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Mycotoxin Production by *Fusarium proliferatum* isolates from rice with *Fusarium* sheath rot disease

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Abstract

Twenty samples of unpolished (rough) rice collected in Arkansas and Texas during the 1995 harvesting season from fields exhibiting *Fusarium* sheath rot disease or panicle blight were previously shown to include 8 samples positive for fumonisin B_1 (FB_1) in the range 2.2–5.2 ppm, and moniliformin (MON), but no beauvericin (BEA), deoxynivalenol, its derivatives or zearalenone were detected. Fifteen cultures of *F. proliferatum* were established from the 20 rough rice samples. Single spore isolates of each culture were grown on rice and tested for the production of fumonisins (FB_1, FB_2, FB_3, etc.), MON and BEA. All 15 isolates produced FB_1, FB_2, MON and BEA in culture on rice. No deoxynivalenol, its derivatives or zearalenone were detected. Seven cultures produced FB_1 at >50 ppm (range 80–230 ppm), with the rest producing FB_1 in the range 14–43 ppm. FB_2 was produced in the range 5–47 ppm, and those cultures which produced the most FB_1 also produced the most FB_2. Of the 15 cultures producing MON, 11 produced it at >100 ppm in the range 188–6018 ppm, with the rest producing in the range 7–64 ppm. BEA was produced in the range 109–1350 ppm. Other derivatives of fumonisins, including FA_1, FA_2 and partially hydrolyzed FB_1, as well as several unknown metabolites including a compound with MW 414, were identified in culture extracts by continuous flow fast atom bombardment with ion spray mass spectrometry (CF/FAB/MS). Further study is needed to identify the factors that control production of FB_1, MON and BEA by *F. proliferatum* in culture and in field samples.

Introduction

Fusarium spp. are known to produce a variety of mycotoxins and phytotoxins which include the fumonisins, moniliformin and beauvericin (Figure 1) [4, 7, 10, 11, 16, 17, 19, 27, 28, 31, 34, 35]. The fumonisins include fumonisin B_1 (FB_1), FB_2, FB_3, FB_4 and other series of derivatives (A-series, C-series). FB_1 causes equine leukoencephalomalacia, pulmonary edema in swine and has been implicated in the causation of human esophageal cancer [21, 25, 30]. The fumonisins are also phytotoxic to various weed and crop species [1, 6, 32], and they may be a virulence factor. Moniliformin is also phytotoxic and causes hemorrhage in rats and poultry [5, 8, 12]. Beauvericin has been found in a toxic culture of *F. proliferatum* from corn and is known to be toxic to brine shrimp and insects [18, 20]. High levels of beauvericin have been observed in naturally contaminated corn in South Africa [36]. All these toxins are known to be significant contaminants of corn, and therefore are a concern for food and feed safety [3, 22, 24, 37-40]. Recently, the presence of FB_1, FB_2, FB_3, and moniliformin was detected in samples of commercial rice from fields exhibiting *Fusarium* sheath rot disease [16–18] associated with *F. proliferatum* [2]. The study described here was carried out to determine if *F. proliferatum* isolated from rice exhibiting *Fusarium* sheath rot disease can pro-

