Analysis of Volatile Compounds for Detection of Molds in Stored Cereals

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ABSTRACT

Volatile fungal metabolites were collected and identified during fungal growth on wheat. This method allows early detection of fungal growth, and representative samples from a large batch of cereals can be taken. The fungi *Aspergillus amstelodami*, *Aspergillus flavus*, *Penicillium cyclopium*, and *Fusarium culmorum* were cultivated on moist wheat (25% mc) in cultivation containers provided with an air inlet and outlet. Volatiles in the headspace gas of the containers were collected on a polymer adsorbent by purging an airstream through the container and were separated and identified by combined gas chromatography and mass spectrometry. The volatile metabolites produced in the greatest quantities were 2-methylfuran, 2-methyl-1-propanol, and 3-methyl-1-butanol. When production of volatiles during different fungal growth phases was investigated, it was found that some compounds were mainly produced during the early stages. The metabolic activity of the fungi was studied by measuring CO_2 content in the cultivation containers. Two days after inoculation of fungi, before growth was visually detectable, a rise in the concentration of CO_2 and other volatiles was readily measurable. The production of terpenes varied greatly between the examined species, and these compounds could possibly be used for the recognition of species.

Fungi are important causes of deterioration in stored cereals. Their activities impair breadmaking properties and nutritive value. There is also always a potential risk of mycotoxins and allergens forming during fungal growth.

Current methods for detection of mold contamination and mold growth have a number of drawbacks. Counting of colony forming units (CFU) is time-consuming and not related to actual activity. Representative sampling is also difficult. The odor of the cereals is often used as a criterion of fungal infection. The major drawbacks of this method are that it is subjective and that many volatiles of fungal origin may not have a characteristic odor.

The search for improved methods is therefore of great importance. One of the most promising new techniques is analysis of volatile compounds in the headspace gas surrounding a sample where fungal infection is suspected. It can be developed into a simple, rapid method with high sensitivity and a good chance of representative sampling. A combination of gas chromatography and mass spectrometry was used by Kaminski et al (1974) to identify a number of volatile compounds produced by different fungi during growth on wheat meal. This method, however, was time-consuming because the volatiles were extracted by a distillation procedure. A much faster method, where a sample of the headspace gas in a flask containing a fungi culture was drawn by a syringe and then directly injected into a gas chromatograph, was employed by Norrman (1977). A further development of the method is the use of a porous polymer to trap the volatiles (Kaminski et al 1985, Harris et al 1986). This method makes it possible to concentrate volatiles that are present in low concentrations in large volumes, and samples can be stored conveniently before analysis (Hyde et al 1983). Abramson et al (1983) used the method for long-term monitoring of storage conditions of cereals. Volatiles associated with microflora in stored wheat include 3-methyl-1-butanol, 1-octen-3-ol and 3-octanon. 3-Methyl-1-butanol can be detected even if the wheat has been ventilated (Sinha et al 1988). Before this method can be used commercially, some important questions have to be clarified. First,

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it is important to choose compounds specific to fungi that do not appear in sound cereals. Such metabolites should be detectable at an early stage of fungal growth. Because different molds have different properties, for example the ability to produce toxins, it is also important to be able to discriminate between species. Even closely related fungi can be discriminated by using the pattern of nonvolatile metabolite formation (Frisvad and Filtenborg, 1983). This study was designed to add information that is essential for the development of a practically applicable method. Volatiles produced by different fungi inoculated into moist wheat were identified, and the possibilities of using the method as a fast and efficient tool for mold detection in cereals were evaluated.

MATERIALS AND METHODS

Two separate experiments were conducted. The first was designed to detect and identify volatiles specifically associated with fungal activities and to differentiate fungi according to their production of volatiles. The second experiment was mainly designed to establish how soon the fungal metabolites could be detected, but also to try a new sampling technique: sampling during continuous airflow through the cultivation container. The materials were the same in both experiments, except for the strain of *Fusarium culmorum*. Due to decreasing ability to produce spores, the strain of *F. culmorum* used in the first experiment had to be exchanged for another one. The methods were also the same, except for the timing and procedure of sampling.

