Volatile profiles and aflatoxin production by toxigenic and non-toxigenic isolates of *Aspergillus flavus* grown on sterile and non-sterile cracked corn

Anthony J. De Lucca, Stephen M. Boué, Carol Carter-Wientjes, Deepak Bhatnagar

Southern Regional Research Center, USDA, New Orleans, USA

▌Abstract

Aspergillus flavus is a saprophytic fungus which can grow on corn and produce aflatoxins which render it unsafe for consumption as food and feed. In this study, aflatoxin and non-aflatoxin producing isolates of *A. flavus* were grown separately on wet (20% water added), sterile or non-sterile cracked corn. Wet and dry cracked corn controls were included as needed. Secondary metabolic volatiles were identified and aflatoxin concentrations determined over a 12-day period. Volatiles unique to the toxigenic *A. flavus* isolates were determined by comparison with volatiles produced by the respective corn controls and the non-toxigenic *A. flavus* isolate. The number and identity of the volatiles produced by these *A. flavus* isolates varied by isolate, whether sterile or non-sterile corn was the substrate, and the sampling day. Overall, most of the volatiles were produced before day 8 after inoculation. Aflatoxin production was 10-fold lower on the sterile corn, compared to the non-sterile corn. Volatiles unique to the aflatoxin producing isolates were identified on both substrates after comparison with those produced by the non-aflatoxin producing isolate, as well as the corn control samples. Results indicate that several factors (substrate, fungal isolate, culture age) affect volatile and aflatoxin production by *A. flavus*.

▌Key words

Aspergillus flavus, aflatoxin, metabolic volatiles, toxigenic, atoxigenic, corn

INTRODUCTION

Aspergillus flavus is a saprophytic, weakly phytopathogenic fungus present on pre-harvest corn together with *Fusarium verticillioides*. Inefficient drying or ingress of water results in stored corn with a high moisture content [1] that allows these fungi to grow and contaminate the corn with mycotoxins, which are toxic secondary metabolites [2]. *A. flavus* produces mycotoxins (aflatoxins) when growing on oilseeds such as corn. Aflatoxin B_1 (AFB1) is the most toxic of all the aflatoxins and is the most potent, naturally occurring hepatocarcinogen [3]. Over 100 countries regulate acceptable aflatoxin levels [4] while the United States bans crops with AFB1 concentrations exceeding 20 ng/g^{-1} from interstate trade [5]. Aflatoxin contamination results in millions of dollars of value loss to corn farmers annually [6].

Fungi produce many volatile compounds such as alkanes, alcohols, ketones, aldehydes, and terpenes [7, 8, 9]. Such secondary metabolic volatiles can be used to differentiate genera, as well as species of the same genus, if the fungi are grown on the same nutrients [7, 10, 11, 12, 13, 14, 15, 16]. Such volatiles appear during mycotoxin production by *Aspergillus* and *Fusarium* species [9].

Volatiles can differentiate toxigenic and atoxigenic isolates of the same species [17]. For example, they can distinguish

between aflatoxin and non-aflatoxin producing *A. flavus* isolates [18, 19]. However, these investigations used different growth substrates resulting in different volatile profiles identified in the two studies. Toxigenic and atoxigenic strains of *F. sambucinum, F. verticillioides* and *F. proliferatum* [12, 20] can also be separated based on volatile profiles. This is also true for the toxin and non-toxin producing isolates of *Penicillium roqueforti* [12, 14, 21].

The purpose of this study was to investigate the classes of volatile compounds produced by aflatoxin and non-aflatoxin producing isolates of *A. flavus* when grown on sterile and non-sterile, wet, cracked corn. AFB1 concentrations were also determined for each sampling period and compared to those for the various isolates grown on the different media.

MATERIALS AND METHODS

Sterile and non-sterile cracked corn medium. Corn kernels (stored at 4°C) were placed in a plastic bag and cracked using a wooden mallet. Afterwards, the cracked kernels were placed in an enamel tray and water added (200 ml per 1,000 g of corn). The corn was occasionally mixed over several hours until the water was imbibed into the kernels. Next, 300 grams of wet, cracked corn was placed in each of three 2-arm 500 ml glass vessels (ProCulture flask, Corning, Lowell, MA, USA), equipped as described below. When the experiments with sterile, cracked corn were performed, the 3 vessels were autoclaved for 1 hour (121°C, 15 lb pressure) on 2 successive days. For experiments with

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non-sterile cracked corn, no sterilization occurred and the corn was immediately inoculated with the test fungus.

Two corn containing vessels were inoculated with an aflatoxin or non-aflatoxin producing isolate of *A. flavus* taken from a 7-day old culture stored at 4°C. The fungal inoculum consisted of 1 ml of a conidial suspension (3×10^6/ml) prepared in phosphate-buffered saline – 0.1% Tween-20. The vessels were then shaken to distribute the inoculum throughout the corn. A third, un-inoculated vessel was used as a corn control and was also shaken.

Analysis of vessel headspace volatile profiles were performed as described below. After volatile analyses were performed, corn samples for aflatoxin analysis were obtained by using a wide-mouth sterile 25 or 50 ml pipette to randomly remove kernels from the vessels by pushing the pipette through the corn and collecting the kernels caught in the pipette. The kernels were then placed in a small paper coin envelope and air-dried (45°C) for 2 days. Next, the samples were analyzed for aflatoxin content.

Analysis of volatile gases present in the headspace of sample vessels.

Separation and identification of volatiles using the Tenax/GC/MS system.