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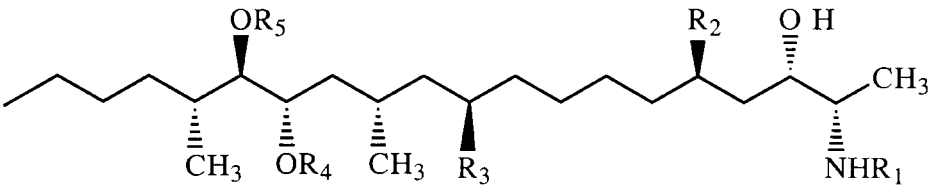
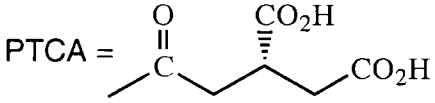
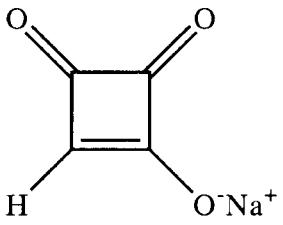
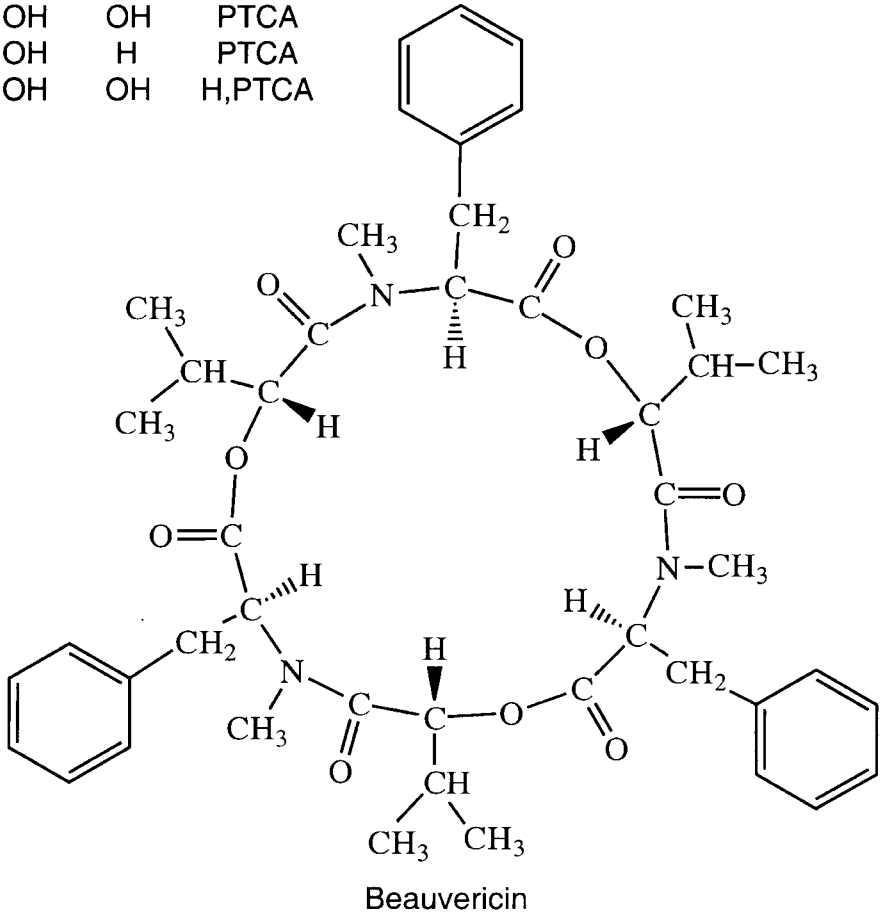


Fumonisin	R_1	R_2	R_3	R_4/R_5
FB_1	H	OH	OH	PTCA
FB_2	H	OH	H	PTCA
FB_3	H	H	OH	PTCA
FB_4	H	H	H	PTCA
FA_1	$COCH_3$	OH	OH	PTCA
FA_2	$COCH_3$	OH	H	PTCA
$PHFB_{1a,b}$	H	OH	OH	H,PTCA





Moniliformin



Beauvericin

Figure 1. Structures of mycotoxins produced by *F. proliferatum* isolates from rice samples from fields with *Fusarium* sheath rot disease.

Table 1. Concentrations of mycotoxins produced by *Fusarium proliferatum* isolates cultured on rice

F. proliferatum culture code numbers	Mycotoxin levels (ppm)*			
	FB_1	FB_2	MON	BEA
FP3 (NRRL 26186)	35	4	947	401
FP4 (NRRL 26185)	200	40	425	350
FP1 (NRRL 26187)	85	8	6018	338
FP2 (NRRL 26188)	25	5	64	154
FP9 (NRRL 26189)	145	11	972	1350
FP14 (NRRL 26190)	43	6	5002	450
FP19 (NRRL 26191)	137	19	13	109
FP45 (NRRL 26192)	135	22	7	109
FP58 (NRRL 26193)	18	5	1100	188
WB2 (NRRL 26197)	185	33	1973	837
WB3 (NRRL 26198)	22	6	37	233
TL1 (NRRL 26194)	14	5	781	157
TL5 (NRRL 26195)	32	6	645	554
TL10 (NRRL 26196)	99	8	128	185
KEITH3 (NRRL 26199)	327	6	506	652

*Average of two independent analyses by HPLC.

duce fumonisins, moniliformin and beauvericin when cultured on rice.

Materials and methods

Rice samples and fungal isolation. The size, sources, and type of the 20 rice samples used in this study have been described in detail [2]. The isolation and identification procedures for the 15 cultures of *F. proliferatum* used in this study have also been described in detail [2, 3, 29].

