ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2010, 17, 301–308

VOLATILE PROFILES OF TOXIGENIC AND NON-TOXIGENIC *ASPERGILLUS FLAVUS* USING SPME FOR SOLID PHASE EXTRACTION

Anthony J. De Lucca, Stephen M. Boué, Carol H. Carter-Wientjes, John M. Bland, Deepak Bhatnagar, Thomas E. Cleveland

Southern Regional Research Center, USDA, New Orleans, USA

De Lucca AJ, Boué SM, Carter-Wientjes CH, Bland JM, Bhatnagar D, Cleveland TE: Volatile profiles of toxigenic and non-toxigenic *Aspergillus flavus* using SPME for solid phase extraction. *Ann Agric Environ Med* 2010, **17**, 301–308.

Abstract: Toxigenic and atoxigenic strains of *Aspergillus flavus* were grown on potato dextrose agar (PDA) and wetted (23% moisture) sterile, cracked corn for 14 and 21 days, respectively. Volatile compounds produced by *A. flavus*, as well as those present in the PDA controls and sterile cracked maize, were collected using solid-phase micro-extraction (SPME) and identified by gas chromatography/mass spectrometry. Results show that growth substrate had a major impact on the number and type of volatiles detected. Growth on sterile cracked maize produced many more volatiles than did potato dextrose agar. There were also differences observed in the type of volatiles produced between toxigenic and non-toxigenic isolates, as well as between isolates of the same toxigenic grouping.

Address for correspondence: A. J. De Lucca, Southern Regional Research Center, USDA, ARS, 1100 Robert. E. Lee Blvd., New Orleans, LA 70124 USA.
E-mail: Anthony.DeLucca@ars.usda.gov

Key words: ***Aspergillus flavus*, secondary metabolic volatiles, aflatoxin, maize.**

INTRODUCTION

Fungi, under conditions conducive to growth, can grow on pre- or post-harvested food and feed resulting in inedible food contaminated with mycotoxins. Mycotoxins are of global concern because of the great risk they pose to human and animal health [14]. Among the most potent mycotoxins are the aflatoxins produced by *Aspergillus flavus*. These toxins are the most potent natural carcinogens known.

Fungi, including *A. flavus*, are known to produce a wide range of volatile compounds during both primary and secondary metabolism [11]. Many metabolic volatiles produced by *A. flavus* have been identified [2, 6, 15]. However, there are differences in these volatiles in the literature. This may be due, in part, to differences in growth substrate [4].

In SPME (solid-phase micro-extraction) extraction, volatiles are adsorbed onto a stationary phase coating a fused silica fibre [10]. Headspace SPME is based on the equilibrium of analytes between the following three phases of the system: polymeric liquid coating, headspace and sample matrix [10]. The amount of analyte adsorbed by the fibre is affected by both the thickness of the stationary phase and the distribution constant [10].

The purpose of this study was to investigate the identities of *A. flavus* produced metabolic volatiles when grown on two different growth media, potato dextrose agar (PDA) and cracked maize. PDA is a defined medium containing freeze-dried aqueous extract of potato and dextrose with agar as a solidifying agent. In contrast, cracked maize is a more natural growth medium containing all the constituents of this grain.

MATERIALS AND METHODS

Growth of fungi on potato dextrose agar. Five millilitres of potato dextrose agar (PDA) was placed in 10 ml screw-capped vials (22.5 × 46 mm, Supelco, Bellefonte,

Received: 25 October 2010
Accepted: 26 November 2010

PA) on which was placed a cap containing a silicone septum. After autoclaving (121°C, 15 lb pressure, 20 minutes) the vials were placed in a slanted position and the agar allowed to cool.

The *A. flavus* isolates (toxigenic isolates: 1000E and AF 13; non-toxigenic isolates: NRRL-5565 or NRRL-5918) were grown on PDA slants for 7 days (30°C) and then stored at 4°C. Each isolate was tested separately in three separate runs using PDA slants (5 ml in a 10 ml screw cap SPME vial [Supelco, Bellefonte, CA]) as a nutrient source. At the beginning of each run, a 3 ml aliquot of 1% potato dextrose broth was added to a stock slant culture of the test isolate which was in storage at 4°C. After preparing a 1 : 20 dilution of the spore suspension, a hemocytometer was used to obtain a count of the spores in this dilution tube. A series of dilutions were performed to obtain a spore suspension (2×10^6/ml). For each sample date, two PDA slant sets were studied. Each set consisted of two 5 ml PDA slants. One set (total 14 slants) was inoculated with the test isolate on day 0 while the other set (total 14 slants) was the PDA sterile control.

