Production of Fumonisin B Analogues and Related Compounds by *Fusarium globosum*, a Newly Described Species from Corn

Eric W. Sydenham,[†] Gordon S. Shephard,*[,†] Sonja Stockenström,[†] John P. Rheeder,[†] Walter F. O. Marasas,[†] and Marthinus J. van der Merwe[‡]

Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, P.O. Box 19070, Tygerberg, 7505 South Africa, and Department of Biochemistry, University of Stellenbosch, Stellenbosch, 7600 South Africa

Fusarium globosum Rheeder, Marasas et Nelson is a recently described species originally isolated from corn kernels harvested in the Transkei region of South Africa. On the basis of morphological criteria, *F. globosum* is closely related to other common fungal contaminants of corn, viz. *F. moniliforme*, *F. proliferatum*, and *F. subglutinans*, and accordingly it has been classified in the section *Liseola*. Species within the section *Liseola* have been reported to produce either the fumonisin B or moniliformin (MON) mycotoxins and, in some cases both. Seventeen isolates of *F. globosum*, cultured on corn, were screened for the production of fumonisins B_1 (FB_1), B_2 (FB_2), B_3 (FB_3), and MON. All isolates produced FB_1 (range 5−325 μg/g), while 15 of 17 also produced FB_2 (range 1−4 μg/g). For 14 of 17 isolates, the levels of FB_3 produced (range 4−24 μg/g) exceeded those of the corresponding FB_2 concentrations. None of the isolates produced detectable levels of MON (<1 μg/g). In addition, several isolates of *F. globosum* also produced two additional fumonisin-like compounds, the mass spectral evidence of which suggests that they may be isomers of FB_1 and FB_2 or FB_3, respectively.

Keywords: *Fumonisin B; Fusarium globosum; corn; maize; fungal contaminants; mycotoxins; carcinogens*

INTRODUCTION

Fusarium globosum Rheeder, Marasas et Nelson, a recently described *Fusarium* species classified in the section *Liseola*, was originally isolated from naturally infected corn kernels (*Zea mays* L.) harvested in the Transkei region of the Eastern Cape Province, South Africa (Rheeder et al., 1996). *F. globosum* is characterized morphologically by the production of microconidia in chains and in false heads, with the globose microconidia often appearing as botryose clusters on monophialidic and/or polyphialidic conidiophores (Rheeder et al., 1996). Since *F. globosum* does not produce chlamydospores, it is closely related to some other members of the section *Liseola* that may occur on corn or other substrates (Nirenberg, 1976), viz. *F. moniliforme* Sheldon [syn. = *F. verticillioides* (Sacc.) Nirenberg], *F. proliferatum* (Matsushima) Nirenberg, *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas, and *F. anthophilum* (A. Braun) Wollenw. (Nelson et al., 1983).

The fumonisins are a group of structurally related mycotoxins produced by *F. moniliforme* and other related *Fusarium* species (Nelson et al., 1992; Thiel et al., 1991b). Those variants thus far characterized may be separated into three main groups identified as the fumonisin A, B, and C series analogues (Bezuidenhout et al., 1988; Gelderblom et al., 1988; Plattner, 1995). Alkali or acid treatment of the individual analogues can yield tricarballylic acid and the corresponding partially or fully hydrolyzed aminopolyol moieties (Badria et al., 1995; Plattner et al., 1990; Sydenham et al., 1995a,b), some of which may occur in processed/treated products (Hopmans and Murphy, 1993) or be the result of metabolic processes (Shephard et al., 1994). However, of the known analogues it is the toxicologically important fumonisins B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) (Gelderblom et al., 1996) that are the most abundant naturally occurring fumonisins. A recent study, conducted under the auspices of the Commission on Food Chemistry of IUPAC, reported a worldwide survey of fumonisin levels in corn-based products intended both for animal and human consumption (Shephard et al., 1996). High fumonisin levels (up to 330 μg of FB_1/g) have been reported in corn-based animal feeds associated with confirmed outbreaks of leukoencephalomalacia (LEM) in horses and pulmonary edema syndrome (PES) in pigs (Shephard et al., 1996). Although mean fumonisin levels in commercially available corn-based human foodstuffs have tended to be very much lower (mainly 1 μg/g or less), corresponding levels in homegrown corn from human esophageal cancer risk areas of the Transkei (South Africa) and the Linxian and Cixian counties of China have been alarmingly high, with concentrations in individual samples similar to those known to have been implicated in confirmed outbreaks of LEM and PES (Shephard et al., 1996).