The sample volume (900 ml) was collected using an Entech model 7100A sample pre-concentrator (Tenax). This unit utilizes a 3-stage trapping system that removes major air components and manages the water and CO_2 content, while collecting volatiles of interest. The collected volatile sample flowed through a Tenax trap held at 50°C. High boiling point volatiles were trapped while other compounds passed through the trapping unit. The trap was then purged with 900 cc of helium to remove residual water. Next, trap 1 was thermally desorbed with 100 cc of helium at 180°C and the released volatiles flowed to the second stage Tenax trap held at 20°C. This dual Tenax trapping configuration allowed for the removal of background water and CO_2, and trapping of heavier molecular weight volatiles. The sample was desorbed at 180°C and then cryo-focused at -60°C before injection into the GC/MS (Hewlett Packard 5890II/5971A). Table 1 shows the conditions used to trap and identify the metabolic volatiles. The volatiles were separated in the GC based upon their retention time on the GC column. After elution from the GC they entered the MS where they were fragmented into subunits that were analyzed for their mass. Compounds

Table 1. Tenax Trapping Conditions and GC/MS Parameters. Adsorbant Concentration with Dry Purge Water Management 7100A Second Trapping Conditions Employed.

Sample Temperature: 25°C
Trap Temperature 1: 50°C
Dry Purge Volume: 900 cc
Desorb Temperature 1: 180°C
Desorb Volume: 100 cc
Trap Temperature 2: 20°C
Desorb Temperature 2: 180°C
Cryofocus Temperature: -60°C
GC/MS (HP 589011/5971A) Conditions
Column DB-5 30m, 0.32mm ID, 1.0u film, flow at 1.0 cc/min
Oven 37°C (1 min) 6°C/min to 100°C, 25°C/min to 240°C (hold 2 min)
MS Scan 35 amu to 500 amu

were identified based upon comparison with standard mass spectra in the library (2008 NIST, Agilent Technologies, Santa Clara, CA, USA). Authentic standard compounds (Sigma Chemicals Inc., St. Louis, MO, USA) were injected to confirm analysis results, and consisted of caryophyllene, ethanol, 1-hexanol, 2-heptanal (E-), 2-hexenal, α-humulene, nonanal, and 2-pentylfuran. Volatiles unique to the toxigenic *A. flavus* isolates were determined by comparison with volatiles produced by the respective corn controls and the non-toxigenic *A. flavus* isolate.

Aflatoxin Analysis. Dried kernels were coarsely crushed with a hammer then transferred to Erlenmeyer flasks (50 ml) and respective dry weights recorded. Methylene chloride (25 ml) was added to each flask then agitated for 30 min. with a wrist-action shaker (Model 75, Burrell Scientific, Pittsburgh, PA, USA). Flask contents were decanted through a filter paper funnel into a 100 ml beaker and allowed to dry passively overnight. The dried beaker residue was dissolved with methylene chloride and transferred into a glass vial (8 ml) and allowed to dry passively overnight.

Dried vial residue was resuspended in 500 μl acetonitrile, transferred to a 2 ml, 0.45 μm filter centrifuge tube (Spin-X; Corning Inc., Corning, NY, USA) and centrifuged at 14,000 rpm for 1 min. Aliquot samples (10 μl) of filtered extract were analyzed by HPLC. AFB1 analysis was performed similar to a previously described procedure [22]. HPLC analyses were performed with a Waters 2695 HPLC combined with a Waters 2475 fluorescence detector. Post-column derivatization was performed with a Photochemical Reactor for Enhanced Detection (PHRED, Aura Industries Inc., New York, NY, USA) system. AFB1 detection wavelength was 365 nm (excitation) and 474 nm (emission). Sample extracts (10 μl) were injected for separation through a Nova-Pak C18 (3.9 mm × 150 mm; 5 μm) reverse-phase column. The analytical column was protected by a guard column containing the same packing. Column temperature was maintained at 38°C. Elution flow rate was 0.8 ml/min with mobile phase solvent consisting of water:methanol:n-butanol (1400:720:15, v/v/v). Retention time for AFB1 was 9.5 min. A calibration curve with high linearity (R^2= 0.9925) was constructed for AFB1 from a series of diluted standards.

RESULTS

Volatiles

Wet, sterile, cracked corn. Few volatiles were detected in the headspace of the sterilized, wet cracked corn not inoculated with *A. flavus* isolates, and comprised of one aldehyde, an ester, and 3 furans (Tab. 2).

Table 2. Volatiles detected in the headspace of wet, sterile cracked, corn.

Retention time	Chemical family	Volatile	Day detected
10.5140	aldehyde	furfural	2, 5
8.6489	ester	ethyl ester (ethyl acetate)	8, 10, 12
5.4424	furan	furan, 2-methyl-	3, 8
5.8151		furan, tetrahydro-	5
13.6918		furan, 2-pentyl-	8

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The unique volatiles produced by the *A. flavus* isolates grown separately on wet, sterile, cracked corn (Tab. 3) were determined by removing the corn control volatiles listed in Table 2 from the original list of *A. flavus* produced volatiles. The fungal volatiles were comprised of a number of chemical groups, including alcohols, aldehydes, alkanes, alkenes, and furans. Single members of the alkyne, amine, carboxylic acid, ester, and terpene groups were also observed. The *A. flavus* test isolates did not produce the same number of volatiles. In the non-aflatoxin isolate, NRRL-5565, only 5 volatile compounds were observed, whereas it was observed that the aflatoxin-producing isolates 1000E and NRRL-3357 produced 13 and 19 volatile compounds, respectively. Of these volatile compounds, the aldehydes, alkenes, alkyne, and 1 carboxylic acid were found only in the headspace of NRRL-3357. Isolate

1000E produced the respective single amine and terpene found. The only toxigenic isolate producing aldehydes and alkenes was *A. flavus* NRRL-3357.