Freshly prepared aliquots (100 µl) of the conidial suspension (2×10^6/ml) were immediately inoculated onto the fungal slant sets. No fungal spores were added to the PDA control slant set. The inoculated and control PDA slants were incubated at 30°C. On days 3, 5, 7, 10, 14, 17, and 21 after inoculation, two vials each from the inoculated and control vials were removed from the incubator. A SPME sampling unit was inserted through the septum of each vial and allowed to collect volatiles. After analysis for volatiles, the inoculated slants and sterile PDA control were analyzed for aflatoxin content (see below for protocol).

Fungal growth on whole sterile maize. Dry, intact corn kernels (300 g) were wrapped in a cotton towel and cracked with a mallet. Next, the damaged maize was wetted with 90 ml of deionized water and allowed to sit quietescently for 3 hrs to allow the maize to imbibe the water. Aliquots (approximately 100 grams) of wetted (23% moisture) maize were then added to each of three 1 l two-armed culture flasks (Corning). One arm was sealed with an intact cap while the other arm was fitted with a bored, 45 mm cap through which a 1/8 inch stainless steel bored-through union (Supleco) on which a stainless steel plug (Supelco) was fastened. The three vessels containing the cracked maize samples were autoclaved (121°C, 15 lb pressure) for 1 hour on two successive days.

At the beginning of each experiment, an aliquot (1 m, 1×10^8 conidia/ml) of the respective isolate (toxigenic *A. flavus* isolates 1000E and AF13; non-toxigenic *A. flavus* isolate NRRL-5565) was added to each of two maize flasks. The third flask served as a sterile cracked maize control. Separate runs for each test isolate were performed three times.

The maize control and inoculated culture flasks were incubated at 30°C. On days 3, 5, 7, 10, and 14 after inoculation, the headspace was sampled by SPME (method below). The sampling time was reduced to 14 days in this experiment because in earlier experiments we found that few volatiles, none of which were unique, were present on day 17 or later after inoculation in the experiments using PDA as a nutrient source.

SPME/HPLC/MS ANALYSIS

Analysis of *A. flavus* volatiles when grown on PDA. The SPME fibre units for volatile analysis used in this study for the volatile analyses were purchased from Supelco (Bellefonte, PA). The sample vials were placed in a VWR (Model 35756) aluminum heating block at 30°C with the SPME fibre inserted into the headspace above the sample. The heating block was seated on a Gerstel Multipurpose Sampler (MPS2, Mülheim-an-der-Ruhr, Germany). The SPME fibres were comprised of polydimethylsiloxane divinylbenzene/carboxen/polydimethyl- siloxane (DCP, 50/30 µm). Adsorption was timed for 30 min. The GC-MS parameters employed are described below.

Analysis of *A. flavus* volatiles when grown on cracked maize. The protocol used to analyze the cracked maize samples differed from that for the small PDA agar vials. On sampling days, the culture vessels were sampled by removing the stainless steel plug from the bored-through union followed by insertion of a desorbed SPME unit. The SPME fibres were then exposed to the headspace atmosphere for 1 hour. Afterwards, the fibres were withdrawn into the unit, and the SPME unit removed from the culture vessel. The stainless steel cap was replaced onto the union and the SPME fibre analyzed by GC-MS.

GC-MS Parameters and Analyses. SPME fibres were desorbed at 230°C for 2 min in the injection port of an HP5890/5989A GC-MS (Hewlett-Packard, Palo Alto, CA) with a HP-5 (cross-linked 5% phenyl methyl silicone, Hewlett Packard, Palo Alto, CA) column (50 m, 0.2 mm i.d., 0.5 µm film thickness). GC-MS runs were 40 min and the fibre remained in the injection port for 20 min after each run. The injection port was operated in splitless mode with a He inlet flow pressure of 42 psi. The initial oven temperature was 40°C, held for 3 min, ramped at 10°C min^{-1} to 60°C, then ramped at 3°C min^{-1} to 150°C, and ramped at 20°C min^{-1} to 250°C and held for 5 min. The HP5989A quadrupole mass spectrometer was operated in the electron ionization mode at 70eV, a source temperature of 200°C, quadrupole temperature of 100°C, interface temperature of 200°C, with a continuous scan from m/z 40 to 500.