Moniliformin (MON) is another mycotoxin produced by some members of the section *Liseola* (Marasas et al., 1984, 1986; Rabie et al., 1982; Leslie et al., 1996), with considerable controversy surrounding its production by *F. moniliforme*, as summarized by Marasas et al. (1986). The natural occurrence of MON in corn is more likely to be due to infection of the substrate with *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun et Marasas, rather than by *F. moniliforme* (Sydenham et al., 1990; Thiel et al., 1982). Consequently, investigations involving the identification and taxonomy of

* Author to whom correspondence should be addressed (telephone +27-21-938-0272; fax +27-21-938-0260; e-mail GSHEPHAR@eagle.mrc.ac.za).

[†] PROMEC.

[‡] University of Stellenbosch.

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Table 1. Fumonisin and Moniliformin Production by Corn Isolates of *F. globosum*

strain (MRC)	mycotoxin concentration (μg/g)			
	FB_1	FB_2	FB_3	MON
6646	325	4	24	ND[a]
6647	260	3	18	ND
6648	200	2	10	ND
6649	80	2	6	ND
6650	5	ND	ND	ND
6651	270	4	20	ND
6652	45	3	18	ND
6653	25	2	8	ND
6654	55	4	18	ND
6655	235	3	15	ND
6656	160	1	10	ND
6657	210	2	13	ND
6658	140	1	7	ND
6659	170	2	11	ND
6660	55	ND	4	ND
6661	15	3	ND	ND
6662	15	2	ND	ND
control corn	0.15	ND	ND	ND

[a] Values given as ND (not detected) correspond to <1 μg/g for cultures and <0.05 μg/g for control corn.

individual biological species within the section *Liseola* (Leslie, 1991; Klittich and Leslie, 1992) may be strengthened by determining the ability of isolates to produce the fumonisins and/or moniliformin (Leslie et al., 1992a,b, 1996). The objective of this study was to determine the ability of newly described *F. globosum* isolates to produce FB_1, FB_2, FB_3, and MON.

MATERIALS AND METHODS

Reagents. All reagents and solvents were of Analar grade obtained from Merck Chemicals (Darmstadt, Germany).

***Fusarium* Isolates and Culture Material.** Seventeen strains of *F. globosum* (Table 1), preserved in the culture collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, South Africa, were used in this study. Each strain was isolated from surface-sterilized, whole corn kernels harvested in the Butterworth district of the Eastern Cape Province, South Africa (Rheeder et al., 1996). Lyophilized conidia of the 17 strains were suspended in sterile water and used to inoculate moistened yellow corn kernels as described by Marasas et al. (1986). The cultures were incubated at 25 °C for 21 days before being dried and ground to fine meals.

Chemical Assays. Production of FB_1, FB_2, and FB_3 by the corn isolates was determined according to the method of Sydenham et al. (1996), with minor modifications. Briefly, test portions of each laboratory sample were extracted with methanol/water by homogenization. The extracts were filtered and, when necessary, adjusted to pH between 5.8 and 6.5, by the addition of 1 M NaOH. Aliquots of the resultant filtrates were purified on solid-phase extraction cartridges containing silica-based strong anion-exchange (SAX) media. The cartridges were washed to remove potential interfering substances and the fumonisins selectively eluted with a 1% solution of CH_3COOH in CH_3OH. The eluate was evaporated to dryness at 60 °C under dry nitrogen and the residue redissolved in CH_3OH. Individual FB_1, FB_2, and FB_3 levels were determined by reversed-phase HPLC coupled with fluorescence detection, as their *o*-phthaldialdehyde (OPA) derivatives (Sydenham et al., 1996). The purified extracts (in CH_3OH) were also used, without derivatization, for electrospray mass spectrometric analysis (as described below).

