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Fumonisin Production by *Fusarium verticillioides* Strains Isolated from Maize in Mexico and Development of a Polymerase Chain Reaction to Detect Potential Toxigenic Strains in Grains

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Fumonisins are mycotoxins produced by *Fusarium verticillioides* (Sacc. Nirenberg) in maize (*Zea mays* L.), a staple crop in Mexico. In this study, we report the isolation and identification of 67 *Fusarium* strains isolated from maize kernels collected in Northwest and Central Mexico. The strains were characterized regarding fumonisin B_1 production and the presence of the *FUM1* gene. *F. verticillioides* was the predominant species isolated in both geographic regions, but the isolates from Northwest Mexico produced higher levels of fumonisin. A polymerase chain reaction (PCR)-based method, to detect a region of the *FUM1* gene involved in fumonisin biosynthesis, was developed and employed to detect mycotoxigenic fungi in pure culture and in contaminated maize. The presence of the *FUM1* gene was associated with fumonisin production in most isolates, except seven that did not synthesize fumonisin but contained the gene in their genome. The PCR method allowed the direct detection of fungal contamination in ground corn and could be employed to screen for the presence of potential mycotoxigenic fusaria.

KEYWORDS: Mycotoxin; fumonisin B_1; *Fusarium verticillioides*; polymerase chain reaction; maize

INTRODUCTION

Fumonisins are sphingoid-like mycotoxins produced by *Fusarium verticillioides* (Sacc. Nirenberg, syn. *moniliforme* Sheldon), formerly known as *Fusarium moniliforme*, that have been reported worldwide in maize and in other agricultural products. Although other *Fusarium* spp. such as *Fusarium proliferatum*, *Fusarium napiforme*, and *Fusarium nygamai* (*1*) produce fumonisins as well, *F. verticillioides* is the most common species isolated from corn (*2*, *3*). This fungus causes ear and stalk rot and has been associated with all of the developmental stages in the maize plant (*4*), as it is seed borne and can be transmitted from seed to seedling, colonizing the developing plant systemically without producing any conspicuous symptoms, until reaching the kernel. Ear and kernel rots are favored by warm, dry weather during the grain-filling period (*3*), causing major damage and losses. Even if the rot is not visible, the fungus might be growing and producing fumonisins and other toxins, reducing grain value. Fumonisin B_1 is the most abundant toxin produced by most *F. verticillioides* isolates and accounts for 70−95% of the total fumonisins (*5*). Natural incidence of this mycotoxin is a health concern because it has been demonstrated to cause leukoencephalomalacia in equines, pulmonary edema in porcines, and has been included in group 2B (possibly carcinogenic in humans) by the International Agency for Research on Cancer (*6*). Although there is no direct evidence on human toxicity, epidemiological studies have shown various degrees of association between high incidence of human esophageal cancer (*7*, *8*) and *F. verticillioides* or fumonisin in the presence in maize. Therefore, recommendations for maximum levels of fumonisin in foods and feeds have been issued by the Food and Drug Administration (*9*). Risk assessment studies have been carried out in several countries considering the average contamination level of sampled products and daily consumption of maize. In The Netherlands, for example, a hypothetical daily intake of 1 μg of fumonisin has been suggested as tolerable, considering that only 8 g of corn are consumed per day per person (*10*). This value is rather low as compared to other countries, like Mexico, where the average consumption of maize is estimated between 222 and 325 g/per day per person (*11*). Most of the maize consumed in Mexico is in the form of tortillas, which are baked from alkaline-cooked corn kernels in a process called nixtamalization (*12*). Fumonisin B_1 has been detected in tortillas acquired in Mexico in levels ranging from 0.2 to 1.8 μg/g (*13*). However, this process might eliminate up to 70% of the initial fumonisin content and generates a hydrolyzed fumonisin (*14*), although the degree of removal depends on nixtamalization conditions. The hydrolyzed product has been detected in levels ranging from 0.01 to 0.1 μg/g in tortillas (*15*) and might be responsible for the toxicity shown in rats fed with nixtamalized contaminated corn (*16*).

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Animal toxicoses, probably due to fumonisin contamination of corn, have also been described in Mexico. In a documented outbreak of equine leukoencephalomalacia in the southwestern state of Oaxaca, where over 100 donkeys died, postmortem examination of three of them revealed macroscopic and microscopic cerebral white matter liquefactive necrosis. Fumonisin B_1 was detected in levels ranging from 0.6 to 28.5 μg/g in 12 out of 14 corn and feed samples taken from the area (*17*), but no fungal isolation was performed. In a systematic 2 year survey carried out in Northwest Mexico in 1999 and 2000, 190 corn samples were collected from the field and from storage facilities and analyzed for fumonisin levels and *Fusarium* spp. presence. The samples yield average fumonisin B_1 contents of 1.5 μg/g in the first year and 2.6 μg/g in the second year, as well as a high incidence (70%) of *F. moniliforme* (*18*). A more limited survey was carried out in Northeast Mexico, where 34 *F. moniliforme* strains were isolated and 33 of them produced fumonisins in cracked maize (*19*). Vegetative compatibility tests identified all of these isolates as the *Gibberella fujikuroi* mating population A, which is known to produce high fumonisin levels (*2*).