Positive identification of a component was performed by comparison of its retention time and mass spectrum with that of an authentic compound (when available). Tentatively identified compounds were uniquely identified on the basis of the mass spectra from the Wiley (v. 7 NIST98) library of mass spectral database (Palisade Corp., Newfield, NY).

RESULTS

***Aspergillus flavus* volatiles produced on PDA.** *A. flavus*, when grown on PDA, was shown to produce a wide range of volatile compounds including esters, ethers, ketones, alcohols, hydrocarbons and sulfur compounds. Table 1 lists the volatiles with compound confirmations at or above 80% produced by the toxigenic and non-toxigenic isolates of *A. flavus*. For sake of clarity, the volatiles present in both the PDA control (not inoculated with *A. flavus*) and the *A. flavus* inoculated PDA were removed from the listings in Table 1.

The data in Table 1 show that when grown on PDA, the non-toxigenic strains produce many more volatiles than the toxigenic isolates studied. However, there were several unique volatiles produced by the toxigenic isolates that were not produced by the non-toxigenic cultures. These volatiles included hexane, styrene, 3-phenoxy-1-propanol, heptadecane, 13-docosenamide, and 9-octadecenamide.

Only a few volatiles were detected in the later stages of growth on PDA. Moreover, the volatiles unique to the toxigenic strains of *A. flavus* appeared no later than day 10 after initiation of incubation. For example, styrene, heptadecane, 13-docosenamide, and 9-octadecemamide, (Z)-, were detected only on day 3, hexane was found on day 7; and 3-phenoxy-1-propanol was detected on day 10.

***Aspergillus flavus* volatiles produced on cracked corn.** A different pattern of metabolic volatile production was observed when sterile cracked maize was the growth

Table 1. *Aspergillus flavus*-produced volatiles detected by SPME-GC/MS when grown on potato dextrose agar[a].

RT[b]	Compound	Isolate(s)[c]	Day Detected	% of total volatiles[d]
5.1375[f]	hexane	1000E	7	25.30
5.3528	ethyl acetate	NRRL-5918	7	0.02
5.5577	2-methyl-1-propannol	NRRL-5918	1, 3	0.25–0.41
5.5600		AF-13	1	0.20
8.4053	3-methyl-1-butanol	NRRL-5918	1, 3, 7	0.64–2.41
8.4100		AF-13	1, 3	0.68
8.5487	2-methyl-(S)-1-butanol	NRRL-5918	3	1.92
		5565	7	0.50
			3	1.87
8.8700	(E)-2-methyl-2-butenal	NRRL-5918	3	0.59
			7	0.29
13.3100	2-methyl-ethylester-butanoic acid	NRRL-5918	3	0.07
15.5800[f]	styrene	AF-13	3	0.13
20.2876	3-octanone[e]	NRRL-5918	3	8.58
20.9700	5,5-dimethyl-1,3-hexadiene	NRRL-5918	3	1.43
25.4700	2,6-dimethyl-5,7-octadien-4-one	NRRL-5918	7	0.10
			3	0.01
25.6300	benzoic acid	NRRL-5918	21	0.21
26.3300	ethyl octanoate	NRRL-5918	3	0.04
27.2800	1-phenoxypropan-2-ol	NRRL-5918	3	0.05
27.2900[f]	3-phenoxy-1-propanol	AF 13	10	0.09
27.7400	3-phenyl-2-propenal	NRRL-5918	3	0.01
29.1000	biphenyl	NRRL-5918	21	0.16
30.1600	3-methyl-1,1”-biphenyl	AF-13	3	0.02
		NRRL-5918	21	0.26
30.8900	3-methyl-2-butanamine	NRRL-5918	21	0.12
30.9400	hexadecane[e]	NRRL-5918	3	0.04
31.7907	2-propen-1-one,1-(4-aminophenyl)-3-phenyl-	NRRL-5565	7	0.16
31.9000[f]	heptadecane[e]	AF-13	3	0.08
32.8960[f]	13-docosenamide	1000E	3	0.74
33.0403[f]	9-octadecenamide, (Z)-	1000E	3	1.97

