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Maize Ear Rot and Moniliformin Contamination by Cryptic Species of *Fusarium subglutinans*

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Fusarium subglutinans causes maize ear rot and contaminates grain with the mycotoxin moniliformin. Previous DNA sequence analysis divided *F. subglutinans* from maize into two cryptic species, designated groups 1 and 2. Here, it was determined whether the two groups differ in the agriculturally important traits of virulence on maize and moniliformin production in planta. Thirty-seven strains from U.S. maize were assigned to groups 1 and 2 by DNA sequence analysis. In field tests, all strains were highly virulent on maize inbred B73 and four maize hybrids. In planta, 82% of group 1 strains and 25% of group 2 strains produced high levels (100−1500 μg/g) of moniliformin. All group 2 strains from more northern states produced little or no moniliformin (0−5 μg/g). These data indicate that moniliformin production is highly variable in *F. subglutinans* from U.S. maize and that production may not be required for the fungus to cause maize ear rot.

KEYWORDS: ***Fusarium subglutinans*; moniliformin; maize ear rot**

INTRODUCTION

Moniliformin (MON), 3-hydroxy-cyclobut-3-ene-1,2-dione, has been reported to be produced only by the fungal genus *Fusarium* (*1*). Precursor feeding experiments indicate that MON is derived from a diketide, but the biosynthetic pathway is unknown (*2*). The original isolation of MON from *Fusarium* culture material in 1973 was guided by a bioassay for toxicity to 1-day-old chickens (*3*). Subsequent studies confirmed that MON was acutely toxic to animals, but at relatively high dosages, with LD_{50} values of 5 and 25 mg /kg of body weight in chickens and mice, respectively, following intraperitoneal injection with the pure compound (*4*, *5*). A variety of studies have also associated MON with subacute and chronic toxicity to animals. In rodents, MON causes significant pathological effects on tissues of the heart (*6*, *7*). Inhibition of free radical scavenging enzymes was proposed as a mechanism for the toxicity of MON to the rodent heart. In addition, MON inhibits a variety of pyruvate-dependent metabolic pathways, including the mitochondrial oxidation of pyruvate and ketoglutarate, which is consistent with the respiratory distress and muscle weakness caused in avian species and rodents by treatment with MON (*8*−*10*). Although purified MON appears to have only moderate toxicity in animal-feeding experiments, *Fusarium* culture materials that contain MON are acutely toxic to animals (*11*). Furthermore, a higher frequency of MON contamination of maize has been associated with the higher rates of Keshan heart disease among human populations in certain regions of China (*12*, *13*). Thus, although MON has not been directly associated with any human or animal disease outbreaks, additional information is needed on toxicity and interactions of MON with other mycotoxins and on contamination of cereal grains with MON and with MON-producing *Fusarium* species.

Although surveys have been limited, MON production appears to be distributed rather widely in the genus *Fusarium*, including species that also produce the trichothecene or fumonisin classes of mycotoxins (*14*). Individual species, however, do vary in the frequency of MON-producing strains and in the level of MON produced. Most notably, many strains of *F. proliferatum* and *F. subglutinans* produce high levels of MON, but their sister species *F. verticillioides* very rarely produces MON. Both *F. proliferatum* and *F. subglutinans* can cause maize (*Zea mays*) ear rot; thus, MON potentially could occur in maize-based foods and feeds. The natural occurrence of MON was first reported in 1982 in South Africa, at levels of 16 and 25 μg/g in two samples of moldy maize (*15*). In South Africa and in Europe, MON contamination of maize has often been associated with the presence of *F. subglutinans* (*15*, *16*). Very little is known, however, about MON contamination of maize in North America. MON was present above the detection limit of 0.05 μg/g, but below 0.2 μg/g, in 11 samples of maize meal purchased at retail markets in the United Kingdom but originating from the United States (*17*). MON was present above the detection limit of 0.02 μg/g in 54% of 134 samples of food grade maize and maize-based products from the United States, but levels were below 0.90 μg/g in all samples (*18*).

F. subglutinans was first described in 1925 as a variety of *F. moniliforme* and emended to *F. subglutinans* in 1983 (*19*). Like its sister species *F. verticillioides* and *F. proliferatum*, *F. subglutinans* is associated with diseases at all stages of maize plant development, infecting the roots, stalks, and ears, and also can be found colonizing symptomless maize plants, especially

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Table 1. Description of *F. subglutinans* Strains in This Study

