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Sodium Bicarbonate Reduces Viability and Alters Aflatoxin Distribution of *Aspergillus parasiticus* in Czapek's Agar†

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The potential of sodium bicarbonate to inhibit growth of and aflatoxin synthesis by *Aspergillus parasiticus* was examined in Czapek's agar (CA), a medium in which fluorescence under UV light indicates aflatoxin production. Incorporation of sodium bicarbonate (SB) into CA at 0.011, 0.022, and 0.033 mol% reduced cell viability 63-, 10^3-, and $>10^7$-fold, respectively. Colonies resulting from surviving cells did not fluoresce under UV light, but thin-layer chromatography analysis of culture extracts detected aflatoxins. Potassium bicarbonate (KB) at 0.011 and 0.022 mol% produced inhibitory effects similar to those of SB, but NaCl and silica had no effect. After 7 days, control cultures had the normal aflatoxin distribution ($B_1 > G_1 > B_2 > G_2$), but this distribution shifted to $B_2 > B_1 \approx G_2 > G_1$ during prolonged incubation. Cultures supplemented with SB and KB contained mostly aflatoxins B_1 and G_1 after 28 days. Both SB and KB raised the pH of CA to 7.5 to 8.5 at the time of growth. Culture growth on CA adjusted to pH 7.5 to 8.5 with NaOH was not inhibited but exhibited reduced fluorescence and elevated levels of aflatoxins B_1 and G_1. Thus, while bicarbonate inhibition of growth could not be attributed to pH elevation, the lack of culture fluorescence on CA-SB and CA-KB and the altered aflatoxin distribution were caused by the ability of SB and KB to elevate pH.

Aflatoxin production by *Aspergillus parasiticus* and *A. flavus* is a serious agricultural problem. The 1979 to 1980 aflatoxin epidemic in corn resulted in a $400 million loss (19). In 1983, corn harvested in Virginia had average aflatoxin levels of 117 ± 360 μg/kg (4). Aflatoxin contamination also occurs in rice, peanuts, barley, oats, rye, sorghum, wheat, cottonseed, and other crops.

Several investigators (7, 11, 17) have found that carbon dioxide-modified-atmosphere storage inhibits aflatoxin production. Because sodium bicarbonate (SB) decomposes to carbon dioxide under high temperature and relative humidity, conditions that normally favor aflatoxin production (1, 10), we hypothesized that SB might inhibit aflatoxin production on corn kernels during storage. SB is inexpensive, easy to handle, and generally recognized as safe for use in foods, properties which are essential in an antiaflatoxigenic agent. The objective of this study was to examine the influence of SB on growth of and aflatoxin synthesis by *A. parasiticus* in a semisynthetic medium model system.

MATERIALS AND METHODS

Inoculum preparation. *A. parasiticus* NRRL 2999 was obtained from J. J. Ellis (Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill.) and maintained on slants of potato dextrose agar (Difco Laboratories, Detroit, Mich.) at 5°C. Spores were generated on potato dextrose agar slants inoculated with *A. parasiticus* and incubated at 30°C for 7 days. Spores were recovered by washing the potato dextrose agar surface with sterile 0.05% Triton X-100, and final spore counts (as determined on potato dextrose agar) were 10^8 CFU/ml. These spores were stored at 5°C and used as inocula for all experiments.

Media. The basal medium was a modification (5) of Czapek's agar (CA) that fluoresces under longwave (365-nm) UV light in the presence of aflatoxigenic aspergilli. The medium was adjusted to pH 5.5 with 1 N NaOH before sterilization at 121°C for 15 min. SB and potassium bicarbonate (KB) (determined to be sterile in preliminary experiments) were aseptically added to sterile CA tempered to 40°C. NaCl and silica (particulate silicon dioxide) were added before the media were autoclaved. All chemicals were from Sigma Chemical Co., St. Louis, Mo., except for SB, which was supplied by the Church and Dwight Co., Inc., Princeton, N.J.

Growth experiments. To determine whether retention of carbon dioxide produced by SB decomposition played a role in inhibiting aflatoxin production, quadruplicate plates of CA containing 0, 0.011, 0.022, or 0.033 mol% SB were inoculated with 10^1 or 10^2 spores per plate. One set of duplicate plates was sealed with Parafilm laboratory film (American Can Co., Greenwich, Conn.), and the other set was left exposed to normal atmosphere. Both sets were incubated at 30°C.