Fungi and Cultivation Media

The following species of fungi were used: *Fusarium culmorum* (W. G. Smith) Sacc. (CBS 171.28) (experiment 1); *Fusarium culmorum* (W. G. Smith) Sacc. (SLU 10-717) (experiment 2); *Aspergillus amstelodami* (Mangin) Thom and Church (SLU 10-904); *Aspergillus flavus* Link (CBS 242.73); *Penicillium verrucosum* var. *cyclopium* (Westling) Samson et al (SLU 10-878).

For production of spores, all fungi except *F. culmorum* were grown on 2% malt extract agar. *F. culmorum* was grown on either oatmeal agar or bran-koji (Fukumoto et al 1983). Winter wheat with an original moisture content of 11.5% was used. The moisture content was raised to 25% on a wet weight basis by adding small amounts of distilled water over 20 hr. Between the water additions, the grain was shaken on a laboratory shaker. After 20 hr, 500 g of wheat was put in a cultivation container and autoclaved at 121°C for 15 min.

Preparation of Spore Suspensions and Inoculation

Spore suspensions were taken out after a growth period of about 10 days. Ten milliliters of a 0.85% NaCl-solution was added to the agar plates or Erlenmeyer flasks containing bran-koji, and spores were liberated mechanically. The spore suspensions were filtered through a cotton cloth and added to the wheat in a cultivation container by means of a sterile plastic syringe. In the first experiment, a spore suspension was also added to 2% malt extract agar that had been poured into a cultivation container.

Equipment for Cultivation and Sampling

The equipment is illustrated in Figure 1. Two-liter Fernbach culture flasks were used as cultivation containers. They were fitted with lids with valves to allow air to flow in and out. To keep the moisture content of the wheat constant during the experiment, the air going in was led through water that had been treated by reversed osmosis, deionization, and charcoal filtration. Glass tubes filled with glass wool were attached to the inlet and outlet to prevent contamination of cultivation container or environment by airborne microbial spores. For collection of volatiles, a porous polymer adsorbent (Chromosorb 102, 100–120 mesh) was used. To prevent volatile contaminants from entering the cultivation container, another adsorbent (Tenax GC, 60–80 mesh) with less tendency to adsorb water was used.

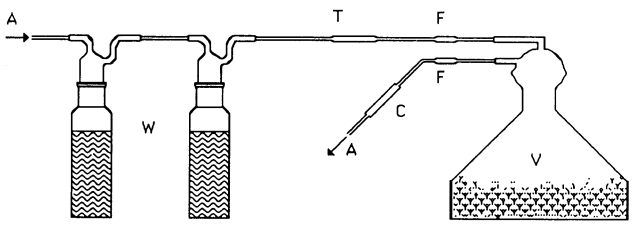

Fig. 1. Equipment for cultivation of fungi and sampling of volatiles. **A** = Airflow; **W** = water-filled flasks for moistening air; **T** = Tenax GC adsorbent; **F** = spore filters; **V** = cultivation container; **C** = Chromosorb 102 adsorbent.

Sampling of Volatiles

The first experiment consisted of at least two trials with each fungus and two trials with uninoculated controls (autoclaved wheat with the same moisture content as the inoculated wheat). The fungi were cultivated for a period of 14 days. Once a day, an airstream of 40 ml/min was led through the cultivation containers and volatiles were adsorbed on Chromosorb 102. The sampling time was 90 min, which gave a total of 3.6 L of air passing through the adsorbent on each occasion. The first sampling period included the first six days of cultivation, the second the following four days, and the third the last four days, that is, three samples for the cultivation period of 14 days. The second experiment consisted of one trial with each fungus and one uninoculated control. The fungi were cultivated for six days, and a continuous air flow of 10 ml/min was led through the cultivation containers (14.4 L/day). Sampling periods were one day long and the cultivation continued for six days.

