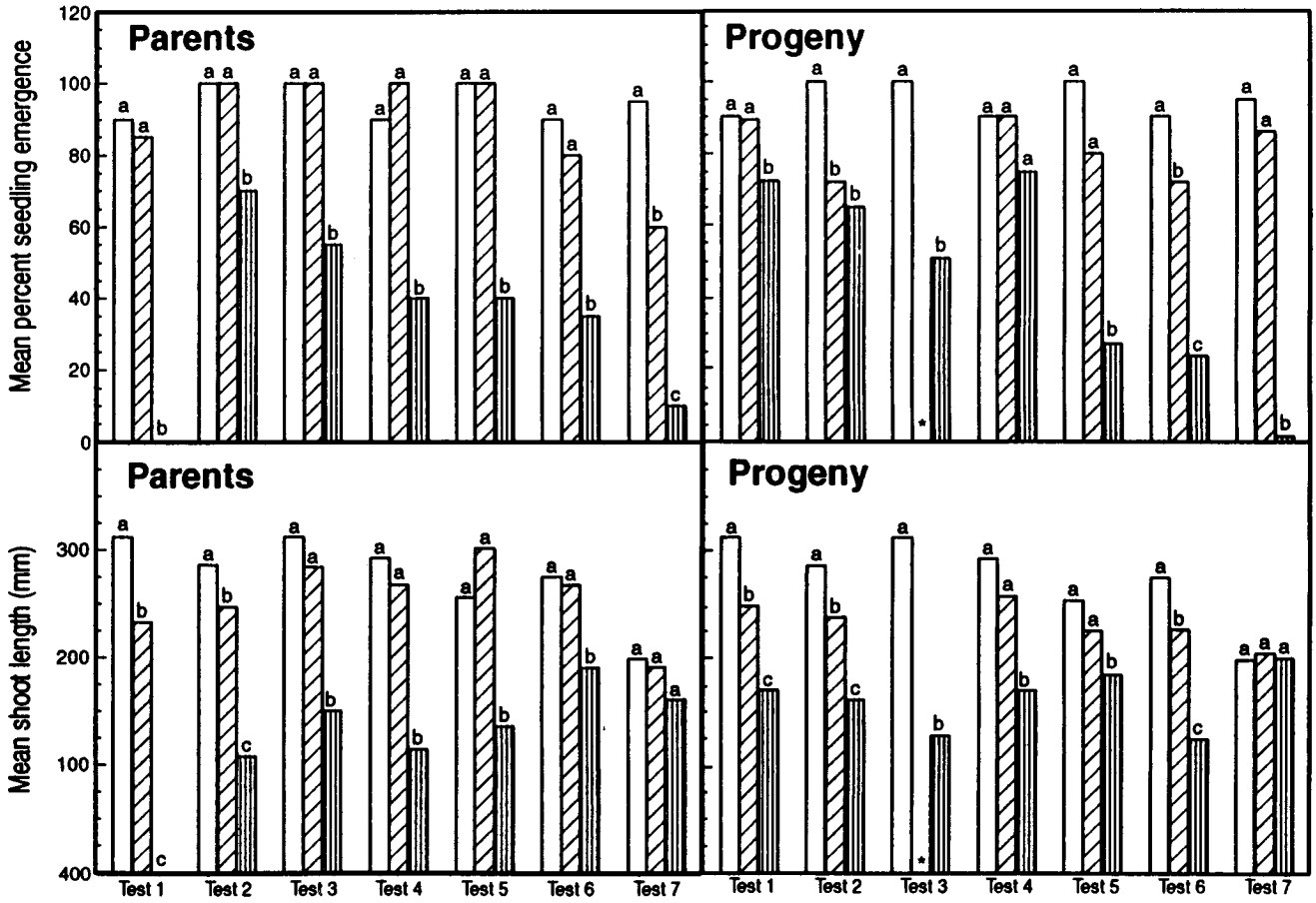

FIG. 2. Segregation of fumonisin production and virulence among random ascospore progeny of cross 57. Maize cultivar Silver Queen and *nit* mutants of *G. fujikuroi* were used for tests 1 through 5. Maize cultivar Golden Bantam and prototrophs were used for tests 6 and 7. Strains producing >10-ppm or ≤10-ppm fumonisin B_1 were scored as high and low producers, respectively. In each experiment, controls and parents M-3125 (high producer) and M-5500 (low producer) were tested simultaneously with the progeny. Tests included the following: 1, one fumonisin producer progeny and five nonproducer progeny, with two pots of 10 seedlings each per treatment; 2 and 7, three each of producer and nonproducer progeny, with two pots per treatment; 3 (*), six producer progeny, with two pots per treatment; 6, four each of producer and nonproducer progeny, with two pots per treatment. Tests 4 and 5 were replicates, with one pot per treatment, including seven producer progeny and nine nonproducer progeny. Open bars, means for control plants; diagonally and vertically striped bars, means for all low-producer and high-producer strains, respectively, parents or progeny. Within each test, bars labeled with the same letter are not significantly different on the basis of *t* tests of least-squares means at $P < 0.05$.

