Grain Preservatives: Effect on Aflatoxin and Ochratoxin Production[1]

E. E. VANDEGRAFT, C. W. HESSELTINE, and O. L. SHOTWELL, Northern Regional Research Laboratory[2], Peoria, Illinois 61604

ABSTRACT

Corn was treated with either 2% ammonia or 1% propionic acid. Both ammonia and propionic acid significantly reduced mold growth and subsequent aflatoxin and ochratoxin formation. Growth and the formation of mycotoxins were inhibited more by propionic acid than by ammonia. Both ammonia and propionic acid remained effective in inhibiting mold growth and aflatoxin production and in preventing ochratoxin production when treated corn stood as long as 19 and 29 weeks, respectively, before inoculation.

High-moisture corn may have to be stored if drying facilities are unavailable or are overloaded. Also, feeding of high-moisture corn has been shown to have advantages over dried corn. Corn at high moistures (20 to 35%) is subject to mold invasion and subsequent mycotoxin formation. Previously, we studied insecticides and fumigants used on stored grain for their effect on aflatoxin and ochratoxin production. None of the insecticides or fumigants reduced mycotoxin formation enough to be considered practical (1,2).

We turned to the chemical preservatives ammonia and propionic acid because they have been shown to prevent mold growth in high-moisture shelled corn (3,4). We applied ammonia to corn at a moisture level that would be encountered under normal storage conditions and propionic acid to high-moisture corn and allowed the corn to stand for various periods of time. We then tested the resistance of the treated corn to mold invasion and subsequent toxin formation by inoculating with aflatoxin and ochratoxin-producing fungi and studying formation of these mycotoxins. The test was made especially severe not only by using a strong inoculum but also by raising the moisture content of the corn to near optimum levels of 36% at the time of inoculation.

MATERIALS AND METHODS

Application of Preservatives

U.S. No. 2 yellow corn containing 12% moisture was placed in a plastic bag, 2.0% NH_3 (w./w.) was added as ammonium hydroxide (29% NH_3), the contents were shaken by hand, and the treated corn was allowed to stand 1 week in the plastic bag to ensure thorough distribution of the NH_3.

Yellow corn containing 28% moisture and treated with 1% propionic acid was furnished by D. B. Sauer, U.S. Grain Marketing Research Center, Manhattan, Kans.

One hundred fifty grams of this chemically treated corn (ammonia or propionic acid) was weighed into Fernbach flasks. The flasks were closed with gauze and allowed to stand in an exhaust hood for 1 to 29 weeks before inoculation.

[1]Presented at the Association of Official Analytical Chemists meeting, Washington, D.C., Oct. 1973.

[2]Agricultural Research Service, U.S. Department of Agriculture. Mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Cultures

For aflatoxin production, the following strains of fungi were furnished by the ARS Culture Collection maintained at the Northern Laboratory: *Aspergillus flavus* NRRL 3251 and NRRL 3357 and *A. parasiticus* NRRL 2999. For ochratoxin production, the strains were *A. ochraceus* NRRL 3174 and *Penicillium viridicatum* NRRL 3712. These fungi have all been shown to produce the two mycotoxins in previous work (5–7).

Potato-dextrose agar inoculated with spores of each organism was incubated for 2 weeks at 25°C. Inoculum was prepared by washing conidia from the agar with distilled water (1).

Fermentation

After the preservative-treated corn stood in an exhaust hood from 1 to 29 weeks, 150 g. of control corn was weighed into Fernbach flasks. Sterile water (30 ml. for propionic acid; 45 ml. for NH_3) was added to the treated corn. Water was added to the control corn to bring the moisture to the same level (36%) as the treated corn. Flasks inoculated with *A. flavus, A. parasiticus,* and *A. ochraceus* were incubated for 6 days at 28°C. on a Gump shaker (200 r.p.m.); flasks inoculated with *P. viridicatum* were incubated for 12 days at 20°C. also on a Gump shaker (200 r.p.m.). On the second and third day of incubation, 10 ml. of sterile water was added to each flask. The water has to be added in increments for mycotoxin production. At the time of harvest, flasks were briefly steamed to kill the fungi. All experiments were run in duplicate.