Media were prepared with 0, 0.011, and 0.022 mol% SB, equimolar concentrations of KB or NaCl, and silica at 1 and 2% (wt/vol) to determine whether the inhibitory activity of SB could be attributed to sodium, bicarbonate, or the physical effect of powder in the medium. These plates were inoculated from serial 10-fold dilutions covering the range of 10^2 to 10^7 spores per plate, incubated at 30°C, and periodically observed for growth and UV light-induced fluorescence in a Chromato-Vue box (Ultraviolet Products, Inc., San Gabriel, Calif.) during the 28-day incubation period. The pH values of uninoculated media were determined at each observation by using a Chemcadet pH meter (Cole Parmer, Chicago, Ill.) with a flat membrane pH electrode (Fisher Scientific Co., Springfield, N.J.) standardized against buffers at pH 3 and 7.

The influence of pH alone was initially examined by aseptically adjusting the pH of CA, CA–0.011 mol% SB, and CA–0.011 mol% KB to an initial pH of 5.5 or 7.5 with sterile 3 N HCl or NaOH, respectively, after the test compounds

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had been added to the sterilized media. These media were inoculated from serial dilutions of the high-spore-count inoculum, incubated, and observed as described above. In additional experiments, CA was prepared at initial pH values ranging from 5.5 to 11.5, inoculated, and incubated for 14 days.

Aflatoxin determinations. Aflatoxins were extracted from the cultures with 20 ml of high-pressure liquid chromatography-grade chloroform for 30 min, transferred to small Erlenmeyer flasks, and evaporated to dryness. The residue was resuspended in 1 ml of chloroform, filtered, and stored at −20°C until analyzed.

Thin-layer chromatography (TLC) of aflatoxins in the chloroform extracts was done in duplicate as described by Nesheim (9), by using Adsorbosil Plus 1 plates (Alltech Associates, Deerfield, Ill.) developed with acetone-chloroform (AC) (10:90, vol/vol), and in separate experiments with diethyl ether-methanol-water (DEMW; 98:1:1, vol/vol) (2). Each plate was also spotted with 10, 20, and 30 μl of a mixed aflatoxin standard (Sigma). After the separation, the intensity of each aflatoxin spot was determined on a dual-wavelength TLC scanner (Shimadzu Corp., Kyoto, Japan). Concentrations were determined by comparing the peak area of the aflatoxin to peak areas of the aflatoxin standards run on the same plate and by averaging the results of duplicate assays. Selected extracts were subjected to two-dimensional TLC by using development with AC in the first direction and development with DEMW in the second direction.

RESULTS

When CA plates containing 0, 0.011, 0.022, or 0.033 mol% SB were inoculated with 10^1 or 10^2 spores per plate and sealed with Parafilm, the control (0% SB) medium had heavy growth after 4 days. Colonies were irregularly shaped, raised, and wrinkled, and they had undulate edges and were cream colored with dark golden yellow centers and margins. These culture plates fluoresced under UV light. Colonies that grew on CA–0.011 mol% SB were of similar shape and elevation but were dark olive green and filamentous with tufts frequently rising from the centers of the colonies. Even after 28 days of incubation, no fluorescence was observed. When the abnormal colonies from CA–0.011 mol% SB plates were streaked onto CA plates and incubated, they formed colonies of normal shape that did fluoresce under UV light. *A. parasiticus* did not grow on CA plates containing 0.022 or

TABLE 1. Influence of SB, KB, NaCl, and silica on growth of and 365-nm UV light-induced fluorescence by *A. parasiticus* NRRL 2999 on CA

