Phytopathol. Mediterr. (2008) 47, 266–271

SHORT NOTES

Volatile metabolites associated with one aflatoxigenic and one nontoxigenic *Aspergillus flavus* strain grown on two different substrates

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Summary. Aflatoxigenic and non-toxigenic *Aspergillus flavus* strains were grown on corn and on peanut substrates. Microbial volatile organic compounds ($MVOC_S$) were collected by trapping headspace volatiles using thermal desorption tubes (TDT) packed with Tenax® TA and Carbotrap™ B. Samples were collected at various fungal growth stages. Trapped compounds were thermally desorbed from the adsorbent tubes, separated by gas chromatography, and identified by mass spectrometry. The fungal stage did not have many differences in the MVOCs but the concentrations of some volatiles changed over time depending on the substrate. Volatiles that were associated with both the aflatoxigenic *A. flavus* strain and the nontoxigenic strain on both substrates included: ethanol, 1-propanol, butanal, 2-methyl-1-propanol, 3-methylfuran, ethyl acetate, 1-butanol, 3-methylbutanal, 3-methyl-1-butanol, propanoic acid-2-methyl-ethyl-ester, 2-methyl-1-butanol, 1-pentanol, 2-pentanol, 3-methyl-3-buten-1-ol, benzaldehyde, 3-octanone, 2-ethyl-1-hexanol and octane. Volatiles that were associated only with the aflatoxigenic *A. flavus* strain included: dimethyl disulfide and nonanal. Volatiles that were associated only with the nontoxigenic *A. flavus* strain included: hexanal, 1- hexanol, 1-octene-3-ol, 1-octen-3-one and 2-pentyl furan.

Key words: mycotoxins, fungi, corn, peanut, MVOCs.

Introduction

Aflatoxins are among the best known natural carcinogens found in the environment and are of great interest in food safety (Abramson *et al*., 1980; Magan and Evans, 2000). These mycotoxins are secondary metabolic products produced primarily by *Aspergillus flavus* (Fries) Link and *Aspergillus parasiticus* Speare (Sinha *et al*., 1988). Aflatoxin contamination of corn, peanut and other agricultural commodities have a significant impact on health and the agricultural economy, especially in the southeastern United States (Börjesson *et al*., 1989) where, over the 1993–1996 crop years, aflatoxin contamination cost farmers, buyers and shellers an annual average of $ 22.7 million (Lamb and Sternitzke, 2001).

Microbial volatile organic compounds ($MVOC_S$) have been used to detect the presence of fungal activity in food and feed during storage (Busby and Wogan, 1979; Larson and Frisvad, 1994; Widstrom, 1996; Pasanen *et al*., 1996, 1997, 1998; Wright *et al*., 2000; Gao *et al*., 2002; Wilson *et al*., 2002), as well as indoor contamination and mycotoxin production (Kaminski *et al*., 1972; Tuma *et al*., 1989; Wilkins and Scholls 1989; Börjesson *et al*., 1992; Zeringue *et al*., 1993; Zeringue, 2000). *Aspergillus flavus* is known to produce strain-specific volatiles such as 3-methylfuran, 3-methylbutanol, 2-methylfuran, nitrometan, 2-methyl-1-propanol, 1-penten-3-ol, 1-octen-3-ol, 1-octanol, cis-2-octen-1-ol, octadiene, dimethylbenzene, ethylbenzene, limonene, thujopsene, 2-octan-

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1-ol, hexanol, acetaldehyde, 3-octanone, 3-octanol, nonanal, alpha-gurjunene, *trans*-caryophyllene, epi-bicyclosequi-phellandrene, eremophilene, beta-cubebene, valencene, epizonaren, gamma-selinene, gamma-cadinene, cadinene, delta-cadinene, gama-gurjunene, alpha-muurolene, naphthalene, aristolen, isocaryophylene and alpha-copaene (Tuma *et al*., 1989; Wilkins and Scholls, 1989; Larsen and Frisvad, 1994; Schnürer *et al*., 1999). The present study was designed to determine differences in the production of $MVOC_S$ by *Aspergillus flavus* aflatoxigenic (AFT) and *A. flavus* non-toxigenic (AFN) strains when they were grown on corn and on peanut substrates.