[a] Only >80% confirmed identities reported. Volatiles from potato dextrose agar control also present in fungal samples have been removed from fungal volatile listings; [b] Retention time; [c] 1000E and AF13, toxigenic; NRRL-5565 and NRRL-5918, non-toxigenic; [d] Range of values; [e] Identification based on the comparison of retention time and mass spectra with standard under the same conditions; [f] Unique for toxigenic strains tested.

substrate. The metabolic volatiles produced by both the toxigenic *A. flavus* isolates 1000E and AF13 as well as those produced by the non-toxigenic isolate NRRL-5565, are listed in Table 2. There was a difference in the number of volatiles produced between the substrates. Many more volatiles were produced by the *A. flavus* isolates on sterile cracked maize than when PDA was the growth substrate. Moreover, the toxigenic isolates produced many more volatiles than were produced on PDA. Also, the toxigenic isolates, particularly 1000E, produced many more volatiles than did the non-toxigenic isolate NRRL-5565. This large number of volatiles is not unique on a vegetative substrate. Approximately 100 volatiles were reported produced by fungi in contaminated canned tomatoes [1].

DISSCUSSION

Differences were observed in the number of total, as well as unique, volatiles produced by the toxigenic *A. flavus* isolates on the two media. On cracked maize, the toxigenic strains, particularly *A. flavus* 1000E, produced many more volatiles than the non-toxigenic isolate. The number of volatiles (92) unique for *A. flavus* 1000E and AF-13 was much higher than those for the non-toxigenic NRRL-5565. It is important to note that nearly all of the 92 volatiles unique to toxigenic *A. flavus* metabolism on cracked maize were not present when PDA was the nutrient source. These results show that volatile production, particularly those unique for the toxigenic isolates, is dependent on the substrate.

Table 2. *Aspergillus flavus* volatiles produced on sterile cracked corn[a].

RT[b]	Compound	Isolate	Day(s) Detected	% of total volatiles[c]
3.6625[f]	ethanol	1000E	5	0.8197
5.0965[f]	hexane[e]	1000E	7	0.3857
5.0147	acetic acid[d]	1000E	3, 10, 14	0.3329–0.3641
		NRRL-5565	3, 5, 10, 14	0.3838–0.3930
		AF-13	2	3.0894
5.2195	2-methyl-furan	1000E	5, 10, 14	0.1020–0.7196
		NRRL-5565	2, 5, 7, 10	0.1870–1.2134
		AF-13	7	0.2958
5.3117[f]	ethyl acetate	1000E	5, 10, 14	0.1640–3.5083
5.5267[f]	2-methylpropan-1-ol	1000E	5, 10, 14	0.5413–1.9735
5.7215[f]	tetrahydrofuran	1000E	3	0.0979
7.3296	heptane[e]	1000E	5, 10	0.0390–0.0789
		NRRL-5565	2, 7, 10, 14	0.1076–0.5242
		AF-13	7, 9	0.0731–0.3806
7.6575[f]	2,5-dimethylfuran	1000E	10	0.0508
7.6882[f]	propanoic acid, ethyl ester	1000E	5	0.1391
8.4051[f]	3-methylbutan-1-ol	1000E	5, 7, 10, 14	0.9507–6.4858
8.4257[f]	1-chloropentane	AF-13	7, 9	0.0731–0.3806
8.5281	2-methylbutan-1-ol	1000E	5, 10	0.5652–2.3549
		NRRL-5565	5, 7	1.5042–1.9426
9.0402[f]	dimethyl disulfide	1000E	10	0.0375
		AF-13	2	0.1459
9.2145[f]	2-methylpropanoic acid	1000E	5, 10, 14	0.1223–0.6555
9.8701f	toluene	1000E	5, 14	0.1589–0.1769
10.0236	2,3-butanediol	1000E	10, 14	0.1945–0.2940
		NRRL-5565	7, 10, 14	0.1536–0.4654
		AF-13	7, 9	0.2058–1.1132
10.4130[f]	3-methylthiophene	1000E	10	0.0738
10.6691	1-octene[d]	1000E	5, 7, 14	0.0908–0.1717
11.0174	octane[e]	1000E	3, 5, 7, 10	0.0520–0.3066
		NRRL-5565	2, 3, 5, 7, 9, 10, 14	0.0709–0.1987
		AF-13	2, 7, 9	0.3683–0.8463
11.0275[f]	hexanal[e]	1000E	5	0.1106
12.1851[f]	1,3-octadiene	1000E	5	0.9745
12.5948	furfural	AF-13	2	0.3780
12.8816[f]	3-methylbutanoic acid	1000E	5, 10, 14	0.0872–0.9759
13.2810[f]	butanoic acid, isopropyl ester	1000E	5, 10, 14	0.0411–1.4557

Table 2 (continuation). *Aspergillus flavus* volatiles produced on sterile cracked corn[a].