Analysis of Volatile Compounds

Before analysis, the adsorbents were dried by a helium flow of 40 ml/min for 20 min. The volatiles were thermally desorbed at 110°C using a helium stream of 20 ml/min for 20 min. The compounds were concentrated in a cold trap (−196°C) of the gas chromatographic concentrating/injection system (Hall 1983). The concentrated compounds were injected onto the column of a gas chromatograph by heating the cold trap to 140°C. The instrumental parameters were as follows: gas chromatograph: Varian 3700 with flame ionization detector; column: fused silica capillary column with chemically bonded methyl-polysiloxan, DB 1, 1.0 μm, 60 m × 0.32 mm i.d.; temperature program: 25–220°C, 2 min initial temperature, 3°C/min; carrier gas: helium, 4 ml/min; data collection system: Hewlett-Packard HP 3367; mass spectrometer: Finnigan 9610-4023 gas chromatography-mass spectrometry system; library of mass spectra: National Bureau of Standards (38,500 spectra).

Analysis of CO_2

The metabolic activity of the fungi was followed by measuring the CO_2-content in the cultivation containers once daily. In the first experiment, the measurement was made before the sampling of volatiles. The airstream coming from the cultivation containers was led through a sampling loop of 90 μl. When 20 ml of air had passed the loop, the contents of the loop were injected onto a gas chromatographic column. The instrumental parameters were as follows: gas chromatograph: HP 5890 gas chromatograph with TCD detector; column: packed column, Chromosorb 101, 60–80 mesh, 2 m × $^1/_8$ in. o.d.; temperature program: isothermally at 25°C; carrier gas: helium, 16 ml/min; data collection system as above.

RESULTS

Results of the First Experiment

Growth and metabolic activity of examined fungi. A significant increase in the CO_2 content was observed in the inoculated containers after two days irrespective of the species inoculated. The production of CO_2 increased until about five days after inoculation, after which it remained constant. The production varied between species, as illustrated in Figure 2. The different fungi also colonized the grain in different ways. *A. amstelodami*

and *F. culmorum* grew extensively over the entire surface of the grain after about three days, whereas the other species grew mostly on damaged kernels.

Analysis of volatiles. The volatiles that increased during fungal growth are presented in Table I. Ethanol, the metabolite produced in the greatest quantities, is not present in the table. The reason for this is that the peaks were cut by the data collection system and an accurate quantification could therefore not be made. Although the differences between replicates were sometimes considerable, the patterns of volatile formation were similar. The figures of *A. flavus* are particularly uncertain, however, and give a mere indication of the magnitude of the production.

The metabolites can be divided into three categories: 1) present in control grain, increase during fungal growth; 2) not present in control grain, produced by several of the tested fungi; and 3) not present in control grain, produced by one of the tested fungi only.

Most of the volatiles were put in category one or two. The compounds that were produced in the greatest quantities were alcohols, alkanes, and terpenes. More than 80% of the total concentration of volatiles present after at least six days of cultivation consisted of alcohols. The only exception was *A. amstelodami* where only about 50% were alcohols. In all cultures, ethanol was the predominant alcohol, constituting more than 90% of the total amount of alcohols. 2-Methyl-1-propanol and 3-methyl-1-butanol were also produced in fairly high concentrations.

Alkanes and terpenes were produced in smaller quantities, less than 1% of the total, and were above all produced by *F. culmorum*, although the production of terpenes by *P. cyclopium* was also detectable. The terpenes eluate quite late and can easily be detected because of the absence of peaks from the uninoculated grain in this area of the chromatogram (Fig. 3).