Medium	Parameter	pH, value, or intensity after the following no. of days of incubation:						
		0	2	4	7	14	21	28
CA	pH	5.39	5.45	5.45	5.42	5.37	5.35	5.18
	Log CFU/ml	—[a]	8.0[b]	8.0	8.0	8.0	8.0	8.0
	Fluorescence[c]	—	—	+3	+4	+5	+5	+5
CA–0.011 mol% NaCl	pH	5.32	5.30	5.37	5.20	5.25	5.15	4.82
	Log CFU/ml	—	7.9	7.9	7.9	7.9	7.9	7.9
	Fluorescence	—	—	+3	+4	+5	+5	+5
CA–0.022 mol% NaCl	pH	5.20	5.26	5.18	5.12	5.09	5.01	4.79
	Log CFU/ml	—	8.0	8.0	8.0	8.0	8.0	8.0
	Fluorescence	—	—	+3	+4	+5	+5	+5
CA–1% silica	pH	5.40	5.57	5.55	5.29	5.38	5.36	5.35
	Log CFU/ml	—	8.0	8.0	8.0	8.0	8.0	8.0
	Fluorescence	—	—	+3	+4	+5	+5	+5
CA–2% silica	pH	5.39	5.44	5.51	5.29	5.29	5.28	5.28
	Log CFU/ml	—	8.0	8.0	8.0	8.0	8.0	8.0
	Fluorescence	—	—	+3	+4	+5	+5	+5
CA–0.011 mol% SB	pH	8.68	8.38	8.00	7.60	7.43	7.23	7.07
	Log CFU/ml	—	—	6.4[d]	6.2	6.2	6.2	6.2
	Fluorescence	—	—	—	—	—	—	—
CA–0.022 mol% SB	pH	8.35	8.58	9.08	7.51	8.21	7.15	9.44
	Log CFU/ml	—	—	—	5.1[d]	4.9	4.9	4.9
	Fluorescence	—	—	—	—	—	—	—
CA–0.011 mol% KB	pH	8.14	ND[e]	7.44	7.45	7.15	6.87	6.52
	Log CFU/ml	—	—	7.2	7.4	7.4	7.4	7.4
	Fluorescence	—	—	—	—	—	—	—
CA–0.022 mol% KB	pH	8.75	ND	9.92	10.05	9.32	9.25	8.55
	Log CFU/ml	—	—	—	—	4.2	4.2	4.2
	Fluorescence	—	—	—	—	—	—	—

[a] —, Not observed.
[b] 100% recovery of initial inocula.
[c] Values of +1 through +5, increasing relative intensity.
[d] Estimated figure.
[e] ND, Not done.

TABLE 2. Influence of SB, KB, NaCl, and silica on aflatoxin distribution in *A. parasiticus* NRRL 2999 cultures on CA after 28 days of incubation

Medium	μg of aflatoxin/ml of medium			
	B_1	B_2	G_1	G_2
CA	1.27	6.24	0	0.85
CA–0.011 mol% NaCl	0.616	6.50	0	0.85
CA–0.022 mol% NaCl	0.06	4.28	0	0.52
CA–1% silica	0.34	6.30	0	0.41
CA–2% silica	0.17	5.37	0	0.39
CA–0.011 mol% SB	5.32	0.27	8.63	0.26
CA–0.022 mol% SB	4.37	0.19	8.66	0.12
CA–0.011 mol% KB	4.73	0	6.35	0.05
CA–0.022 mol% KB	0	0	0	0

0.033 mol% SB which were inoculated with 10^2 spores (data not shown). Similar results were obtained on unsealed plates.

When media were inoculated with greater numbers of spores (i.e., serial dilutions of inocula containing 10^7 or 10^8 spores per ml), both SB and KB influenced viability and aflatoxin distribution. SB (0.011 mol%) delayed growth and reduced viability by almost 100-fold (Table 1). KB (0.011 mol%) also delayed growth but was somewhat less inhibitory. At 0.022 mol%, SB and KB delayed growth even further and reduced viable counts by more than 1,000-fold. SB and KB elevated the pH of CA. Fluorescence was not observed in cultures grown on any medium containing SB or KB. However, TLC analysis of chloroform extracts from these cultures detected aflatoxins in all cultures except for those containing 0.022 mol% KB (Table 2). Total aflatoxin levels were somewhat higher than levels in controls. In contrast to the aflatoxins in the controls, aflatoxins B_1 and G_1 were predominant. Neither NaCl nor silica affected *A. parasiticus* growth or aflatoxin synthesis (Table 1). After 2 days of incubation, medium with these additions had colony counts and fluorescence similar to medium with CA. Under these conditions, total aflatoxin synthesis was 4.85 to 8.36 μg per ml of medium (Table 2) as determined by development with AC. Aflatoxin B_2 accounted for most of the aflatoxin. Although low levels of aflatoxins B_1 and G_2 were observed, no aflatoxin G_1 was detected. These aflatoxin distributions were confirmed with the DEMW development system and two-dimensional TLC (data not shown). In time course experiments, we found that the distribution of aflatoxins in control cultures was $B_1 > G_1 > B_2 > G_2$ after day 7 but that it had shifted to $B_2 > B_1 \approx G_2 > G_1$ by day 14 of incubation.

