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$C_{15}H_{24}$ Volatile Compounds Unique to Aflatoxigenic Strains of *Aspergillus flavus*

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Headspace volatiles from eight strains of *Aspergillus flavus* (four aflatoxigenic strains and four nonaflatoxigenic strains), grown for 1, 2, 3, 4, 8, and 10 days in submerged cultures, were collected in Tenax GC traps. The traps were desorbed onto a 50-m gas-liquid chromatography capillary column by heat and gas purge from an external direct injector device. The column was interfaced with a mass spectrometer data acquisition system. Peaks were identified by comparing retention times and mass spectra with those obtained from authentic compounds and by using a computer-assisted mass spectral data base. Aflatoxigenic strains of *A. flavus* produced several $C_{15}H_{24}$ compounds (e.g., alpha-gurjunene, *trans*-caryophyllene, and cadinene) which peaked in 3-day cultures and were not present in earlier (1- and 2-day) or later (8- and 10-day) cultures. None of these volatiles were detected in nonaflatoxigenic strains of *A. flavus*. There was an apparent correlation between the release of $C_{15}H_{24}$ volatile compounds and the initiation of aflatoxin biosynthesis, and a correlation between decline of aflatoxin synthesis and the disappearance of the $C_{15}H_{24}$ compounds unique to aflatoxigenic *A. flavus* also existed.

Aflatoxins are polyketide-derived secondary metabolites produced by the imperfect fungi *Aspergillus flavus* (Fries) Link and *Aspergillus parasiticus* Speare (2). Aflatoxin biosynthesis has been extensively studied (for reviews, see references 3 and 13). An accepted generalized scheme for aflatoxin B_1 production may be outlined: acetate → polyketide → anthraquinones → xanthones → aflatoxins. Research on characterization of the enzymes required at each step during this biosynthetic conversion has been initiated only recently, and many investigators are presently working in this area (3). Aflatoxin B_1 is prevalent in cottonseed (*Gossypium hirsutum* L.) grown in desert regions of the southwestern United States (15, 18). These fungal metabolites, especially aflatoxin B_1, are toxic to both humans and animals and are among the most carcinogenic of all natural compounds (4, 14). *A. flavus* infection of cottonseed with subsequent aflatoxin contamination reduces both the quality and economic value of the crop and is, therefore, a major potential health problem for both animals and humans.

Kaminski et al. (16, 17) identified the primary volatiles from steam distillates of *A. flavus* grown on moistened wheat meal as 1-octen-3-ol and 2-octen-1-ol and suggested that these two alcohols were responsible for the musty odor characteristic of certain fungi. Additionally, these researchers identified 3-methylbutanol, 3-octanol, 3-octanone, and 1-octanol in the same steam distillates. Fifty compounds from vacuum steam volatile concentrates from three strains of *Aspergillus clavatus* grown on standard media have been identified and analyzed by capillary gas-liquid chromatography-mass spectrometry (capillary gas-liquid chromatography-MS) (24); the major compounds were identified as oct-1-en-ol, 4-methylbenzaldehyde, phenylacetaldehyde, and 2-methyl phenol. In that study, Seifert and King (24) suggested that the chromatographic patterns of the observed volatiles from these fungal cultures were distinctive for each strain and could be used to characterize these fungi.

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Investigations in this laboratory are directed toward the elimination of aflatoxin contamination of cottonseed through the enhancement of natural defense systems present in the cotton plant. Earlier studies from this laboratory have focused on the possible role of volatiles in *A. flavus*-cotton plant interactions and the possibility that certain volatiles may be involved in the defense of the host cotton plant against *A. flavus* infection and aflatoxin contamination (29–31).

In this study, the volatile profiles of atoxigenic and toxigenic *A. flavus* strains were compared to identify any correlation between aflatoxin biosynthesis and a shift in production of volatile compounds by fungal cultures.