sample source				DNA group[a]		
strain no.[b]	location	year	sample type	TEF	HB26	MAT type[c]
M-1041	MI	1978	maize soil debris	2	2	NF
M-1042	MI	1978	maize soil debris	2	2	2
M-1185	PA	1980	sweet maize	2	2	2
M-1318	NY	1982	animal feed maize	1	1	2
M-1851	MN	1984	maize stalk	2	2	2
M-1972	PA	1984	maize seed	2	2	2
M-3693	IL	1986	maize	2	2	2
M-3696	IL	1986	maize	2	2	1
M-3763	OH	1986	maize	2	2	2
M-5119	KS	1988	maize	2	2	2
M-5126	KS	1988	sorghum	2	2	2
M-6924	KS	1992	maize	2	2	2
M-7330	WI	1993	maize seed	1	1	NF
M-7331	WI	1993	maize seed	2	2	2
NSM 39	NM	1999	San Felipe Pueblo white maize seed	2	2	2
NSM 56	AZ	1999	Apache mix maize seed	2	2	2
NSM 107	NM	1999	Acoma Pueblo blue maize seed	2	2	1
NSM 136	AZ	1999	Apache mix maize seed	1	2	2
NSM 188	NM	1999	Santo Domingo Pueblo white maize seed	2	2	2
NSM 194	NM	1999	Hispanic Pueblo red maize seed	1	2	NF
NSM 197	NM	1999	Velarde Pueblo blue and white maize seed	2	1	2
NSM 198	NM	1999	Velarde Pueblo blue and white maize seed	1	1	NF
NSM 221	AZ	2001	Hopi white maize seed	1	1	2
NSM 224	AZ	2001	Hopi white maize seed	1	1	2
NSM 225	NM	2001	Isleta Pueblo white maize seed	1	1	2
NSM 226	NM	2001	Isleta Pueblo white maize seed	1	1	2
NSM 228	AZ	2001	Hopi yellow maize seed	1	1	NF
NSM 229	AZ	2001	Hopi yellow maize seed	1	2	NF
NSM 237	AZ	2001	Hopi yellow maize seed	1	1	2
NSM 249	AZ	2001	Hopi blue maize seed	1	1	2
NSM 254	AZ	2001	Hopi blue maize seed	1	1	NF
NSM 263	NM	2001	Santo Domingo Pueblo blue maize seed	1	1	2
NSM 264	NM	2001	Santo Domingo Pueblo blue maize seed	1	1	NF
NSM 266	NM	2001	Santo Domingo Pueblo blue maize seed	2	2	2
NSM 269	NM	2001	Hernandez Pueblo multicolor maize seed	1	1	2
NSM 270	NM	2001	Santo Domingo Pueblo posole maize seed	2	2	2
NSM 271	NM	2001	San Felipe Pueblo mix maize seed	2	2	2

[a] TEF indicates partial sequence of the translation elongation factor 1-α gene, and HB26 indicates a single nucleotide polymorphism of the locus HB26, as described under Materials and Methods. [b] Strain designations: M indicates a strain from the Fusarium Research Center, and NSM indicates a strain isolated for this study. [c] MAT type indicates the mating type as determined by crosses to tester strains of *F. subglutinans.* NF = not fertile.

seeds (*20*). Unlike these two sister species, *F. subglutinans* does not usually produce fumonisins or contain fumonisin biosynthetic genes (*21*). However, *F. subglutinans* is highly toxigenic and, in addition to MON, produces the mycotoxins beauvericin, fusaproliferin, and fusaric acid (*14*). Strains of *F. subglutinans* that produce MON in vitro have been isolated from maize or animal feeds in Argentina, Canada, Peru, Poland, Slovakia, South Africa, and Iowa in the United States (*11*, *22*−*30*).

The taxonomy of the genus *Fusarium* has been revolutionized by the application of biological species concepts based on sexual cross-fertility and phylogenetic species concepts based on DNA sequence polymorphisms. By these methods, *F. subglutinans* from maize has been distinguished from the morphologically similar species *F. circinatum*, which causes pitch canker of pine, and *F. mangiferae*, which causes mango malformation (*31*). Phylogenetic analysis of sexually fertile strains of *F. subglutinans* from maize and related *Zea* species (teosintes) revealed that the biological species *F. subglutinans* is divided into two phylogenetically distinct major groups, which have been designated groups 1 and 2 (*31*−*33*). Phylogenetic concordance analysis indicated that groups 1 and 2 were reproductively isolated in nature, even though the groups were interfertile in the laboratory and both groups were present at the same time and place in samples of maize and teosinte collected in a field near Texcoco, Mexico. Because *F. subglutinans* groups 1 and 2 are morphologically indistinguishable but do not appear to form hybrids in nature, they can be defined as cryptic species.

The major aim of the present study was to determine whether the two cryptic species of *F. subglutinans* differ in the agriculturally important traits of virulence on maize and production of MON. The approach was to characterize a collection of 37 *F. subglutinans* strains from maize from Arizona, New Mexico, and the more northern United States for genetic variability, for ability to cause maize ear rot in field tests, and for MON production in infected ears. Such an analysis should provide additional information on the distribution of the two cryptic species of *F. subglutinans* on maize in the United States and on their potential impact on maize production.

MATERIALS AND METHODS

Fungal Strains. Strains of *F. subglutinans* used in this study are described in **Table 1**. Fourteen strains (designated with the prefix M) were obtained from the Fusarium Research Center, The Pennsylvania State University, University Park, PA. Twenty-three strains (designated with the prefix NSM) were isolated from maize seed samples obtained in 1999 and 2001 from Native Seeds/SEARCH, Tucson, AZ, a nonprofit organization for conservation of Native American landraces of plants from the greater southwest region. For fungal isolations, seeds were surface disinfested by placing them in 0.5% sodium hypochlorite for 1 min and were rinsed twice in sterile water. Fifty seeds were tested for

each of the 1999 samples and 100 seeds for each of the 2001 samples. Surface-disinfested seeds were softened in sterile water for ≈1 h, then split open with a sterile knife, and placed, cut surface down, on a *Fusarium*-selective agar medium containing 1% pentachloronitrobenzene (*19*). Seeds were incubated for 5−7 days, and then one colony per seed was purified by re-isolation from a single spore and identified to species using morphological criteria (*19*).