Non-sterile, cracked corn. Many volatiles were identified as being present in the non-sterilized, wet and dry cracked corn controls, and were detected before day 8 with only a few detected on days 10 or 12 (Tab. 4). The largest number (33) belonged to the alkane group and were comprised mainly of propane, pentane, hexane, heptane, nonane, decane and their analogs. The headspace of the non-sterilized corn contained 20 alkenes, the second largest chemical family present. Most of these were also of the C_5 - C_{10} families mentioned above or their analogs.

Table 3. Volatiles[1] produced by aflatoxin and non-aflatoxin producing isolates of *Aspergillus flavus* grown on sterile, cracked corn

Retention time	Chemical family	Volatile	Isolate[2]	Day Detected
6.1568	alcohol	2-hexanol*	1000E	10
7.1399		cyclobutanol*	3357	4
7.2229		2-(3,3-diphenyl-propylamino)-ethanol*	1000E	10
27.0850		ethanol*	1000E	10
6.4051	aldehyde	2-butenal*	3357	5
7.2020		2,4-hexadienal, (E,E)-*	3357	2,4
9.2514		methacrolein*	3357	8
10.3072		furfural	5565	2, 4, 5
6.8705	alkane	cyclopentane, 1,3-dimethyl-, cis-*	1000E	1
7.7088		cyclohexane, methyl-*	1000E	1
8.5683		cyclopentane, 1,2-dimethyl-, cis-*	3357	5
			1000E	1
15.9066		2,6-dimethyl-decane*	3357	8,10
16.4241		octacosane*	3357	3
18.8357		eicosane, 3-methyl*	1000E	3,4
18.8461		tetracosane*	3357	3,8
19.2290		pentadecane*	1000E	3,4
19.6121		pentadecane, 1-methoxy*	1000E	10
19.6741		nonacosane*	1000E	3,4
6.7364	alkene	propene*	3357	4
8.6823		1-butene,3-methyl-*	3357	12
8.4648		2-pentene, (E)-*	3357	1
14.0126		cyclopropene*	3357	3
7.5022	alkyne	3-heptyne*	3357	2
8.6719	amine	3-propoxyamphetamine*	1000E	10
11.6631	aromatic	styrene*	3357	2,3,4,5
8.8893	carboxylic acid	3-butenoic acid*	3357	4, 5
14.6335	diene	1,2-pentadiene*	3357	1
14.6336		1,4-pentadiene*	3357	1
5.6081	ester	ethyl acetate	5565	8, 10, 12
5.4321	furan	2-methyl-furan	5565	8
5.8151		tetrahydrofuran	5565	5
11.6423		2-n-butyl furan*	1000E	10
7.5540	ketone	2-cyclopenten-1-one, 2-methyl*	3357	3
14.6440		2,5-cyclohexadiene-1,4-dione,2,5-dimethyl*	3357	4
14.5921	terpene	d-limonene*	1000E	1
11.5491	triene	bicyclo[4.2.0]octa-1,3,5-triene*	5565	2

[1] ≥ 80% confidence.
[2] NRRL-5565 does not produce aflatoxin; NRRL-3357 and AF1000E produce aflatoxin.
* Volatiles unique to these aflatoxin-producing isolates compared to NRRL-5565 and the sterile corn control.

Table 4. Volatiles[1] produced by non-sterile, cracked corn (wet and dry controls) not inoculated with aflatoxin or non-aflatoxin producing isolates of *Aspergillus flavus*

Retention time	Chemical family	Volatile	Day Detected
8.8271	acid	acetic acid, 2-methylpropyl ester	3
10.7315		butanoic acid, 2-methyl-,ethyl ester	2,3
5.8981	alcohol	3-buten-1-ol	2
8.9924		2-hexyn-1-ol	2,4
11.3939		1-butanol, 3-methyl, acetate	2,4
13.1432		2-decanol	2
13.1743		2-hexadecanol	2,3
10.3694	aldehyde	2-hexenal, 3-methyl-	2
12.6359		2-(5-methyl-furan-2-yl)-propionaldehyde	10
15.9999		nonanal	2,3
17.8108		decanal	5
21.1334		tetradecanal	2,4
21.1644		octadecanal	1,3
4.5939	alkane	pentane	2,3
6.9537		pentane, 1-methoxy-	3
7.1295		heptane	3
9.1687		cis-1-butyl-2-methylcyclopropane	2,3,4
9.3757		octane	2,3,4,5
9.5517		octane, 2,7-dimethyl-	2,5
9.8932		heptane, 2,4-dimethyl	2,3
9.9965		bicyclo[2.2.2]octane	4,8
10.2762		cyclopropane,(1-methylethylidene)	2,10
10.6280		propane, 1-methoxy-2-methyl-	2
10.8557		hexane, 3-ethyl-	2
11.4042		cyclopropane, propyl	1,2,3,4,5,8
11.8288		nonane	3
12.5119		hexane, 1,6-dimethoxy	2
12.6152		octane,2,6-dimethyl	10
12.7185		heptane, 3-ethyl-2-methyl-	3
13.0914		nonane, 4-methyl-	2,3
13.2051		nonane, 2-methyl-	2,3
13.3397		nonane, 3-methyl-	2,3
13.9711		decane	8,10
14.3437		decane, 4-methyl-	8,10
14.9959		tetradecane, 2,6,10-trimethyl-	2,4
15.0063		eicosane, 10-methyl-	2
15.0476		octane, 5-ethyl-2-methyl	2
15.1820		decane, 2-methyl-	10
15.3168		heptadecane, 8-methyl-	2
15.7938		decane,3-methyl-	4,8
15.8550		undecane,4,6-dimethyl-	3
15.8648		undecane, 2-methyl	2,4
16.5275		butane, 1-methoxy-2-methyl-	2
17.6765		heptadecane,2,6,10,14-tetramethyl	2,5
18.9808		eicosane	5
21.1646		eicosane, 9-cyclohexyl	1,3
4.7179	alkene	1,4 pentadiene	2,4
6.0945		1,2-dimethyl cyclopropene	1,2,3,4,5
6.9329		1-heptene	3,8

