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PRODUCTION OF FUMONISINS AND FUSARINS BY *FUSARIUM MONILIFORME* FROM SOUTHEAST ASIA[1]

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ABSTRACT

A liquid culture method for the screening of *Fusarium moniliforme* for the production of fusarins and fumonisins was used to test strains from Southeast Asia. An analytical method for fumonisins from liquid media was developed after evaluation of five solid phase extraction minicolumns under various conditions. Strains isolated mainly from maize from Indonesia, the Philippines and Thailand produced fusarins A, C and F as well as fumonisins B_1 and B_2. Among the strains, the ratio of fusarins to fumonisins production varied considerably indicating a genetic difference between strains. This is the first report of fumonisins B_1 and B_2 from *F. moniliforme* from all three countries. Fusarins are reported for the first time from strains from Indonesia and the Philippines.

Key Words: fumonisins, fusarins, *Fusarium moniliforme*, mycotoxins

Fusarium moniliforme Sheldon is a common contaminant of staple crops including corn, millet and sorghum. Various animal diseases have been associated with ingestion of heavily contaminated maize for ca 100 yr. A number of toxins, including moniliformin, fusaric acid, fusarins and fumonisins have been reported from cultures of this fungus (Nelson, **1992**).

Fumonisins are a group of mycotoxins discovered in 1988 from cultures of *F. moniliforme* isolated from maize in southern Africa (Bezuidenhout et al., **1988**; Gelderblom et al., **1988**). The discovery followed studies of the association of esophageal cancer and ingestion of *F. moniliforme*-contaminated maize in southern Africa. Fumonisins have since been shown to be hepatotoxic and hepatocarcinogenic in rats, and fumonisin B_1 (FB_1) has also been demonstrated to cause a neurological disease in equine species called Equine Leukoencephalomalacia. Six fumonisins (FB_1, FB_2, FB_3 and FB_4; FA_1 and FA_2) have been isolated so far, only two of which (FB_1 and FB_2) are considered to occur in quantity in contaminated maize (Sydenham et al., **1992**; Thiel et al., **1992**).

Fusarin C was discovered as a naturally occurring compound in diseased maize from southern Africa (Gelderblom et al., **1984**). This compound is a potent in vitro mutagen and macrophage inhibitor (Farber and Scott, **1989**; Gelderblom et al., **1986**). Fusarin C is a member of a family of compounds of varying biological activities which also includes fusarins A, D, E and F (Savard and Miller, **1992**).

Strains of *F. moniliforme* isolated from maize from the USA, Australia, Zimbabwe, Nigeria, Transkei, and Nepal have been shown to produce fumonisins B_1 and B_2 (Thiel et al., **1991a**, **b**; Nelson et al., **1991**, **1992**). These studies have employed fermentations on moistened, autoclaved corn kernels. Generally fumonisin B_2 was produced at concentrations about 25% of those of fumonisin B_1 in such fermentations. In the case of fusarins, only fusarin C has been extensively studied. The production of fusarin C by

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F. moniliforme has been shown for strains from Canada, the U.S.A., South Africa, Egypt, Denmark, Czechoslovakia, Finland, Norway, Sweden, England, Switzerland, Germany, the Netherlands, Russia, Honduras, Japan, and Taiwan (Gelderblom et al., **1984**; Farber and Sanders, **1986a, b**; Savard and Miller, **1992**; Thrane, **1988**; Tseng et al., **1990**). From countries in Southeast Asia, there is only one report of a *F. moniliforme* strain from Thailand producing fusarins A, C and F (Savard and Miller, **1992**).

The present study determined the production of fusarins and fumonisins from *F. moniliforme* strains from Southeast Asia: 9 strains from Indonesia, 7 from the Philippines and 11 from Thailand. A liquid culture method was used.

MATERIALS AND METHODS

Fungi.—*Fusarium moniliforme* strains were isolated from corn or sorghum from different provinces in Indonesia, the Philippines and Thailand. Nine strains were isolated from Yogyakarta, Indonesia. Strains FRR 4318, FRR 4319, C10-3I, FRR 4320, and C19-9I were isolated from maize; strains FRR 4317, S1-11I, FRR 4316, and S1-17I were isolated from sorghum. Strains isolated from maize grown in the Philippines were: FRR 4324, isolated from General Santos; FRR 4329, isolated from Banga (South Cotabato); FRR 4321, isolated from Cagayan; M56-9P, isolated from Koronadal; FRR 4322, isolated from Urdaneta; M60-8P, isolated from Santiago; and M61-6P, isolated from Tuguegarao. Maize samples from Thailand yielded: FRR 4299, M96-12T and M96-13T, isolated from Lop Buri Province; FRR 4301, FRR 4300, FRR 4302, M114-6T, M114-11T and M114-12T, isolated from Nakkon Sawan Province; and FRR 4303 and M123-4T, isolated from Loei Province.