To determine their sexual fertility, mating population, and mating type, *F. subglutinans* strains were crossed to standard tester strains of three species obtained from the Fusarium Research Center. Tester strain numbers were as follows: *F. subglutinans* M-3693 (*MATE-2*) and M-3696 (*MATE-1*), *F. thapsinum* M-6563 (*MATF-2*) and M-6564 (*MATF-1*), and *F. verticillioides* M-3125 (*MATA-1*) and M-3120 (*MATA-2*). Strains were tested twice as males on carrot agar medium as described (*34*) except that incubation conditions were constant light at 20 °C for up to 6 weeks. Crosses were scored as fertile when ascospores were observed upon microscopic examination of the contents of enlarged perithecia.

Fungal inoculum for field tests was prepared from strains incubated as described above on V-8 juice (Campbell Soup Co., Camden, NJ) agar for 1−2 weeks. Inoculum was produced by washing spores from plates using modified Bilay's medium (*35*) to a final concentration of 1×10^6 or 5×10^6 spores/mL. Spore suspensions were prepared in the laboratory and applied in the field on the same day. Production of MON was assessed in a cracked maize medium consisting of 10 g of maize and 4 mL of water autoclaved in a 50-mL Erlenmeyer flask. Cultures were inoculated with a plug from an agar culture and incubated in the dark at 25 °C for 4−14 days.

DNA Isolation, Amplification, and Sequencing. For sequence analyses, genomic DNA was purified with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) protocol from lyophilized mycelia. Sequencing templates were amplified from genomic DNA by PCR, and amplification products were purified by the UltraClean (MoBio Laboratories, Solana Beach, CA) method. Sequencing reactions were done with the BigDye Terminator Cycle (Perkin-Elmer Applied Biosystems, Foster City, CA) protocol. Following the cycle sequencing procedure, reactions were passed through a Sephadex G-50 column, dried under vacuum, suspended in formamide, and subjected to electrophoretic analysis with a 3730 DNA Analyzer (Applied Biosystems). To confirm species identification of *F. subglutinans* strains, translation elongation factor 1 gene (*TEF1*) sequence data were submitted to the FUSARIUM-ID database (*36*). Phylogenetic analysis of *TEF1* sequences was done with the Phylogenetic Analysis Using Parsimony (PAUP) program, version 4.0b10 (Sinauer Associates, Sunderland, MA).

For analysis of the single nucleotide polymorphism (SNP) in locus HB26, a 210-bp fragment was amplified from genomic DNA and then sequenced with primers HB3 (5′-ACAATGGCTTTCTTGATACC-3′) and HB5 (5′-AGTGTCAGAAAGTAAAAGCC-3′). The SNP is located at position 69 of locus HB26 and consists of a T/A in group 1 and a G/C in group 2 (*33*). For analysis of the translation elongation factor 1 gene (*TEF1*) sequence, a ≈700-bp fragment of the gene was amplified from genomic DNA by PCR with primers EF1 (5′-ATGGGTAAGGARGACAAGAC-3′) and EF2 (5′-GGARGTACCAGTSATCATGTT-3′) (*36*). The amplification products were sequenced with primers EF1, EF2, and EF22 (5′-AGGAACCCTTACCGAGCTC-3′).

Field Tests. Three field tests of *F. subglutinans* were conducted in Peoria County, Illinois: test 1 was of 20 fungal strains on maize inbred B73 in 2003, test 3 was of the same 20 fungal strains on four maize hybrids in 2004, and test 2 was of 17 additional fungal strains on maize inbred B73 in 2004. Seeds of maize inbred B73 were obtained from M. Muhitch (Peoria, IL). The four hybrids comprised two sets of widely used, commercial hybrids: each set contained one transgenic hybrid that carried a gene for Bt toxin and a corresponding nontransgenic hybrid. Hybrids were (A) Pioneer 34N43, (B) Pioneer 34N44 Bt, (C) Dekalb 60-15 CN, and (D) Dekalb 60-16 Bt.

Field tests 1 and 2 each consisted of a plot of maize inbred B73 that contained 15 rows each 1 m apart and 20 m long. The plot was divided into three replicate subplots, each five rows wide and 20 m long. Each subplot was a randomized complete block with 21 treatments in 2003 and 18 treatments in 2004. Field test 3 consisted of plots of four maize hybrids that each contained 24 rows ≈1 m apart and 24 m long. The plots were separated from each other by 3-m borders of cultivated ground, and the entire field test was surrounded by 3 m of cultivated ground. Each plot was divided into three replicate subplots, each eight rows wide and 24 m long. Each subplot was a randomized complete block with 21 treatments.

For all field tests, 2 mL of fungal spore suspension in Bilay's medium was injected into the center of the silk channel and above the cob of the primary ear 4−6 days after silk emergence. The concentration of spores per milliliter was 5×10^6 for tests 1 and 2 and 1×10^6 for test 3. Control ears were injected with Bilay's medium. For each treatment, there were three replicates, with 10 ears per replicate. After physiological maturity and after some drying had occurred, ears from the treated plants were hand-picked, husked, and air-dried in the laboratory for at least 1 month.