seedlings, pots of seedlings treated with parent M-3125 or parent M-5500, and pots of seedlings treated with progeny strains. The virulence tests did not always include equal numbers of fumonisin producer and nonproducer progeny because, for many progeny, virulence was tested before fumonisin production was assayed.

The data presented in Fig. 2 illustrate that the coefficients of variation between tests performed on different days were low for control seedlings (5% for seedling emergence and 12% for shoot length). For seedlings treated with the fumonisin-nonproducing parent, M-5500, coefficients of variation between tests were also low (16% for seedling emergence and 15% for shoot length). Strain M-5500-treated seedlings and control seedlings were significantly different from each other in seedling emergence in test 7 only and were significantly different in shoot length in tests 1 and 2 only. Virulence data for seedlings treated with the fumonisin-producing parent, M-3125, were more variable from day to day; coefficients of variation were 62% for seedling emergence and 25% for shoot length for tests performed on different days. Statistical analysis by *t* test indicated that seedlings treated with strain M-3125 had significantly ($P \leq 0.05$) lower emergence scores than both control seedlings and M-5500-treated seedlings in all tests and lower shoot lengths in six of seven tests. These results confirmed the earlier conclusion that strain M-3125 is more virulent than M-5500 on maize seedlings.

For genetic analysis of the importance of fumonisin in virulence expression, progeny were grouped by fumonisin phenotype (producer or nonproducer) for statistical analysis of each of the individual tests, 1 through 7. Although fumonisin production segregated as a discontinuous variable among the progeny of cross 57, virulence did not segregate into two discrete, discontinuous classes of high- and low-level virulence in any of the tests. The data presented in Fig. 2 show, however, that the group of seedlings treated with fumonisin-producing progeny was significantly ($P \leq 0.05$) different from control seedlings and from the group of seedlings treated with fumonisin-nonproducing progeny in seedling emergence in five of seven tests and in shoot length in six of seven tests.

To compare virulence data for progeny tested on different days, seedling emergence and shoot length data for each pot of seedlings treated with parent or progeny strains were normalized to the seedling emergence and shoot length data of control seedlings tested on the same day. Data were expressed as

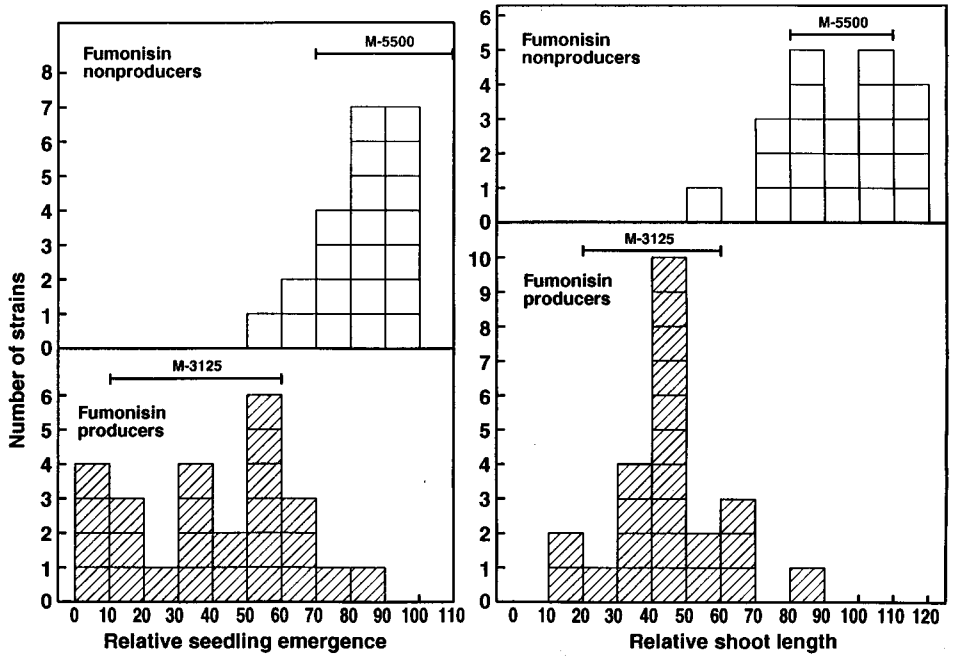

FIG. 3. Frequency distribution histograms of virulence of progeny from cross 57 producing high and low levels of fumonisin. The experiments are described in the legend to Fig. 1. Each square represents the mean for all tests of one progeny strain. The means ± standard deviations of virulence ($n = 12$) for parent strains M-3125 and M-5500 are shown (horizontal lines). Virulence data for each strain were normalized to data for the control seedlings tested at the same time.