MATERIALS AND METHODS

Fungal strains and growth conditions. Four wild-type aflatoxigenic isolates of *A. flavus* (SRRC 1187, 1000A, 2089, and 1299) obtained from Arizona cottonseed and four nonaflatoxigenic isolates (NRRL 3537, 5918, 5565, and 5917) obtained from the National Center for Agricultural Utilization Research (U.S. Department of Agriculture, Peoria, Ill.) were cultured on potato dextrose agar petri plates. The spores were harvested after 7 days of incubation at 28°C and placed in sterile, deionized water with 1% Triton X-100 added to give a final spore suspension containing 10^8 spores per ml for each strain. The spores from each strain were inoculated into 150 ml of Adye and Mateles liquid growth medium (1) in 1.2-liter glass storage bottles (K-323255; Kontes) fitted with Teflon inlet and outlet valves; the inlet tube extended to 30 cm from the bottom of the flask and 10 cm above the level of the culture medium. These cultures were maintained at 28 ± 0.5°C. In a separate experiment to study the production of $C_{15}H_{24}$ compounds and aflatoxins in cultures maintained at 40°C, the toxigenic strain SRRC 2089 was inoculated into four separate 1.2-liter glass storage bottles fitted with Teflon inlet and outlet valves as described above.

Volatile trapping and desorbing procedure. A glass tube, 84 mm in length with a 9-mm outside diameter and a 1-mm wall

thickness (Tek Lab, Baton Rouge, La.), packed with 0.10 g of Tenax GC (60/80 mesh) held in place with glass wool plugs at each end, was preconditioned for 24 h at 240°C with a helium flow of 20 cm^3/min. Microbe-free (filtered) (0.2-μm-pore-size filter; Pall Ultipor) compressed air entered the inlet tube of the bottle containing the liquid culture, which in turn purged the volatiles to the outlet tube where the Tenax glass trap was positioned, fastened with a short piece of Tygon tubing, to receive the volatiles. The flow of air at the outlet end of the glass trap was adjusted to 20 cm^3/min, and purging and trapping of the volatiles from each sample continued for 1½ h. The glass tube was marked at the end that was attached to the outlet tube to designate the inlet for volatiles on the Tenax trap; later the tube was positioned so that the mark was closest to the gas chromatography (GC) column, designating the injection site once the tube was positioned in the external, closed-inlet system. After the trapping procedure was completed, the glass tubes were sealed with corks, placed in Teflon, screw-cap test tubes, and stored in a freezer. Most tubes were analyzed within 3 days. Volatiles from spore-inoculated samples and noninoculated samples (controls) were collected from each of the 1-, 2-, 3-, 4-, 8-, and 10-day-incubated cultures.

GC-MS analysis. Volatiles derived from the *A. flavus* cultures or non-fungus-inoculated media (controls), trapped in Tenax tubes, were thermally desorbed (200°C, 3 min) in an external, closed-inlet system (Scientific Instrument Service, River Ridge, La.) (19). Desorbed volatiles were introduced into a Hewlett-Packard (HP) 5890A-5971A GC-MS system. Prior to desorption of the volatiles, the column temperature was lowered by CO_2 to −30°C in order to cryogenically focus the desorbed compounds onto an HP-5 cross-linked 5% phenyl methyl silicon capillary column (50 m by 0.2 mm by 0.5 μm thick). The GC oven was programmed for an initial temperature of −30°C, which was held for 3 min for introduction of the trapped volatiles onto the column, followed by an increase to 30°C at a rate of 3.0°C/min. Oven programming continued with heating to 150°C at a rate of 5.0°C/min and then to 250°C at a rate of 15°C/min. The temperature was held at 250°C for 5 min. Helium linear velocity was adjusted to 30 cm/s, electron ionization was set at 70 eV, and electron multiplier voltage was set at 2,180 V. The percent peak area was determined by electronic integration with an HP G1030MS ChemStation.