For field tests 1 and 2, all ears were harvested and disease severity ratings were recorded in the laboratory. For field test 3, disease severity ratings for all ears were evaluated in the field, but only ears from selected treatments were harvested for further evaluation. Each ear was individually evaluated using a disease severity rating scale based on visual estimation of ear rot as the percentage of visibly damaged seeds on an ear as follows: 1 = 0%, 2 = 1−3%, 3 = 4−10%, 5 = 26−50%, 6 = 51−75%, and 7 = 76−100% (*37*). Each ear was individually 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. Nonsymptomatic and symptomatic fractions of each ear were separately weighed.

Disease severity rating, kernel weight, percent ear rot by weight, and moniliformin data were statistically analyzed using the program SAS for Windows version 9.1.3 (SAS Institute Inc., Cary, NC). For data shown in **Table 2**, treatment means within a year were analyzed using a single-factor analysis of variance. If a significant *F*-test statistic was obtained at $p \leq 0.05$, differences of least-squares means with a Bonferroni adjustment were used as a multiple-comparison test to determine which treatments were different. Group means within a year were analyzed using a single-factor analysis of variance. Because there were only two groups, a significant *F*-test statistic at $p \leq 0.05$ indicated that the group means were statistically significantly different from one another. For **Table 3**, data for each hybrid were analyzed by single-factor analysis of variance to compare MSN, M, and control groups. If a significant *F*-test statistic was obtained at $p \leq 0.05$, Duncan's new multiple-range test was used to determine differences between the three groups.

Moniliformin Analysis. For MON analysis, symptomatic and asymptomatic kernels from all 10 replicate ears from each block were pooled, weighed, and ground. Ground samples were extracted and analyzed according to the method of Munimbazi and Bullerman (*38*). Briefly, 20 g of ground maize was extracted with 100 mL of water containing 1% of the ion-pair reagent tetrabutylammonium hydrogen sulfate (TBAHS). For laboratory-cultured samples of maize, the extraction method was the same, except that a lower amount (10 g) of maize was extracted. A portion of the aqueous solution was extracted with dichloromethane, into which the MON−TBA ion pair partitions. After drying, the dichloromethane extract was reconstituted with water and applied to a strong anion exchange (SAX) solid-phase extraction column. The SAX column was rinsed with water and the MON was eluted with 0.05 M sodium phosphate (pH 5). The isolated MON was then separated from interfering substances by reverse-phase high-performance liquid chromatography (RP-HPLC) and was detected by absorbance at 229 nm using previously described procedures (*38*, *39*). To validate the method in our laboratory, spiking and recovery studies were conducted and were published previously (*39*). Control ground maize containing <0.05 μg of MON/g was spiked with the appropriate volume of a stock solution of MON to give levels ranging from 0.2 to 5 μg of MON/g of maize. The spiked maize was held overnight at ambient temperature to ensure the spiking solution had dried. The spiked samples were then extracted and analyzed as described above. Recoveries averaged 87.8 ± 6.4% ($n = 16$).

RESULTS

Isolation and Identification of *F. subglutinans*. To obtain fungal strains for field tests, a highly diverse selection of

Table 2. Disease Severity Visual Ratings, Kernel Weight, Percentage Ear Rot by Weight, and Moniliformin Levels in Maize Kernels Following Application of *F. subglutinans* to Maize Inbred B73 in Field Tests in 2003 and 2004

year and group	treatment[a]	disease severity visual rating[b]	kernel wt (g)	percentage ear rot by wt[b]	moniliformin in kernels[c] (μg/g)
2003, 1	M-1318	6.9 a	23 d	92 ab	344
	M-7330	6.9 a	21 d	100 a	509
	NSM 194	6.8 a	16 d	95 a	535
	NSM 221	6.5 ab	26 cd	92 ab	261
	NSM 224	6.5 ab	35 bcd	91 ab	276
	NSM 226	6.6 ab	26 cd	99 a	320
	NSM 228	6.3 ab	32 bcd	79 abc	255
	NSM 229	6.4 ab	32 bcd	81 abc	331
	NSM 237	6.5 ab	39 bcd	84 abc	307
	NSM 249	5.7 abc	54 abc	77 abc	84
	NSM 254	6.5 ab	29 cd	83 abc	448
	group 1 mean	6.5 A	30 A	88 A	309
2003, 2	M-1185	6.0 abc	35 bcd	53 bc	0
	M-3693	5.1 c	61 ab	46 c	1.2
	M-3696	6.1 abc	27 cd	73 abc	1.2
	M-5126	6.6 ab	23 cd	89 ab	5.3
	M-6924	6.4 ab	33 bcd	86 abc	1.7
	NSM 56	6.2 abc	25 cd	79 abc	63
	NSM 107	6.8 a	21 d	98 a	1315
	NSM 197	5.5 bc	32 bcd	65 abc	80
	NSM 270	6.7 a	20 d	86 ab	842
	group 2 mean	6.2 B	31 A	75 B	125
2003	control	1.9 d	78 a	4 d	13
2004, 1	NSM 136	3.9 cd	50 abc	41 bcd	82
	NSM 198	4.8 bcd	48 abc	58 a–d	116
	NSM 225	5.9 ab	44 abc	63 a–d	159
	NSM 263	5.8 ab	46 abc	70 abc	212
	NSM 264	6.1 a	44 abc	75 abc	247
	NSM 269	3.6 d	66 ab	30 cd	33
	group 1 mean	5.0 B	50 A	56 A	191
2004, 2	M-1041	5.8 ab	42 abc	33 bcd	2.8
	M-1042	5.2 a–d	38 abc	41 bcd	0
	M-1851	5.6 abc	31 bc	50 a–d	0
	M-1972	5.6 abc	46 abc	42 a–d	0.3
	M-3763	5.9 ab	26 c	47 a–d	0.5
	M-5119	6.0 ab	41 abc	63 a–d	0
	M-7331	5.8 ab	47 abc	60 a–d	0.4
	NSM 39	6.6 a	30 bc	87 a	1039
	NSM 188	5.7 abc	45 abc	76 ab	767
	Nsm 266	6.1 ab	37 abc	70 abc	1529
	NSM 271	5.2 a–d	49 abc	22 d	39
	group 2 mean	5.8 A	39 B	54 A	307
2004	control	1.5 e	71 a	13 d	58

