Mycotoxin Production Affected by Insecticide Treatment of Wheat

E. E. VANDEGRAFT, O. L. SHOTWELL, M. L. SMITH, and C. W. HESSELTINE, Northern Regional Research Laboratory[1], Peoria, Illinois 61604

ABSTRACT

Insecticide treatment of wheat both increased and decreased aflatoxin and ochratoxin production depending on the fungal strains used—*Aspergillus flavus, A. parasiticus, A. ochraceus*, and *Penicillium viridicatum*. Some strains were affected the same by phosphine and carbon tetrachloride:carbon disulfide treatment while others were affected oppositely. In some experiments the effect of insecticide treatment was significant on wheat sterilized by autoclaving at about 25% moisture but not on unsterilized. Mycotoxin production by most molds is enhanced on wheat sterilized by autoclaving before inoculation.

The effects of insecticides on fungi in stored grain and on production of metabolites by these fungi have not been so thoroughly investigated as the effects of insecticides on soil fungi (1–6). During storage of agricultural commodities, conditions can become favorable for both growth of fungi and production of mycotoxins. The action of several chemicals, including fungicides and insecticides, on molds from the series *Aspergillus flavus* has been reported (7–10). Although aflatoxin synthesis by *A. flavus* in a synthetic medium was inhibited by dimethyl sulfoxide, the aflatoxin was not degraded (11). Chemicals used in foods or feed as preservatives or stabilizers were screened for either prevention or reduction of *A. flavus, A. parasiticus*, aflatoxin accumulation, or any combination of these in peanut pods. Results varied from complete inhibition of the fungi with no aflatoxin produced to aflatoxin found in amounts up to twice that of the control (12).

We studied two insecticides used to protect stored grain, namely, phosphine and carbon tetrachloride:carbon disulfide (CCl_4:CS_2) (80:20 w./w.). Specifically, we investigated their effects on aflatoxin production by strains of *A. flavus* and

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

A. parasiticus and ochratoxin production by *A. ochraceus* and *Penicillium viridicatum* growing on slightly cracked wheat at about 25% moisture content both sterilized by autoclaving and nonsterilized. Sterilized wheat was used to produce maximum yields of mycotoxins. Stored wheat would not be sterilized.

METHODS AND MATERIALS

Insecticide Treatment of Wheat

Hard red winter wheat (Parker variety) was slightly cracked; that is, the surface was slightly abraded, but kernels were left whole. This cracking was to allow the mold spores to infect the kernel readily. The wheat was cracked after insecticide treatment but before sterilization by autoclaving.

Wheat (25 lb.) was treated with 300 mg. phosphine for 48 hr. at 21° to 27° C., aerated 5 min., and then stored in a metal container at 0° C. CCl_4:CS_2 (80:20 w./w., 3.9 ml.) was used to treat another 25 lb. of wheat during a 72-hr. exposure at 21° to 27° C. and aerated before the grain was stored in a metal container at 0°.

Cultures

The four strains of *A. flavus*, NRRL 3251, NRRL 3353, NRRL 3357, and NRRL 3517; two of *A. parasiticus*, NRRL 2999 and NRRL 3145; one of *A. ochraceus*, NRRL 3174; and one of *P. viridicatum*, NRRL 3712, used in this study have been shown to produce mycotoxins in previous work (13,14,15).

Inoculum was prepared in tubes of potato-dextrose agar with spores of each organism incubated for 2 weeks at 25° C. From sporulated slants, a spore suspension was prepared by adding 10 ml. sterile tap water to each test tube, scraping spores from the slant with a sterile loop, and combining the spore suspension from eight tubes (16).

Fermentation

The slightly cracked wheat (150 g./2.8 liter Fernbach), wet with 45 ml. tap water, was inoculated with 8 ml. of the spore suspension. The 150 g. of insecticide-treated or untreated wheat was prepared with and without sterilization by autoclaving before inoculation. 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. on a Gump shaker (200 r.p.m.). At the time of harvest, the flasks were briefly steamed to destroy the fungi. Duplicate flasks were run for each set of conditions. Each mycotoxin value is the average of the assays of two fermentations. In two instances, the fermentations were done more than one time. The results for phosphine done four times with NRRL 2999 were in agreement and for CCl_4:CS_2 done two times with NRRL 3251 also were in agreement.