Materials and methods

Fungal cultures

The cultures were obtained from the culture collection of the USDA/ARS located at The Northern Regional Research Laboratory (NRRL), Peoria, IL, USA. The aflatoxigenic strain *Aspergillus flavus* (NRRL 3357) and the non-toxigenic strain (NRRL 1957) were used in the study (Hara *et al*., 1974). *Aspergillus flavus* strain NRRL 3357 has been shown to produce both aflatoxins B1 and B2 on peanut and corn. Wilson and Bell (1984) reported that strain NRRL 3357 grown on corn for 10 days produced 3500 ppb B1 and 356 ppb B2, while grown on peanut for 10 days produced 40,000 ppb B1 and 9,000 ppb B2.

Substrates and conditions

Shelled peanuts with 20% moisture content were autoclaved at 121ºC for 30 min. Corn moisture was adjusted to 25% water content, and autoclaved at 121ºC for 30 min. A moisture contents of 20% peanut and of 25% for corn were chosen because they are the minimum moisture contents reached during harvesting. The moisture content of peanut and corn had been determined by measuring the weight of corn and peanut seeds before and after drying at 103ºC for 72 h (Baxter, 1979). Corn and peanut were placed in plastic bags and de-ionized water was added to adjust moisture. Bags were held in a cold room at 4ºC for 3 days. Bags were shaken three times per day. The moisture equilibration was carried out in the cold to delay microbial deterioration before the initiation of the experiments.

Autoclaved peanuts (600 g) were placed in sterile 3.785 l glass jars and inoculated with the *A. flavus* aflatoxigenic (AFT) strain or the non-toxigenic (AFN) strain. Autoclaved corn (650 g) was placed in sterile 3.785 l jars and inoculated with the *A. flavus* AFT or AFN strains. The jars with the corn and peanut substrates were hermetically closed with an inlet and outlet opening for air, and were placed in a water bath at 30ºC. Each experiment (peanut or corn) was conducted in three replicates with a non-inoculated control.

Thermal desorption tubes and water trap

Volatile compounds were trapped in thermal desorption tubes (TDT) (Tekmar™). The thermal desorption tubes were filled with a combination of Tenax® TA 80/100 mesh (70 mg) (Supelco, Bellfonte, PA, USA) and Carbotrap™ B 20/40 mesh (300 mg) (Supelco). To avoid moisture and fungal spore contamination in the TDTs; a water trap column was used at the TDT inlet glass tube measuring 7 cm long and 0.5 cm in diameter containing sodium sulfate Na_2SO_4 (Fisher Scientific, Pittsburgh, PA, USA). Silane treated glass wool (Supelco) was used to plug the TDTs and the water traps.

Tubing

All connections were made using 6.35 mm diameter Tygon® tubing with a Teflon interior lining (Cole-Parmer Instrument Co., Vernon Hills, IL, USA).

Condition of thermal desorption tubes and water traps

Thermal desorption tubes were baked for 8–10 h at 255ºC and purged with a constant flow of 40–50 ml min^{-1} high-purity nitrogen. Water traps were baked at 255ºC for 12 h.

Sampling

Collection of volatiles began after 5–6 hours of substrate inoculation and continued daily for 3 to 25 days, depending on the experiment. Volatiles were collected from one to 24 h day^{-1}, giving a total sample air volume of 12 to 57.6 l. Zero-grade air was purged (10–200 ml min^{-1} l^{-1}, 24h, depending on the experiment) through the inlet of the glass jar and the outlet was attached to the water trap and the TDT.