Most compounds were produced during the whole period of cultivation, and in increasing amounts. Exceptions were only found during cultivation of *F. culmorum*, where for instance 2,2,5-trimethylhexan was produced almost exclusively during the first six days of cultivation. During growth of *P. cyclopium* on malt

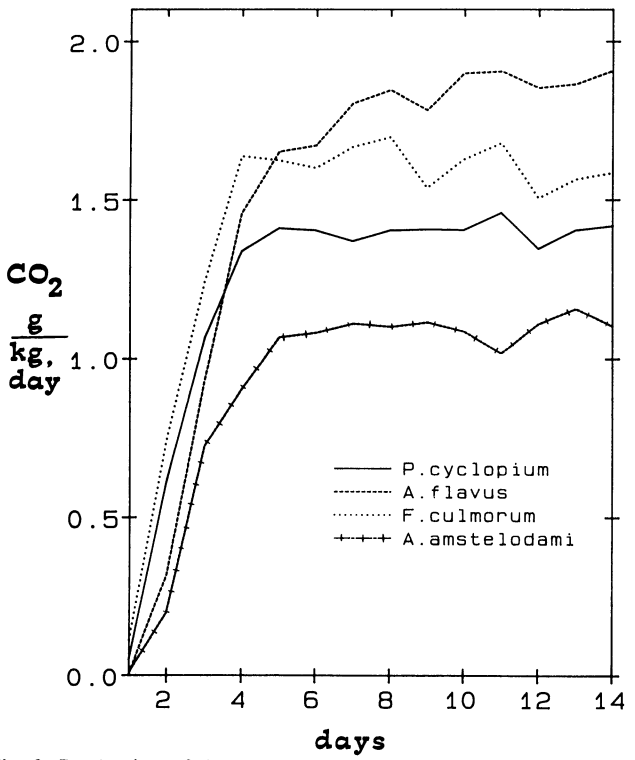

Fig. 2. Production of CO_2 measured as grams of CO_2 per kilogram of wheat per day in cultivation containers inoculated with different fungi. The CO_2 was measured once daily and the graphs were made from mean values of two replicates.

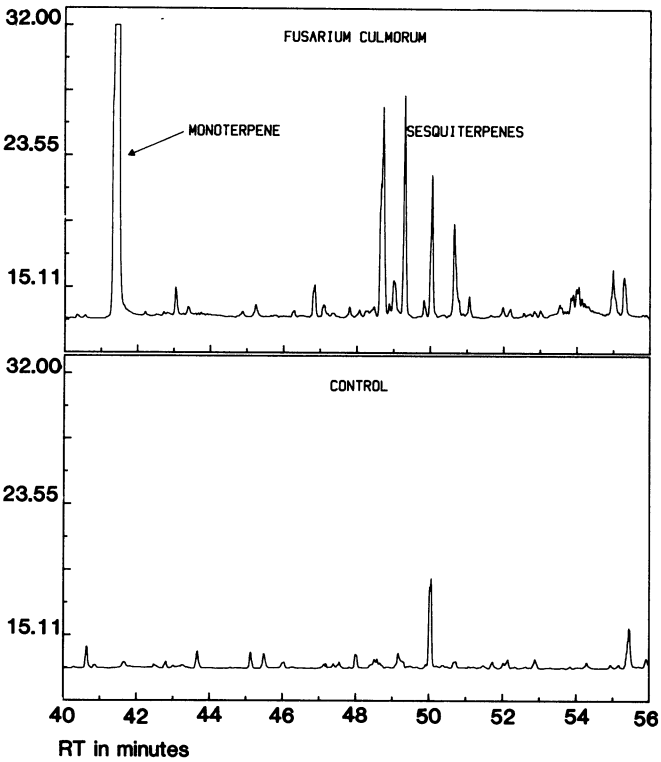

Fig. 3. Comparison of late fractions of chromatograms showing the slowest eluting volatile compounds in the cultivation container inoculated with *F. culmorum* and the uninoculated control.