RT[b]	Compound	Isolate	Day(s) Detected	% of total volatiles[c]
13.9674[f]	4-methyloctane	1000E	5, 10, 14	0.1645–0.5261
14.5407[f]	3-methyl-1-butyl acetate	1000E	3, 5, 7, 10, 14	0.1691–1.9420
15.2682	methoxy-phenyl oxime[d]	1000E	10	0.1104–0.2403
		NRRL-5565	3, 5, 7, 9, 10, 14	0.2963–1.5612
		AF-13	2, 7, 9	0.7312–1.9429
15.5654	bicycle[4.2.0]octa-1,3,5-triene	1000E	5, 14	0.0965–0.3969
		NRRL-5565	3, 5, 7, 9, 10, 14	0.0871–1.3803
15.5755	styrene	1000E	5, 14	0.0965–0.3869
		NRRL-5565	5, 7, 9, 10, 14	0.1081–0.9807
16.1389[f]	2-butoxyethanol	1000E	5	1.1344
16.4975[f]	2,3-dimethylbutane	1000E	10	0.3822
16.7023[f]	butyrolactone	1000E	5	0.6410
16.9892[f]	2-butanoic acid	1000E	10, 14	0.0464–0.8888
19.2017[f]	benzaldehyde[d, e]	1000E	5	0.3130
		AF-13	2	0.1743
20.6254	2-pentylfuran[d, e]	1000E	5, 10, 14	0.8217–1.5169
		AF-13	2, 7	1.7260–2.6985
20.8509[f]	hexanoic acid, ethyl ester	1000E	14	0.2533
21.1580[f]	octanal[e]	1000E	10	0.0228–0.3138
21.1684[f]	ethanol,2-(2-ethoxyethoxy)-	1000E	5	0.1994
21.8855[f]	nonane, 2,5-dimethyl	1000E	5	0.1285
21.9060[f]	7-oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-	AF-13	2	0.1478
22.9984[f]	decane, 2,5-dimethyl-	1000E	5	0.2597
22.2233	1-hexanol, 2-ethyl	1000E	5	0.1380–0.2597
		NRRL-5565	3, 7	0.5140–0.9869
22.4796[f]	limonene	AF-13	2, 7	0.0638–0.1073
22.6238[f]	eucalyptol	AF-13	2, 7	0.0750–0.5085
22.9917[f]	benzeneacetaldehyde	1000E	14	0.1035
23.1964	tridecane[e]	1000E	5	0.0730
		NRRL-5565	5, 7, 14	0.201–0.0559
23.2477	decane[e]	NRRL-5565	2, 7, 9	0.1339–0.7504
23.2579[f]	decane,2,4,6-trimethyl-	1000E	7	0.3401
23.2579[f]	1-iodo-2-methylnonane	1000E	10	0.0901
23.2580[f]	tetradecane,2,6,10-trimethyl-	1000E	5	0.1373
23.2580	heneicosane	1000E	10	0.2507
		NRRL-5565	7, 14	0.0247–0.0533
23.2581	hexadecane[e]	1000E	3, 7	0.1984–0.6425
		NRRL-5565	2, 5, 14	0.0368–0.0726
23.2581[f]	octane,2,3,6-trimethyl	1000E	5	2.2523
23.3196	decane,4-methyl-	1000E	5, 14	0.0654–0.1484
		NRRL-5565	3, 7	0.391–0.0458
23.3604[f]	3(2H)-furanone,4-methoxy-2,5-dimethyl-	1000E	5	0.0412
23.5243[f]	2-octen-1-ol, (Z)-	1000E	14	0.5559
23.7394	acetophenone	1000E	3, 5, 7, 10, 14	0.0789–1.0408
		NRRL-5565	2, 3, 5, 7, 9, 10, 14	0.2580–1.4665
		AF-13	2, 7	0.2300–0.7778
24.1389[f]	benzyl alcohol	1000E	5, 7, 10	0.0789–1.0408
24.2926[f]	bicyclo[4.1.0]hept-2-ene,3,7,7-trimethyl-	AF-13	7	0.0857
24.3028[f]	bicycle[3.1.0]hexan-3-ol,4-methyl-1-91-methylethyl)-	AF-13	7	0.1075
24.3029[f]	heptanoic acid, ethyl ester	1000E	14	0.0720

Table 2 (continuation). *Aspergillus flavus* volatiles produced on sterile cracked corn[a].