Analytical procedures

Analyses were performed using a Hewlett-Packard Model 6890 gas chromatograph (GC) (Palo Alto, CA, USA) and a model 5973 mass spectrometer (MS) fitted with a thermal desorption unit (Tekmar-

Dohrmann 6000, Cincinnati, OH, USA). Samples were desorbed at 225°C for 10 min and concentrated in a cryo trap which had been cooled down to -160°C. The cryo trap was then heated to 240°C for 4 min. Volatiles were transferred by a transfer line into a cryo focuser where they were concentrated at -150°C. Injections were performed for 1 min by heating the cryo focuser to 225°C. The cryo trap was then baked at 250°C for 10 min. A capillary GC column (Phenomenex, 5% phenyl/95% dimethyl-polysiloxane, 60 m × 0.25 mm i.d., with a 0.25 μm film) was used with helium as the carrier gas. The helium (ultra-high purity) carrier flow was set at a constant linear velocity of 40 cm s^{-1}. The GC oven was held initially at 32°C for 4 min, then ramped at 2.5°C min^{-1} to 65°C, at 5°C min^{-1} to 225°C for a total run time of 45.2 min. The HP 5973 MS was operated in scan mode from m/z 25 to 500 under EI conditions at 70 eV. The ion source was held at 250°C. Acquired data were analyzed using the Hewlett-Packard Chemstation software, which incorporated the NIST02 and Wiley96 database mass spectral libraries. Volatiles were identified using a tentative identification from a mass spectral library search. Compounds were considered to be identified if their mass spectra matched 90% or greater with the mass spectra in the libraries in at least two out of the three replicates.

Results and discussion

Volatiles in headspace gases produced by autoclaved un-inoculated peanuts during seven days of storage at 30°C with a continuous zero-air flow rate of 10 ml^{-1} min^{-1} were 1-butanol, 1-hexanol, 2,3-butanedione, 2-methyl-1-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 3-methyl-butanal, benzealdehyde, ethanol, hexanal, Table 2. The fungal volatiles produced by the AFT and the AFN strains when grown on the peanut substrate with a continuous zero-air flow of 40 ml min^{-1} for seven days are shown in Table 1. No differences in volatiles were found between the AFT and AFN strains. The predominant MVOCs were alcohols (2-methyl-1-propanol, 3-methyl-1-butanol and ethanol) and one aldehyde (3-methylbutanal).

The AFT and AFN strains that were grown on the peanut substrate for seven days with a continuous air flow rate of 10 ml min^{-1} likewise did not show any differences in volatile profiles (Table 1). The prevalent MVOCs were five alcohols (1-propanol, 1-pentanol, 2-methyl-1-propanol, 3-methyl-1-butanol and ethanol), an aldehyde (3-methylbutanal) and a hydrocarbon (octane). Although differences between AFT and AFN were not detected, the number of MVOCs increased after the air flow was reduced from 40 to 10 ml min^{-1}.

Aspergillus flavus AFT and AFN that had grown on the peanut substrate with discontinuous air flow, 200 ml min^{-1} for 1 hour day^{-1} for five days showed differences in their MVOC composition (Table 1). A total of seventeen MVOCs were detected, 11 alcohols (1-propanol, 1-butanol, 1-hexanol, 1-octene-3-ol, 1-pentanol, 2-ethyl-1-hexanol, 2-methyl-1-propanol, 2-pentanol, 3-methyl-1-butanol, 3-methyl-3-buten-1-ol, ethanol), 4 aldehydes (benzaldehyde, butanal, hexanal, nonanal), and two others (dimethyl disulfide, ethyl acetate). Discontinuous air flow increased the production of MVOCs corresponding to data presented by Larsen and Frisvad, 1995. Differences were detected between AFT and AFN. Nonanal and dimethyl disulfide volatile compounds were related only to the AFT, while 1-hexanol, 1-octene-3-ol, hexanal, 1-octen-3-one and 2-pentyl furon were related only to AFN. These results are similar to data presented by Ito *et al.* (1990) that showed production of alcohols and aldehydes increased significantly when there was a deficiency in oxygen. We found that the aldehydes especially increased, showing amounts 100 times higher than those of the control. Our study also showed that under the conditions of the experiment, an air circulation of 10 ml min^{-1} or more appeared to keep MVOC production by the AFT and AFN strains the same.