TABLE I
Volatile Compounds in Headspace Gases (in ng/L of air)[a] Produced by Different Fungi During 14 Days of Cultivation

	A. amstelodami			*A. flavus*			*F. culmorum*			*P. cyclopium*		
Volatile Compound	**1-6[b]**	**7-10[b]**	**11-14[b]**	**1-6**	**7-10**	**11-14**	**1-6**	**7-10**	**11-14**	**1-6**	**7-10**	**11-14**
2-Methylfuran	11[c]	76[c]	160[c]	...	...	...	...	...	...	35	59[d]	45[d]
2-Methyl-1-propanol	23	19	21	350	2,100	3,200[d]	1,200	3,500	3,600	310	880	1,700
2-Pentanon	23	42	64	...	...	...	...	...	...	...	...	...
3-Methyl-1-butanol	...	...	...	83	480	870[d]	...	...	...	90	170	400
3-Octen-2-ol	6	12	17	...	...	...	...	...	...	39[d]	26	16
1-Octen-3-ol	9	12	16	...	...	...	...	...	...	31[d]	7[d]	4[d]
Monoterpenes	...	...	...	...	...	...	44	170	260	...	...	...
Sesquiterpenes	...	...	...	...	...	...	49[d]	36	33	2	3[d]	3
2,4-Dimethylhexan	...	...	...	...	...	...	68[d]	97	280	...	...	...
2,3,5-Trimethylhexan	...	...	...	...	...	...	17[d]	4	2	...	...	...

[a]Mean of two replicates for all fungi except *P. cyclopium* where three replicates were used.
[b]Numbers represent ranges of days.
[c]2-Methylfuran and methylbutenol (mixture of 2-methyl-3-buten-2-ol and 3-methyl-2-buten-1-ol) could not be separated.
[d]Difference between replicates more than 100%.

extract agar, terpenes were produced in higher concentrations and some sesquiterpenes not found during growth on wheat were newly detectable. The production of other metabolites did not, however, seem to differ much between the media.

TABLE II
Volatiles in Headspace Gases (in ng/L of air) Produced by Different Fungi During Six Days of Cultivation with Continuous Air Flow

Fungus/ Volatile Compound	Days 1	2	3	4	5	6
Control						
2-Methylfuran	11	4	···	···	···	···
2-Methyl-1-propanol	2	1	···	···	···	···
2-Pentanon	7	6	···	···	···	···
3-Methyl-1-butanol	7	2	···	···	···	···
Aspergillus amstelodami						
2-Methylfuran	22	10	13	19	39	58
Methylbutenol[a]	···	···	···	···	···	···
2-Methyl-1-propanol	4	6	5	6	5	6
2-Pentanon	10	15	28	19	17	10
2-Methyl-1-butanol	41	81	9	2	1	···
3-Octen-2-ol	2	1	6	6	6	5
1-Octen-3-ol	1	3	10	10	10	7
Aspergillus flavus						
2-Methylfuran	20	11	12	42	89	119
2-Methyl-1-propanol	4	24	12	48	84	76
3-Methyl-1-butanol	16	86	29	8	5	2
Fusarium culmorum						
Ethyl acetate	20	51	33	72	132	143
2-Methyl-1-propanol	3	30	89	145	201	202
Monoterpenes	6	2	7	8	15	17
Sesquiterpenes	2	4	24	25	36	34
Penicillium cyclopium[b]						
2-Methylfuran	···	64	37	83	92	100
2-Methyl-1-propanol	···	5	84	200	140	120
3-Methyl-1-butanol	···	35	20	17	9	7
3-Octen-2-ol	···	6	47	48	16	12
1-Octen-3-ol	···	6	36	45	28	21
Sesquiterpenes	···	2	3	6	7	5

[a]Could not be separated from 2-methylfuran. Mixture of 2-methyl-3-buten-2-ol and 3-methyl-2-buten-1-ol.
[b]This concentration of volatiles day 1 was not measured for this fungus.