RT[b]	Compound	Isolate	Day(s) Detected	% of total volatiles[c]
24.3129[f]	undecane,4-methyl-	1000E	7	0.0294
24.5897[f]	tetracosane, 3-ethyl-	1000E	3, 5	0.0472–0.0860
24.5283	nonanal[5]	1000E	5	0.2083–0.2985
		NRRL-5565	2, 14	0.1809–0.6504
24.8970[f]	phenylethyl alcohol	1000E	10	0.0399
26.3208[f]	octanoic acid, ethyl ester	1000E	14	0.1137
26.3822[f]	dodecane	AF-13	7	0.0676
26.5369[f]	decanal[e]	1000E	5	0.3034
26.9046[f]	ethanol, 2-phenoxy-	1000E	5	0.1777
27.1300[f]	2-phenylcyclopropionamide, N-(4-phenylazo)phenyl-	1000E	7, 14	0.0710–0.2912
27.2325[f]	benzeneacetic acid, ethyl ester	1000E	10, 14	0.1266–0.3318
27.2939[f]	1-propanol, 3-phenoxy-	1000E	5	0.8860
27.3862[f]	decane, 1-iodo-	1000E	5	0.1268
27.4475[f]	nonanoic acid	1000E	5	0.5234
27.6319[f]	tridecane, 6-propyl-	1000E	5, 10	0.0437–0.0717
27.6319[f]	dodecane,2,6,11-trimethyl-	1000E	10	0.0643
27.6320[f]	tetracosane	1000E	5	0.6139
27.7445	nonane[e]	1000E	10, 14	0.0410–0.1248
		NRRL-5565	7, 9	0.0594–0.0890
27.7446	eicosane[e]	1000E	3, 5, 7, 10, 14	0.0879–0.5803
		NRRL-5565	2, 3, 5, 9, 10, 14	0.0505–0.3148
		AF-13	7	0.0754–0.0755
27.7447[f]	dotriacontane	1000E	5	0.1129
27.8161	pentadecane[e]	1000E	5	0.0681–0.0900
		NRRL-5565	2, 10, 14	0.0698–0.1559
27.8164[f]	Nonadecane[e]	1000E	7	0.0506
27.8265[f]	2-propanol, 1-[2-(2-methyloxy-1-methylethoxy)-1-methylethoxy]-	1000E	5	0.1066
27.9188[f]	docosane, 9-butyl-	1000E	7	0.0543
27.9802[f]	isobornyl acetate	1000E	5, 10	0.1001–0.1052
		AF-13	2, 7	0.3535–0.6819
28.2055[f]	hexadecane, 3-methyl-	1000E	10	0.0316
28.2055[f]	Nonane, 4,5-dimethyl	1000E	14	0.0311
28.2055	Dodecane[e]	1000E	5, 7, 10	0.032–0.2762
		NRRL-5565	2, 5, 7, 9	0.0361–0.5567
28.2056	docosane	NRRL-5565	3, 7, 14	0.1833–0.5791
28.2669[f]	2-methoxy-4-vinylphenol[3]	1000E	5, 10	0.0288–0.0418
		AF-13	2, 7	0.0547–0.7124
28.2875[f]	1-nonene	1000E	5	0.0721
28.3183[f]	octadecane, 5,14-dibutyl	1000E	5	0.1756
28.3286[f]	docosane, 9-butyl-	1000E	7	0.0529
28.6153[f]	n-decanoic acid	1000E	5	0.2777
28.6461[f]	benzeneacetic acid	1000E	7	0.2087
29.0250[f]	tetradecane	1000E	5	0.3537
29.1889[f]	oleyl alcohol	1000E	5	0.1975
29.3835[f]	1,1'-biphenyl,2-methyl-	1000E	5	0.1975
29.5269[f]	hexanedioic acid, bis(1-methylethyl)ester	1000E	5	0.1241
29.8137[f]	cyclododecane	AF-13	7	0.1379
29.8240[f]	cyclodecane	1000E	5	0.4215
		AF-13	2	0.2836

Table 2 (continuation). *Aspergillus flavus* volatiles produced on sterile cracked corn[a].