The initial methanolic extracts were also used for purification using immunoaffinity chromatography. Short chromatographic columns (of 5 mm i.d.) were packed with a suspension of fumonisin−antibody-based resin in a phosphate buffer solution (PBS) (Vicam, Watertown, MA) to yield a chromatographic bed of approximately 30 mm. The methanolic extracts were diluted with PBS, mixed, and filtered. An aliquot of the filtrate was applied to the immunoaffinity column and allowed to pass under gravity. The immunoaffinity column was then washed with PBS (10 mL) and dried by passing air through the column. The retained fumonisin analogues were eluted with CH_3OH (5 mL), the eluate being reduced in volume, under nitrogen and at 60 °C, to yield a final volume of approximately 1 mL. The extracts were retained for further analyses.

MON levels were determined according to the method of Scott and Lawrence (1987), with minor modifications. Test portions were extracted with CH_3CN/H_2O and defatted with *n*-hexane, and aliquots were partitioned on disposable C_{18} and neutral alumina columns. The purified extracts were separated by paired-ion HPLC, in which the column eluate was monitored at 229 nm, while UV spectra were collected between 200 and 350 nm using diode array detection, for confirmatory purposes.

Mass Spectral Confirmation. The production of fumonisins was unequivocally confirmed by electrospray mass spectrometry (ESMS) using a VG Quattro triple-quadrupole mass spectrometer equipped with an electrospray interface (Thermo Instruments, Manchester, U.K.). The solvent [CH_3CN/H_2O (60:40) adjusted to pH 3 by the addition of CF_3COOH] was delivered by a Pharmacia LKB Model 2249 pump (Pharmacia, Uppsala, Sweden). The temperature of the electrospray interface was set at 80 °C, with the capillary voltage at 3.5 kV and a counter electrode potential (cone voltage) of 30 V. For analysis of the total extracts, the CH_3OH solutions were diluted 1:10 with the above solvent and 10 μL was injected via loop injection directly into the ESMS, at a flow rate of 10 μL min^{-1}. Data were collected over the mass range m/z 400−1000. Extracts were, in addition, analyzed by on-line HPLC-ESMS using a Spherisorb 5 μm ODS-1 microbore column (150 × 2.0 mm i.d.) with the above solvent as mobile phase, which was delivered at a flow of 50 μL min^{-1}. The resultant data were collected over the same mass range or as single ion monitoring (SIM) recordings, at specific masses.

RESULTS AND DISCUSSION

Table 1 summarizes the results of the fumonisin and moniliformin analyses conducted on the *F. globosum* corn cultures. Fumonisin B_1 was produced by each strain at levels ranging from 5 to 325 μg/g, while 15 of 17 also produced FB_2 (at between 1 and 4 μg/g) (Table 1). With the exception of three isolates (MRC 6650, MRC 6661, and MRC 6662), the cultures produced FB_3 at between 4 and 24 μg/g (i.e. at levels that were higher than the corresponding FB_2 concentrations). None of the isolates produced detectable levels of MON (i.e. >1 μg/g). Conversely, the control material was found to be contaminated with a trace level of FB_1 [0.15 μg/g (Table 1)], while FB_2, FB_3, and MON were not detected (i.e. <0.05 μg/g).

Figure 1, the HPLC chromatogram obtained for the purified extract of MRC 6646 (after OPA derivatization), illustrates the presence of five distinct peaks, in which peaks 1, 4, and 5 correspond to the chromatographic positions of FB_1, FB_3, and FB_2, respectively. With the exception of six isolates (MRC 6650, MRC 6652, MRC 6653, MRC 6660, MRC 6661, and MRC 6662; Table 1), all others produced chromatographic peaks corresponding to peaks 2 and 3 (Figure 1). Although the magnitude of peak 2 differed between the individual isolates, those of peak 3 approximated (in most cases) the corresponding FB_3 peak, as shown in Figure 1. Aliquots of the original aqueous methanolic extracts prepared for MRC 6646 and MRC 6647 were, in addition, purified on FumoniTest immunoaffinity columns prior to analyses by HPLC. The resultant chromatograms of these latter extracts included all five peaks previously identified in Figure 1. Given that the immunoaffinity column