The volatiles in the headspace gases produced by autoclaved corn during twenty-five days of storage at 30°C with an air flow rate of 50 ml min^{-1} for 4h day^{-1} were 1-butanol, 1-pentanol, 2,3-butanedione, 2-methyl-1-butanol, 2-methyl-1-propanol, 2-pentanone, 3-methyl-1-butanol, 3-methylbutanal, benzealdehyde, ethanol, ethyl acetate, heptanol, hexanal.

Fungal volatiles in the headspace gases produced by the AFT and AFN strains grown on the corn substrate for twenty-five days were mainly alcohols (1-propanol, 1-butanol, 1-octen-3-one, 1-pentanol, 2-methyl-1- propanol, 3-methyl-1-butanol, ethanol), and furans (2-pentyl furan, 3-methyl furan), and others (Table 1). All of the other volatiles were the same for the AFT and AFN strains.

Table 1. MVOCs in headspace gases associated with *Aspergillus flavus* aflatoxigenic (AFT) and non-aflatoxigenic (AFN) strain growth on peanut and corn substrate with continuous or discontinuous air flow.

Fungal volatile	Substrate	AFT+AFN[a]	AFT[b]	AFN[c]
1-butanol	Peanut-200[f], corn-50[g]	+		
1-hexanol	Peanut-40[d], peanut-200	+		
1-octen-3-one	Corn-50			+
1-octene-3-ol	Peanut-200			+
1-pentanol	Peanut-10[e], peanut-200, corn-50	+		
1-propanol	Peanut-10, peanut-200, corn-50	+		
2-ethyl-1-hexanol	Peanut-200	+		
2-methyl-1-propanol	Peanut-40, peanut-10, peanut-200, corn-50	+		
2-methyl-1-butanol	Peanut-10	+		
2-pentanol	Peanut-200	+		
2-pentyl furan	Corn-50			+
3-methyl furan	Corn-50	+		
3-methyl-1-butanol	Peanut-40, peanut-10, peanut-200, corn-50	+		
3-methylbutanal	Peanut-40, peanut-10, corn-50	+		
3-methyl-3-buten-1-ol	Peanut-200	+		
3-octanone	Corn-50	+		
Benzaldehyde	Peanut-200	+		
Butanal	Peanut-200	+		
Dimethyl disulfide	Peanut-200		+	
Ethanol	Peanut-40, peanut-10, peanut-200, corn-50	+		
Ethyl acetate	Peanut-40, peanut-10, peanut 200, corn-50	+		
Hexanal	Peanut-200			+
Nonanal	Peanut-200		+	
Octane	Peanut-10	+		
Propanoic acid-2- methyl-ethyl-ester	Corn-50	+		

[a] AFT+AFN – volatiles common to both fungal strains.
[b] AFT, *Aspergillus flavus* aflatoxigenic.
[c] AFN, *Aspergillus flavus* non-aflatoxigenic.
[d] Peanut-40, continuous air flow 40 ml/min/24h (57.6 l/sample)
[e] Peanut-10, continuous air flow 10 ml/min/24h (14.4 l/sample)
[f] Peanut-200, air flow 200 ml/min/1hr/day only during the sampling period (12.0 l/sample).
[g] Corn-50, air flow 50 ml/min/4hr/day only during the sampling period (12.0 l/sample).

Table 2. MVOCs in headspace gases associated with uninoculated peanut and uninoculated corn substrate only, or also associated with AFT and AFN strain.