[a] Strains applied as treatments are described in **Table 1**. Control treatments were Bilay's medium. Inoculation 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 (*37*). Percentage ear rot was determined by weighing symptomatic and nonsymptomatic kernels from each ear. Treatment means in each column within a year followed by the same lower case letter are not significantly different based on differences of least-squares means with a Bonferroni adjustment at $p \leq .05$. Group means in each column within a year followed by the same upper case letter are not significantly different based on analysis of variance *F*-test statistics at $p \leq 0.05$. [c] Moniliformin levels in kernels were determined by RP-HPLC as described under Materials and Methods.

landraces of maize from the greater southwest region of the United States and Mexico was surveyed for contamination with *F. subglutinans* by culturing seeds on a *Fusarium*-selective medium. Fungal colonies typical of *Fusarium* were recovered from 102 of 135 maize seed samples. The great majority of fungal colonies contained chains of microconidia, a morphological trait of *F. proliferatum* and *F. verticillioides*, both of which are common contaminants of maize seed. These strains were not identified further because the focus of the study was *F. subglutinans*, which produces microconidia in false heads, never in chains (*19*). Fungal strains with morphology typical of *F. subglutinans* were recovered from 28 of the 135 maize seed samples. Among 13 samples of flour and flint maize from Native American pueblos along the Rio Grande River in New Mexico, from 1 to 5% of seeds were infected with *F. subglutinans*. In five samples of flour maize from the Apache and Hopi tribes of Arizona, from 4 to 10% of seeds were infected. Among 10 samples of flour and flint maize from the Tarahumara tribe of Chihuahua, Mexico, from 2 to 25% of seeds were infected. A total of 54 strains of *F. subglutinans* were isolated from maize from Arizona and New Mexico, and 61 strains were isolated from Mexican maize. However, because our ultimate goal was to conduct field tests and because foreign plant pathogens cannot be field-tested in the United States, the Mexican strains of *F. subglutinans* were not investigated further in this study.

DNA sequence data from a 210-bp portion of locus HB26 was used for a preliminary classification of *F. subglutinans* strains from the United States as group 1 or group 2. On the basis of a single nucleotide polymorphism, 25 strains from Arizona were group 1 and 1 strain was group 2; 10 strains from New Mexico were group 1 and 14 were group 2. Ten additional U.S. *F. subglutinans* strains obtained from a culture collection were classified by HB26 sequence data as group 1 (2 strains) or group 2 (8 strains). From this collection, 9 strains from Arizona, 14 strains from New Mexico, and 8 strains from more northern states (Michigan, Minnesota, New York, Pennsylvania, and Wisconsin) were selected for additional DNA analysis of the translation elongation factor 1-α (TEF) gene using the FUSARIUM-ID database (*36*). Six additional strains (M-3693, M-3696, M-3763, M-5119, M-5126, and M-6924), which previously had been characterized as *F. subglutinans* group 2 by DNA sequence analysis of six genes, were also included in the TEF gene analysis (*33*). TEF gene sequence analysis confirmed the identification of all 37 strains as *F. subglutinans*. Furthermore, phylogenetic analysis based on the TEF gene sequence also split the 37 strains into two groups, which were congruent with groups 1 and 2 as defined in the previous study (*33*) and, for all but 4 strains, congruent with groups 1 and 2 as defined by the preliminary HB26 single-nucleotide polymorphism analysis (**Table 1**). For 2 strains from Arizona and 2 from New Mexico, the HB26 single nucleotide marker and TEF gene sequence were discordant, and when this occurred, the strain was moved to the group assigned by the more conclusive TEF gene sequence.