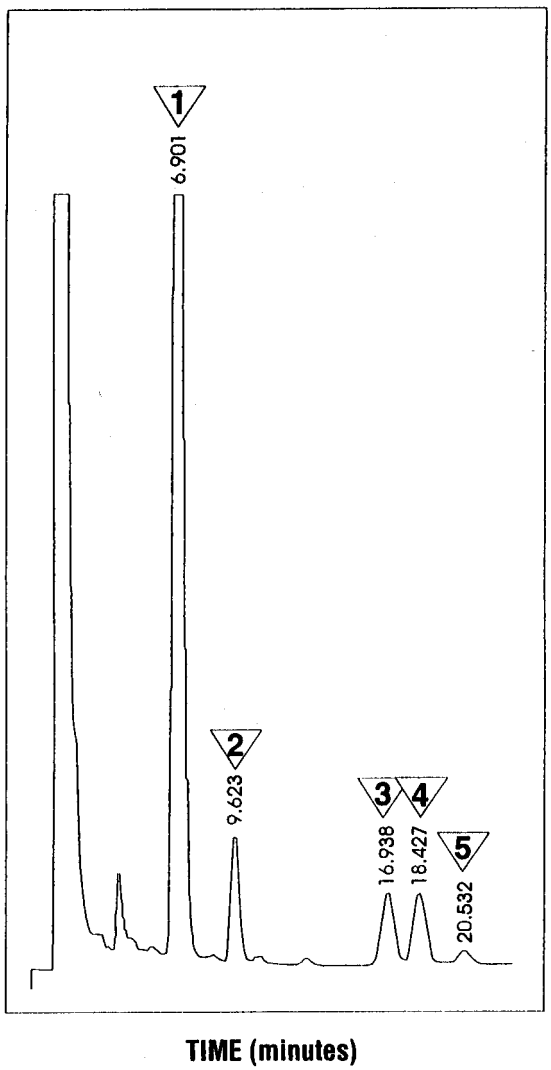

Figure 1. HPLC chromatogram of MRC 6646 (as its OPA derivative) showing the chromatographic positions of (1) FB_1, (2) unknown, (3) Unknown, (4) FB_3, and (5) FB_2.

might be expected to exhibit superior selectivity for the retention of fumonisin-like compounds than would silica-based SAX media, it was considered that the unidentified peaks might also be fumonisin-related compounds.

Several ionization and sample introduction techniques have been investigated for the MS confirmation of underivatized fumonisins and/or their corresponding hydrolysis products. These have included flow injection thermospray mass spectrometry (Korfmacher et al., 1991), liquid chromatography−ion spray mass spectrometry (Chen et al., 1992), direct probe fast-atom bombardment mass spectrometry (Chen et al., 1992; Holcomb et al., 1993; Korfmacher et al., 1991), and liquid chromatography−particle-beam mass spectrometry (Young and Lafontaine, 1993). More recently, several groups have investigated the use of either flow injection or liquid chromatography linked with electrospray mass spectrometry (Doerge et al., 1994; Korfmacher et al., 1991; Musser, 1996; Plattner, 1995; Sydenham et al., 1995b), while Caldas et al. (1995) reported that when used in the positive ionization mode, the technique yielded abundant protonated molecular ions for those toxin variants containing amino groups (such as the fumonisin B analogues). Accordingly, purified extracts prepared from MRC 6646 and MRC 6647 were analyzed by flow injection ESMS, which resulted in the observation of major ions at m/z 722 (consistent with the protonated molecular ion of FB_1) and m/z 706 (consistent with the protonated molecular ions of both FB_2 and FB_3). Subsequent HPLC-ESMS of the same extracts yielded total ion chromatograms (between m/z 400 and 1000) with single base ions at m/z 722 for peaks 1 (= FB_1) and 2 (= unidentified), the SIM of which is shown in Figure 2A, and corresponding base ions at m/z 706 for peaks 3 (= unidentified), 4 (= FB_3), and 5 (= FB_2) (SIM shown in Figure 2B). However, under the prevailing chromatographic conditions peaks 3 and 4 were partially fused, as opposed to their complete baseline resolution as OPA derivatives (Figure 1). The confirmed masses of peaks 2 and 3 do not correspond with any of the other known fumonisin A, B, or C analogues or with the *n*-acetyl keto derivative of FB_1 (FAK_1) recently isolated from corn cultures of *F. proliferatum* (Musser et al., 1995). On the basis of our preliminary observations it is proposed that peaks 2 and 3 may correspond to isomers of FB_1 and FB_2 or FB_3, respectively. However, additional studies would be required to elucidate the structures of these two compounds. For example, MS/MS experiments could be used to demonstrate the consecutive loss of the tricarballylic acid esters from the protonated molecules, which would indicate that the unknown compounds were of the fumonisin class.