Volatile	Uninoculated substrate (control)		Inoculated substrate (AFT and/or AFN)
1-butanol	Peanut	+	+
1-hexanal	Peanut, corn	+	+
1-pentanol	Corn	+	+
2-methyl-1-butanol	Peanut, corn	+	+
2-methyl-1-propanol	Peanut, corn	+	+
3-methyl-butanal	Peanut, corn	+	+
3-methyl-1-butanol	Peanut, corn	+	+
Benzaldehyde	Peanut, corn	+	+
Ethanol	Peanut, corn	+	+
Ethyl acetate	Corn	+	+
Hexanal	Peanut, corn	+	
2-methyl propanal	Peanut	+	-
2-pentanone	Corn	+	-
2,3-butanedione	Peanut, corn	+	-
2,3-pentanedione	Corn	+	-
Heptanol	Corn	+	-
Isobutene	Peanut	+	-

Air flow was 50 ml min^{-1} for 4 hr day^{-1}, for twenty five days during the MVOC sampling period. Table 1 shows the total MVOCs from both substrates (peanut and corn) related to both the AFT and the AFN strains and their differences in MVOCs production. The predominant MVOCs were alcohols and aldehydes, followed by ketones, furans, sulfides, alkanes, esters and aromatic compounds. According to other authors (Kaminski *et al.,* 1972; Jelen and Wasowicz, 1998; Magan and Evans, 2000), the predominant MVOCs related to *A. flavus* were the alcohols, which is in agreement with our results. Different varieties of air were introduced into the jars so that all or most possible variations in the fungal volatile profiles could be seen.

Conclusion

Profile differences in the MVOCs of toxigenic and nontoxigenic *Aspergillus flavus* were not found when the air flow rate through the glass jar was continuous at 10 ml min^{-1} or greater. When the circulation of air flow was increased to over 10 ml min^{-1}, we detected fewer MVOCs, and they were mainly alcohols. There were differences in the volatile profiles of toxigenic and nontoxigenic *Aspergillus flavus* isolates when the air flow was less than 10 ml min^{-1}. The production of different alcohols was also increased when the air flow was reduced. The production of volatiles differing between AFT and AFN was not consistent among substrates. In addition, differences in volatile production may be strain-specific and not attributable to the production of aflatoxin. The present study is a first step in understanding the production of volatiles under various storage conditions and indicates that it is possible to detect volatile differences in aflatoxigenic grains and peanuts in storage. Further studies are needed to verify that different strains of aflatoxigenic fungi produce similar volatiles, and to develop this method in order to detect AFT in stored products.

Literature cited

Abramson D., R.N. Sinha and J.T. Mills, 1980. Mycotoxin and odor formation in moist cereal grain during granary storage. *Cereal Chemistry* 57, 346–351.

Baxter J.F., 1979. *Agricultural Engineering Yearbook*: *Standardization in ASAE: Moisture Measurement - Grain and Seeds*. American Society of Agriculture Engineers, St. Joseph Michigan, USA, 355 pp.

Börjesson T., U. Stöllman, P. Adamek and A. Kaspersson, 1989. Analysis of volatile compounds for detection of molds in stored cereals. *Cereal Chemistry* 66, 300–304.

Börjesson T., U. Stöllman and J. Schnurer, 1992. Volatile metabolites produced by six fungal species compared with other indicators of fungal growth on cereal grains. *Applied and Environmental Microbiol*ogy 58, 2599–2605.

Busby W.F. and G.N. Wogan, 1979. Food-borne mycotoxins and alimentary mycotoxicosis. In: *Food Borne Infections and Intoxications*, *2nd ed.* (H.P. Reimann, F.L. Bryan, ed.), Academic Press, Inc. New York, NY, USA 519–610.

Gao P., F. Korley, J. Martin and B. Chen, 2002. Determination of unique microbial volatile organic compounds produced by five Aspergillus species found in problem buildings. *American Industrial Hygiene Association Journal* 63, 135–140.

Hara S., D.I. Fennel and C.W. Hesseltine, 1974. Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. *Applied and Environmental Microbiol*ogy 27, 1118–1123.

Ito K., K. Yoshida, T. Ishikawa and S. Kobayashi, 1990. Volatile compounds produced by the fungus *Aspergillus oryzae* in rice *koji* and their changes during cultivation. *Journal of Fermentation and Bioengineering* 70, 169–172.

Jelen H. and E. Wasowicz, 1998. Volatile fungal metabolites and their relation to the spoilage of agricultural commodities. *Food Review International* 14, 331–426.