Fermentations.—Cultures were initially grown on 2% malt extract agar slants for 7–10 days. A slant was macerated in 27 ml of sterile water. Aliquots (2.5 ml) of the resulting suspension were added to 250 ml erlenmeyer flasks containing 50 ml of inoculation medium made up of ultrapure water (1 L), NH_4Cl 3 g, $FeSO_4 \cdot 7H_2O$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 2 g, KH_2PO_4 2 g, peptone 2 g, yeast extract 2 g, malt extract 2 g (all Difco), and glucose 20 g. After 48 h of incubation in the dark at 28 C on a rotary shaker (220 rpm, 3.81 cm throw), the suspension was macerated and employed as follows:

1) Fusarins: For fusarins production, 2.5 ml aliquots were added to two 250 ml erlenmeyer flasks containing 50 ml of production medium consisting of ultrapure water (1 L), $(NH_4)_2HPO_4$ 1 g, KH_2PO_4 3 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 5 g, sucrose 40 g, and glycerol 10 g (final pH 6.2). The flasks were covered in aluminum foil and incubated as above for 10 days. These conditions had previously been shown to be optimal for fusarins production (Savard and Miller, **1992**).
2) Fumonisins: The remaining macerate of the inoculation medium was centrifuged at 10 000 × *g* for 10 min, 50% of the supernatant removed and the pellet was resuspended by vortexing. A 3.5 ml aliquot of the resulting suspension was added to each of three 250 ml flasks containing 50 ml of production medium and put on the rotary shaker as above for 14 days. These conditions had been previously shown to be optimal for fumonisins production (Blackwell et al., **1993**).

Fusarins analysis.—All operations were performed in aluminum-foil-wrapped glassware. The contents of duplicate flasks of each strain were combined and filtered under vacuum through Whatman no. 41 paper. The filtrate was extracted with ethyl acetate (2 × 75 ml). The organic extract was dried over Na_2SO_4, filtered and concentrated to dryness. A Varian 5000 High Performance Liquid Chromatograph (HPLC) was used along with a BioRad Biodimension high speed scanning ultraviolet (UV)-visible detector. While the full UV spectrum of each peak was stored in memory, wavelengths of 225, 280 and 365 nm were monitored simultaneously for the chromatographic profile. Analysis for fusarins was performed with a Lichrosorb 5-μM, RP-18, 250 × 4.6-mm column and an H_2O-methanol (35:65) solvent system at 1 ml/min. Evaluation of the concentration of fusarins was based on the absorption of a standard fusarin C sample at 365 nm (Savard and Miller, **1992**).

Fumonisins analysis.—The cultures were filtered as above and analysis was performed on each replicate flask respectively as follows. A 1-ml aliquot of filtrate was applied to a Bond Elut Certify II (200 mg, Varian) column preconditioned by aspiring methanol (6 ml) and water (6 ml) under vacuum. The minicolumns were then washed with water (6 ml) and methanol (6 ml). Fumonisins were then eluted with 0.1% trifluoroacetic acid (TFA) in methanol (2.5 ml).

To optimize fumonisins recovery and sample clean-up, two elution solvents (0.1% acetic acid and 0.1% TFA in methanol) were tested on five prepared solid phase extraction minicolumns (TABLE I). The effect of culture filtrate pH was then examined on the two giving the best results, SAX (400 mg, chloride form, Analytichem) and Certify II columns (TABLE II). The pH was adjusted with ammonium hydroxide.

FB_1 and FB_2 were quantified by HPLC using a variation of the analytical method developed by Scott and Lawrence (**1992**). The TFA methanol fraction from the clean-up column was concentrated to dryness and taken up in 1 ml of methanol. A 20-μl aliquot was transferred to a 2-ml vial and dried under a stream of nitrogen. The residue was redissolved in 100 μl of 0.05 M sodium borate buffer, pH 8.3 (adjusted with 1 N HCl). A freshly prepared solution of 4-fluoro-7-nitrobenzofurazan [100 μl of 22 mM NBD-F (Molecular Probes, Inc.) in 95% ethanol] was added. After heating for 70 sec at 70 C, the solution was cooled and made up to 500 μl with a 1:1 mixture of HPLC mobile phases A (0.05 M NaH_2PO_4/methanol adjusted to pH 6.3 with 2 N NaOH, 1:1) and B (acetonitrile/H_2O, 8:2). A 20-μl aliquot (corresponding to 8 ng of FB_1 and FB_2 for standard solutions) was injected in duplicate onto a Lichrosorb 5-μm,