Table 4 (Continuation). Volatiles[1] produced by non-sterile, cracked corn (wet and dry controls) not inoculated with aflatoxin or non-aflatoxin producing isolates of *Aspergillus flavus*

Retention time	Chemical family	Volatile	Day Detected
8.8167		1,3-pentadiene,2,4-dimentyl-	3,10
8.9718		1,4-hexadiene, 2-methylpropyl ester	5,8,10
9.1479		2-hexene, (Z)-	2
9.2824		1-butene, 3-methyl	3
10.0378		cyclooctine	4
10.2346		2-pentene, (Z)-	3,10
11.1040		p-xylene	2
11.4040		1-hexene	3
11.5700		styrene	2,3,4,8,10
11.7252		1,3,5,7-cyclooctatetraene	2,8
12.3877		1-butene, 4-methoxy	2
12.3979		17-pentatriacontene	10
14.4474		2,6,-dimethyl-1,3,5,7-octatraene, E,E-	3,5
14.6647		cyclohexene, 4-methylene-1-(1-methylethyl)	2,4,8
15.2338		naphthalene, decahydro-	2,10
21.2887		cyclohexane, 3-methyl-6-(1-methylethenyl)-(3R-trans)-	10
22.7066		adamantane	8
7.3471	alkyne	3-heptyne	2,3,4
8.9718	diene	1,3-pentadiene, 2,3-dimethyl	5,8
9.1582		1,3-cyclopentadiene, 1,2-dimethyl	3,4
9.1892		1,4-cyclohexadiene, 1-methyl	2,3
11.0421		1,3-cyclopentadiene, 5-(1-methylethylidene)	2,3
11.5700		bicycle[4.2.0]octa-1,3,5-triene	1,2,3,4,5,8,10
14.1782		1,4-cyclohexadiene, 1-methyl-4-(1-methylethyl)	1,2,3,4
16.6519		1,3-cyclohexadiene, 1-methyl-4-(1-methylethyl)-	8,10
6.3224	ester	ethyl acetate	1,2,8,10
5.4116	furan	3-methyl furan	1,2,3,8,10
5.8357		tetrahydrofuran	1,3
7.1711		2,5-dimethylfuran	1,3,4,5,8,10
7.4089		2,4-dimethylfuran	2,3,10
8.2371		2,5-dihydrofuran	2
13.7125		2-pentyl-furan	1,2,3,10,12
6.8915	ketone	2-pentanone	2,8
13.1018		3-hexenone, 6-methoxy	2
10.1726	lactone	bicyclo[3.1.1] heptan-2-one	3,8
15.7826		8-nonen-2-one	2
19.1774		2-undecanone	2
8.6821	polyene	1,3,5-cycloheptatriene	3
12.5222	terpene	1R-.alpha.pinene	2,4,5
13.0085		camphene	3
14.1161		alpha.-phellandrene	2,10
14.5612		limonene	2
14.6335		d-limonene	1
22.9654		epizonarene	5

[1] ≥ 80% confidence. Volatiles may be produced by naturally-occurring microorganisms growing on the wet, non-sterile corn.

In contrast to the headspace of the sterilized corn controls, 6 terpenes (alpha-pinene, camphene, alpha-phellandrene, limonene, d-limonene, and epizonarene) were identified in the headspace of the non-sterile corn controls. It should be noted that a number of these volatiles could be due to the growth of naturally-occurring microorganisms present on the wet, non-sterile corn, and not emitted by the corn itself.

The volatiles produced by the toxigenic and atoxigenic isolates of *A. flavus* are listed in Table 5. This listing was obtained by removing any volatile present in Table 4 (wet and dry non-sterile, cracked corn controls), mostly comprised of volatiles unique to the tested toxigenic isolates.

Table 5. Volatiles[1] produced by a non-aflatoxin, as well as aflatoxin-producing isolates of *Aspergillus flavus* using wet, non-sterile cracked corn as the growth medium