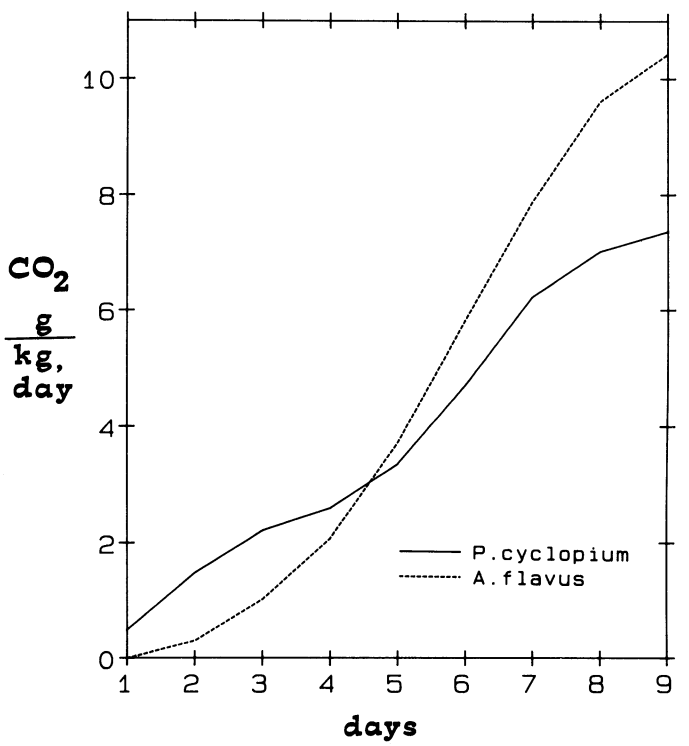

Fig. 4. Comparison of production of CO_2 measured as grams of CO_2 per kilogram of wheat per day in cultivation containers inoculated with *P. cyclopium* and *A.flavus*.

Results of the Second Experiment

The CO_2 production rose continuously, but the concentrations of CO_2 did not reach such high levels as in the first experiment. The CO_2 content in the culture of *A. flavus* just reached 20% compared with 25% in the first experiment. The production of CO_2 is presented in Figure 4. The visual appearance of the fungal growth did not differ from that in the first experiment, except for *F. culmorum* which grew more slowly during this trial.

The total concentration of volatiles did not rise during fungal growth. The concentration of some compounds diminished rapidly, whereas that of others rose.

The reduction of volatiles during fungal growth was especially obvious for the aldehydes 2-methyl-1-butanal and 3-methyl-1-butanal (Fig. 5). In general, the same volatiles as in the first experiment were produced during fungal growth (Table II).

Differences occurred, except for *F. culmorum*, in the first part of the chromatogram only. This was probably a result of the much lower production of ethanol in this experiment, allowing compounds that were previously masked by ethanol to be detected. An example of this was the production of 2-methylfuran by *A. flavus*. In the case of *F. culmorum*, the production of monoterpenes was different from the first experiment. This may possibly be attributed to the use of a different strain of *F. culmorum* in this experiment.

Some compounds appeared predominantly at the early stages of fungal growth. An example of this was 3-methyl-1-butanol, which was produced by *A. flavus* and *P. cyclopium*.