the means of all replicate tests of each strain and plotted in frequency distribution histograms for fumonisin-producing and -nonproducing parents and progeny (Fig. 3). Comparison of the inheritance pattern of fumonisin production with the inheritance pattern of seedling virulence indicated two important points. First, highly virulent, fumonisin-nonproducing progeny were not recovered in this cross. Second, some fumonisin-producing progeny were less virulent than the fumonisin-producing parent, M-3125. These data support the hypothesis that fumonisin plays a role in virulence on maize seedlings. These data also suggest that other virulence genes may be segregating on cross 57.

Recovery of *nit* mutants from infected seedlings. In cross 57 virulence experiments 1 through 4, *Fusarium* spp. were reisolated from maize leaf tissue from each pot of seedlings infected with *nit* mutants and tested for complementation with the strain used for inoculation. In these experiments, no *Fusarium* spp. were recovered from control seedlings and no wild-type strains of *G. fujikuroi* were recovered from any of the leaf pieces. *nit* mutants were recovered from leaves from pots of seedlings infected with both of the parent strains and with 30 of the 32 progeny strains, although *Fusarium* spp. were not recovered from every leaf piece plated on selective medium. Complementation tests indicated that the reisolated *nit* mutants were usually identical to the inoculated strain in vegetative compatibility. Among *G. fujikuroi* colonies recovered from individual leaf pieces, rates of overall recovery of inoculated strains were 100, 71, 95, and 84% for experiments 1 through 4, respectively. The contaminating *nit* mutants probably originated from other inoculated pots in the growth chamber.

In order to validate the use of *nit* mutants for the tracking experiments, it was necessary to demonstrate that the majority of progeny tested were vegetatively incompatible with most of the other progeny. Parent M-5500 and 11 progeny were self-compatible but unable to complement any other strain and thus belonged to unique VCGs. Parent M-3125 and the remaining 40 progeny were members of one of seven VCGs. The recovery of 18 VCGs among 51 progeny is consistent with the hypothesis that parents M-3125 and M-5500 differ at five or more *vic* loci. There was also little evidence of linkage between VCG and fumonisin production phenotype. For example, ratios of progeny producing high levels of fumonisin to those producing low levels of fumonisin were 7:3, 2:5, 5:0, 4:2, and 4:2 for the five largest VCGs. Mating type is known to be unlinked to *fum1* (26).

Effects of adding fumonisin B_1 and of autoclaving inocula on symptom expression. A final series of experiments was performed to further investigate the role of fumonisins in production of disease symptoms on maize seedlings (Table 4). To detect fumonisin-specific activity, purified fumonisin B_1 was

TABLE 4. Effects of adding fumonisin B_1 and of autoclaving inocula on disease symptom expression of strains M-3125 and M-5500

Strain	Treatment[a]	Seedling virulence[b]	
		% Emerged of 90 planted	Mean shoot length (mm)
None (control)	None	81	246 ab
	Fumonisin B_1	72	224 bc
M-5500	None	58	213 c
	Autoclaving	80	267 a
	Fumonisin B_1	38	215 bc
M-3125	Autoclaving	46	175 d
	None	14	134 d

[a] Prior to seedling assays, each gram of fungal inoculum and control cornmeal was mixed with 1 ml of water (no treatment) or with 2.0 g of fumonisin B_1 in 1 ml of water.

[b] Results of three replicate tests of all treatments, with 30 seedlings of cultivar Golden Bantam per treatment per test. The assay was as described in Table 1 footnote *b*. Values followed by the same letter are not significantly different on the basis of *t* tests of least-squares means at $P < 0.05$.

added to a final level of 2,000 ppm to the maize meal inocula used for virulence assays. The addition of fumonisin to maize meal alone or to maize meal inoculated with the fumonisin-nonproducing strain M-5500 resulted in decreased emergence and seedling height compared with comparable treatments without added fumonisin. Neither fumonisin treatment, however, reproduced the level of symptoms produced by the fumonisin-producing strain M-3125. These results suggest that strain M-3125 has abilities in addition to fumonisin biosynthesis that are responsible for its ability to induce seedling blight symptoms.