Retention time	Chemical family	Volatile	A. flavus Isolate[2]	Day Detected
5.4426	acid	2-nonenoic acid*	167B	4
8.4337		propanoic acid, 2-methyl-,ethyl ester*	13A	10,12
			9B	1,3,8
10.8558		butanoic acid, 3-oxo, ethyl ester*	167B	2,3,4
			1000E	2,3
11.9943		2-octenoic acid, (E)-*	167B	4
13.2156		formic acid, decyl ester*	167B	4
15.3685		benzoic acid*	13A	4
16.0826		acetic acid, heptyl ester*	13A	1,5
8.3509	acetal	1,3-dioxo-lane, 2,4,5-tri-methyl-*	9B	3
			13A	2,10
			3357	4
5.4322	alcohol	3-buten-1-ol, 3-methyl-*	167B	3
			13A	3
6.8915		(E)-1,3-butadien-1-ol	167B	4
			5565	1
9.5205		2-pentyn-1-ol*	167B	4
11.2593		1-hexanol*	13A	5
11.4145		2,2-dimethyl-1,3-butanediol*	1000E	2
11.4973		3,3-dimethylbutane-2-ol*	1000E	2
12.3150		2-heptadecanol*	1000E	2
14.0435		2-undecanol*	1000E	2
14.9234		1-hexadecanol, 2-methyl-*	167B	4
15.2545		5-octen-1-ol, (Z)-*	13A	10
15.7513		2-nonanol*	1000E	2
15.8134		2-dodecanol*	1000E	2
15.8446		ethanol, 2-(octadecyloxy)-*	1000E	2
15.9168		1-hexaosanol*	9B	5
			13A	4
16.1551		phenol,4-(1-methylethyl)-*	13A	5
16.2481		phenol,3,4,5-trimethyl-*	13A	5
16.5688		phenol,4-ethyl-*	13A	10
16.7657		phenol,3,4-dimethyl-*	167B	10
17.3866		phenol,4-(1-methylethyl)-, acetate*	1000E	3
17.8008		9-octadecen-1-ol, (E)-*	167B	3
21.4957		benzyl alcohol	5565	1
7.5955	aldehyde	2-butenal, 2-ethenyl*	1000E	2,8
7.9576		methacrolein*	167B	3
9.1997		2-heptenal, (E)-	5565	2
9.6448		2-hexenal*	167B	4
10.2139		furfural*	13A	1
13.8159		2,4-hexadienal, (E,E)-*	13A	4
14.0230		octanal*	167B	3
15.4306		hexanal, 5-methyl-*	167B	4
16.2896		2-nonenal, (Z)-*	167B	4
16.9519		benzaldehyde, 4-(methyoxyethyl)-, acetate*	1000E	2
5.7321	alkaloid	pyrrolidine,1-[8-(3-octyloxiranyl)-1-oxooctyl]-*	167B	1
4.7283	alkane	cyclopropane*	13A	10
5.1320		pentane, 2-methyl-*	167B	2
			1000E	1
5.2873		pentane, 3-methyl-*	1000E	1
5.5150		hexane*	1000E	2,3
			167B	3,4
7.0158		butane, 1-methoxy-3-methyl-	1000E	2,3,4
			3357	5
8.2784		cyclopropane, 1,1-dimethyl-	167B	3,4
8.6614		cyclopropane, 1,2-dimethyl-, cis	167B	3,4
9.5619		butane, 1-(ethenyloxy)-*	1000E	3
10.2653		cyclohexane-ethyl-	5565	
10.6590		cyclopropane, butyl-*	167B	4
10.8765		octane, 4-methyl-*	13A	2,3,5
10.9175		tridecane, 5-methyl-*	1000E	3
12.5636		octane,2,3-dimethyl-*	167B	4
12.9362		octane,4-ethyl*	167B	3
12.9981		cyclopropane, 1-ethyl-2-heptyl-*	167B	2
13.0914		octane,2,5-dimethyl*	167B	3
13.1018		undecane, 2,6-dimethyl-*	167B	4

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Table 5 (Continuation). Volatiles[1] produced by a non-aflatoxin, as well as aflatoxin-producing isolates of *Aspergillus flavus* using wet, non-sterile cracked corn as the growth medium

Retention time	Chemical family	Volatile	A. flavus Isolate[2]	Day Detected
13.1327		cyclohexane, 1,1,2,3-tetramethyl-*	1000E	2,3
13.1638		octadecane,1-(ethenyloxy)-*	1000E	2
13.1842		tridecane, 6-methyl-*	167B	3
			1000E	3
13.6814		1-hexacosene*	167B	4
13.9710		dodecane, 2,6,11-trimethyl-*	9B	10
14.3436		octane, 1-methoxy-*	1000E	2
14.3644		nonane, 2,6-dimethyl-*	167B	4
14.6854		cyclooctane, methyl-*	167B	4
14.8923		9-eicosene, (E)-*	167B	3
14.9544		octacosane*	167B	2
14.9856		hexadecane*	167B	4
			1000E	3
14.9957		nonane, 4,5-dimethyl-*	167B	4
15.0162		decane, 2,5,6-trimethyl-*	1000E	3
15.0166		heneicosane, 11-decyl-*	13A	5
15.0269		heptadecane*	13A	5
15.0371		tetradecane, 4-ethyl-*	13A	2
15.1097		heptadecane, 2,6-dimethyl-*	167B	3
15.1508		tetracyclo[3.3.1.1(1,8).0(2,4)] decane*	13A	3
15.1510		octadecane, 2-methyl-*	13A	2
15.1640		decane,4-ethyl-*	13A	4
15.2028		pentadecane, 7-methyl-*	13A	10
15.2029		octadecane,2,6-dimethyl-*	167B	3
15.2030		tridecane, 7-methyl-*	167B	3
15.2442		hexadecane, 3-methyl-*	9B	10
15.4406		heptadecane, 4-methyl-*	167B	3
15.4512		dodecane, 4-methyl-*	1000E	4
15.8752		nonadecane	5565	3
15.8444		undecane, 5-methyl-*	13A	3
			167B	3
15.8652		pentadecane, 4-methyl-*	167B	4
15.8653		decane, 3,6-dimethyl-*	13A	4
15.8860		heptane, 2,6-dimethyl-*	13A	5
			1000E	2
15.9170		decane, 3,7-dimethyl-*	9B	10
16.0309		hexadecane, 2,6,10,14-tetramethyl-*	9B	10
19.0117		cyclopropane, 1,2-dimethyl-, trans-*	167B	4
19.1047		eicosane, 9-octyl-	5565	2
19.3843		heptadecane, 2-methyl-*	167B	1,2
19.3948		tridecane*	9B	3
6.0945	alkene	1,2-dimethyl cyclopene*	9B	10
6.9329		1-heptene*	167B	4
7.5955		2-butene, 2-methyl-*	167B	2
			9B	4
8.3303		2-pentene, (E)-*	167B	4
			1000E	3,4
			5565	8,12
8.9512		1,3,5-hexatriene, 3-methyl-	9B	5
			13A	1,4,5
			167B	2,4
			1000E	2
9.1789		1-octene*	167B	2
9.1892		2-octene, (Z)-	1000E	3
11.1040		o-xylene*	9B	2
11.9323		1,3,6-octatriene*	167B	4
12.5118		1-decene, 4-methyl-*	167B	3
12.5532		1-cyclohexene-1-methanol*	13A	5
12.9048		cyclohexene,1-methyl-3-(1-methylethenyl)-*	167B	3
12.9879		cyclopentane,1,1,2-trimethyl-*	1000E	3
12.9982		3-nonene,2-methyl-*	167B	4
13.1432		3-decene*	1000E	3
13.6606		cyclopentane, (1-methylbutyl)-*	167B	3
13.7331		hexene, 1-butyl*	1000E	3
14.2404		1-docosene*	13A	8
14.3230		bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-*	1000E	2
15.7203		1-nonene*	167B	3
15.7205		1-pentadecene*	13A	8,10
18.2457		2,4,6-octatriene, 2,6-dimethyl-*	167B	4