Determination of Fusarium *species.* A total of 15 *Fusarium* isolates were used in this study. They were identified as *F. proliferatum*. The identification was confirmed by the Fusarium Research Center, Pennsylvania State University [3, 29]. Stock cultures were stored on silica gel, soil or lyophilized and placed in (i) the mycoherbicide collection at the Southern Weed Science Research Unit, Stoneville, MS; and (ii) the fungal collection, USDA-ARS, NCAUR, Peoria, ILL under the NRRL code numbers given in Tables 1 and 2.

Media and single-spore isolation. The procedure used in this study was essentially the same as described previously [3]. Briefly, potato dextrose agar (PDA, Difco) was prepared according to the manufacturer's instructions. Agar/water plates contained 7.5 g agar in 500 ml water. *Fusarium proliferatum* cultures were transferred to 1 ml of sterile Tween 20 in water ($1:10^4$, v/v) with a sterile wire loop. The suspensions were mixed on a vortex mixer, and streaked on agar/water plates with a sterile loop. Plates were incubated at room temperature overnight. For each culture, three germinated spores were picked and used to inoculate PDA slants and plates. Cultures were incubated at room temperature.

Mycotoxin production. Mycotoxins were produced by all isolates of *F. proliferatum* on solid rice as described previously [4, 7, 34]. Similar results were obtained with corn cultures. Briefly, 120 ml water was added to 200 g rice in 2800 ml Fernbach flasks, allowed to stand at least 2 hours and autoclaved. Fungi were grown on V8 agar plates at 28 °C for 2 weeks for use as inocula. V8 agar contained 1 can V8 juice (455 ml), 5.25 g $CaCO_3$, 1400 ml water, 10 g agar. Each culture plate was flooded with 5 ml sterile water, and scraped with a pipet to suspend the fungus. The suspensions were transferred to the autoclaved grain. The cultures were mixed by gentle shaking to break up clumps and incubated at 28 °C for 3 weeks. During the first week, each culture was mixed daily by gentle shaking.

Extraction of fumonisins and moniliformin. Fumonisins and MON were extracted under the same conditions. Similar extraction efficiencies were observed for extraction of fresh fungus-infested culture material and cultures that were air dried to facilitate shipping and subsequently rehydrated by absorption of an equal weight of water prior to extraction. Fresh fungus-infested culture material (30 g samples in triplicate) was extracted with 100 ml acetonitrile:water ($80:20$, v/v) or 50% methanol in water by shaking on a wrist shaker for 1 h. For cultures FP3 and FP4, 200 g samples in triplicate were extracted as described above after rehydration with 120 ml water. Extracts were obtained by filtering through 12.5 cm #588 filter paper (Schleicher and Schuel), and stored at room temperature until used for fumonisin or moniliformin purification [4, 7, 9, 34]. Subsequent studies have avoided storage of methanol-containing extracts at room temperature to reduce the possibility of forming fumonisin methyl esters.

Fumonisin sample preparation. Samples were prepared for HPLC analysis using SPE ODS-3 columns (Whatman Solid Phase Extraction Device ODS-3, 500 mg/6 ml, cat. no. 6801-0307) on a vacuum manifold with solvent flow rate = 2 ml/min [33]. Columns were preconditioned with 5 ml methanol followed by 5 ml 1% KCL. To prepare fumonisin samples, 2 ml

Table 2. Proportion of other fumonisins relative to FB_1 produced in rice cultures of *F. proliferatum* isolates obtained from rice with *Fusarium* sheath rot disease

F. roliferatum culture code number	Other fumonisin as a % of FB_1*						
	FB_2	FB_3	FA_1	FA_2	FB_4	$PHFB_{1a,b}$	
FP3 (NRRL 26186)	30.4	2.0	9.5	3.6	1.7	4.6	6.0
FP4 (NRRL 26185)	36.3	3.8	7.9	3.0	2.1	2.7	3.4
FP1 (NRRL 26187)	33.7	6.1	5.7	1.9	0.9	5.0	6.4
FP2 (NRRL 26188)	49.3	7.2	10.7	3.6	5.8	2.1	2.2
FP9 (NRRL 26189)	30.9	12.6	4.0	1.1	6.8	9.5	12.5
FP14 (NRRL 26190)	49.1	6.0	5.6	3.2	7.5	2.2	2.5
FP19 (NRRL 26191)	32.1	29.2	12.8	4.3	7.0	3.9	4.4
FP45 (NRRL 26192)	88.7	3.6	0.8	1.1	18.4	6.8	9.6
FP58 (NRRL 26193)	39.2	7.0	0.9	0.5	5.5	2.4	2.9
WB2 (NRRL 26197)	24.2	8.7	1.9	0.5	5.2	45.2	55.8
WB3 (NRRL 26198)	37.1	3.9	3.6	1.4	0.1	5.6	7.9
TL1 (NRRL 26194)	45.4	18.1	2.1	9.3	19.2	6.0	8.4
TL5 (NRRL 26195)	76.2	6.3	3.0	2.3	16.3	11.6	14.5
TL10 (NRRL 26196)	43.2	4.1	1.4	1.1	3.9	2.2	3.1
KEITH3 (NRRL 26199)	44.2	3.5	3.0	1.8	9.1	3.1	4.0
Range	24–89	2–29	1–13	1–9	0.1–19	2–45	2-56