Maize is grown all over Mexico with very distinct environmental and production conditions. In Central and Southern Mexico, mostly open-pollinated landraces and locally adapted hybrids are grown in rain fed, low-input agriculture. In contrast, hybrid seed is employed with high pesticide and fertilizer input and frequently under irrigation in Northwest Mexico (*20*). Associated with this high variability in maize genotypes and growing conditions, *F. verticillioides* populations would be expected to show high variability as well in several phenotypic characteristics including fumonisin B_1 production.

Studies on fumonisin production potential of a given strain typically include isolation of *F. verticillioides* in a selective media and then obtaining single-spore isolates for identification based on morphological characters (*21*). Such isolates are inoculated in a suitable substrate for fumonisin production such as cracked corn or rice grain, and the fumonisin produced is analyzed by qualitative and quantitative methods (*1*, *19*). In this work, we report a highly specific polymerase chain reaction (PCR) method using primers on the β-cetoacyl reductase domain of the polyketide synthase enzyme (*FUM1* gene) that was able to distinguish fumonisin-producing strains from nonproducing strains and species.

MATERIALS AND METHODS

Maize Samples. Maize samples were either collected in the field or directly with the grower in local markets. The collection site in Northwest Mexico was circumscribed to the Yaqui Valley, which is an important agricultural area in the state of Sonora. Samples were obtained in 2000 and consisted mainly of hybrid maize. Maize samples from Central Mexico were collected in 1999 and 2000 and mainly came from the State of Mexico and represented locally adapted landraces.

Isolation and Identification of *Fusarium* spp. The *Fusarium* sp. was isolated from both moldy and asymptomatic kernels. Briefly, kernels were surface disinfected by immersion in a 0.5% NaClO solution for 2 min, decanted, and washed with sterile water for another 2 min. The washing was repeated twice, and the seeds were air-dried on a sterile filter paper. Two selective media were employed for *Fusarium* spp. isolation, pentachloronitrobenzene (PCNB) agar (*21*) and benzoxazolinone agar (BOA). The latter has been reported as highly selective for *F. verticillioides* isolation (*22*). Up to 18 seeds were placed on each plate containing the selective media and incubated for 5 days at room temperature under fluorescent light. Fungal colonies were transferred to PDA agar and incubated under the same conditions for 5−7 days. Single-spore isolates were obtained by preparing a spore suspension of the PDA culture in 10 mL of sterile water and streaking a loophole on water agar according to Nelson et al. (*21*). Species identification was performed using the morphological criteria described by Nelson et al. (*21*). All single-spored strains were preserved in sterile soil according to Windels et al. (*23*).

Fumonisin B_1 Production and Analysis. Potential fumonisin production for each strain was evaluated by inoculating autoclaved rice medium with each single-spore isolate according to Abbas et al. (*24*). Briefly, 15 g of long-grain parboiled rice was mixed with 9 mL of distilled water in a 50 mL flask and allowed to stand for 1 h at room temperature. The flasks were autoclaved for 1 h at 121 °C twice with a 24 h interval. Once the media was cooled, it was inoculated with each isolate and incubated for 4 weeks at room temperature (24−27 °C) in the dark. At the end of the incubation period, the moldy grain was collected and dried in an incubator at 47−50 °C.

A small-scale method was adapted for rapid fumonisin extraction from the dry grain. A subsample of 0.5 g was transferred to a 15 mL Falcon tube containing 5 mL of acetonitrile−water (1:1; v/v). The tubes were kept at room temperature for 12 h, placed in an orbit shaker at 200 rpm for 1 h, and centrifuged for 20 min at 2000*g* in a Sorvall RT6000D benchtop centrifuge, and the supernatant was recovered for analysis.

A preliminary qualitative analysis of fumonisin B_1 content was performed by thin-layer chromatography (TLC) on silica gel plates employing ethyl acetate−acetic acid−water (6:3:1; v/v/v) as the mobile phase and visualized with a 0.5% *p*-anisaldehyde solution (*25*). R_f values of the spot were compared with a commercial standard of fumonisin B_1 (Sigma Chemical Co., St. Louis, MO). If a spot was detected on the crude extract, it was analyzed directly by high-performance liquid chromatography (HPLC); otherwise, it was cleaned-up and concentrated as described below for contaminated maize samples. HPLC analysis of fumonisins was performed according to Sydenham et al. (*26*) employing a Shimadzu LC-10AD delivery module coupled to a Shimadzu RF-10AXL fluorescence detector (Shimadzu Corporation, Kyoto, Japan) set at 335 nm excitation/440 nm emission wavelengths. Fumonisins were derivatized with *o*-phthaldialdehyde (Sigma Chemical Co.) reagent [2.5 mg OPA dissolved in 50 μL of ethanol and 2.5 μL of 2-mercapthoethanol in 2.45 mL of 3% borate buffer, pH 10.5] and separated on a 15 cm × 4.6 mm i.d., 5 μm, SuperCosil LC-18 analytical C_{18} column (Supelco, St. Louis, MO) under isocratic conditions with a flow rate of 1.5 mL/min. The mobile phase employed consisted of methanol−0.1 M NaH_2PO_4 in a 68:32 v/v ratio, pH 3.3. Purified fumonisin B_1 (Sigma Chemical Co.) was employed to prepare a stock solution (1 mg/mL) in 1:1 acetonitrile−water and diluted to obtain standard solutions with decreasing concentrations. These solutions were employed to construct calibration curves (3, 12, 24, and 50 ng/mL).