Two days after inoculation, it was possible to distinguish clearly

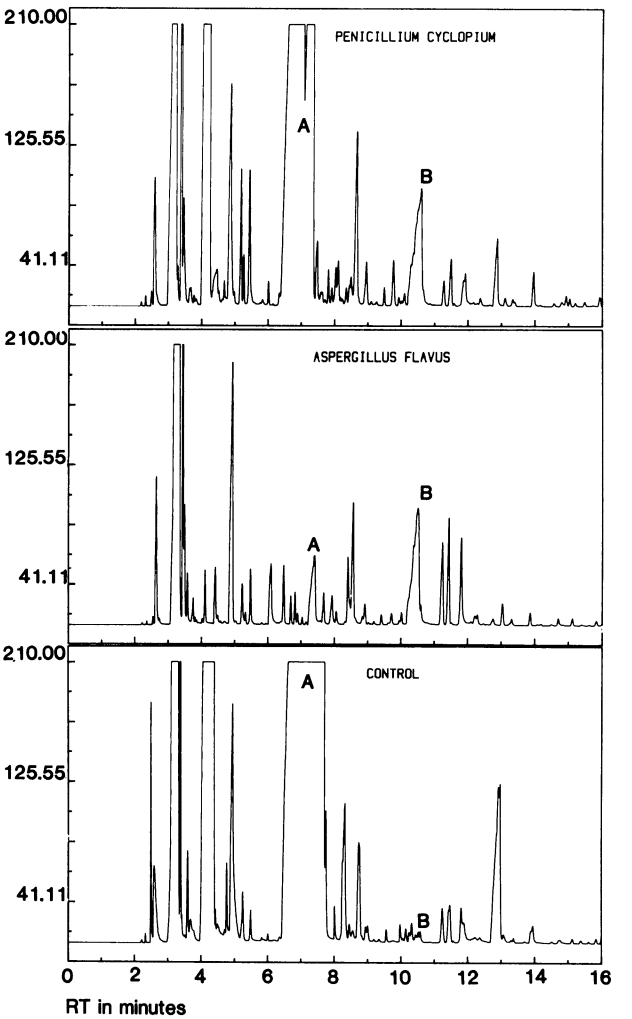

Fig. 5. Comparison of the first part of the chromatograms showing the volatile compounds of uninoculated control and two days after inoculation of *P. cyclopium* and *A. flavus*. A = 3-Methylbutanal and 2-methylbutanal; B = 3-methyl-1-butanol.

the chromatograms from those of uninoculated control wheat (Fig. 5). This occurred before the mold growth could be detected by visual inspection.

DISCUSSION

Some of the volatiles found have previously been reported by other scientists. There are numerous reports of production of 3-methyl-1-butanol and 1-octen-3-ol by fungi (Kaminski et al 1974, Harris et al 1986, Vanhaelen et al 1978, Richard-Molard et al 1976). 2-Methyl-1-propanol was reported by Karahadian et al (1985), but 3-octen-2-ol and 2-methylfuran were not found in the literature. Terpenoid compounds can be produced by a number of fungi (Latrasse et al 1985), but the ones found in this investigation have, as far as we know, not been reported previously. The production of terpenoid compounds by *F. culmorum* is not surprising since the trichothecene mycotoxins produced by *Fusarium* species consist of a sesquiterpene skeleton. The terpenes found could be intermediates in the synthesis of trichothecenes.

The differences in the pattern of volatiles between the experiments could either be attributed to the small amounts of ethanol in the second experiment or, in the case of *F. culmorum*, the use of different strains. The fact that different strains within one species can give rise to entirely different patterns of volatile metabolites is consistent with previous reports (Seifert and King 1982, Sprecher 1980, Sprecher and Hanssen 1982). The production of ethanol and possibly also other alcohols, was stimulated by the high levels of CO_2 in the first experiment. It is possible that the long intervals between aeration combined with intense fungal metabolism led to depletion of oxygen and that the high levels of alcohols were reached as a result of anaerobic metabolism.

In the second experiment, it was obvious that different metabolites were produced at different phases of fungal growth. 3-Methyl-1-butanol was found to be associated with early stages of fungal growth, which is consistent with earlier findings (Karahadian et al 1985). The reason for its presence in the first experiment may have been a change to anaerobic metabolism, with an increased production of 3-methyl-1-butanol. This study shows that metabolites specifically associated with fungal growth are produced at early stages, before the growth is visually detectable. They are produced in sufficiently high amounts to allow their detection in quite small air samples. An identification of species may not be possible at this early stage, however, since the compounds produced in the highest amounts at early stages are produced by several species. Previous findings revealed major differences regarding production of terpenes both between species and strains of fungi (Collins 1975, Sprecher and Hanssen 1982). In this study, the most striking differences between the studied species were production of terpenes, and the main difference between the strains of *F. culmorum* was also the production of terpenes. This indicates that it should be possible to use volatile terpenes, not only for recognition of species, but also to identify strains. It is also obvious that recognition of species cannot be conducted unless the differences between strains are known and taken into account. Besides recognition of species, future research should concentrate on detection of the metabolites that are produced early during fungal growth. It is also essential to examine the production of volatiles when different environmental factors, such as water content or species of cereal, are varied.

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