To determine whether symptom expression was associated only with the presence of viable propagules of *G. fujikuroi*, maize meal inocula were autoclaved before the testing. Fumonisins in a maize matrix have been shown to be heat stable (6). Autoclaving for 30 min killed all fungal propagules and abolished symptom expression of the fumonisin-nonproducing strain M-5500 on maize seedlings but had little effect on symptom expression of the fumonisin-producing strain M-3125. The observation that purified fumonisin B_1 did not cause as many blight symptoms as the autoclaved culture material of strain M-3125 (Table 4) is indirect evidence that additional heat-stable toxins may be involved.

DISCUSSION

Maize seeds planted in sand infested with *G. fujikuroi* mating population A showed extensive symptoms of seedling blight. Both fumonisin-producing and fumonisin-nonproducing field strains and ascospore progeny were able to colonize seedling leaf tissues and cause disease symptoms. These data indicate that fumonisin production is not required for virulence, at least as defined by seedling blight. On the other hand, the genetic analysis indicated that only fumonisin-producing progeny produced a high level of virulence. Although fumonisin-producing, weakly virulent recombinant progeny were obtained, fumonisin-nonproducing, highly virulent progeny were not. In contrast, a survey of field strains identified a rare fumonisin-nonproducing Nepalese strain that was quite virulent. Thus, fumonisin appears to play a role in virulence, but fumonisin production is not necessary or sufficient for virulence on maize seedlings. Fumonisin could therefore be classed as a virulence factor in maize seedling blight, because it can affect "the amount of extent of disease caused" (27). In this regard, fumonisins appear to be similar to the trichothecene toxins, which are virulence factors in *Fusarium* diseases of some plants (4). Fumonisins also resemble the trichothecenes in the host nonspecificity of their phytotoxicity and in their ability to cause a variety of diseases in animals (16, 19).

The diverse toxicological effects of fumonisins on a variety of animals have led to extensive research on their biochemical mode of action. The acute equine neurotoxicity of fumonisins and their structural similarity to the sphingolipid precursor sphingosine led Wang et al. (25) to propose and subsequently demonstrate that fumonisins affect sphingolipid metabolism. Although sphingolipids are particularly rich in neuronal tissues, they are also important membrane components of many other types of eukaryotic cells, including plant cells, and appear to be involved in the regulation of cell growth and differentiation (17). Thus, fumonisin inhibition of sphingosine metabolism could be at the core of a wide range of physiological effects, including tumor promotion in animal cells and toxicity to plant cells.

The retention of phytotoxicity in autoclaved culture material of the fumonisin-producing strain M-3125 indicates that colonization of seedling tissue is not necessary for expression of some blight symptoms. The observation that autoclaved culture material of the fumonisin-nonproducing strain M-5500 produced no symptoms suggests that the phytotoxicity was not due to autoclaving per se but was specific to strain M-3125. Although fumonisin is heat stable and could be responsible for the phytotoxicity of autoclaved culture material, exogenously added fumonisin had only a limited effect on seedlings treated either with the maize meal control or with the fumonisin-nonproducing strain M-5500. Strains M-3125 and M-5500 differ in many ways besides fumonisin production. They were recovered by different investigators (P. T. Spieth and P. E. Nelson, respectively) from maize grown in different geographic areas (California and Nepal) at different times (late 1970s and late 1980s). Although both strains carry the spore killer-sensitive allele (5, 8), they differ in mating type and at several of the *vic* loci that determine vegetative compatibility and at numerous restriction fragment length polymorphism markers scattered throughout the genome (26). Thus, it will not be surprising if these strains also differ in their abilities to synthesize additional phytotoxins, a hypothesis that we are presently testing.

Fumonisins are highly toxic to a wide range of organisms, but their specific function, if any , in the survival of the fungi that produce them is not obvious. In common with many other fungal secondary metabolites, fumonisins apparently are not essential for fungal growth or reproduction in vitro. Field strains of mating population A that do not produce fumonisins are very rare (5, 14, 15, 20) but appear to grow as vigorously as fumonisin-producing strains and to retain sexual fertility. The classical genetic analysis in the present study has provided preliminary evidence that fumonisins are virulence factors, but this type of evidence is relatively inconclusive. Localization of the *fum1* locus by restriction fragment length polymorphism mapping (26) should facilitate the development of a molecular genetic analysis of the importance of fumonisins as virulence factors of *G. fujikuroi* mating population A on maize.

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