Primer Design. Two sets of primers were employed in this study. A set of universal primers for fungal DNA consisted of primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAA-AAGTCGTAACAAGG-3′), which amplified a 600 bp DNA fragment containing the internal transcribed spacer (ITS) sequences and the 5.8S ribosomal RNA gene (*27*). These primers are based on the sequences of the 28S and 18S ribosomal RNA genes that show a high degree of conservation. For the detection of fumonisin-producing strains, specific primers were designed using the Primer3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) based on the sequence of the *F. verticillioides FUM1* gene (AF155773) reported by Proctor et al. (*28*). Several primer combinations were tested, and a primer set [FUM53F (5′-CTTGAACGCGGAGCTAGATTAT-3′) and FUM53R (5′-ATCCGTGTATGCATATGTCGAG-3′)] based on the sequence of the exon 3 that amplifies a 354 bp fragment was employed in a PCR using 50−100 ng of fungal DNA.

DNA Extraction and Purification. Each strain was grown on PDA in 10 cm diameter Petri dishes for 9−10 days at room temperature under fluorescent light. A volume of 10 mL of 0.5% Tween-20 solution was added to each plate and shaken at 75−100 rpm for 2 h at room temperature. The spore and mycelia suspension was collected and transferred to a 25 mL centrifuge tube and centrifuged for 20 min at 600*g* in a Jouan MR1812 refrigerated centrifuge. The supernatant was discarded, and the pellet was washed with 10 mL of sterile water and centrifuged again under the same conditions. The pellet was resuspended in 1 mL of sterile water, transferred into a 2 mL Eppendorf tube, and

centrifuged for 10 min at 10000*g*. The pellet was frozen in liquid nitrogen, and 1 mL of DNAzol (Invitrogen, Carlsbad, CA) was added to the tube and incubated at room temperature until thawed. Then the tube was centrifuged at 10000*g* for 10 min at room temperature, and the supernatant containing the DNA was transferred to a fresh tube containing 0.5 mL of 2-propanol (−20 °C), which was mixed by inversion and incubated at room temperature for 3 min before a third centrifugation step (10 min at 7000*g*). The supernatant was discarded, and the pellet was washed with 1 mL of 70% ethanol. The pellet was air-dried for 15 min and dissolved in 20 μL of 8 mM NaOH and incubated for 15 min at 37 °C, then centrifuged at 7000*g* to eliminate the insoluble material. The supernatant neutralized by adding 1 M Tris-HCl, pH 8.0, so this solution had approximately a final concentration of 5%.

PCR. PCR for amplification of the *FUM1* DNA fragment was carried out in an Applied Biosystems GeneAmp System 9700 thermocycler (Applied Biosystems, Foster City, CA) programmed for one cycle of 3 min at 94 °C, 27 cycles of 40 s at 94 °C, 40 s at 56 °C, 40 s at 72 °C, and one cycle of 7 min at 72 °C. Reactions were carried out in volumes of 20 μL containing 50−100 ng of template DNA in 1 μL, 0.3 μM each primer (20 μM), 0.02 of platinum *Taq* DNA polymerase, 2 μL of 10X PCR buffer, 2 μL of $MgCl_2$ (25 mM), and 0.4 μL of dNTPs (10 mM) mix. Amplification of the ITS region was performed with the same temperature program as described above, but with 30 cycles, the primer concentration was adjusted to 0.5 μM. PCR products were resolved in a 1.5% agarose ethidium bromide gel in 40 mM Tris acetate and 2 mM EDTA buffer. The 100 bp DNA ladder (Promega Corp., Madison, WI) was used as a molecular size marker. DNA was purified from 1 week old cultures of *Fusarium* sp. and employed for PCR procedures. To guarantee that the purified DNA was an adequate substrate for amplification and free of inhibitors, all DNAs were tested first with the generic primers ITS4 and ITS5, all of them yielding a 600 bp fragment (Sánchez-Rangel, results not shown).