Kernels of corn, 5 from each flask, for the 19-week ammonia experiment and the 29-week propionic acid experiment were surface-sterilized for 1 min. with 1% sodium hypochlorite, washed twice with sterile water, plated on potato-dextrose-agar, then incubated at 28°C. for 1 week.

Extractions and Column Chromatography

Aflatoxin was extracted from the ammonia-treated, propionic acid-treated, and control corn according to a modified method by Lee (8). The corn was mixed with water in a Waring Blendor 5 min. The aqueous slurry of ammonia-treated corn was acidified to pH 5.7 with 6N HCl. After standing 20 min., chloroform was added to the aqueous slurry, which was mixed an additional 5 min.

Ochratoxin was extracted from both ammonia-treated and propionic acid-treated corn by mixing 5 min. with acetonitrile:water and hexane in a Waring Blendor (7). The acetonitrile:water:hexane slurry from the ammonia-treated corn was acidified to pH 5.7 with 6N HCl and then blended an additional 2 min. The acetonitrile:water layer was washed with hexane, concentrated, and saved for thin-layer chromatography.

Aflatoxin-containing extracts from the ammonia-treated corn were partially purified by a modification of the column chromatography method developed by Eppley (9). An acetone:benzene (5:95, v./v.) wash was added after the hexane. For thin-layer chromatography, residues were dissolved in benzene:acetonitrile (98:2, v./v.) for aflatoxins and glacial acetic acid:benzene (1:99, v./v.) for ochratoxin.

TABLE I. EFFECT OF 1% PROPIONIC ACID TREATMENT OF CORN[1] ON AFLATOXIN AND OCHRATOXIN PRODUCTION (γ/g.)

	1-Week Standing[2]					11-Week Standing		29-Week Standing	
	Aspergillus flavus		*A. parasiticus*	*A. ochraceus*	*Penicillium viridicatum*	*A. parasiticus*	*A. ochraceus*	*A. parasiticus*	*A. ochraceus*
Corn	NRRL 3251	NRRL 3357	NRRL 2999	NRRL 3174	NRRL 3712	NRRL 2999	NRRL 3174	NRRL 2999	NRRL 3174
	Aflatoxin B-1			Ochratoxin A		Aflatoxin B-1	Ochratoxin A	Aflatoxin B-1	Ochratoxin A
Untreated	89[3]	94	100	37	6.0	471	954	188	325
Treated	ND[4]	ND	ND	ND	ND	ND	ND	ND	ND
	Aflatoxin M-1	Aflatoxin G-1		Ochratoxin B		Aflatoxin G-1		Aflatoxin G-1	
Untreated	1.3	ND	77	1.5	0.4	201		143	
Treated	ND	ND	ND	ND	ND	ND		ND	

[1]150 g./Fernbach, 28°C., incubate 6 days, Gump shaker, 200 r.p.m. except NRRL 3712, 20°C., 12 days.
[2]Fernbach flasks closed with gauze and allowed to stand in an exhaust hood for 1, 11, and 29 weeks before inoculation.
[3]Geometric means of duplicate flasks.
[4]ND = not detected.

TABLE II. EFFECT OF 2% AMMONIA TREATMENT OF CORN[1] ON AFLATOXIN AND OCHRATOXIN PRODUCTION (γ/g.) 1 WEEK STANDING

Corn	*A. flavus* NRRL 3251	*A. flavus* NRRL 3357	*A. parasiticus* NRRL 2999	*A. ochraceus* NRRL 3174	*P. viridicatum* NRRL 3712
	Aflatoxin B-1			Ochratoxin A	
Untreated	98[2]	108	147	0.19	0.9
Treated	ND[3]	0.004	0.013	ND	ND
	Aflatoxin M-1	Aflatoxin G-1		Ochratoxin B	
Untreated	1.2	ND	89	ND	0.9
Treated	ND	ND	ND	ND	ND

[1]150 g./Fernbach, 28°C., 6 days, Gump shaker, 200 r.p.m., except NRRL 3712, 20°C., 12 days. Fernbach flasks were closed with gauze and kept in an exhaust hood.
[2]Geometric means of duplicate flasks.
[3]ND = not detected.