TABLE I

AVERAGE FUMONISIN B_1 RECOVERY (%) FROM AQUEOUS SOLUTION[a]

	NH_2[b]	DEA[c]	PSA[d]	SAX[e]	Certify-II[f]
0.1% Acetic acid in methanol	13.5 ± 2.8	0	0	52.1 ± 3.9	47.8 ± 16.2
0.1% Trifluoroacetic acid in methanol	16.3 ± 7.3	0	0	84.1 ± 15.4	92.1 ± 8.8

[a] All analyses were done in triplicate.
[b] Aminopropyl weak anion exchange.
[c] Diethylaminopropyl weak anion exchange.
[d] Ethylenediamine-N-propyl (primary/secondary amine) weak anion exchange.
[e] Strong anion exchange.
[f] Hydrophopic anion-exchange resin.

RP-18, 250 mm × 4.6-mm column on a Varian Vista 5500 HPLC with a Varian Vista CDS-401 data system. An 11 min linear gradient of 0 to 100% B followed by a 2 min plateau was run at a flow rate of 1 ml/min. The derivatized fumonisins were then detected by their fluorescence at 490 nm after excitation at 450 nm (detector: Waters model 420-E). FB_1 and FB_2 were identified by their retention times of 10.3 min and 11.3 min, respectively, at 20 C. A reagent blank produced two main peaks at 6.3 min and 12.3 min.

To determine the optimal conditions for the preparation of the fumonisin derivatives, the NBD-F reaction was run at different pH values adjusted with sodium borate (8, 8.5, 9 and 9.5) and KH_2PO_4/NaOH (6, 7 and 8). The time (60, 70, 90 and 120 sec) and temperature (60, 70, 80 and 90 C) of the derivatizing reaction were also varied.

Isocratic (65:35, 70:30, 75:25 and 80:20) and gradient (6, 8 and 11 min) solvent systems based on mixtures of mobile phases A and B were also tested for the optimization of resolution and FB_1 and FB_2 retention times. A standard curve was created with FB_1 and FB_2 which was used to calculate the concentrations in each sample.

Statistical analysis was performed using Systat version 5.0 (Evanston, Illinois.)

RESULTS

Fumonisins analysis.—Recovery studies of FB_1 (10 μg) from neutral aqueous solutions showed that weak anion-exchange resins (NH_2, DEA and PSA) did not retain FB_1, while recoveries from SAX and Certify II columns were comparable (TABLE I). The strength of the acid in the eluting solvent also played a major role. While only 50% of fumonisin B_1 was recovered with 0.1% acetic acid in methanol, 85–90% was recovered with the same volume (14 ml) of 0.1% TFA in methanol. The effect of pH on the recovery of fumonisins was studied in more detail for the SAX and Certify II columns (TABLE II). The pH of solutions of FB_1 (10 μg) dissolved in toxin-free liquid medium was adjusted to 2.7, 4 and 5.7. In this case, lower pHs were found to greatly reduce the recovery of FB_1 from SAX columns, while recovery from Certify II columns was always quantitative. Fractionation of the eluent from the latter column also showed that all FB_1 was eluted in the first 2.5 ml of 0.1% TFA in methanol instead of the 14 ml necessary for other columns and eluents.

Fumonisins derivatized with NBD-F were stable for up to 30 min in a mixture of HPLC mobile phases A and B (1:1) at room temperature (loss of 10 and 20% fluorescence response after 2.5 and 6 h, respectively) and up to 1 day when stored at −12 C. The combination of the Certify II clean-up and the NBD-F derivatization pro-

TABLE II

AVERAGE FB_1 RECOVERY (%) FROM FUMONISIN PRODUCTION MEDIUM

	SAX			Certify-II		
	pH 2.7	pH 4.0	pH 5.7	pH 2.7	pH 4.0	pH 5.7
Load	21.4 ± 20.9	4.9 ± 3.0	0	0	0	0
Water wash	67.7 ± 47.7	0	0	0	0	0
MeOH wash	8.2 ± 0.9	0	0	0	0	0
0.1% TFA/MeOH	5.8 ± 2.0	55.7 ± 9.8	83.3 ± 7.1	103.2 ± 4.5	114.7 ± 4.5	125.1 ± 14.8
	n = 3[a]	n = 3	n = 2	n = 9	n = 3	n = 2

[a] Number of replicate analyses.