Analysis of Contaminated Samples. Fifty grams of cracked maize kernels was placed in a 250 mL Erlenmeyer flask and moistened with 15 mL of deionized water and kept for 1 h at room temperature. The flasks were autoclaved for 1 h at 121 °C twice with a 24 h interval. Once the media was cooled, it was inoculated with strain MY6, a high fumonisin producer, and a second flask with strain MZ3-1 that did not produce fumonisin B_1. The flasks were incubated for 4 weeks at room temperature (24−27 °C) in the dark. The moldy grain was then collected and dried in an incubator at 47−50 °C. A 20 g subsample of this maize was ground to a fine powder in a grinding mill (Bel-Art products, Pequannock, NJ). This flour was blended at different ratios (10, 1%; 0.1, 0.01, 0.001, and 0.0001%; w/w) with noncontaminated ground corn for fumonisin and DNA extractions according to the following protocols. Samples were extracted and cleaned according to Hopmans and Murphy (*29*). Briefly, 10 g of the ground material was placed in a 250 mL flask with 50 mL of acetonitrile−water (1:1; v/v) and shaken at 200 rpm for 30 min at room temperature. The extract was filtered through a Whatman 4 filter paper, and a 1 mL aliquot was collected and passed through a conditioned SPE C18 Sep-Pak column (Waters, Milford, MA). Fumonisin was eluted with 1 mL of acetonitrile−water (70:30 v/v), and the eluate was analyzed by TLC and HPLC as described above.

DNA was extracted from 100 mg of the ground flour in a 2 mL Eppendorf tube containing 0.5 mL of 25 mM Tris and 50 mM EDTA solution, pH 7.5. A volume of 7.5 μL of lyticase (20 mg/mL) was added to the tube that was placed in a shaker for 1 h at room temperature. The tube was centrifuged at 10000*g* for 10 min in a MC12 Sorvall microcentrifuge, and the pellet was immersed in liquid nitrogen for 3 min, 1 mL of DNAzol was added, and the protocol was completed as described above. The same procedures were performed with naturally contaminated maize samples that were ground to detect fumonisin B_1 and the DNA of fumonisin-producing fungi.

RESULTS

In this work, 67 *Fusarium* strains were isolated and observed for identification. Fifty-four isolates (80.6%) were identified as *F. verticillioides* based on morphological characters, and a few other species of low incidence were identified as well: *Fusarium avenaceum* (four isolates), *Fusarium nivale* (two isolates), *Fusarium oxysporum* (two isolates), *Fusarium subglutinans* (two isolates), and *Fusarium chlamydosporium*, *Fusarium semitectum*, and *Fusarium sporotrichioides* with one isolate each (**Table 1**). *F. verticillioides* was recovered with high frequency, either from kernel or ear samples by using both PCNB agar and BOA agar. Fumonisin production was evaluated in all strains using a natural substrate medium that allows the synthesis of the mycotoxin, showing a wide variation in this parameter. By using the criteria of Nelson (*30*), 11 out of 13 strains isolated from the Northwest produced high levels (above 500 μg/g), one intermediate (below 500 μg/g), and one low producer (below 50 μg/g). In contrast, strains isolated from Central Mexico consisted mostly of low producers and nonfumonisin producers and only three intermediate producers. No fumonisin production was detected in any of the other isolated *Fusarium* species.

The specificity of the FUM53F/FUM53R primer set to detect the *FUM1* gene was tested with all purified DNA samples. This primer set yielded a unique 354 bp fragment (**Figure 1A**) in 37 of the 67 DNAs tested. The identity of the amplicon was confirmed by sequencing the cloned DNA fragment obtained from MY6 strain, showing an identical sequence to that reported (AF155773) by Proctor et al. (*28*). In this 354 bp DNA fragment, an *SstI* site (GAGCTC) was identified at position 284 (**Figure 1B**). Upon restriction with this enzyme, two fragments were obtained, a 288 bp DNA fragment and a 66 bp fragment. The former migrated slightly faster than the intact product, thus providing a confirmatory test for all of the amplified products (**Figure 1C**). Fumonisin production was then associated with the presence of the *FUM1* gene (**Table 1**). The specific PCR amplification product was detected in all 30 strains that produced fumonisin but also in seven *F. verticillioides* isolates (PAL1, AZU3, H135-3, MOR2, MZ2-2, JIQ5-5, and CRIB-2) in which the toxin was not detected. All of these strains came from maize samples collected in Central Mexico.

To test the feasibility to employ the primers in a diagnostic test that would allow the detection of fumonisin-producing fungi in maize, blends containing known ratios of contaminated maize were analyzed for fumonisin production and the presence of the *FUM1* gene. Cracked maize kernels inoculated with strain MY6 contained 3100 μg/g fumonisin B_1 and upon 10-fold dilutions with noncontaminated maize, fumonisin B_1 levels were reduced down to 0.14 μg/g in the 0.01% blend. The fungal biomass was unmistakably detected by PCR in the inoculated maize and in the first two dilutions, and a fainter band was still detected in the 0.1% blend corresponding to fumonisin levels approximately of 2 μg/g (**Figure 2**).