Results determined by CF/FAB/MS with an 0.5 μl aliquot of extract injected. $PHFB_{1a}$ and $PHFB_{1b}$ are the two possible forms of partially hydrolyzed FB_1. The two peaks for $PHFB_{1a}$ and $PHFB_{1b}$ have not been unequivocally assigned.

of sample extract was mixed with 5 ml 1% KCL and applied to a column. The column was washed with 5 ml 1% KCL followed by 2 ml of 10% acetonitrile in 1% KCL. Fumonisins were eluted from the column with 4 ml of 70% acetonitrile in water into a 10 ml silane-treated vial, and dried in a heating block at 60 °C under a stream of nitrogen. Samples were stored at room temperature until HPLC analysis [33].

HPLC analysis of fumonisins. Samples for analysis of all fumonisins were resuspended in 1 ml methanol. The following reagents were added with mixing in order: sodium borate buffer (1 ml of 0.05 M, pH 9.5), NaCN solution (0.5 ml of 13 mg/100 ml water) and naphthalene-2,3-dicarboxaldehyde reagent (0.5 ml of 2 mg/8 ml methanol). Samples were then incubated in a heating block at 48 °C for 15 min and allowed to cool to room temperature. The samples were diluted with 7 ml of mobile phase water : acetonitrile : acetic acid (40 : 60 : 1), filtered through a 0.2 μm filter (Gelman 13 mm diameter, 0.2 μm pore size, acrodisc PTFE) and an aliquot injected onto the HPLC column.

Moniliformin sample preparation and HPLC analysis. A 10 ml aliquot of each extract (acetonitrile : water, 80 : 20) was transferred to silane-treated 125 ml round bottom flasks and concentrated to near dryness (<1 ml) on a rotary evaporator at a bath temperature of 40–50 °C. The residues were resuspended in 25 ml methanol, transferred to 125 ml separatory funnels with the aid of a 5 ml methanol rinse, and extracted with 3–40 ml hexane. The lower phase of each sample was concentrated to near dryness in a silane-treated flask and suspended with swirling and vortexing in 5 ml mobile phase [900 ml water: 100 ml acetonitrile: 10 ml ion pair (24 ml tetrabutyl ammonium hydroxide in 50 ml 1.1 M K_2HPO_4)]. Suspensions were allowed to settle for 10–15 min at room temperature, and a 1 ml aliquot of the supernatant filtered through a 0.2 μm polyvinylidene fluoride (PVDF) syringe filter (Whatman 13 mm diameter, 0.2 μm pore size, Cat. no. 6777-1302) and the sample injected onto an octadecylsilane column (4.6 mm internal diameter by 250 mm) as described previously by Scott et al. [34]. Moniliformin was detected by UV absorption at 229 and 254 nm. Off scale samples were diluted 1/100 with mobile phase and reinjected [9, 34].

Beauvericin sample preparation and HPLC analysis. The method of Plattner and Nelson [31] was used. Briefly, 25 g samples of culture material were extracted with 125 ml ethyl acetate followed by 125 ml of 50% acetonitrile in water. Beauvericin levels in fungus-infested grains and rice samples were determ-

ined by HPLC on a C_{18} column (methanol : water 67 : 33, v/v) with detection by UV absorption at 204 nm.

Sample preparation and analysis of the unknown metabolite with molecular weight of 414. Extraction and detection of the novel *Fusarium* metabolite with molecular weight of 414 and molecular formula of $C_{21}H_{38}N_2O_6$ was as described in detail by Vesonder et al. [41].