Identification of $C_{15}H_{24}$ compounds. Identification of *trans*-caryophyllene, α-copaene, γ-gurjunene, and isocaryophyllene was based on the retention times and mass spectra of authentic compounds (Fluka Chemical Corp.). These $C_{15}H_{24}$ compounds, without authentic standards, were tentatively identified by computer matching of the unknown spectra with reference mass spectra in the HP G1035A no. AA9 Wiley 130K mass spectral data base (27). The probability-based-matching library search system was utilized to search mass spectral data bases by using the probability-based-matching algorithm developed by McLafferty et al. (20) and Stauffer et al. (26). A compound was identified on at least three separate runs before its presence was recorded.

Aflatoxin analysis. Separate cultures were prepared by using the same strains of *A. flavus*, grown as described for the volatile-trapping tests. Cultures of each strain incubated for 1, 2, 3, 4, 8, and 10 days were collected so that a time-based relationship between emitted sesquiterpenes and aflatoxin production could be derived. Quantities of 10^8 spores from each *A. flavus* strain were inoculated into 150 ml of growth medium. After the desired length of incubation, the metabolites from the mycelia and the medium were extracted by the following procedure. A 75-ml volume of acetone was added to the culture to stop the fungal growth, and the mixture was kept at room temperature for 2 h with occasional shaking. The acetone-culture mixture was placed in a separatory funnel, and 75 ml of $CHCl_3$ was added to extract the aflatoxins. The funnel was vigorously shaken, the $CHCl_3$ layer was separated through a bed of sodium sulfate, and the filtrate was evaporated to dryness. Residues were solubilized in $CHCl_3$ for thin-layer chromatography. Extracts and standards were spotted on prescored 250-μm-thick silica gel 60 thin-layer chromatography plates (20 by 20 cm) (5763-7; EM Science/Merck & Co., Ind.) and developed with a mixture of diethylether, methanol, and water (96:3:1, vol/vol/vol). The aflatoxins B_1, B_2, G_1, and G_2 on developed thin-layer chromatography plates were quantified by fluorometric scans (excitation wavelength, 360 nm) (model CS-930 scanning densitometer; Shimadzu Scientific Instruments, Inc., Tokyo, Japan) as described earlier (31). The results were determined by calculating the mean for the four aflatoxins (B_1, B_2, G_1, and G_2), and standard deviations were estimated from three determinations for each culture.

RESULTS

No $C_{15}H_{24}$ compounds were detected from culture flasks incubated for 1, 2, 3, 4, 8, and 10 days and containing only growth medium, i.e., controls without fungal culture inoculations. Preliminary experimental GC-MS programs did not show any differences in the occurrence of low-molecular-weight compounds between aflatoxigenic and nonaflatoxigenic liquid cultures of *A. flavus*. No $C_{15}H_{24}$ volatile compounds from 1- and 2-day-incubated cultures were detected in initial volatile-trapping and volatile-purging tests. Headspace volatiles from 3-, 4-, 8-, and 10-day-incubated aflatoxigenic and nonaflatoxigenic liquid cultures of *A. flavus* were trapped in Tenax tubes and were thermally desorbed onto a capillary GC column interfaced to a mass spectral detector; differences in the volatiles' profiles were determined. Under the conditions used in these studies, profile differences between the volatiles of the various aflatoxigenic strains of *A. flavus* tested with respect to the presence of $C_{15}H_{24}$ compounds (sesquiterpenes) were found; these compounds appeared on the total ion chromatograms at retention times between 50 and 53 min. A total of 19 airborne sesquiterpenes were identified either by their retention times and comparison with mass spectra of authentic compounds or by computer matching of their mass spectra with the Wiley library of mass spectra (28). A typical total ion chromatogram of an aflatoxigenic strain, with an expanded view of peaks between retention times of 50 and 53 min, is shown in Fig. 1. The expanded ion chromatograms (Fig. 1) and the data included in Table 1 clearly demonstrate that all the aflatoxigenic strains produce airborne sesquiterpenes which peak in 3-day-old fungal cultures and are essentially undetected in cultures incubated for 8 and 10 days. A strain producing extremely low levels of toxin (NRRL 5917), generally considered nonaflatoxigenic, did produce detectable levels of airborne sesquiterpenes (Table 1). However, other nonaflatoxigenic strains of *A. flavus* (NRRC 3537, 5918, and 5565) which do not produce any detectable aflatoxin did not produce airborne sesquiterpenes. Figure 2 demonstrates a typical ion chromatogram of headspace volatiles from 3- and 4-day-old cultures of a nonaflatoxigenic strain of *A. flavus* (NRRL 5918), showing that no sesquiterpene peaks were apparent at retention times between 50 and 53 min.

