Effect of Carbon Dioxide, Temperature, and Relative Humidity on Production of Aflatoxin in Peanuts

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Abstract

Effects of carbon dioxide (CO_2) in combination with reduced relative humidities (RH) and temperatures on growth and aflatoxin production by *Aspergillus flavus* in peanuts were investigated. Sound mature kernels of Early Runner peanuts were surface disinfested, inoculated with *A. flavus*, and incubated at various temperatures, RH, and CO_2 concentrations. Visible growth, aflatoxin production, and free fatty acid (FFA) formation by *A. flavus* was inhibited at approximately 86% RH by 20% CO_2 at 17C and by 60 and 40% CO_2 at 25C. Aflatoxin and FFA levels decreased as RH decreased from approximately 99% to 92% to 86%. At a constant temperature, an increase in CO_2 concentration caused a decrease in aflatoxin and percentage FFA; and, at a given CO_2 concentration, lowering the temperature resulted in a decrease in aflatoxin and percentage FFA.

Introduction

A COMMON SEED-STORAGE FUNGUS, *Aspergillus flavus* Link ex Fries received only routine attention until 1960 when aflatoxin, a carcinogenic substance produced by this fungus, was found in peanut meal. Since that time, the factors affecting growth and aflatoxin production by *A. flavus* in agricultural commodities have been widely investigated (10,11). Several studies (2–6,9) have been conducted on the relation of temperature and moisture to the production of aflatoxin in peanuts, but only one report (7) has been made of studies on the effect of atmospheric gases. Landers et al. (7) used various levels of carbon dioxide (CO_2), oxygen (O_2), and nitrogen (N_2) to inhibit growth and production of aflatoxin and free fatty acids (FFA) by *A. flavus* at optimal relative humidity (RH) and favorable temperature. This paper reports the influence of CO_2 on growth and production of FFA and aflatoxin by *A. flavus* when used in association with reduced RH and temperature.

Materials and Methods

A. flavus, isolate Ala-6, was obtained from peanuts in Alabama in 1964 and has been a consistent aflatoxin producer (3). Sound, mature kernels of peanuts (*Arachis hypogaea* L., cultivar Early Runner) were obtained from the 1965 crop at the Wiregrass Substation, Headland, Alabama. Peanuts were incubated at temperatures of 30C, 25C, or 17C (± 1) in various experiments with compressed air (0.03% CO_2, 21% O_2, 79% N_2) serving as a check at each RH in each experiment. Gas mixtures were obtained in size H cylinders from Davis-Dyar Supply Co., Opelika, Alabama, and were certified to be within a tolerance of $\pm 0.5\%$ volume. Gas mixtures were introduced by means of oxygen therapy regulators (National Welding Equipment Co., Richmond, Calif.). Concentrations of CO_2 and O_2 were verified for accuracy by measurement with a Burrell Kwik-Chek gas analyzer (Burrell Corp., Pittsburgh, Pa.) and a YSI model 52 oxygen meter (Yellow Spring Instrument Co., Yellow Springs, Ohio), respectively.

Figure 1 shows a diagram of the apparatus used in this investigation. Saturated water solutions of ammonium dihydrogen phosphate and potassium chromate were used to maintain RH of approximately 92% and 86%, respectively. Water was used to maintain a RH of approximately 99%. Erlenmeyer flasks (500-ml) containing 400 ml of demineralized water or saturated salt solution were placed between each gas cylinder and the 2 culture vessels to maintain the desired RH. Flasks of the same solutions were also used to seal the outlets. Gas mixtures passed from the regulator into the solution in flask and then to the bottom of a 1.25-liter culture vessel containing 200 g of peanut kernels dispersed in 9 layers on wire baskets. Peanut kernels had been previously surface-disinfested with 1% sodium hypochlorite for 2 min and atomized with a spore suspension prepared from 2-week-old Petri dish cultures of *A. flavus*. It was calculated that the peanuts in each culture vessel were sprayed with approximately 2×10^7 spores. The gas was released in 125 ml of solution in the bottom of the culture vessel, through the inoculated peanuts, and into a second culture vessel where the cycle was repeated. The gas passed from the second culture vessel to an outlet seal and then into the atmosphere of the laboratory. At the beginning of each experiment the culture vessels were flushed with the gases at 500 cc/min for 30 min, then the gas flow was reduced to 150 cc/min for the remainder of the 14-day incubation period.