Silane treatment of glassware. Silane treatment of glassware was carried out as recommended by Romer Labs, Inc. Briefly, glassware was submerged in or filled with 5% dichlorodimethylsilane in iso-octane, and dried in a fume hood for at least 1 h. The glassware was then rinsed twice with methanol and dried at 100 °C for 1 h.

Identification of Mycotoxins. The identity of FB_1, FB_2, FB_3, FB_4, FA_1, FA_2, $PHFB_{1a}$, $PHFB_{1b}$, and related compounds in this group was confirmed by continuous flow fast atom bombardment with ion spray mass spectrometry (CF/FAB/MS) interfaced to microcapillary HPLC [2, 26] (Figure 2). FB_1 and FB_2 were also confirmed by the thin layer chromatography (TLC) procedure of Rottinghaus et al. [33]. Moniliformin was identified based on relative mobility or retention time using TLC (4) or HPLC [34], respectively, and comparison to an authentic standard. BEA was identified based on comparison of the retention time to that of an authentic standard using HPLC [31]. The presence of BEA in two cultures was also confirmed in the laboratory of Dr. R. Krska using their method [22, 23]. Mycotoxins used as TLC standards were obtained from Sigma Chemical Co., St. Louis, MO, except moniliformin and the unknown metabolite with molecular weight of 414, which were gifts from R. F. Vesonder.

Results

All 15 cultures established from 20 rice samples obtained from fields with *Fusarium* sheath rot disease were identified as *Fusarium proliferatum*. Each of the 15 isolates produced FB_1 in the range 14–200 ppm when grown in culture on solid rice medium. Seven of the 15 isolates produced >50 ppm FB_1 ranging from 85–200 ppm. Each isolate also produced FB_2, ranging from 5–40 ppm, and those isolates which produced the highest levels of FB_1, also produced the highest levels of FB_2 (Table 1). Each of the 15 isolates produced additional fumonisins, which were identified by TLC and CF/FAB/MS as FB_3, FB_4, FA_1, FA_2, and $PHFB_1$ isomers a and b. The relative amounts of these fumonisins are presented in Table 2. The culture which contained the largest number of fumonisin-like metabolites by TLC analysis (PF4) was analyzed further by capillary LC/FAB/MS in full scan mode, and shown to also contain FB_1 monomethyl ester (4 isomers) and FB_1 dimethyl ester (Figure 2).

All 15 *F. proliferatum* isolates produced moniliformin ranging from 7–6018 ppm. Eleven cultures produced moniliformin levels of >100 ppm with a range of 128–6018 ppm. Those producing <100 ppm had a range of 7–64 ppm (Table 1). All 15 *F. proliferatum* isolates produced beauvericin ranging from 109–1350 ppm. Nine of the isolates produced >200 ppm BEA ranging from 233–1350 ppm (Table 1). All 15 *F. proliferatum* isolates also produced a new metabolite with molecular weight of 414 and molecular formula of $C_{21}H_{38}N_2O_6$, the extraction and detection of which has been described in detail by Vesonder et al. [41].

Discussion

We report here the observation that *F. proliferatum* isolates from field samples of rice with *Fusarium* sheath rot disease are capable of producing fumonisins and moniliformin in culture. This result is consistent with *F. proliferatum* being the source of fumonisins and moniliformin detected in naturally contaminated rice samples from fields with *Fusarium* sheath rot disease. Whether *F. proliferatum* is the primary cause of *Fusarium* sheath rot disease or a strong secondary invader of sheaths and panicles damaged by primary bacterial infections is not clear [2, 13–15]. Each of the isolates also produced large amounts of beauvericin when cultured on rice, although it was not detected in any field samples. Presumably, culture conditions were much more favorable for BEA production, and moderately more favorable for MON production by *F. proliferatum*, than the conditions which the fungus experienced on viable grains in the field. The production of FB_1 monomethyl esters and FB_1 dimethyl ester may be an artifact of the isolation conditions, which included methanol as a solvent for extraction and subsequent purification. This possibility can be eliminated by isolation with acetonitrile-containing solvents instead of methanol.