The use of various macro- and microconidial morphological characteristics to delimit *Fusarium* species in the section *Liseola* has resulted in the establishment of alternative classification systems and nomenclature for the identification of individual species (Snyder and Toussoun, 1965; Nelson et al., 1983; Nirenberg, 1989). This has subsequently led to confusion within the scientific literature due to, on occasions, the inability of investigators to accurately identify the strains being examined, rather than differences between the abilities of the various species to produce selected mycotoxins. Miller et al. (1991) used the trichothecene-producing abilities of isolates of *F. graminearum*, *F. culmorum*, and *F. crookwellense* as a basis for the chemotaxonomic verification of individual species, and it has been proposed that a similar system be considered for the classification/verification of species within the section *Liseola* (Leslie et al., 1992b).

On the basis of the classification system of Nelson et al. (1983), Table 2 compares the fumonisin- and MON-producing abilities of the 17 isolates of *F. globosum*, with the corresponding abilities of other important *Fusarium* species in the section *Liseola*. Although the majority of *F. proliferatum* isolates appear to produce the fumonisins, Nelson et al. (1992) recorded 12 of 19 that did not produce detectable levels of FB_1. Ross et al. (1992) identified several isolates of *F. proliferatum* that produced only FB_2 and/or FB_3 rather than FB_1. It is therefore possible that some of the non-FB_1 producers screened by Nelson et al. (1992) may have synthesized other fumonisin B analogues. In addition to producing the fumonisins, isolates of *F. proliferatum* have also been shown to produce MON (Table 2). Conversely, none of the 24 isolates of *F. subglutinans* produced detectable levels of either FB_1 or FB_2 (Nelson et al., 1992; Thiel et al., 1991b), while a majority of isolates have been shown to produce MON (Table 2). With respect to isolates of *F. anthophilum*, only 3 of 18 have been found to produce fumonisins at relatively low levels (Nelson et al., 1991), while 6 of 8 isolates produced MON