Cadinene isomers were common in all toxigenic strains

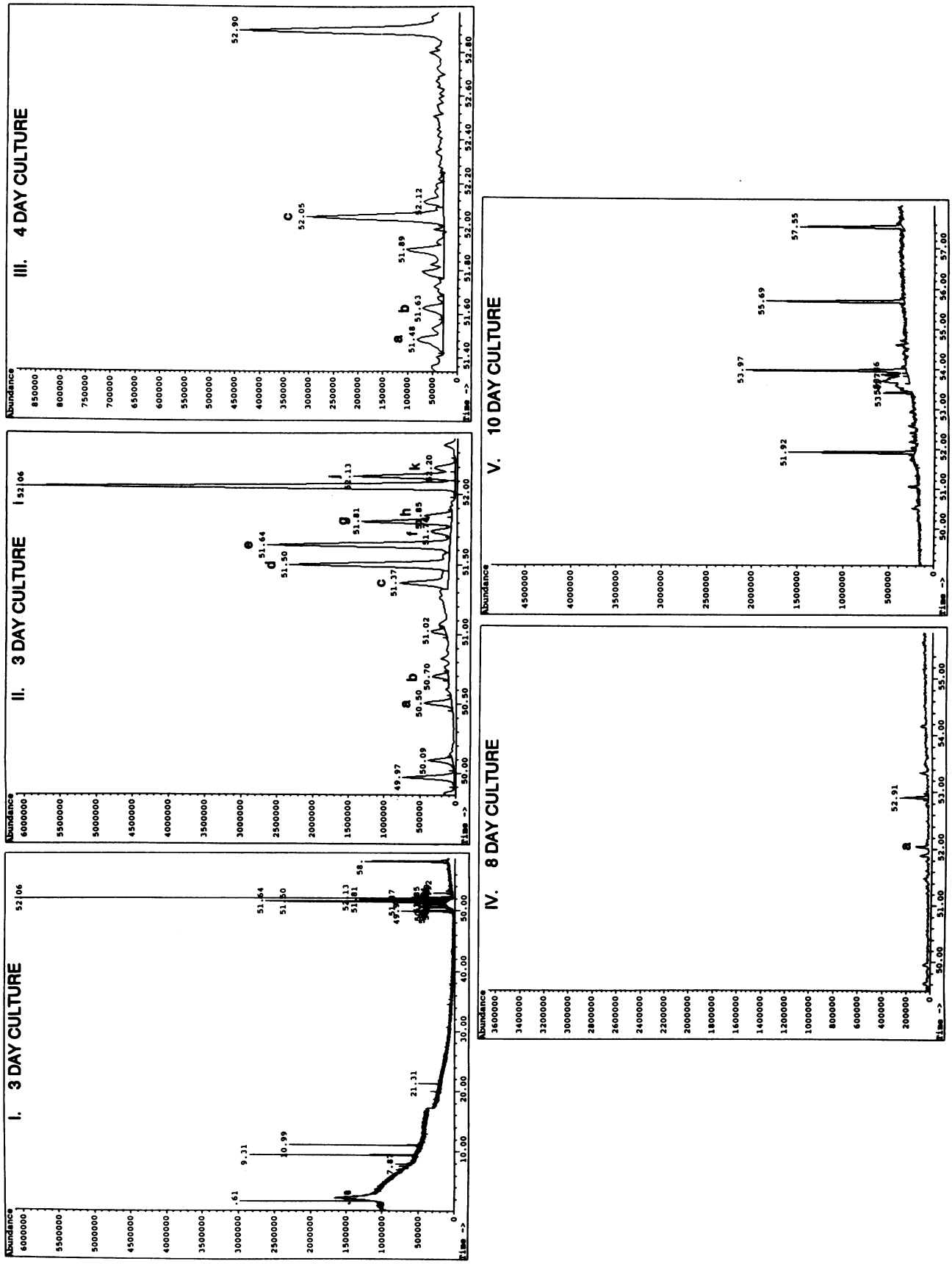

FIG. 1. Total ion chromatograms of volatiles from a typical aflatoxigenic strain of *A. flavus* (SRRC 2089). Panels II to V give an expanded view of peaks at retention times between 50 and 53 min. Letters a through k represent compounds identified by the same letters in Table 1.

TABLE 1. $C_{15}H_{24}$ volatile compounds and total aflatoxins emitted from liquid cultures of *A. flavus*

A. flavus strain	Culture age (days)	Volatile $C_{15}H_{24}$ compound found[a]	CAS no.[b]	RT[c]	% Peak area[d]	Quality of match[e]	Total aflatoxins (μg/g [dry wt] of mycelia)[f]
SRRC 2089	3	(a) Alpha-gurjunene	489-40-7	50.50	1.23	93	155 ± 2
		(b) *trans*-Caryophyllene	87-44-5	50.70	0.64	97	
		(c) Epi-bicyclosequi-phellandrene	000-00-0	51.37	2.86	94	
		(d) Eremophilene	10219-75-1	51.50	7.20	90	
		(e) Beta-cubebene	13744-15-5	51.64	7.43	82	
		(f) Valencene	4630-07-3	51.74	0.98	94	
		(g) Epizonaren	000-00-0	51.81	4.05	96	
		(h) Gamma-selinene	515-17-3	51.85	0.97	94	
		(i) Gamma-cadinene	39029-41-9	52.06	16.59	91	
		(j) Cadinene	523-47-7	52.13	3.86	90	
		(k) Delta-cadinene	483-76-1	52.20	1.16	89	
	4	(a) Gamma-gurjunene	2256-17-5	51.48	0.08	90	29 ± 0.2
		(b) Alpha-muurolene	17627-24-6	51.63	0.04	92	
		(c) Gamma-cadinene	39029-41-9		0.22	94	