The influence of pH was first examined by adjusting CA and either CA–0.011 mol% SB or CA–0.011 mol% KB to initial pHs of 5.5 and 7.5 after the appropriate component was added (Table 3). The pH of CA containing either bicarbonate salt rose from 7.5 to >8.8 within a few hours of preparation; these media did not support *A. parasiticus* growth. Bicarbonate-free CA that had been adjusted to an initial pH of 7.5 exhibited colony counts similar to those of CA at pH 5.5 but with decreased fluorescence. Growth and fluorescence were similar for all media which were adjusted to an initial pH of 5.5. Aflatoxin levels in CA and CA-SB at pH 5.5 were similar. Levels in CA-KB (pH 5.5) were about 50% lower (Table 4).

When growth was examined on basal CA over a wide range of pHs, initial pHs as high as 10.5 had little effect on viability (Table 5). Although no fluorescence was observed in CA at pH > 8.5 (data not shown), aflatoxins were detected on all media exhibiting growth. Total aflatoxin levels decreased as pH increased, and levels of B_1 and G_1 became greater than those of B_2 and G_2.

TABLE 3. Plate counts, pH, and fluorescence reaction of *A. parasiticus* on CA, CA–0.011 mol% SB, and CA–0.011 mol% KB with initial pH of 5.5 and 7.5

Medium and pH (variable)	Parameter	pH, value, or intensity after the following no. of days of incubation:						
		0	2	4	7	14	21	28
CA, 5.5	pH	5.50	5.50	5.53	5.50	5.42	5.37	5.39
	Log CFU/ml	—[a]	7.0[b]	7.0	7.0	7.0	7.0	7.0
	Fluorescence[c]	—	—	+5	+5	+5	+5	+5
CA, 5.5 (0.011 mol% SB)	pH	5.92	5.92	5.88	5.81	5.67	5.70	5.70
	Log CFU/ml	—	7.0	7.0	7.0	7.0	7.1	7.1
	Fluorescence	—	—	+5	+5	+5	+5	+5
CA, 5.5 (0.011 mol% KB)	pH	5.73	5.76	5.72	5.78	5.33	5.66	5.61
	Log CFU/ml	—	6.9	6.9	6.9	6.9	6.9	6.9
	Fluorescence	—	—	+5	+5	+5	+5	+5
CA, 7.5	pH	7.09	7.07	7.08	6.69	6.99	6.87	6.89
	Log CFU/ml	—	7.0	7.0	7.0	7.0	7.0	7.0
	Fluorescence	—	—	+1	+2	+1	+1	+1
CA, 7.5 (0.0113 mol% SB)	pH	8.86	9.13	9.29	9.20	8.99	8.89	8.99
	Log CFU/ml	—	—	—	—	—	—	—
	Fluorescence	—	—	—	—	—	—	—
CA, 7.5 (0.011 mol% KB)	pH	8.86	9.02	8.85	8.62	8.33	8.10	7.99
	Log CFU/ml	—	—	—	—	—	—	—
	Fluorescence	—	—	—	—	—	—	—

[a] —, Not observed.
[b] 100% recovery of initial inocula.
[c] Values of +1 through 5, increasing relative intensity.

TABLE 4. Aflatoxin distribution of *A. parasiticus* after 28 days on CA, CA–0.011 mol% SB, and CA–0.011 mol% KB adjusted to initial pHs of 5.5 or 7.5 after addition of the test compound

pH/medium	μg of aflatoxin/ml of medium			
	B_1	B_2	G_1	G_2
5.5				
CA	2.14	8.68	0.08	2.45
CA–SB	3.24	9.65	0.26	2.10
CA–KB	0.55	4.85	0.04	1.22
7.5				
CA	9.67	0.51	9.75	0.51
CA–SB	0[a]	0	0	0
CA–KB	0[a]	0	0	0

[a] No growth observed.