Retention time	Chemical family	Volatile	A. flavus Isolate[2]	Day Detected
18.5251		2-methyl-1-butene*	167B	3,4
5.8564	alkyne	2-hexyne*	9B	3,5
			167B	3
			1000E	3,5
5.8877		methoxy-1-buten-3-yne*	13A	3,5
6.1566		4-methyl-2-pentyne*	167B	10
6.3947		1,5-hexadien-3-yne	5565	2
6.3983		2,4-hexadiyne*	167B	10
7.3471		3-hexyne*	167B	10
8.4129		1-pentyne*	13A	5
			167B	4
8.9823		1-hepten-3-yne	5565	3,5
4.9353	amide	butanmide,3-methyl-*	1000E	2
8.2057		1,octamine,N-methyl-*	9B	4
15.3167		hydroxylamine, O-decyl-*	167B	3
13.7745	antra-cycline	esorubin hydrochloride*	167B	3
4.8319	diene	1,2-pentadiene*	9B	10
7.0677		1,2-pentadiene,4,4-dimethyl-*	9B	1
8.1956		2,3-pentadiene, 2,4-dimethyl-*	167B	10
8.9096		1,3-pentadiene,2,3-dimethyl-	5565	12
8.8994		1,4-hexadiene, 5-methyl-*	167B	2,3,4
8.9306		2,4-hexadiene, 3-methyl*	167B	5
9.0133		2,5-heptadiene, (E,E)-*	1000E	4
9.0342		1,3-cycloheptadiene*	13A	4
14.4886		1,3,8-p-menthriene	5565	2
16.5586		1,4-cyclohexadiene,6-isopropenyl-1,2,3,4-tetramethyl-	5565	3
5.6495	furan	furan, 3-methyl-	5565	1,3,8
7.1813		furan, 2-ethyl-*	3357	1
10.5659		furan, 4-methyl-2-propyl-*	9B	10
22.0962		2,3-dihydrofuran	5565	4
6.3224	hydro-carbon	benzene*	9B	3,8
11.1662		benzene, 1,3-dimethyl*	1000E	2
15.1097		benzene, 1,2,3,4-tetramethyl-*	167B	3
15.4617		benzene, 4-ethyl-1,2-dimethyl-*	167B	2
15.6686		benzene, 1,3-diethyl-*	1000E	3
15.9170		benzene-1,3,5-trimethyl*	1000E	10
15.9273		benzene, 1-ethyl-4methyl-	5565	8
15.9376		benzene, 1-ethyl-methyl-	5565	3
16.3516		benzene, (2-methoxyethyl)-*	1000E	2
7.8851	ketone	butyl methyl ketone	5565	4
			9B	10
5.8463		2H-pyran-2-one, tetrahydro-6,6-dimethyl-*	13A	8
			167B	8
9.9863		2-cyclohexen-1-one, 2-methyl-*	1000E	3
12.4807		3-heptanone,4-methyl-*	13A	8
12.5635		cyclobutanone,2,3,3-trimethyl-*	167B	4
17.7387		1-propanone,1-(5-methyl-2-furanyl)-*	167B	3
18.5149		2-propanone, 1-methyoxy-*	1000E	2
8.2784	lactone	cyclo-butanone, 2-ethyl-*	13A	4
			167B	4
			1000E	3
8.3095		2-pentanone, 3-methyl-*	9B	10
			13A	1
			1000E	1
13.6499		3-octanone	5565	4
12.5221	terpene	1R.-alpha-pinene	5565	3
12.5636		4-carene (1S,3S,6R)-(-)-*	13A	3
20.7091		.alpha.-cubebene*	167B	2
21.6510		(+)-epi-bicyclosesquiphellandrene*	167B	10
22.1168		bicyclogermacrene*	1000E	3
22.3859		cedrene*	13A	8
22.9654		epizonarene	5565	3

[1] ≥ 80% confidence.
[2] non-aflatoxin producing isolate (5565=NRRL-5565); aflatoxin-producing isolates: 9B, 13A, 167B, 1000E, 3357 = NRRL-3357.
*compounds unique to aflatoxin-producing isolates of *A. flavus*.