	8	(a) Gamma-cadinene	39029-41-9		0.96	90	1.8 ± 0.06
	10	ND					
SRRC 1000A	3	(a) Naphthalene 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methyl ethyl), (1 alpha, 4a alpha, 8a alpha)	30021-74-0	50.69	0.06	94	31 ± 0.6
		(b) Bicyclo[4,4,0]dec-1-en,2-isopropyl-5-methyl-9-methylene	000-00-0	50.99	0.25	90	
		(c) Aristolen	27862-07-3	51.12	0.25	83	
		(d) Gamma-cadinene	39029-41-9		0.28	96	
		(e) Epizonaren	000-00-0		0.27	96	
		(f) Epi-bicyclosesqui-phellandrene	000-00-0		0.19	80	
		(g) Cadinene	523-47-7		0.13	91	
		(h) Delta-cadinene	483-76-1		0.05		
	4	(a) Epizonaren	000-00-0		1.61	95	1 ± 0.02
		(b) Gamma-cadinene	39029-41-9		1.79	94	
	8	ND					
	10	ND					
SRRC 1187	3	(a) Isocaryophyllene	87-44-5	51.46	1.78	88	298 ± 6
		(b) Beta-cubebene	489-39-4		2.51	90	
		(c) Alpha-copaene	3856-25-4	51.76	2.75	86	
		(d) Gamma-cadinene	39029-41-9		11.50	98	
		(e) Cadinene	523-47-7		3.66	90	
	4	(a) Alpha-copaene	3856-23-4	52.00	2.50	83	52 ± 0.4
	8	ND					43 ± 3
	10	ND					32 ± 1.6
SRRC 1299	3	(a) Gamma-gurjunene	22567-17-5		0.45	95	1,084 ± 10
		(b) Bicyclo[4,4,0]dec-1-en,2-isopropyl-5-methyl-9-methylene	000-00-0		0.83	86	
		(c) 6,10,11,11-Tetramethyltricyclo [6.3.0.1E2,3]undec-1(7)-ene	489-39-4	51.34	0.65	93	
		(d) Gamma-cadinene	39029-41-9		0.35	95	
		(e) Cadinene	523-47-7		0.77	97	
	4	(a) Gamma-gurjunene	22567-17-5		0.35	94	571 ± 2
	8	ND					
	10	ND					