The results obtained in this study indicate that rice produced in areas experiencing *Fusarium* sheath

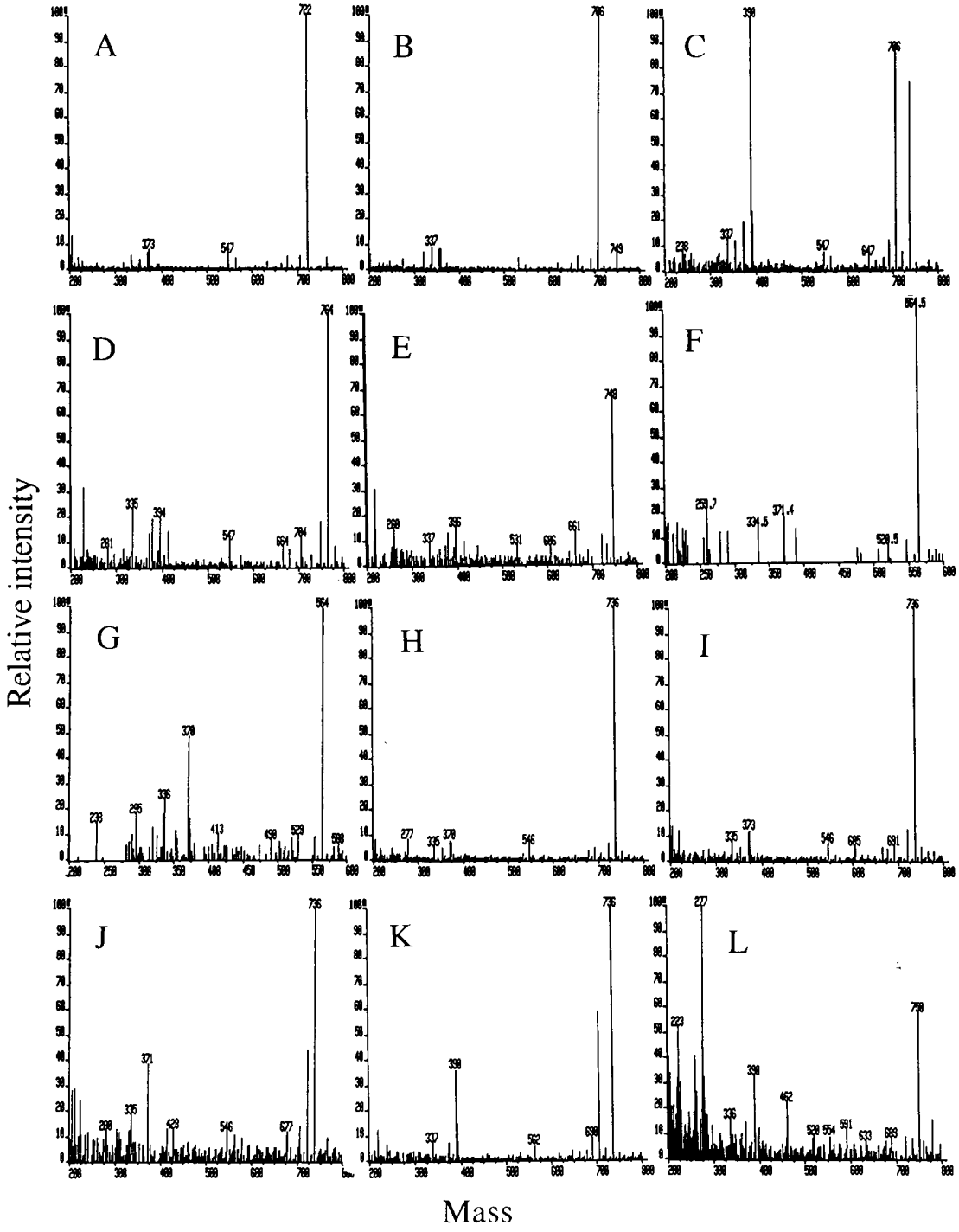


Figure 2. Product ion fragments of (A) FB_1 [$(M + H)^+$ = 722]; (B) FB_2 [$(M + H)^+$ = 706]; (C) FB_3 [$(M + H)^+$ = 706]; (D) FA_1 [$(M + H)^+$ = 764]; (E) FA_2 [$(M + H)^+$ = 748]; (F) $PHFB_1$, isomer 1 [$(M + H)^+$ = 564]; (G) $PHFB_1$, isomer 2 [$(M + H)^+$ = 564]; (H) FB_1 monomethylester isomer 1 [$(M + H)^+$ = 736]; (I) FB_1 monomethylester isomer 2 [$(M + H)^+$ = 736]; (J) FB_1 monomethylester isomer 3 [$(M + H)^+$ = 736]; (K) FB_1 monomethylester isomer 4 [$(M + H)^+$ = 736]; and (L) FB_1 dimethylester [$(M + H)^+$ = 750].

rot disease should be monitored for fumonisins and moniliformin.

Acknowledgements

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