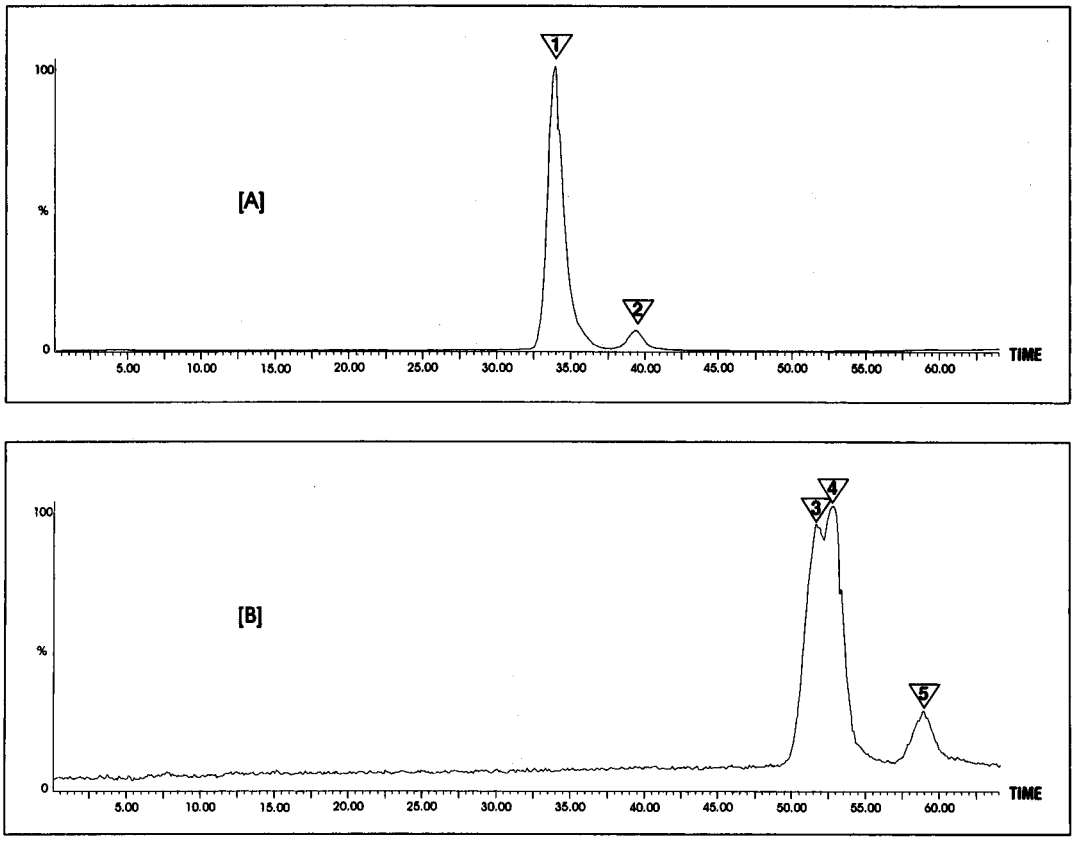

Figure 2. HPLC-ESMS single ion monitoring (SIM) recordings of MRC 6646 (A) at *m/z* 722 and (B) at *m/z* 706: (1) FB_1; (2) unknown; (3) unknown; (4) FB_3; (5) FB_2.

Table 2. Comparative Fumonisin- and Moniliformin-Producing Abilities of *Fusarium* Species in Section *Liseola*[a]

Fusarium species	no. of strains tested	fumonisins		moniliformin		ref
		no. of positive	no. of negative	no. of positive	no. of negative	
F. globosum	17	17	0	0	17	Table 1
F. proliferatum	31	19	12	–	–	Nelson et al., 1992
	4	4	0	–	–	Thiel et al., 1991b
	3	3	0	–	–	Ross et al., 1990
	16	16	0	–	–	Ross et al., 1992
	4	3	1	–	–	Chelkowski and Lew, 1992
	26	26	0	12	14	Logrieco et al., 1995
	8	–	–	8	0	Marasas et al., 1986
F. subglutinans	23	0	23	–	–	Nelson et al., 1992
	1	0	1	–	–	Thiel et al., 1991b
	3	0	3	–	–	Chelkowski and Lew, 1992
	59	–	–	48	11	Marasas et al., 1986
	6	–	–	6	0	Rabie et al., 1982
F. anthophilum	17	3	14	–	–	Nelson et al., 1992
	1	0	1	–	–	Thiel et al., 1991b
	2	1	0	–	–	Chelkowski and Lew, 1992
	8	–	–	6	2	Marasas et al., 1986
F. moniliforme	90	61	29	–	–	Nelson et al., 1991
	7	7	0	–	–	Thiel et al., 1991b
	40	40	0	–	–	Ross et al., 1992
	10	10	0	–	–	Thiel et al., 1991a
	26	26	0	–	–	Sydenham et al., 1992
	20	20	0	–	–	Chelkowski and Lew, 1992
	17	16	1	–	–	Chelkowski et al., 1995
	36	–	–	26	10	Rabie et al., 1982
	58	–	–	13	45	Marasas et al., 1986

[a] Fumonisin-positive samples include those cultures that produced either FB_1, FB_2, or FB_3. In those cases where the original data were reported as trace levels of contamination, the corresponding result is reflected as a negative value. The presence of a dash (–) indicates that no relevant data are available.

(Table 2). These latter data illustrate the necessity to screen relatively large numbers of isolates to unequivocally determine the toxin-producing ability of individual fungal species. While a large number of isolates of *F. moniliforme* have been reported to produce significant amounts of the fumonisins, Nelson et al. (1991) still

Table 3. Comparative Fumonisin and Moniliformin Production by A and F Mating Populations of *Gibberella fujikuroi (F. moniliforme)*[a]

mating population	no. of strains tested	fumonisins		moniliformin		ref
		no. of positive	no. of negative	no. of positive	no. of negative	
A	25	25	0	−	−	Leslie et al., 1992a
	17	17	0	−	−	Leslie et al., 1992b
	31	30	1	−	−	Desjardins et al., 1994
	28	27	1	−	−	Moretti et al., 1995
	20	19	1	3	17	Leslie et al., 1996
F	12	12	0	−	−	Leslie et al., 1992b
	9	3	6	−	−	Moretti et al., 1995
	20	3	17	20	0	Leslie et al., 1996