Continued on following page

TABLE 1—*Continued*

A. flavus strain	Culture age (days)	Volatile $C_{15}H_{24}$ compound found[a]	CAS no.[b]	RT[c]	% Peak area[d]	Quality of match[e]	Total aflatoxins (μg/g [dry wt] of mycelia)[f]
NRRL 5917	3	(a) Delta-cadinene	483-76-1		2.89	93	4 ± 0.2
	4	(a) Delta-cadinene	483-76-1		4.70	94	4 ± 0.1
	8	ND					0.8 ± 0.06
	10	ND					ND
NRRL 5565, 5918, and 3537	3	ND					ND
	4	ND					ND
	8	ND					ND
	10	ND					ND

[a] $C_{15}H_{24}$ molecular weight, 204. Letters correspond to peaks shown in Fig. 1. ND, none detected.
[b] Chemical Abstract Services (CAS) number.
[c] Retention time (RT) in minutes.
[d] Determined by autointegration; initial area reject = 0.
[e] Quality of the match between the MS scans of known and unknown compounds on the basis of a probability-based-matching algorithm. Values less than 50 mean that substantial differences exist between the unknown and reference compounds. Values greater than 90 indicate very good matches (20, 23).
[f] Mean of the sum of the weights of the four aflatoxins (B_1, B_2, G_1, and G_2) ± standard deviation after three quantitative determinations for each culture.

(Table 1). These compounds ranged from >1 to 17% of the relative peak areas among the different strains examined. Each strain exhibited its own fingerprint in terms of the sesquiterpene present and the relative amounts (percentage of peak areas) of that sesquiterpene. Among the toxigenic strains, SRRC 2089 emitted more diverse sesquiterpenes with an overall higher relative percentage than the other strains; a 47% total peak area was represented in SRRC 2089 (3-day-incubated culture) by the sesquiterpenes emitted, 22% was represented in SRRC 1187 (3-day-incubated culture), 3% was represented in SRRC 1299, and 2% was represented in SRRC 1000A (3-day-incubated culture).