As with the other samples, the majority of compounds identified were C_4 - C_{10} based compounds, such as butane through decane or their analogs. An exception were the terpenes (4-carene, alpha-cubebene, epibicyclosesquiphellandrene, bicyclogermacrene and cedrene) produced by the toxigenic isolates 13A, 167B and 1000E, while NRRL-3357 did not produce any detectable levels of terpenes. The non-toxigenic isolate, NRRL-5565, produced 2 terpenes, alpha-pinene and epizonarene.

A greater number of chemical families were found in the headspace of *A. flavus* isolates grown on non-sterile, cracked corn than those produced by these fungi when grown when wet, sterile corn. Of the former, the largest number of fungal volatiles produced were alkanes (49), followed by alkenes (23), alcohols (21), and dienes (11). The other groups listed had 10 or less members identified.

There was a wide disparity in the number of volatiles that each fungus produced. The *A. flavus* toxigenic isolates produced the following number of volatiles: 167B (72), 1000E (44), 13A (38), 9B (20) and NRRL-3357 (3). The non-toxigenic isolate, NRRL-5565, produced 20 volatiles.

Aflatoxin concentrations. Table 6 shows the levels of aflatoxin produced on the wet sterile, cracked corn by the toxigenic and atoxigenic isolates of *A. flavus*. No aflatoxin was detected on any of the un-inoculated corn controls. As expected, the atoxigenic isolate NRRL-5565 did not produce any aflatoxin. The toxigenic isolates produced varying amounts of aflatoxin, as measured each sampling day. Aflatoxin concentrations in the corn sampled ranged daily from 0 ng/g to as much as 8.8×10^7ng/g. However, the highest concentration generally found was 10^4ng/g corn.

Similar results were observed for the non-sterile, wet corn inoculated with the toxigenic and atoxigenic isolates of *A. flavus* (Tab. 7). The corn inoculated with the toxigenic isolates also had wide disparities for each sampling period. Isolate 1000E had aflatoxin ranges of $0.0-1.1 \times 10^7$ng/g corn. Isolates 1000E and NRRL-3357 had higher ranges with most samples generally containing 10^6ng/g daily. This was approximately 100-fold higher than the average high aflatoxin content observed in the inoculated sterile corn. Aflatoxin was not found on each sampling day in corn inoculated with toxigenic isolates 9B, 13A, and 167B. Some wet, cracked corn controls contained aflatoxin, although the dry controls did not.

Table 6. Aflatoxin levels (ng/g) in wet, sterile corn (control) and *Aspergillus flavus* inoculated wet, sterile corn.

A. flavus isolate*	Sample type	Day							
		1	2	3	4	5	8	10	12
1000E	Inoculated	$0-3.6 \times 10^1$	$1.9-5.0 \times 10^2$	$0.2-1.5 \times 10^4$	$0.02-2.8 \times 10^4$	$0.14-8.8 \times 10^4$	$0.7-4.7 \times 10^7$	$0.02-1.1 \times 10^5$	$0.5-4.6 \times 10^6$
	Wet Control	0	0	0	0	0	0	0	0
NRRL-3357	Inoculated	$0.0-4.0 \times 10^2$	$0.2-1.8 \times 10^4$	$0.4-1.4 \times 10^6$	$0.9-1.8 \times 10^4$	$1.4-2.4 \times 10^4$	$0.8-2.0 \times 10^4$	$0.8-2.0 \times 10^4$	$1.0-2.1 \times 10^4$
	Wet Control	0	0	0	0	0	0	0	0
NRRL-5565	Inoculated	0	0	0	0	0	0	0	0
	Wet control	0	0	0	0	0	0	0	0

*1000E and NRRL-3357 are aflatoxin producing isolates; NRRL-5565 is a non-aflatoxin producing isolate.

Table 7. Aflatoxin levels (ng/g) in wet or dry non-sterile corn (control) and *Aspergillus flavus* inoculated wet, non-sterile corn

A. flavus isolate*	Sample type	Day							
		1	2	3	4	5	8	10	12
1000E	Inoculated	$0-1.6 \times 10^4$	$0-2.2 \times 10^7$	$0-3.7 \times 10^6$	$0-3.7 \times 10^6$	$0-4.3 \times 10^6$	$0-1.1 \times 10^7$	$0-1.1 \times 10^7$	$0-2.3 \times 10^6$
	Wet Control	0	0	$0-1.0 \times 10^6$	$0-8.8 \times 10^5$	$0-2.0 \times 10^6$	$0-9.7 \times 10^5$	0	0
	Dry Control	0	0	0	0	0	0	0	0
9B	Inoculated	0	0	$0-1.6 \times 10^5$	$0-1.3 \times 10^5$	0	$0.02-5.2 \times 10^5$	$0-2.2 \times 10^5$	$0-2.2 \times 10^6$
	Wet Control	0	0	0	0	0	0	0	0
	Dry Control	0	0	0	0	0	0	0	0
NRRL-3357	Inoculated	$0-1.6 \times 10^4$	$0-2.7 \times 10^6$	$0-5.2 \times 10^6$	$0-4.7 \times 10^6$	$0-3.2 \times 10^6$	$0-3.4 \times 10^6$	$0-5.0 \times 10^6$	$0-2.2 \times 10^6$
	Wet Control	$0-7.1 \times 10^3$	0	0	0	0	0	0	0
	Dry Control	0	0	0	0	0	0	0	0
13A	Inoculated	0	0	0	0	0	0	$0-9.5 \times 10^4$	$0-9.2 \times 10^6$
	Wet Control	0	0					0	0
	Dry Control	0	0	0	0	0	0	0	0
167B	Inoculated	0	0	0	$0-7.9 \times 10^5$	$0-9.6 \times 10^3$	$0-1.9 \times 10^6$	$0-3.6 \times 10^6$	0
	Wet control	0	0	0	0	0	0	0	0
	Dry Control	0	0	0	0	0	0	0	0
NRRL-5565	Inoculated	0	0	0	0	0	0	0	0
	Wet control	0	0	0	0	0	0	0	0
	Dry Control	0	0	0	0	0	0	0	0