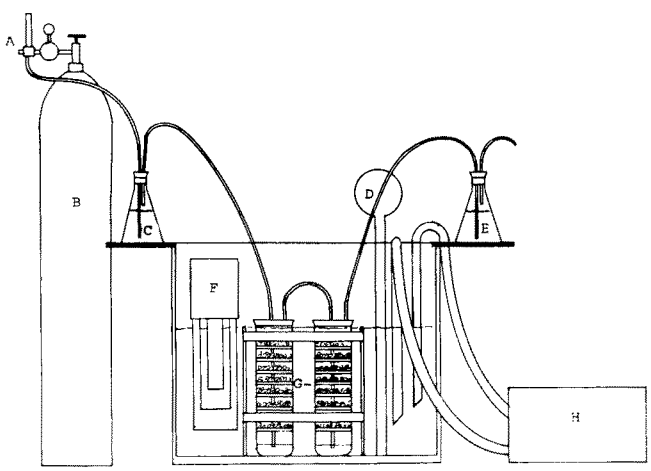

FIG. 1. Diagram of the apparatus used to study the influence of various temperatures, relative humidities, and CO_2 concentrations on aflatoxin production and free fatty acid formation in peanuts inoculated with *A. flavus*. (A) oxygen therapy regulator, (B) gas cylinder, (C) scrubber flask (water or saturated salt solution), (D) agitator unit, (E) outlet seal (water or saturated salt solution), (F) heater unit, (G) culture vessel, (H) cooling unit.

TABLE I
Effect of Various Gas Mixtures and Relative Humidities (Kernel Moisture) on Production of Free Fatty Acids and Aflatoxin by *Aspergillus flavus* on Peanuts Stored for 14 Days at 25C

Gas conc. (%)			RH	KMC	FFA	Aflatoxin (μ g/g)				
CO_2	O_2	N_2	(%)	(%)	(%)	B_1	B_2	G_1	G_2	Total
0.03	21	79	99	22.4	69.2	68.3	6.9	102.1	29.1	206.3
0.03	21	79	92	26.5	58.5	53.4	7.1	93.2	31.6	185.2
0.03	21	79	86	15.0	44.1	17.5	3.1	39.6	11.9	72.1
60	20	20	99	24.5	8.1	.1	+[a]	.1	+	.2
60	20	20	92	20.0	3.3	+	0	+	0	+
60	20	20	86	11.8	.6	0	0	0	0	0
Untreated check				6.0	.5	0	0	0	0	0

[a] + = .001–.049 μ g/g aflatoxin.

Moisture content of the peanut kernels was determined according to the official American Oil Chemists' Society method Ab 2–49 (1) with the following modifications. A 15-g sample (wet wt) of kernels was placed in a tared metal can, heated at 135C for 20 min, dried to constant weight at 70C, and placed in an oven for 3 hr at 130C. Tabular data on kernel moisture content (KMC) represent an average of 6 replications, except in the first table where each value is an average of 2 replications. Aflatoxin determinations were made on duplicate 30-g samples of dried, ground kernels from each of the 2 replicate culture vessels by the aqueous acetone method of Pons and Goldblatt (8). This method is accurate to within 1–2 μ g/kg of aflatoxin. FFA were determined on duplicate 7.05-g samples of oil by American Oil Chemists' Society method Ab 5–49 (1). FFA were calculated as percentage of oleic acid of the total fat extracted and data are the average of 4 replications.

Results

The influence of a gas mixture of 60% CO_2, 20% O_2, and 20% N_2 on production of aflatoxin and FFA by *A. flavus* on peanuts stored for 14 days in various RH at 25C is shown in Table I. Aflatoxin production decreased approximately 10% and 65% in air as RH was lowered from 99% to 92%, and 86%, respectively. Treatments with 60% CO_2 reduced aflatoxin production 99.6–99.8%. In 60% CO_2 aflatoxin production decreased from 0.25 μ g/g at 99% RH to 0.01 μ g/g at 86% RH. Aflatoxin production at 86% RH in 60% CO_2 was no greater than the amount sometimes detected in untreated kernels assayed immediately upon removal from cold storage. Visible growth and sporulation decreased in 60% CO_2 and air as RH decreased. No visible growth or sporulation was observed in 60% CO_2 at 86% RH, but both were abundant in air at 86% RH. Percentage FFA decreased with decreased RH in both 60% CO_2 and air. The percentage FFA produced in peanuts in the 60% CO_2 and 86% RH was only 0.05% more than that found in the untreated kernels. The percentage reductions in FFA with decreasing levels of RH were not as great as the reduction in aflatoxin production.

A gas mixture of 40% CO_2, 20% O_2, and 40% N_2 substantially decreased production of aflatoxin and FFA by *A. flavus* on peanuts stored for 14 days in 99% and 86% RH at 30C when compared with air (data not presented). Although aflatoxin and FFA were lower in 86% RH than 99% RH in both 40% CO_2 and air, the level of control was not comparable to that obtained with 60% CO_2 at 25C.

The influence of a gas mixture of 40% CO_2, 20% O_2, and 40% N_2 on the production of aflatoxin and FFA by *A. flavus* on peanuts stored in various RH at 25C is shown in Table II. Aflatoxin production in air decreased approximately 80% and 95% when RH was lowered from 99% to 92% and 86% RH, respectively. In 40% CO_2, aflatoxin production was decreased approximately 85% when RH was lowered from 99% to 92%. When RH was further lowered to 86% RH, aflatoxin production was prevented. In both air and 40% CO_2, growth and sporulation decreased as RH was lowered. In 40% CO_2 at 86% RH, fungus growth was negligible and percentage FFA approximated that of uninoculated checks.