DISCUSSION

SB reduced *A. parasiticus* plate counts by almost 100-fold at 0.011 mol% and by more than 1,000-fold at 0.022 mol%. KB was also inhibitory, which suggested that bicarbonate was the inhibitory moiety. High viability in the presence of NaCl ruled out inhibition by the sodium ion. Inhibition on unsealed plates suggested that carbon dioxide was unimportant, since it would diffuse during the 28-day incubation. Growth inhibition could not be attributed to pH elevation because growth on CA was not inhibited at initial pHs as high as 10.5. At an initial pH of 7.5, SB and KB inhibited growth and elevated medium pH, but neither KB nor SB reduced counts when the initial pH was 5.5. Thus, it appears that inhibition was due to bicarbonate ion (the dominant form of SB at pH 7.5) rather than to carbon dioxide (the dominant form at pH 5.5) (3). Benzoic acid, cinnamon, and sodium acetate are other food ingredients that are fungicidal to *A. parasiticus* (13–15).

The production of unusual pigments by colonies grown on CA-SB and CA-KB suggested the accumulation of precursors in the aflatoxin biosynthetic pathway (8) and gave credence to the negative results of the Czapek's agar aflatoxin screen under UV light. Benzoic acid-inhibited cultures accumulate yellow (13) and orange (14) pigments. The orange pigment accumulated by dichlorovos-inhibited cultures (18) has been identified as the aflatoxin precursor versiconal acetate (12). Subsequent identification of aflatoxins in CA-SB and CA-KB cultures suggests that the pigments we observed were not aflatoxin precursors. However, the pigments and the unusual filamentous tufted colony shape indicate that SB and KB had some other influence on fungal physiology.

Although *A. parasiticus* grown on CA-SB and CA-KB did not fluoresce under UV light, aflatoxin was detected by TLC. Similar results were obtained by using media adjusted to high pHs with NaOH (Table 5) and also by using pure aflatoxin B_1 in buffer at pH 9 (6). These data indicate that Czapek's agar should not be used to screen compounds that alter the pH of the medium. Low levels of false-negatives have been reported previously for the Czapek's agar screening method (16).

The relatively high aflatoxin B_2 levels in the control cultures after 28 days suggested the possibility of an artifact in the TLC analysis. In some media, *A. parasiticus* NRRL 2999 produces blue fluorescent pyrazines that comigrate with aflatoxin B_2 during development with AC but that migrate with the solvent front during development with DEMW (2). Reanalysis of our samples with DEMW yielded results similar to those obtained by development with AC; two-dimensional TLC also confirmed the high B_2 levels. In extracts from 7-day cultures, however, the level of B_1 was greater than that of B_2. This finding indicated that the unusual aflatoxin distribution found in the control cultures was caused by the conversion of B_1 to B_2 during the prolonged incubation required to obtain growth and sporulation of cultures containing 0.022 mol% SB or KB.

The conversion of B_1 to B_2 did not occur in alkaline CA, regardless of whether the pH was elevated by adjustment with NaOH or by addition of SB or KB. The instability of aflatoxin B_1 at pH 9 (6) might explain the low total aflatoxin levels in cultures whose pH had been elevated with NaOH but makes the high total aflatoxin levels in the SB and KB cultures (Table 2) somewhat perplexing. It is possible that bicarbonate either inhibited degradative enzymes or actually stimulated aflatoxin synthesis in these cultures. The only treatment that supported growth without producing detectable levels of aflatoxins was that with 0.022 mol% KB. This finding might be due to the very high pH (>9.0) during growth or to the fact that less time elapsed between the initial observation of growth and the end of the 28-day incubation. Although silica gel did not affect aflatoxin distribution or concentration, some increase was observed with 0.022 mol% NaCl, in agreement with previous observations (14).

In summary, although surviving *A. parasiticus* produced aflatoxins, SB did delay growth and reduce cell viability in a medium model system. The applicability of these results to inhibition of aflatoxin production in corn kernels during storage is currently being examined in this laboratory.

TABLE 5. Influence of pH on growth of *A. parasiticus* and on aflatoxin production in CA after 14 days of incubation

Initial pH of medium	Log CFU/ml	Final pH[a,b]	Aflatoxin (μg/ml)[c]			
			B_1	B_2	G_1	G_2
5.5	7.8	5.42	1.3	3.9	1.9	2.3
6.5	7.9	6.67	ND	ND	ND	ND
7.5	8.0[d]	7.54	2.9	0.1	4.2	0.2
8.5	7.9	8.07	0.6	0	0.3	0.2
9.5	7.8	8.43	1.0	0	1.1	0.1
10.5	7.7	8.67	0.1	0	0.4	0
11.5	0	NA	0	0	0	0

[a] NA, Not applicable because no growth occurred.
[b] pH at time colonies first grew on plate.
[c] ND, Not done.
[d] 100 % of original inoculum.

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