TABLE III. INFLUENCE OF 2% AMMONIA TREATMENT OF CORN[1] ON AFLATOXIN PRODUCTION BY *A. PARASITICUS* NRRL 2999 AND OCHRATOXIN PRODUCTION BY *A. OCHRACEUS* NRRL 3174 (γ/g.)

Standing Time[2] weeks	Aflatoxin B-1		Ochratoxin A	
	Untreated	Treated	Untreated	Treated
5	270[3]	0.018	6.8	ND[4]
7	232	0.032	8.5	ND
19	254	25	101	18

[1]150 g./Fernbach, 28°C., 6 days, Gump shaker, 200 r.p.m.,
[2]Fernbach flasks closed with gauze and allowed to stand in an exhaust hood for 5, 7, and 19 weeks before inoculation.
[3]Geometric means of duplicate flasks.
[4]ND = not detected.

Thin-Layer Chromatography

Thin-layer plates (20 × 20 cm.) were coated with Adsorbosil-1 to a thickness of 0.5 mm., air-dried 30 min., activated at 110°C. for 2 hr., and stored in a desiccating cabinet.

Extracts and standard aflatoxins or ochratoxins were spotted on thin-layer plates. The developing solvent for aflatoxins B-1 and G-1 was water:acetone:chloroform (1.5:12:88, v./v./v.); for aflatoxin M-1, 2-propanol:acetone:chloroform (5:10:85, v./v./v.) (10); and for ochratoxins A and B, glacial acetic acid:benzene (10:90, v./v.). Aflatoxins and ochratoxins were determined quantitatively using a Schoeffel SD3000 densitometer. The excitation and emission wavelengths were 362 and 435 nm. for aflatoxins and 310 and 470 nm. for ochratoxins.

RESULTS AND DISCUSSION

Chemical preservatives that completely inhibit mold growth obviously would prevent mycotoxin formation. However, if the preservative is only partly effective, or its effectiveness decreases with time, mold growth can occur, with possible formation of mycotoxins. From a practical viewpoint, direct measurements of mycotoxin formation provide a useful indication of preservative effectiveness. In our studies unnaturally heavy inocula were used to provide an extreme test of mold inhibition. The severity of the test was further increased by raising moisture level of the corn to near optimum value of about 36% before inoculation.

Propionic acid prevented mold growth in all cases, as indicated by the absence of aflatoxin and ochratoxin formation, as well as mold counts. Table I shows the effects of propionic acid treatment on aflatoxin and ochratoxin production on corn after standing 1, 11, and 29 weeks before inoculation. Kernels from the 29-week experiment were surface-sterilized and plated on agar. There was no evidence of mold growth from any of the treated kernels, but untreated kernels had luxuriant growth of *A. parasiticus* or *A. ochraceus*.

Ammonia treatment, however, was completely effective initially, but permitted some mold growth after storage. However, aflatoxin and ochratoxin production was drastically reduced for all of the five strains of fungi selected (Table II). Ochratoxins A and B were not produced on the ammonia-treated corn that stood 1 week. When the same treated corn was inoculated with NRRL 3251, no aflatoxin B-1 or M-1 was produced and when inoculated with NRRL 2999, no aflatoxin G-1 was formed. Aflatoxin B-1 production was severely reduced with the strains NRRL 3357 and NRRL 2999 (Table II).