Identification of the 37 strains as *F. subglutinans* was also investigated by determining their sexual fertility as males with standard, female-fertile, tester strains of *F. verticillioides* (sexual stage: *G. moniliformis*, *G. fujikuroi* mating population A); *F. proliferatum* (sexual stage: *G. intermedia*, *G. fujikuroi* mating population D); and *F. subglutinans* (sexual stage: *G. subglutinans*, *G. fujikuroi* mating population E). None of the strains were fertile with tester strains of *F. verticillioides* or *F. proliferatum*. Ten of 17 group 1 strains and 19 of 20 group 2 strains were fertile with tester strains of *F. subglutinans*, confirming their identification as this biological species. Distribution of mating types was highly skewed, with a *MAT1*:*MAT2* ratio of 2:27 (**Table 1**).

Table 3. Disease Severity Visual Ratings of Maize Ears Following Application of *F. subglutinans* to Four Maize Hybrids in a Field Test in 2004 (Values Are Means Followed by Range in Parentheses)

treatment[a]	hybrid A[b]	hybrid B	hybrid C	hybrid D
group 1 (11 strains)	4.7 (4.0–5.5) a	5.0 (4.0–5.6) a	5.6 (5.1–6.4) a	5.4 (4.8–6.1) a
group 2 (9 strains)	4.9 (4.4–5.6) a	5.0 (4.0–6.0) a	5.4 (4.2–6.1) a	5.5 (4.8–6.4) a
NSM group (13 strains)	4.9 (4.0–5.6) a	5.0 (4.4–6.0) a	5.7 (5.1–6.4) a	5.5 (4.8–6.4) a
M group (7 strains)	4.6 (4.2–4.9) a	5.0 (4.2–5.6) a	5.3 (4.2–5.9) a	5.4 (4.8–6.0) a
control	1.8 b	1.2 b	1.8 b	1.0 b

[a] Strain groups are as described in **Table 1**. [b] Disease severity was scored as described in **Table 2**. Group means for each hybrid followed by the same letter are not significantly different based on Duncan's multiple-range test at $p \leq 0.01$.

Although the sample size is rather limited, geographic distribution of *F. subglutinans* groups 1 and 2 in the United States appears to be highly skewed. Among maize samples from the more northern states of Illinois, Kansas, Michigan, Minnesota, New York, Ohio, Pennsylvania, and Wisconsin, group 1 strains were relatively rare. Group 2 strains were relatively rare in maize landraces from the Apache and Hopi tribes in Arizona. Group 1 and 2 strains were represented roughly equally in landraces from Native American pueblos in New Mexico. Both strain groups co-occurred in individual maize samples from the White Mountain Apache Reservation, from the Santo Domingo Pueblo, and from the Velarde Pueblo.

Maize Ear Rot. The ability of *F. subglutinans* groups 1 and 2 to cause ear rot was compared by injecting spores into the silk channel of maize inbred B73 in field test 1 in 2003 and in field test 2 in 2004 (**Table 2**). Fungal strains were selected to represent the two genetically defined cryptic species and some of the geographical areas in which they occur. Disease was assessed on each individual ear by three parameters: a visual rating of disease severity, total kernel yield per ear, and percentage ear rot by weight. In field test 1, 11 strains of group 1 and 9 strains of group 2 were tested. Disease levels in control ears were low, with a disease severity visual rating of 1.9 and percentage ear rot by weight of 4%. In test 1, 18 of 20 strains of both groups 1 and 2 were significantly different from controls ($p \leq 0.05$) by three disease parameters; all strains were significantly different from controls by at least two parameters. The disease severity visual rating was significantly higher at 6.5 for group 1 than at 6.2 for group 2, of a maximum possible rating of 7.0. The kernel yield was not significantly different at 30% of control ears for group 1 and 31% for group 2. The percentage ear rot by weight was significantly higher at 88% for group 1 than at 75% for group 2. Thus, in test 1, all strains of groups 1 and 2 were virulent, and group 1 was significantly more virulent than group 2 by two of three parameters tested, although the differences were minor.

In field test 2, an additional 6 strains of group 1 and 11 strains of group 2 were tested on maize inbred B73 (**Table 2**). Disease levels in control ears were more variable than in test 1, with a disease severity visual rating of 1.5 and percentage ear rot by weight of 13%, which masked some differences between controls and strain treatments. Disease levels in fungal treated ears were generally lower in test 2 than in test 1. All 17 strains of both groups 1 and 2 were significantly different from controls by disease severity visual rating, and 7 strains were significantly different from controls by at least two parameters. The disease severity visual rating was significantly higher at 5.8 for group 2 than at 5.0 for group 1. The kernel yield was significantly lower at 39% of control ears for group 2 than at 50% for group 1. The percentage ear rot by weight was not significantly different at 56% for group 1 and 54% for group 2. Thus, in test 2, all strains of groups 1 and 2 were virulent, and group 2 was significantly more virulent than group 1 by two of three parameters tested, although the differences were minor.

In field test 3, the 20 strains of *F. subglutinans* that had been assessed in test 1 on maize inbred B73 were assessed further on four commercial maize hybrids. Disease was assessed on each individual ear of each hybrid by a visual rating of disease severity, for a total of 120 ears per treatment (**Table 3**). Disease levels in control ears were low, with disease severity visual ratings of 1.0−1.8 for all hybrids. All strains of both groups 1 and 2 were virulent on all four hybrids. The average disease severity visual ratings for groups 1 and 2 on the four hybrids were not significantly different, with a rating of 5.2 for group 1 and 5.3 for group 2. Strains also were grouped on the basis of geographic origin, with one group comprising 13 NSM strains from Arizona and New Mexico and the other group comprising 7 M strains from the more northern states (**Table 1**). The average disease severity visual rating for these groups on the four hybrids was again not significantly different, with a rating of 5.3 for the NSM strains and 5.1 for the M strains (**Table 3**).