Kaminski E., L.M. Libbey, S. Stawicki and E. Wasowicz, 1972. Identification of the predominant volatile compounds produced by *Aspergillus flavus*. *Applied and Environmental Microbiol*ogy 24, 721–726.

Lamb M.C. and D.A. Sternitzke, 2001. Cost of aflatoxin to the farmer, buying point, and sheller segments of the southeast United States peanut industry. *Peanut Science* 28, 59–63.

Larsen T.O. and J.C. Frisvad, 1994. Production of volatiles and presence of mycotoxins in conidia of common indoor *Penicillia* and *Aspergilli*. In: *Health Implications of Fungi in Indoor Environments* (R.A. Samson, M.E Flannigan, A.P. Verhoeff, O.C.G. Adan, E.S. Hoekstra, ed.) Elsevier, North Holland Biomedical Press, Amsterdam, Netherlands, 251–279.

Larsen T.O. and J.C. Frisvad, 1995. Comparison of different methods for collection of volatile chemical markers from fungi. *Journal of Microbiological Methods* 24, 135–144.

Magan N. and P. Evans, 2000. Volatiles as an indicator of fungal activity and differentiation between species, and the potential use of electronic nose technology for early detection of rain spoilage. *Journal of Stored Products Research* 36, 319–340.

Pasanen P., A. Korpi, P. Kalliokoski and A.L. Pasanen, 1997. Growth and volatile metabolite production of *Aspergillus versicolor* in house dust. *Environment International* 23, 425–432.

Pasanen P., A. Korpi, J.P. Kasanen and A.L. Pasanen, 1998. Critical aspect on the significance of microbial volatile metabolites as indoor air pollutants, *Environment International* 24, 703–712.

Pasanen A.L., S. Lappalainen and P. Pasanen, 1996. Volatile

organic metabolites associated with some toxic fungi and their mycotoxins. *Analyst* 121, 1949–1953.

Schnürer J., J. Olsson and T. Börjesson, 1999. Fungal volatiles as indicators of food and feed spoilage. *Fungal Genetics and Biology* 27, 209–217.

Sinha R.N., D. Tuma, D. Abramson and W.E. Muir, 1988. Fungal volatiles associated with mold grain in ventilated and non-ventilated bin-stored wheat. *Mycopathologia* 101, 53–60.

Tuma D., R.N. Sinha, W.E. Muir and D. Abramson, 1989. Odor volatiles associated with microflora in damp ventilated and non-ventilated bin-stored bulk wheat. *International Journal of Food Microbiology* 8, 103–119.

Widstrom N.W., 1996. The aflatoxin problem with corn grain. *Advances in Agronomy* 56, 219–280.

Wilson D.M., W. Mubatanhema and Z. Jurjevic, 2002. Biology and ecology of mycotoxigenic *Aspergillus* species as related to economic and health concerns. *Advances in Experimental Medicine and Biology* 504, 3–17.

Wilkins C.K. and S. Scholls, 1989. Volatile metabolites of some barley storage molds. *International Journal of Food Microbiology* 8, 11–17.

Wilson D.M. and D.K. Bell, 1984. Aflatoxin production by *Aspergillus flavus* and *A. parasiticus* on visibly sound rehydrated peanut, corn and soybean seed. *Peanut Science* 11, 43–45.

Wright M.S., D.M. Greene-McDowelle, H.J. Zeringue, D. Bhatnagar and T.E. Cleveland, 2000. Effect of volatile aldehydes from *Aspergillus* resistant varieties of corn on *Aspergillus parasiticus* growth and aflatoxin biosythesis. *Toxicon* 38, 1215–1223.

Zeringue H.J., 2000. Identification and effect of maize silk volatiles on cultures of *Aspergillus flavus*. *Journal of Agricultural and Food Chemistry* 48, 921–925.

Zeringue H.J., D. Bhatnagar and T.E. Cleveland, 1993. $C_{15}H_{24}$ Volatile compounds unique to aflatoxigenic strains of *Aspergillus flavus*. *Applied and Environmental Microbiology* 59, 2264–2270.

Accepted for publication: August 28, 2008