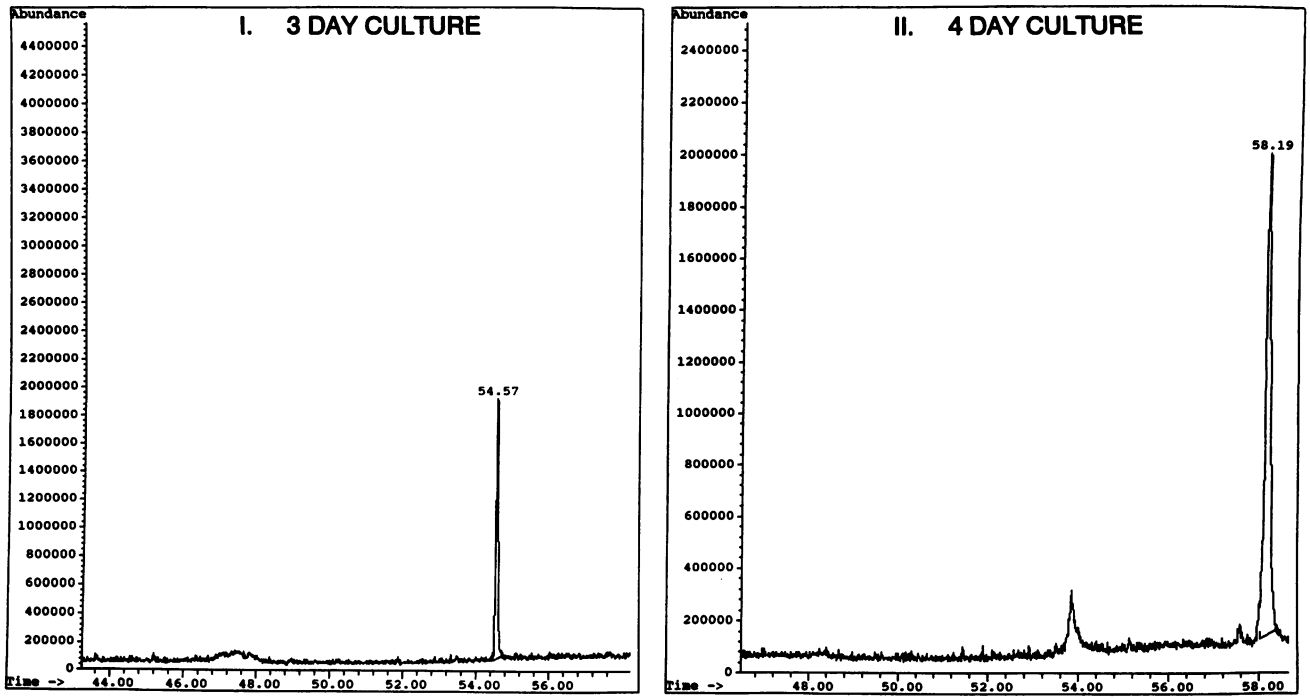

FIG. 2. Total ion chromatograms of volatiles from a typical nonaflatoxigenic strain of *A. flavus* (NRRL 5918).

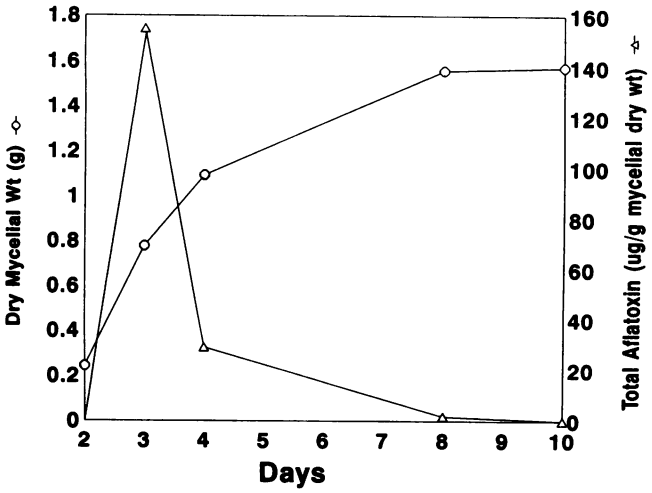

FIG. 3. Dry biomass and toxin production of SRRC 2089 maintained at 28 ± 0.5°C over a 10-day period.

Separate liquid cultures containing the same strains of *A. flavus* were prepared to monitor the production of aflatoxins on the same time bases as for the production of headspace volatiles. A growth curve comparing biomass with total aflatoxin production of *A. flavus* (SRRC 2089) is shown in Fig. 3. Total aflatoxins were present in greatest concentrations in cultures incubated for 3 days (Table 1); there was a decline in aflatoxin production thereafter. These results are similar to those reported earlier (7–12), which indicated that peak aflatoxin concentrations are attained within 72 h, followed by a rapid decline in accumulations of aflatoxins. The decline in aflatoxin levels in later stages of growth has been attributed to fungal degradation of the toxin in culture coupled with a plateau in toxin production; the factors affecting this phenomena are mycelium age, aeration, fragmentation of mycelia, aflatoxin concentration (10), temperature, and pH (12).