The same procedure was applied to naturally contaminated kernels from maize ears collected in the Yaqui Valley that contained 32 μg/g fumonisin B_1 (**Figure 3**, lane 4) and showed a strong amplification signal. In contrast, two maize samples collected in Chalco, Edo, Mexico, did not contain fumonisin B_1 and were negative for the *FUM1* gene (**Figure 3**, lanes 2 and 3).

DISCUSSION

F. verticillioides is a plant pathogen of maize that occurs with high incidence wherever this crop is grown; however, there are few studies on the incidence of this fungus in maize in Mexico despite the enormous importance of this crop. The use of the species selective BOA media contributed to increase the frequency of isolation of *F. verticillioides*, although other species such as *F. avenaceum*, *F. nivale*, *F. subglutinans*, *F. oxysporum*,

Table 1. Fumonisin B_1 Production and Presence of the *FUM1* Gene in Several *Fusarium* Species Isolated from Maize in Northwest and Central Mexico

strain	geographic origin	source	species identification	fumonisin B_1 production (μg/g)	*FUM1* gene
MY1	Yaqui Valley, Son	ear	*F. verticillioides*	1718	+
MY2	Yaqui Valley, Son	ear	*F. verticillioides*	1152	+
MY3	Yaqui Valley, Son	ear	*F. verticillioides*	1699	+
MY4	Yaqui Valley, Son	ear	*F. verticillioides*	1940	+
MY5	Yaqui Valley, Son	ear	*F. verticillioides*	1.3	+
MY6	Yaqui Valley, Son	ear	*F. verticillioides*	3658	+
L-189-1	Yaqui Valley, Son	ear	*F. verticillioides*	3062	+
L-189-2	Yaqui Valley, Son	ear	*F. verticillioides*	4047	+
L-189-3	Yaqui Valley, Son	ear	*F. verticillioides*	1617	+
L-176-1	Yaqui Valley, Son	ear	*F. verticillioides*	454	+
L-176-2	Yaqui Valley, Son	ear	*F. verticillioides*	1223	+
L176-3	Yaqui Valley, Son	ear	*F. verticillioides*	1257	+
L-176-4	Yaqui Valley, Son	ear	*F. verticillioides*	1534	+
PAL1	Chalco, Edo Mex	kernel	*F. verticillioides*	ND[a]	+
PAL2	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	−
CHQ1	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	−
CHQ2	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	−
CHQ5	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	−
CHQ6	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	−
AZU2	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	−
AZU3	Chalco, Edo Mex	kernel	*F. semitectum*	ND	+
H135-1	Chalco, Edo Mex	kernel	*F. verticillioides*	174	+
H135-2	Chalco, Edo Mex	kernel	*F. verticillioides*	99	+
H135-3	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	+
H135-4	Chalco, Edo Mex	kernel	*F. verticillioides*	53	+
MOR2	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	+
MOR3	Chalco, Edo Mex	kernel	*F. verticillioides*	0.21	+
CRI	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	−
CRIB-1	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	−
CRIB-2	Chalco, Edo Mex	kernel	*F. verticillioides*	ND	+
CRIB-3	Chalco, Edo Mex	kernel	*F. verticillioides*	3.7	+
PALB-1	Chalco, Edo Mex	kernel	*F. verticillioides*	1	+
PALB-2	Chalco, Edo Mex	kernel	*F. verticillioides*	0.9	+
MORB-1	Chalco, Edo Mex	kernel	*F. verticillioides*	1.4	+
MORB-2	Chalco, Edo Mex	kernel	*F. verticillioides*	0.9	+
MORB-3	Chalco, Edo Mex	kernel	*F. subglutinans*	ND	−
MEM-1	Acambay, Edo Mex	ear	*F. avenaceum*	ND	−
MEM-2	Acambay, Edo Mex	ear	*F. verticillioides*	ND	−
MEM-3	Acambay, Edo Mex	ear	*F. subglutinans*	ND	−
MEM-4	Acambay, Edo Mex	ear	*F. avenaceum*	ND	−
MCH-1	Acambay, Edo Mex	ear	*F. verticillioides*	ND	−
MCH-2	Acambay, Edo Mex	ear	*F. chlamidosporium*	ND	−
MCH-3	Acambay, Edo Mex	ear	*F. verticillioides*	ND	−
MCH-4	Acambay, Edo Mex	ear	*F. nivale*	ND	−
MCH-5	Acambay, Edo Mex	ear	*F. verticillioides*	ND	−
MEMB-1	Acambay, Edo Mex	ear	*F. verticillioides*	0.7	+
MEMB-2	Acambay, Edo Mex	ear	*F. verticillioides*	ND	−
MEMB-3	Acambay, Edo Mex	ear	*F. verticillioides*	ND	−
MEMB-4	Acambay, Edo Mex	ear	*F. verticillioides*	ND	−
MZ1-1	Aculco, Edo Mex	ear	*F. oxysporum*	ND	−
MZ1-2	Aculco, Edo Mex	ear	*F. oxysporum*	ND	−
MZ2-1	Aculco, Edo Mex	ear	*F. nivale*	ND	−
MZ2-2	Aculco, Edo Mex	ear	*F. verticillioides*	ND	+
MZ2-3	Aculco, Edo Mex	ear	*F. avenaceum*	ND	−
MZ2-4	Aculco, Edo Mex	ear	*F. verticillioides*	ND	−
MZ3-1	Aculco, Edo Mex	ear	*F. sporotrichioides*	ND	−
MZ3-4	Aculco, Edo Mex	ear	*F. verticillioides*	ND	−
MZ2B-2	Aculco, Edo Mex	ear	*F. verticillioides*	3.6	+
MZ2B-3	Aculco, Edo Mex	ear	*F. verticillioides*	0.9	+
MZ3B-3	Aculco, Edo Mex	ear	*F. verticillioides*	ND	−
MZ3B-4	Aculco, Edo Mex	ear	*F. verticillioides*	ND	−
JIQ5-1	Jiquipilco, Edo Mex	kernel	*F. avenaceum*	0.2	+
JIQ5-2	Jiquipilco, Edo Mex	kernel	*F. verticillioides*	<0.1	+
JIQ5-3	Jiquipilco, Edo Mex	kernel	*F. verticillioides*	0.1	+
JIQ5-4	Jiquipilco, Edo Mex	kernel	*F. verticillioides*	0.6	+
JIQ5-5	Jiquipilco, Edo Mex	kernel	*F. verticillioides*	ND	+
GUA4-1	Jiquipilco, Edo Mex	kernel	*F. verticillioides*	0.2	+