TABLE III

FUMONISINS AND FUSARINS IN SOUTHEAST ASIAN STRAINS OF *FUSARIUM MONILIFORME*

Strain[a]	Fusarins (mg/L)				Fumonisins (mg/L)		
	F	C	A	Total	B_1	B_2	Total
FRR 4316	0.16	0.38	0.11	0.65	0.29	0.46	0.75
S1-11I	0.54	0.7	0.24	1.5	0.23	0.43	0.66
S1-17I	0.74	1.2	0.29	2.2	17	6.2	23
FRR 4317	2.8	5.0	1.7	9.5	0.54	10.4	10.9
C19-9I	0.16	0.48	0.06	0.7	20	3.6	24
C10-3I	0.56	1.6	0.32	2.5	79	20	99
FRR 4318	1.6	2.7	0.77	5.1	84	19	103
FRR 4319	1.6	3.6	0.76	6.0	105	18	123
FRR 4320	6.0	8.3	3.0	17	147	45	192
FRR 4324	1.0	2.4	0.46	3.9	51	13	64
M56-9P	4.2	3.4	1.5	9.1	38	9.1	47
FRR 4329	2.3	7.5	1.2	11	34	7.2	41
M61-6P	5.5	5.5	1.5	12	24	4.7	29
FRR 4322	6.8	11	2.8	21	140	21	161
FRR 4321	20	26	6.9	53	71	14	85
M60-8P	23	25	7.5	55	72	17	89
FRR 4301	2.7	7.2	1.1	11	23	4.9	28
FRR 4299	4.8	6	1.8	13	6.9	1.8	8.7
M96-13	7.2	18	3.9	29	124	22	146
M96-12	15	11.4	4.2	31	54	4.0	58
FRR 4300	13	12.6	5.1	31	n.t.[b]	n.t.	n.t.
FRR 4303	12	18	4	34	47	8.6	56
M114-6	18	18	5.4	41	30	5.6	36
M114-12	24	36	7.2	67	18	3.1	21
FRR 4302	36	45	13	94	20	1.8	22
M123-4	49	54	18	121	71	20	91
M114-11	75	75	24	174	24	5.1	29

[a] FRR: Culture Collection of CSIRO Division of Food processing, North Ryde 2113, Australia.
[b] Not tested: n.t.

cedures for quantitating FB_1 and FB_2 gave excellent recoveries (TABLE II).

Toxin production.—All strains of *F. moniliforme* tested produced fusarins A, C and F as well as fumonisins B_1 and B_2 (TABLE III). Total fusarins (A, C, F) production varied from less than 1 mg/L to 174 mg/L. The best producing strains were isolated from Thailand. All strains tested produced FB_1 and FB_2 in amounts from 0.5 to 192 mg/L. Typically, FB_2 was produced at 7–26% of FB_1 (TABLE III). The best producing strains were isolated from Indonesia and the Philippines. Fusarins and fumonisins analysis from replicate cultures tested to date gave standard deviations of ca 16% and 25%, respectively.

Total fusarins and fumonisins production of each strain are shown in FIG. 1. The concentrations of fusarins and fumonisins produced were not correlated. The productions of fusarins A, C and F were highly correlated to each other as were the productions of fumonisins B_1 to B_2 (TABLE IV). There was a difference in the fumonisins and fusarins production between the strains isolated from corn from Indonesia, the Philippines and Thailand. Fumonisins : fusarins ratios were ca 15:1; 6:1 and 1:1, respectively.

DISCUSSION

The use of liquid fermentations to determine the production of fusarins and fumonisins is a relatively simple process. Recoveries of these compounds from liquid media were good (TABLE II; Savard and Miller, **1992**). Repeatability of the results for fumonisins from a single strain was comparable to one report from fermentations for fumonisins on autoclaved corn (Leslie et al., **1992**). However, such replications are apparently not normally made (Leslie et al., **1992**; Nelson et al., **1991**; Thiel et al., **1991a**). The present method provides such replications which allows analysis of the relative performance of each strain.