RT[b]	Compound	Isolate	Day(s) Detected	% of total volatiles[c]
30.0493[f]	eicosane, 10-methyl-	1000E	10	0.0285
30.0494[f]	octacosane	AF-13	2	0.0619–0.1962
30.0494[f]	nonacosane	1000E	10	0.0240
30.3259[f]	2,2'-dimethylbiphenyl	1000E	5	0.9562
30.4591[f]	1,4-methanoaphthalene	1000E	5	0.9662
30.5411[f]	4,7-methano-1H-indene,3a,4,7,7,7a-tetrahydro-	AF-13	2	0.4196
31.2068[f]	17-pentatriacontene	1000E	5	0.9426
32.0058[f]	1,2-benzisothiazole	1000E	5	1.6642–4.2279
32.1697[f]	5,6,7-trimethoxy-1-indanone	1000E	5	3.4189
32.9994[f]	2-dodecen-1-yl(-)succinic anhydride	1000E	5	1.5138
33.2454[f]	Isopropyl myristate	AF-13	2	0.5509
33.5117[f]	benzenamine	1000E	3, 5	0.0648–0.1040
		AF-13	7	0.1864
34.0647[f]	1-eicosanol	1000E	5	1.2157
34.6383[f]	1,2-benzisothiazole	1000E	5	0.9189
34.7203[f]	2,6-bis(1,1-dimethylethyl)-	1000E	5	0.9352
36.9030[f]	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	1000E	3	14.8687

[a] Summary of 3 runs. Identification quality >80%. Isolates 1000E and 13A are toxigenic; NRRL-5565 is non-toxigenic; [b] Retention time; [c] Range of values from multiple runs; [d] Also present in sterile cracked corn control; [e] Identification based on the comparison of retention time and mass spectra with standards under the same conditions; [f] Unique for toxigenic isolates tested.

These results were very different than those observed with PDA as a substrate. The non-toxigenic isolates grown on PDA produced more volatiles than did the toxigenic strain. There were also appreciably fewer unique volatiles produced by the toxigenic isolates compared to those produced by the non-toxigenic isolates. Of all the unique volatiles observed, only styrene was produced by toxigenic isolates on both media. Styrene has been reported earlier as being produced by *Penicillium* species [9, 13]. We believe that this is the first report of *A. flavus* producing this compound.

When PDA was the growth medium, the majority of the *A. flavus* metabolic volatiles were detected only during days 3–7 of incubation. In addition, the great majority of the *A. flavus* volatiles produced on PDA were not observed consistently during the entire length of the studies, especially during the latter stages of the life of the culture. This is consistent with previous reports which observed that certain *A. flavus* metabolic volatile compounds appeared on some days but not on others [3, 6, 15]. This inconsistent appearance of volatiles may be due to different metabolic pathways being "switched on or off" at different stages of culture growth, with the preponderance of volatiles observed during the early days of growth when the most active period of growth and metabolic activity occurs.

Fungi, including *A. flavus*, are known to produce a wide range of volatile compounds during both primary and secondary metabolism [2, 6, 13, 15]. Different growth substrates can affect the metabolic volatiles produced by *A. flavus*. Several publications have shown that several species of *Aspergillus*, including *A. flavus*, produce different volatiles based on the type of building materials and synthetic media used as growth substrate [5, 6]. In addition, earlier publications reported that *A. flavus* metabolic volatile compounds appeared on some days but not others [3, 7, 17]. The reported volatiles in these publications differ greatly from those observed in the current study in which PDA and sterile cracked maize were used as the growth medium.

The instrumental protocol employed to study the fungal produced volatiles also play crucial roles in the identification of such volatiles. For example, the current study, which employed SPME as the volatile trapping agent, only identified one terpene, limonene, when cracked maize was the substrate, and no terpenes when PDA was the substrate. This is similar to an earlier study of volatiles produced by several *Aspergillus* species in which there was also only detected a single terpene, limonene, by SPME [8]. In comparison, when Tenax was the volatile trapping agent, a number of different fungi, including *A. flavus*, were found to produce mono-, di-, tri- and sesqui-, terpenes [7, 12, 15].

Results shown here and in earlier reports indicate that a comprehensive listing of volatiles produced by the same fungal species must take into account differences in isolates, substrate composition and instrumental analysis employed.

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