[a] Not detected.

and *F. semitectum* were also recovered. *F. verticillioides* recovery was reported to increase from 71% in PCNB agar to 86% in this media (22). A total of 67 *Fusarium* strains were isolated, and most of them (80%) were identified as *F. verticillioides*. This finding is in agreement with other studies in Northern Mexico, such as the report of Desjardins et al. (*19*) who isolated 34 strains from maize, and all of them were identified as *F. verticillioides*. More recently, Cortez-Rocha et al. (*18*) reported that *Fusarium* is the genus most frequently isolated from maize (67−70%) in Sonora, Mexico, with *F. moniliforme* (*syn. F. verticillioides*) as the species more prevalent among the isolates.

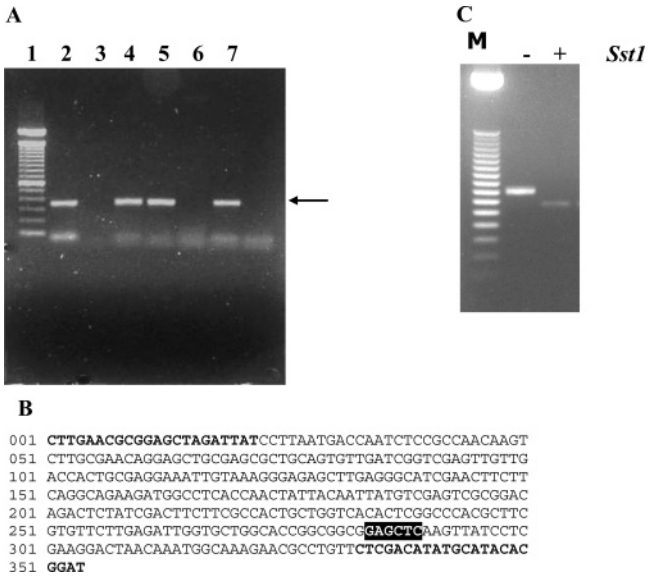

Figure 1. PCR amplification of the *FUM1* gene using purified genomic DNA of various *Fusarium* strains. (**A**) PCR amplification products using the primers FUM53F and FUM53R. Lane 1, 100 bp ladder; lanes 2, 4, 5, 7, positive samples corresponding to strains MY2, L189-1, PAL1, and H135-1, respectively; and lanes 3 and 6, negative samples corresponding to strains MZ3-1 and CHQ-2, respectively. (**B**) Nucleotide sequence of the 354 bp PCR amplification product. Sequences corresponding to FUM53F and FUM53R primers are bolded, and the *SstI* restriction site is highlighted in black. (**C**) Mobility shift of the PCR amplification product upon digestion with *SstI* restriction enzyme. Lane M, 50 bp ladder.