In a gas mixture of 20% CO_2, 20% O_2, and 60% N_2, production of aflatoxin and FFA in peanuts stored at 3 RH at 25C decreased about 50–60% in comparison to those stored in air (data not presented). Practically no reduction in fungus growth was noted.

The influence of a gas mixture of 20% CO_2, 20% O_2, 60% N_2 on the production of aflatoxin and FFA by *A. flavus* on peanuts stored in various RH at 17C is shown in Table III. In air at 17C, aflatoxin production decreased approximately 80% when RH was lowered from 99% to 92%. A further decrease in RH to 86% resulted in the production of 0.21 μ g/g of aflatoxin in air. The largest amount of aflatoxin produced in 20% CO_2 was 0.174 μ g/g at 99% RH with a negligible amount at 92% and 86% RH. Thus, 20% CO_2 with a slight reduction in RH virtually eliminated aflatoxin production in peanuts for 14 days at 17C, whereas at 25C this concentration of CO_2 gave little or no control over toxin production. Growth and sporulation were noted in all air treatments and in 20% CO_2 at 99% RH; however, little or no visible growth occurred in 20% CO_2 at 92% and 86% RH at 17C. In 20% CO_2 at 92% and 86%

TABLE II
Effect of Various Gas Mixtures and Relative Humidities (Kernel Moisture) on Production of Free Fatty Acids and Aflatoxin by *Aspergillus flavus* on Peanuts Stored for 14 Days at 25C

Gas conc. (%)			RH	KMC	FFA	Aflatoxin (μ g/g)				
CO_2	O_2	N_2	(%)	(%)	(%)	B_1	B_2	G_1	G_2	Total
0.03	21	79	99	26.8	19.4	53.7	11.0	107.0	24.8	196.6
0.03	21	79	92	21.7	6.4	7.5	1.4	23.5	5.0	37.4
0.03	21	79	86	17.1	3.0	2.3	.4	7.4	1.7	11.8
40	20	40	99	29.2	7.0	1.1	.1	2.2	.4	3.8
40	20	40	92	24.8	1.5	+[a]	0	.2	+	.3
40	20	40	86	17.4	.3	0	0	0	0	0
Untreated check				4.4	.3	0	0	0	0	0

[a] + = .001–.049 μ g/g aflatoxin.

TABLE III
Effect of Various Gas Mixtures and Relative Humidities (Kernel Moisture) on Production of Free Fatty Acids and Aflatoxin by *Aspergillus flavus* on Peanuts Stored for 14 Days at 17C

Gas conc. (%)			RH (%)	KMC (%)	FFA (%)	Aflatoxin (μ g/g)				
CO_2	O_2	N_2				B_1	B_2	G_1	G_2	Total
0.03	21	79	99	24.3	10.7	18.6	.8	33.0	4.7	57.1
0.03	21	79	92	17.3	3.3	.7	.1	1.5	.3	2.5
0.03	21	79	86	15.5	.6	+[a]	+	.1	+	.2
20	20	60	99	21.6	1.0	.1	+	.1	+	.2
20	20	60	92	17.6	.3	0	0	+	0	+
20	20	60	86	15.8	.5	+	0	+	0	+
Untreated check				6.1	.3	+	0	+	0	+

[a] + = .001–.049 μ g/g aflatoxin.

RH, percentage FFA was approximately the same level as that of the untreated check.

Discussion

This investigation showed that concentrations of 20% and 40% CO_2 in combination with reduced temperatures, or reduced RH, or both, prevented aflatoxin formation in peanuts. Landers et al. (7) found that growth and aflatoxin production of *A. flavus* were inhibited at 40%, 60%, and 80% CO_2 at 15C and 99% RH. They also found that at 99% RH, aflatoxin was formed at 20% CO_2 and 15C, as was the case at 17C in this study; it was further noted here that with a reduction of RH to 92% and 86% no growth or aflatoxin production occurred. At higher temperatures (25C) aflatoxin was formed at 40% CO_2 and 92% RH, but not at 86% RH. Also, at 25C a concentration of 60% CO_2 inhibited aflatoxin at 92% RH. As in previous investigations (4–7) percentage FFA was used as an index of fungus growth, since mycelial growth and sporulation are difficult to measure in a natural substrate. CO_2 reduced the growth of *A. flavus* as evidenced by decreases in percentage FFA and in aflatoxin production in comparison to that in air. At a given temperature in air or in a given CO_2 concentration, lowering the relative humidity or the KMC of the substrate also reduced fungus growth and indirectly FFA and aflatoxin. Thus, prevention of fungus growth and aflatoxin production was accomplished with several different concentrations of CO_2 in combination with certain temperatures and RH. Aflatoxin elaboration was prevented in simulated storage conditions with 20% CO_2, 17C, and 86% RH.

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