In field test 3 of the four maize hybrids, not all ears were harvested for further assessment. The five treatments selected for further assessment were controls, a MON-producing group 1 strain (NSM 229) and three group 2 strains that included a MON nonproducer (M-1185) and two MON producers (NSM 56 and NSM 107) (**Table 4**). All four fungal strains were virulent on all four hybrids, whether disease was assessed by disease severity visual rating (average of 5.2), kernel yield (average of 51% of controls), or percentage ear rot by weight (average of 35%).

Moniliformin in Maize Ear Rot. The high levels of ear rot produced by *F. subglutinans* in the field tests allowed a thorough investigation of the production of MON in maize under field conditions. Background contamination of control ears with MON occurred at 13, 58, and 2 μg/g in tests 1, 2, and 3, respectively, but in general did not obscure differences that resulted from the different strain treatments (**Tables 2** and **4**). In the two tests on maize inbred B73, 5 group 2 strains from New Mexico produced very high levels of MON, ranging from 767 to 1529 μg/g. Eighty-five percent of the 17 group 1 strains, including the only 2 group 1 strains from the more northern states, also produced relatively high levels of MON, ranging from 116 to 535 μg/g. In contrast, all 12 group 2 strains from the more northern states produced little or no MON (0−5 μg/g), even in ears with >80% ear rot by weight.

MON production phenotypes of 8 *F. subglutinans* strains were tested further by culture on a substrate of autoclaved cracked maize kernels. Uninoculated substrate contained no detectable MON. A time-course experiment demonstrated that strain NSM 107, which produced 1315 μg/g of MON in planta, produced 15, 1200, and 2694 μg/g of MON in vitro after incubation for 4, 8, and 14 days, respectively. Therefore, the other 7 strains were analyzed for MON production after 14 days of incubation.

Table 4. Disease Severity Visual Ratings, Kernel Weight, Percentage Ear Rot by Weight, and Moniliformin Levels in Maize Kernels Following Application of *F. subglutinans* to Maize Hyrids in a Field Test in 2004

treatment and group[a]	hybrid	disease severity visual rating[b]	kernel wt (g)	percentage ear rot by wt[b]	moniliformin in kernels[c] (μg/g)
NSM 229, 1	A	4.2	164	17	13
	B	4.7	119	23	35
	C	5.9	125	50	94
	D	4.8	162	27	58
	mean	4.9	142	29	50
M-1185, 2	A	4.5	157	18	0.3
	B	5.0	109	20	0
	C	5.1	149	31	0
	D	5.6	92	54	0.3
	mean	5.0	127	31	0.2
NSM 56, 2	A	5.1	108	26	30
	B	4.8	125	24	9.5
	C	6.1	112	62	35
	D	6.1	67	66	32
	mean	5.5	103	44	27
NSM 107, 2	A	5.8	91	37	391
	B	5.4	93	33	315
	C	5.7	118	53	797
	D	4.4	161	25	348
	mean	5.3	116	37	463
control	A	1.9	245	5	3.3
	B	1.1	238	0	0
	C	2.2	236	9	2.7
	D	1.1	246	0	0.6
	mean	1.6	241	4	1.6

[a–c] Footnotes *a–c* as in **Table 2**. Data are from one replicate of 10 ears from each of the four hybrids for each of the five treatments.

Five strains (M-1185, M-3693, M-3696, M-5126, and M-6924) that produced little or no MON in planta also produced no detectable MON in vitro. Strain M-7330, which produced 509 μg/g of MON in planta, produced 421 μg/g in vitro. Strain NSM 254, which produced 448 μg/g of MON in planta, produced 193 μg/g in vitro. Thus, MON production on autoclaved maize kernels was generally consistent with MON production in infected maize ears.

DISCUSSION

In a previous phylogenetic analysis, two cryptic species of *F. subglutinans* were identified from maize and wild *Zea* species (teosintes) in North America and also from maize in South Africa (*31*−*33*). In those studies, the geographical distribution of group 2 was relatively homogeneous; strains with group 2 genotypes were present in all regions tested—in Guatemala, Mexico, and South Africa and in Illinois, Kansas, and Ohio in the United States. In contrast, group 1 was not found in the United States: strains with group 1 genotypes were found only in Mexico and South Africa. The authors proposed that the absence of group 1 from maize in the United States was likely to be an artifact of a sample size of only 10 strains.