Headspace volatiles from 3-, 4-, 8-, and 10-day liquid cultures of an aflatoxigenic strain of *A. flavus* (SRRC 2089) incubated at 40°C were collected in Tenax tubes. These volatiles were thermally desorbed onto a capillary GC column interfaced to a mass spectral detector, and the resulting total ion chromatograms were searched for $C_{15}H_{24}$ compounds as described in Materials and Methods. Three-day-old cultures gave only <1% peak area for gamma-cadinene, tentatively identified by computer matching; no other $C_{15}H_{24}$ compounds were found in this 3-day culture or in the 4-, 8-, and 10-day liquid cultures of *A. flavus* SRRC 2089. Total aflatoxin determinations of the same cultures yielded 0.5 ng of aflatoxins per g (dry weight) of mycelia in the 3-day-old cultures. No aflatoxins were found in the 4-, 8-, and 10-day cultures.

DISCUSSION

In this study, we have observed a correlation between the production of sesquiterpenes ($C_{15}H_{24}$ compounds) and the initiation of the biosynthesis of aflatoxins. Additionally, as the concentration of aflatoxin declined, so did the amounts of the emitted volatile sesquiterpenes. These sesquiterpenes were not produced by atoxigenic strains of *A. flavus* (NRRL 3537, 5918, and 5575). However, a putative nonaflatoxigenic strain of *A. flavus* (NRRL 5917) produces trace amounts of aflatoxin; this strain was the only atoxigenic strain tested that produced the sesquiterpenes characteristic of toxigenic strains during aflatoxin elaboration. Under conditions in which aflatoxin synthesis was absent (e.g., in the early stages of fungal growth) or inhibited (i.e., when toxigenic cultures were maintained at 40°C) (21, 23), the $C_{15}H_{24}$ compounds were not detected in the collected headspace volatiles of the toxigenic strains of *A. flavus* (SRRC 1187, 1000A, 2089, and 1299). Therefore, it is more than a coincidence that these volatile sesquiterpenes appear during idiophase in the life cycle of the fungus and in concert with the initiation and production of secondary metabolites (in this case, aflatoxins).

Studies from this laboratory have shown that enzymes catalyzing the final two steps in aflatoxin biosynthesis were produced in the late growth phase of *A. parasiticus* cultures, immediately preceding the onset of aflatoxin synthesis (3). Thus, the biosynthesis of sesquiterpenes and production of at least two of the various enzymes involved in the complex aflatoxin biosynthetic pathway appear to be regulated by events occurring after fungal primary metabolism has declined. The fact that we were unable to show a lack of correlation between sesquiterpene and aflatoxin biosynthesis under various conditions of aflatoxigenicity (by comparing aflatoxin producer and nonproducer strains, varying the temperature, or comparing times of induction of the metabolites) suggests that the syntheses of these different families of secondary metabolites are regulated at a common precursor step. Both secondary metabolite families, the sesquiterpenes and the aflatoxins (synthesized via the terpenoid and polyketide biosynthetic pathways, respectively), are produced from acetate units perhaps accumulating in excess when primary metabolism slows, but whether the apparent commonality in metabolic regulation of these two diverse pathways occurs as early as the acetate step in the biosynthetic sequence is unknown.

The correlation between sesquiterpene production and aflatoxin production by toxigenic fungal cultures provides a parameter for researchers attempting to understand the molecular regulation of aflatoxin biosynthesis. This is particularly significant with the recent isolation of regulatory genes (*afl-2* in *A. flavus* [22] and *apa-2* in *A. parasiticus* [5]) and structural genes (5, 25, 28) involved in the pathway. Our laboratory is currently examining the expression of these genes in fungal cultures exposed for various durations to atmospheres of headspace volatiles ($C_{15}H_{24}$ sesquiterpenes) from toxin-producing fungal cultures. Significant information on regulatory elements of aflatoxin biosynthesis may be obtained if a direct correlation between production of these sesquiterpenes and expression of regulatory (structural) genes or genes resulting in the production of these volatile compounds is observed.

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