*9B, 13A, 167B, 1000E and NRRL-3357 are aflatoxin producing isolates; NRRL-5565 is a non-aflatoxin producing isolate.

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DISCUSSION

Results of this study show that few volatiles were detected by the Tenax/GC/MS instrument in the headspace of corn treated with steam and heat (121°C, 15 lbs pressure) for 1 hour on each of 2 consecutive days. In an earlier study using solid phase microextraction (SPME) fibers to trap headspace volatiles, fewer and different volatiles were detected in the headspace of similarly treated corn. This disparity in results may be due to SPME and Tenax differing in their ability to bind compounds based on polarity and volatility [23, 24].

The non-sterilized corn kernel controls, both wet and dry, emitted many more volatiles than the autoclaved kernels. A number of volatiles, mostly terpenes, have been isolated from damaged kernels treated with regurgitant of *Spodoptera* species [25]. Among the naturally occurring compounds in corn kernels tentatively identified are limonene, α-terpineol, 1,-8 cinole, β-ionone, citral, (E,E)-2,4-nonadienal and (E,E)-2,4-decadienal [26]. Volatiles can differ considerably among corn varieties and depend on climatic conditions and nutrient availability [27].

The use of sterilized cracked corn as a growth substrate allowed a separate view of volatiles produced by toxigenic and atoxigenic isolates of *A. flavus* without the affect of other fungi on *A. flavus* metabolism. However, results showed that the number of volatiles and their family groups produced by the *A. flavus* isolates grown on autoclaved corn were much fewer in number than those produced on the non-autoclaved kernels. For example, *A. flavus* isolate 1000E was observed to produce 13 volatile compounds, comprising alcohols, alkanes, an amine, one furan, and a terpene when grown on sterilized corn kernels. In contrast, it produced 44 volatiles (acids, alcohols, aldehydes, alkanes, alkenes, 1 alkyne and 1 amide, dienes, hydrocarbons, ketones, and terpenes) on the non-sterile corn. Overall, the volatiles produced by the same isolates differed when these fungi were grown on both sterile and non-sterile cracked corn. For example, NRRL-3357 produced a number of aldehydes on sterile corn but none when grown on the non-sterile corn. The isolate 1000E produced many more alcohols, alkanes and alkenes on the non-sterile corn than the sterilized corn. The differences observed could be due to the growth of the naturally-occurring fungi affecting the metabolism of the inoculated *A. flavus* isolates. It is known that the presence of other fungi can affect the biosynthesis of aflatoxin and inhibit the growth of *A. flavus* [28, 29, 30]. In addition, the heating from autoclaving of the cracked corn to achieve sterile conditions may also affect the nutrients available for fungal metabolism and lead to major differences in the volatile profiles of the test isolates.

Fungi were observed growing on the wet, but not the dry, non-sterile corn controls. Removing these volatiles from the complete list of volatiles produced on non-sterile corn, resulted in the listing of only those volatiles unique to the toxigenic isolates grown on the non-sterile corn (Table 5).

Results show that volatiles were not uniformly produced in number and type by the tested *A. flavus* isolates. For example, toxigenic isolates 167B, 1000E, and 13A produced 72, 44, and 38 volatiles, respectively, comprising many different chemical families. The toxigenic NRRL-3357 only produced 3 volatiles, while 20 volatiles were detected in the headspace of the non-toxin producer NRRL-5565. These results indicate that metabolic differences occur in *A. flavus* species, whether or not they produce aflatoxin.

Aflatoxin values for the toxigenic isolates varied widely in the non-sterilized samples, but much less so when grown on sterilized corn. For the non-sterile corn samples, it was not unusual to detect little or no aflatoxin on a given day during 1 experiment, only to obtain high levels on the same sampling day of a different experimental set. This could be due to the effects of other fungi coexisting in the microenvironment with the inoculated toxigenic *A. flavus* isolates. Earlier studies showed that other fungi present in corn kernels affect the metabolism of aflatoxin-producing strains of *A. flavus* [28, 29]. This variability could be also due to the random sampling of kernels from within the test vessel that have no *A. flavus* growth. Sampling variability is usually the largest source of variation due to the distribution of contamination among the individual kernels [31, 32].

Peak aflatoxin production occurred on day 8 in the wet, non-sterile corn, as well as in the sterile corn. In addition, these values were 10-fold higher in the former than in the latter samples. It is possible that these differences are due to the nutrients available to the *A. flavus* isolates from corn in the respective corn sample types, or competition with the other fungi coexisting in the non-sterilized corn.

The data presented here suggest that the aflatoxin and non-aflatoxin producing isolates produce secondary metabolic volatiles, including those that are unique for the toxigenic isolates, whose number and type depend on whether wet, sterile or non-sterile cracked corn is the growth substrate.

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