In the present analysis of 31 additional strains of *F. subglutinans* from the United States, the range of group 2 was extended to the states of Arizona, Michigan, Minnesota, New Mexico, Pennsylvania, and Wisconsin. The range of group 1 was extended to the United States—to Arizona, New Mexico, New York, and Wisconsin. Nonetheless, among the combined collection of 54 strains from the Americas, the geographical distribution of group 1 remains skewed. Strains with group 1 genotypes comprise more than half of the strains from the greater southwest region (Guatemala and Mexico, Arizona, and New Mexico), but only 10% of strains from the more northern United States (from Kansas north to Minnesota and east to New York). In the previous study of six strains from South Africa, four were group 1 and two were group 2 (*33*). The reasons that groups 1 and 2 are present at different frequencies in the greater southwest region and in South Africa than in the more northern United States remain unknown. From a biogeographical perspective, studies of the occurrence of groups 1 and 2 among *F. subglutinans* from maize in Canada and South America would be of particular interest.

Although *F. subglutinans* occurs worldwide on maize, there have been relatively few studies of its ability to cause maize ear rot and produce MON in planta. The first experimental inoculations of maize ears with this fungus were performed from 1932 to 1934 at the Botanic Gardens, Sydney, Australia, by E. T. Edwards, who showed that injecting immature ears with spores from two Australian strains of *F. subglutinans* was a highly effective method of producing ear rot (*20*). South African scientists demonstrated that inserting toothpicks infested with *F. subglutinans* (three strains) into maize ears (a total of eight ears) caused ear rot and MON contamination in planta at an average of 188 μg/g in symptomatic kernels (*28*). No MON was detected in nonsymptomatic kernels from inoculated maize ears. In field tests in Canada in 1989, 1990, 1994, and 1995, a strain of *F. subglutinans* (DAOM 194909) caused severe ear rot on maize hybrids following injection of spores into ears or into the silk channel above the immature ears (*40*, *41*). However, levels of MON in the infected ears were not reported.

As far as we are aware, our study is the first to survey genetically defined strains of *F. subglutinans* for maize ear rot and MON production in planta. The majority of strains tested caused high levels of ear rot on maize inbred B73 and on four maize hybrids, when ears were inoculated at the susceptible early stage of silk development. Genetically defined groups 1 and 2 from the United States were similar in their ability to cause ear rot when tested under field conditions in Illinois, but the relative virulence of groups 1 and 2 from regions outside the United States remains unknown. Due to restrictions on field release of foreign plant pathogenic fungi, *F. subglutinans* strains from Canada, Europe, Mexico, and South Africa could not be included for comparison in the maize field tests in this study.

More than half of the 37 strains of *F. subglutinans* tested produced MON at >100 μg/g of kernel dry weight in planta. There was no strict association between production of high levels of MON and phylogenetic group, geographic region, or time since strain collection. Most MON-producing strains of both groups 1 and 2 originated from recent collections of maize landraces from Arizona and New Mexico, but also included strain M-1318, which was isolated from animal feed in New York in 1982, and strain M-7330, which was isolated from maize in Wisconsin in 1993. Some strains produced very high levels of MON in planta; in particular, five group 2 strains from maize from New Mexico produced MON at 767−1529 μg/g of kernel dry weight. In addition, three strains that produced MON at a range of 448−1315 μg/g in planta were confirmed to produce MON at a range of 193−2694 μg/g of culture dry weight on an autoclaved maize substrate. Previous studies also have found that *F. subglutinans* isolated from maize can produce

high levels of MON on autoclaved maize substrates. For example, 40 of 51 strains of *F. subglutinans* from South African maize produced MON in vitro at an average level of 650 μg/g of culture dry weight (*27*). Eight strains of *F. subglutinans* from Peruvian maize produced MON in vitro at an average level of 150 μg/g of culture dry weight (*26*). These results indicate a significant potential for MON contamination of maize in which *F. subglutinans* is present.

Strains of *F. subglutinans* that produce little or no MON in vitro have been isolated from maize from Argentina, Poland, South Africa, and other regions (*23*, *27*, *29*). In particular, 15 strains of *F. subglutinans* from Canadian maize produced MON at an average of only 9 μg/g on an autoclaved maize substrate (*22*). In our study as well, all 12 group 2 strains from the more northern United States produced little or no MON in planta, even in samples with high levels of ear rot, and 5 of these strains were confirmed to produce no MON in vitro. These data indicate that MON production is a highly variable trait in *F. subglutinans* isolated from maize in North America, but further work is needed to clarify relationships between genetic variation, MON production, and biogeography.

Although MON is produced by several *Fusarium* species pathogenic to maize, the importance of MON in *Fusarium*−maize interactions is not known. In earlier studies, MON was toxic to maize callus culture ($ED_{50} = 100$ μM) or when placed at high levels (200 μg) into the leaf whorl of 1-week-old maize seedlings (*3*, *42*). Recently, MON was tested at the relatively high concentration of 100 μM (equivalent to 20 μg per seedling) for toxicity to 20 diverse genotypes of maize (*43*). Seedling germination and growth were inhibited by nearly half in two maize landraces, but inbred B73 and the remaining 17 landraces were not affected by MON. In the present study, 5 strains of *F. subglutinans* from Illinois, Kansas, and Pennsylvania that produced little or no MON in planta or in vitro were able to cause maize ear rot following silk channel inoculation. These results indicate that production of MON may not be required for *F. subglutinans* to cause maize ear infection and ear rot. A more rigorous test of the importance of MON in maize ear rot would use strains that are identical except for a gene that confers MON production. To this end, a search for MON biosynthetic genes is underway in our laboratory.

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