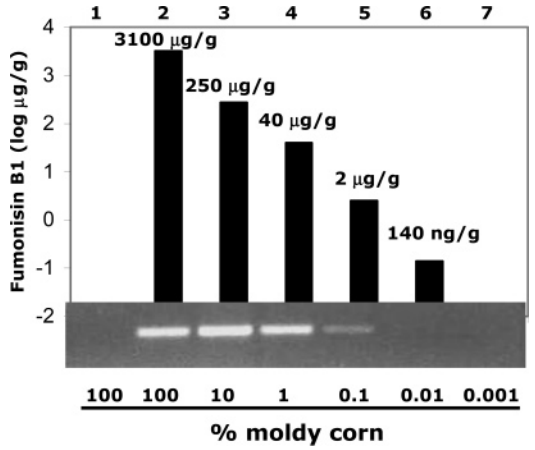

Figure 2. Association between levels of fumonisin B_1 quantified by HPLC (upper panel) and detection of the *FUM1* gene by PCR (lower panel). Corn was inoculated with a nonfumonisin producing strain (MZ3-1) (lane 1) or with a high-producer strain (MY6). The latter was blended at different ratios with noncontaminated ground corn (lanes 2–7), and all ground samples were analyzed for fumonisin and the *FUM1* gene.

Fumonisin production was very variable among strains because some of them did not produce fumonisin B_1 or synthesized it at very low levels (0.1 μg/g), while other strains produced high amounts of the toxin (4000 μg/g). These levels are comparable to those reported by Desjardins et al. (*19*) in *F. verticillioides* strains isolated from maize in Northeastern Mexico in which 97% of the strains produced fumonisin B_1 in levels ranging from 10 to 5800 μg/g. A worldwide survey of *F. verticillioides* strains isolated from different regions in distinct geographic regions described production ranging from 10 to 6400 μg/g. It is noteworthy from our study that two distinct populations of *F. verticillioides* were characterized with contrasting phenotypes regarding fumonisin B_1 production. While most isolates from Northwest Mexico could be classified as high producers (*30*), and probably belong to mating population A (*2*), most strains from Central Mexico could be classified as low fumonisin producers. This distinction can be partially explained by the fact that all strains isolated from Northwest Mexico came from corn ears collected in the field, while samples from Central Mexico included field ear samples and kernel samples obtained in local markets. In Central Mexico, *F. subglutinans*, a species that does not produce fumonisins, is frequently isolated in some fields in maize (*31*) and thus might compete effectively with toxigenic strains of *F. verticillioides*.

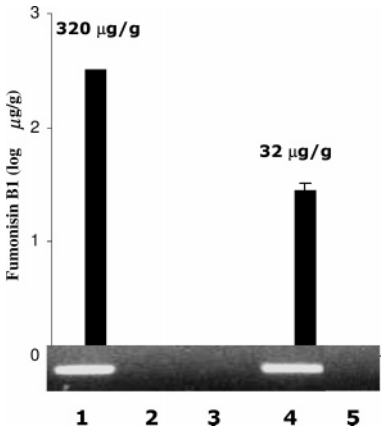

Figure 3. Natural incidence of fumonisin in maize, quantified by HPLC (upper panel), and of *F. verticillioides*, detected by PCR amplification of the *FUM1* gene (lower panel). Lane 1, Maize kernels inoculated with a fumonisin-producing strain (MY3); lanes 2 and 3, maize samples collected in central Mexico; lane 4, maize samples collected in the Yaqui Valley; and lane 5, maize kernels inoculated with a nonproducing strain (CHQ5).

The association between the presence of the *FUM1* gene and fumonisin production was tested with all of the identified isolates, and we found that in all of the producing strains, the *FUM1* gene was positive as well. Proctor et al. (*28*) found that the *FUM1* gene is relevant in fumonisin biosynthesis because its expression precedes the toxin accumulation, and deletion of the gene disrupts fumonisin B_1 production at a 99% level. In our study, we detected seven *F. verticillioides* strains that did not produce fumonisin B_1 at any detectable level but contained the *FUM1* gene in the PCR reaction. Although fumonisin B_1 production was evaluated in a natural substrate favorable for toxin biosynthesis, other factors might regulate the metabolic route that results in a wide variation of fumonisin levels. A cluster of 15 fumonisin biosynthetic genes has been identified and characterized in *F. verticillioides*. Most of the genes within the cluster have a similar expression pattern that is consistent with fumonisin production in culture (*32*), and disruption of some of these genes, like *FUM1*, renders a mutant with reduced fumonisin-producing ability (*28*). The identification of the *FUM* loci allowed its search in fumonisin producing and nonproducing species, and the results indicated that nonproducing species lack these genes. However, there are isolates of *F. fujikuroi* and *F. nygamai* that are positive for the *FUM1* gene and three other genes of the FUM cluster but do not produce fumonisins (*33*). Such phenotypes could be explained by a mutation in the FUM cluster or by altered expression of the FUM genes. Until now, there are three identified factors that regulate fumonisin production and very likely through the expression of genes within the FUM cluster: FCC1 gene codes for a cyclin-like protein that regulates fumonisin production and fungal conidiation (*34*); PAC1, which appears to be a transcriptional repressor of