[a] Fumonisin-positive samples include those cultures that produced either FB_1, FB_2, or FB_3. In those cases where the original data were reported as trace levels of contamination, the corresponding result is reflected as a negative value. The presence of a dash (−) indicates that no relevant data are available.

observed 29 of 90 isolates that were considered to be nonproducers of FB_1, although 26 of these were contaminated with trace levels of the toxin (i.e. between 1 and 10 μg of FB_1/g) (Nelson et al., 1991). In addition, the data presented in Table 2 do not unequivocally establish the MON-producing ability of *F. moniliforme* isolates.

Consequently, the data in Table 2 would suggest that the fumonisin- and/or MON-producing criteria cannot, on their own, be used to unequivocally delimit individual *Fusarium* species in the section *Liseola*. The general confusion regarding both the taxonomic and mycotoxicological data on *F. moniliforme* is of concern, since it is one of the fungi most frequently recovered from cereal grains (such as corn and sorghum) intended for animal and human consumption. Accordingly, several workers have investigated the use of the sexual stage of fungal isolates to distinguish species within the section *Liseola* (based on mating tests), which has resulted in the identification of six mating populations (designated A−F, respectively) (Hsieh et al., 1977; Klittich et al., 1992; Kuhlman, 1982; Leslie, 1991). However, these studies have also resulted in additional taxonomic problems since, based on the classification system of Nelson et al. (1983), members of both the A and F populations belong to the *F. moniliforme* anamorph (Leslie et al., 1992b). In Table 3, the fumonisin- and MON-producing abilities of isolates in the A and F mating populations of *F. moniliforme* are compared. With respect to their toxin-producing profiles, these data indicate a clearer definition between the two populations. Most members of the A mating population (which are mainly recovered from corn) produce significant levels of the fumonisins [up to 10 330 μg/g total FB_1, FB_2, and FB_3 (Leslie et al., 1996)] while few produce detectable levels of MON [maximun 175 μg/g (Leslie et al., 1996)]. Conversely, members of the F mating population (commonly recovered from sorghum) tend to be non- or low-producers of the fumonisins (Leslie et al., 1992b, 1996), but high producers of MON [85−10 345 μg/g (Leslie et al., 1996)]. Of the 12 members of the F mating population reported to produce the fumonisins (Table 3), only 1 produced in excess of 10 μg of FB_1/g, while 6 of 12 produced levels of $\leq$3 μg of FB_1/g (which was similar to the residual concentration in the corn substrate used to prepare the cultures) (Leslie et al., 1992b). Similarly, the 3 of 9 fumonisin producers identified by Moretti et al. (1995) produced only $\leq$3 μg of FB_1 + FB_2/g (Table 3). These observations may explain some of the results reported by Nelson et al. (1991), in which a significant number of the 26 strains of *F. moniliforme* reported to produce trace levels of FB_1 (Table 1) were originally isolated from sorghum.

While the continued development and use of definitive classification systems may improve the ability of investigators to correctly identify toxigenic *Fusarium* species, there are other sources of potential error regarding the toxin-producing abilities of selected species. The use of corn for the preparation of cultures (rather than a "defined" medium) can present its own problem, since this substrate can be naturally contaminated with residual levels of the target toxin. Concurrent analysis of the control corn, used for the preparation of cultures, may yield an indication of this probable background level, but these data do not necessarily reflect the likely distribution of that contamination throughout the sample lot. Under these conditions, and in the absence of a recognized "production threshold", it can often be difficult to unequivocally determine the toxin-producing ability of isolates. The reporting of the presence or absence of a particular metabolite may also be influenced by the analytical method used for its determination. Alternative methods exhibit different selectivities and analyte recoveries, which in turn influence the detection limit of the separate methods. It is also important that, having determined the toxin production by fungal isolates, the investigators confirm their observations and verify the identity of the toxin using suitable methods, such as diode array and/or mass spectrometry.

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