Small amounts of aflatoxin were observed when ammoniated corn that stood 5 and 7 weeks was inoculated with NRRL 2999 and incubated (Table III). No ochratoxin was produced on the corn that stood 5 and 7 weeks. As corn stood longer (19 weeks), it became more vulnerable to mold invasion and subsequent toxin formation.

Kernels from each flask of the 19-week ammonia experiment were plated on agar. Untreated kernels had a great deal of mold growth. Ammonia-treated kernels had much less mold growth than untreated kernels. Three of five kernels that had been inoculated with *A. ochraceus* did not show mold growth; from a duplicate flask, there was mold growth on all five kernels but retardation of *A. ochraceus* outgrowth was obvious. Ochratoxin A production was 101 γ per g. on untreated and 18 γ per g. on the ammonia-treated corn that stood 19 weeks (sensitivity = 0.030 γ per g.). Untreated kernels inoculated with *A. parasiticus* had a great deal of mold growth. Outgrowth of *A. parasiticus* from the ammonia-treated kernels was greatly reduced. Aflatoxin B-1 production was reduced from 254 to 25 γ per g. with ammonia treatment (sensitivity = 0.005 γ per g.). After standing 19 weeks, ammonia loses some of its protective action and capacity to inhibit new infections by mold.

The chemical preservatives, ammonia and in particular propionic acid, should find practical application in preventing the formation of mycotoxins in stored corn.

Acknowledgments

We thank R. J. Bothast for assistance with the fermentations; D. I. Fennell for identification of the

microorganisms; and W. F. Kwolek (Biometrician, North Central Region, U.S. Department of Agriculture, stationed at the Northern Laboratory) for statistical analysis of the data.

Literature Cited

1. VANDEGRAFT, E. E., SHOTWELL, O. L., SMITH, M. L., and HESSELTINE, C. W. Mycotoxin production affected by insecticide treatment of wheat. Cereal Chem. 50: 264 (1973).
2. VANDEGRAFT, E. E., SHOTWELL, O. L., SMITH, M. L., and HESSELTINE, C. W. Mycotoxin formation affected by fumigation of wheat. Cereal Sci. Today 18: 412 (1973).
3. JONES, G. M. Preservation of high moisture corn with volatile fatty acids. Can. J. Anim. Sci. 50: 239 (1970).
4. BOTHAST, R. J., LANCASTER, E. B., and HESSELTINE, C. W. Ammonia kills spoilage molds on corn. J. Dairy Sci. 56: 241 (1973).
5. VAN WALBEEK, W., SCOTT, P. M., HARWIG, J., and LAWRENCE, J. W. *Penicillium viridicatum* Westling: A new source of ochratoxin A. Can. J. Microbiol. 15: 1281 (1969).
6. HESSELTINE, C.W., SHOTWELL, O.L., SMITH, M.L., ELLIS, J.J., VANDEGRAFT, E. E., and SHANNON, G.M. Production of various aflatoxins by strains of the *Aspergillus flavus* series. Proc. First U.S.-Jap. Conf. Toxic Micro-Organisms, 1968, p. 202. U.S. Dept. Interior (1970).
7. HESSELTINE, C. W., VANDEGRAFT, E. E., FENNELL, D. I., SMITH, M. L., and SHOTWELL, O. L. Aspergilli as ochratoxin producers. Mycologia 64: 539 (1972).
8. LEE, W. V. Quantitative determination of aflatoxin in groundnut products. Analyst 90: 305 (1965).
9. EPPLEY, R. M. Screening method for zearalenone, aflatoxin, and ochratoxin. J. Ass. Offic. Anal. Chem. 51: 74 (1968).
10. STUBBLEFIELD, R. D., SHOTWELL, O. L., and SHANNON, G. M. Aflatoxins M_1 and M_2: Thin-layer chromatography and physical and chemical properties. J. Ass. Offic. Anal. Chem. 55: 762 (1972).

[Received January 18, 1974. Accepted June 19, 1974]