fumonisin biosynthetic genes (*35*); and ZFR1, which encodes a polypeptide that contains a DNA binding motif consisting of a Zn(II)2Cys6 binuclear cluster. The latter belongs to a protein family that has been characterized as regulators of secondary metabolism in other fungi (*36*). Interestingly, none of these genes lies within the FUM cluster, or their disruption blocked fumonisin production in the mutants. The incidence of atoxigenic strains of a given toxigenic fungal species has been documented in *F. verticillioides* (*5*) and in other fungi, like *Aspergillus flavus* (*37*). In this study, we identified seven *F. verticillioides* isolates that either did not produce fumonisin B1 or synthesized it at very low or nondetectable levels but contained the *FUM1* gene. It would be interesting to compare the expression levels of this gene in these isolates with high producers, to discriminate between a possible mutation in the FUM locus and alteration in a regulator. The presence of this type of isolates requires confirmatory tests to be performed for fumonisins in positive samples for the *FUM1* gene.

Since the discovery of fumonisins, several analytical methods have been developed for its quantitative analysis in certain agricultural products and derived foods. In this study, we used a preliminary TLC analysis and a quantitative analysis based on detection of the fluorescent fumonisin B_1 derivative separated by HPLC (*26*). Besides the detection of the toxic metabolite, it is relevant to establish the presence of the toxin-producing fungi. We designed a set of primers for detecting a fragment of the *FUM1* gene that showed a strong association with fumonisin production. By using this detection principle, we developed a diagnostic assay to detect the toxigenic fungi in grain samples. The detection and classification of mycotoxigenic species within the genus *Fusarium* is based on the microbiological isolation and identification by morphological criteria. This procedure is time-consuming and requires trained personnel for correct species identification. Moreover, *Fusarium* taxonomy is highly complicated and the correct identification of the morphological structures depends on the exact growing conditions as established in published manuals (*21*). This constraint limits the number of samples that could be analyzed and hence the scope of a survey. Molecular identification based on the presence of an essential gene for fumonisin production, in most cases, could replace the tedious labor of identifying the fungus and determining the toxin production profile. When the molecular method was compared with the classical analytical method of fumonisin detection in a heavily contaminated sample, we found good correlation between the two methods and detection of fungal biomass equivalent to fumonisin levels of 2 μg/g. Fumonisin detection is highly sensitive and could go down to 50 ng/g; however, these numbers have a limited practical value as the recommended guidance levels lie between 2 and 4 μg/g for human foods and from 20 to 100 μg/g for animal feeds (*9*). When the test was applied to a limited number of naturally contaminated samples, the *FUM1* gene was readily and specifically detected in a sample containing 32 μg/g fumonisin B_1; thus, it has potential applications for wider surveys to search for the presence of fumonisin-producing isolates in distinct geographical regions. For example, the method reported here might be employed for evaluating the efficacy of management strategies for controlling *Fusarium* kernel and ear rot and to identify natural resistance to the disease in hybrid lines and races. Another type of study would be to evaluate the incidence of mycotoxigenic fungi in genetically engineered corn expressing the *Bacillus thuringiensis* cry proteins as it has been reported that this type of corn contains lower levels of fumonisins than their nontransgenic counterparts (*38*). Although the diagnostic test developed is qualitative, it established the principles for the development of quantitative tests by using real-time quantitative PCR (RTQ-PCR), as well as to explore other regions of the FUM loci for the design of primers.

Because detection of fumonisin-producing isolates is a labor- and time-consuming task that requires taxonomical identification and chemical analysis, alternative methods to quickly and reliably discriminate this phenotype have been developed. A first approach was by identifying polymorphism in the ITS1 region of ribosomal genes in fumonisin-producing and nonproducing strains and developing specific primers (*39*). This strategy yielded a nonspecific method, which was partially corrected by adding an enzyme-linked immunosorbent assay step with a labeled oligonucleotide. Another set of primers, developed on the basis of the intergenic spacer region too, discriminated fumonisin-producing *F. verticillioides* strains that are mainly associated with cereal crops, from nonfumonisin producing strains that are associated with bananas (*40*). A different approach was taken by Jiménez et al. (*41*), who used random amplified polymorphic DNA analysis to categorize *Fusarium* strains into low and high fumonisin-producing isolates, but no clear distinction could be made betweeen fumonisin producers and nonproducers.

A more specific assay has been achieved with primers based on nucleotide sequences of genes involved in fumonisin production such as reported by González-Jaen et al. (*42*) who designed primers that amplified the β-cetoacyl reductase domain of the polyketide synthase that is located in exon five of the *FUM1* gene. The assay was tested in 16 *Fusarium* strains and showed an excellent association between the fumonisin production and the presence of the amplification product.

The methods and experience reported in this study could be employed to perform wider studies in other geographical regions within Mexico to determine the occurrence and distribution of toxigenic *Fusarium* strains in maize, as it a staple crop with high consumption per capita. The data gathered in these studies plus the wealth of information on fumonisins present in the scientific literature might contribute to establish guidelines for fumonisin levels in maize in Mexico.

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