This report expands knowledge of the toxigen-

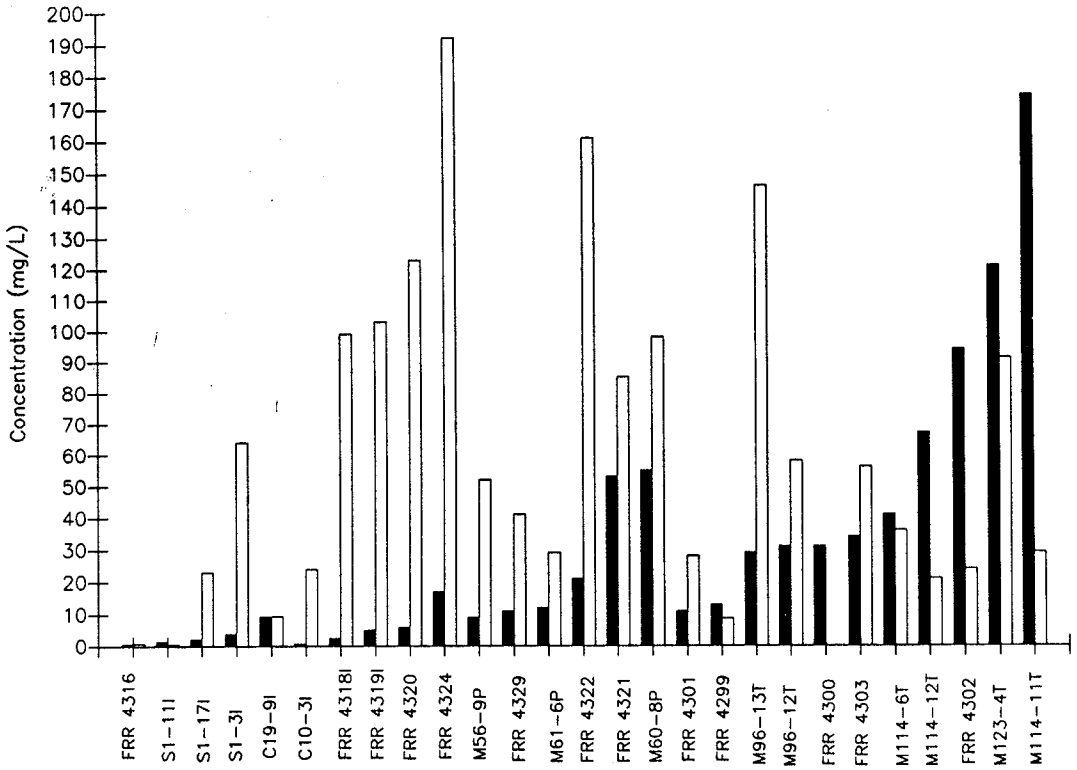

FIG. 1. Production of fusarins (solid bars) and fumonisins (empty bars) by Southeast Asian strains of *F. moniliforme* in liquid medium.

ic potential of Southeast Asian strains of *F. moniliforme*. Data on the production of fusarins A, C and F of one strain from Thailand and on fusarin C production from a few strains from Taiwan have been reported (Savard and Miller, **1992**; Tseng et al., **1990**). There were no previous studies on fumonisins production from this area. Yields of fusarins from these strains were generally comparable to those from one South African and 5 North American strains of *F. moniliforme* as well as 16 European strains tested under the same conditions (Rapior et al., **1993**; Savard and Miller, **1992**). Fumonisins production from these Southeast Asian strains was also comparable to yields from North American and European strains of *F. moniliforme* (Miller et al., unpub. data; Rapior et al., **1993**).

As reported by others, the ability of strains to produce fumonisins is apparently widespread (Nelson et al., **1992**; Ross et al., **1990**; Thiel et al., **1991a, b**). The ratio of fumonisin B_1 to B_2 in cultures in the present study is similar to that found in other reports (Ross et al., **1991**). However, this study has revealed that marked differences can occur in the proportion of the fusarins to the fumonisins produced between strains from

TABLE IV

PEARSON CORRELATION MATRIX FOR FUSARINS AND FUMONISINS PRODUCTION

	A	C	F	B_1	B_2
Fusarin A	1.000				
Fusarin C	0.894	1.000			
Fusarin F	0.934	0.977	1.000		
Fumonisin B_1	−0.230	−0.163	−0.202	1.000	
Fumonisin B_2	−0.165	−0.109	−0.152	0.889	1.000

different geographic regions. Most of the strains from Thailand were distinct in this regard. This is analogous to geographically distinct metabolite patterns from strains of *F. graminearum* isolated from different parts of the world (Miller et al., **1991**). Various studies have suggested that the toxicity of extracts of *F. moniliforme* cultures is highly variable (e.g., Norred et al., **1991**; Ross et al., **1991**). A partial explanation of this may lie in the variable co-occurrence of fusarins.

Recent studies of the fungi associated with various commodities from Thailand have shown that *F. moniliforme* is quite common in maize (Pitt et al., unpubl. data). Work currently underway indicates a similar situation in Indonesia and the Philippines. It will be of interest to examine the toxigenic potential of more of these strains and determine the natural occurrence of fusarins and fumonisins in the commodities concerned.

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