Assay Procedure

Aflatoxin was extracted by grinding and mixing the molded wheat (25 g.) in a Waring Blendor for 5 min. with water (250 ml.) followed by mixing the aqueous slurry with chloroform (250 ml.) for 5 more min. The water:chloroform slurry was centrifuged, filtered, and treated with anhydrous sodium sulfate (17). Ochratoxin was extracted from molded wheat (20 g.) by grinding and mixing

with acetonitrile:water (90:10 v./v., 200 ml.) and hexane (100 ml.) for 5 min. in a Waring Blendor. The slurry was filtered and extracted with hexane (15). Extracts of aflatoxin and ochratoxin were concentrated on a rotary evaporator to near dryness, transferred to a small vial, and then taken to dryness under nitrogen on a steam bath. For thin-layer chromatography, residues were made up to 10 ml. with chloroform for aflatoxin and glacial acetic acid:benzene (1:99 v./v.) for ochratoxin.

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.

Properly diluted extracts (3 μl.) were spotted in triplicate on thin-layer plates. Standard aflatoxins (3 μl.) (2.5 γ B-1 per ml., 2.0 γ G-1 per ml., and 2.0 γ M-1 per ml.) and ochratoxins (4 μl.) (10.00 γ A per ml. and 10.01 γ B per ml.) were spotted in triplicate. The development 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.) (18); and for ochratoxins A and B, glacial acetic acid:benzene (10:90 v./v.). Aflatoxins and ochratoxins were determined quantitatively by densitometry (Schoeffel SD3000). The excitation and emission wavelengths were 362 and 435 nm. for aflatoxins and 310 and 470 nm. for ochratoxins.

RESULTS

When wheat was treated with phosphine (Table I), a statistically significant increase occurred in yield of aflatoxin B-1 by NRRL 3251 and a significant decrease of B-1 and G-1 by NRRL 3145. Significant increases in yields of ochratoxins A and B by NRRL 3174 were also noted on treated wheat. Yields of mycotoxins were almost always higher on sterilized than on unsterilized wheat. Interactions of sterilization and insecticide treatment affecting mycotoxin production were noted with NRRL 3353 and NRRL 3357.

When wheat was treated with CCl_4:CS_2 (Table II), statistically significant increases occurred in yields of aflatoxins B-1 and G-1 by NRRL 2999, aflatoxin G-1 by NRRL 3145, and ochratoxin A by NRRL 3712; but the yield of ochratoxin B by NRRL 3174 decreased. Sterilization of wheat by autoclaving at about 25% moisture caused a significant increase in yield of all mycotoxins with the following exceptions: aflatoxins B-1 and G-1 by NRRL 3353 and ochratoxin A by NRRL 3174. The effect of insecticide treatment on mycotoxin production depended on sterilization in the following experiments: aflatoxins B-1 and G-1 by NRRL 3353, aflatoxin B-1 by NRRL 3357, and ochratoxin B by NRRL 3712. The interactions were significant between sterilization and insecticide treatment in these exceptions.

We studied aflatoxin M-1 production by NRRL 3251 on insecticide-treated and untreated wheat. One of the highest producing organisms of M-1 found in a survey of *A. flavus* is NRRL 3251 (14). Higher yields were obtained than in the survey and on the rice used in the original isolation (19). We mention this since a demand exists for M-1 for feeding studies and standards; however, means for producing large quantities of M-1 are not available. Yields of M-1 on insecticide-

TABLE I. EFFECT OF PHOSPHINE-TREATMENT OF WHEAT[a] ON AFLATOXIN AND OCHRATOXIN PRODUCTION (γ per g.)

Wheat	*Aspergillus flavus* NRRL 3251	*Aspergillus flavus* NRRL 3353	*Aspergillus flavus* NRRL 3357	*Aspergillus flavus* NRRL 3517	*A. parasiticus* NRRL 2999	*A. parasiticus* NRRL 3145	*A. ochraceus* NRRL 3174	*Penicillium viridicatum* NRRL 3712
	Aflatoxin B-1						Ochratoxin A	
Sterilized								
Untreated	2,850[b]	0.8	190	390	1,030	150	2,890	1,740
Treated	3,380*	5.3	260	430	910	120*	3,380*	1,680
Unsterilized								
Untreated	620	1.9	100	90	220	30	1,720	1,030
Treated	860*	0.3	70	70	170	20*	2,340*	1,360
	Aflatoxin G-1						Ochratoxin B	
Sterilized								
Untreated	ND[c]	0.6	ND	ND	540	230	80	540
Treated	ND	4.1	ND	ND	470	180*	120*	490
Unsterilized								
Untreated	ND	0.8	ND	ND	120	40	30	230
Treated	ND	0.1	ND	ND	100	20*	60*	280

[a]150 g./Fernbach, 28° C., 6 days, Gump shaker, 200 r.p.m., except NRRL 3712, 20° C., 12 days.

[b]Geometric means of duplicate flasks.

[c]ND = not detected.

TABLE II. EFFECT OF CARBON TETRACHLORIDE:CARBON DISULFIDE-TREATMENT OF WHEAT[a] ON AFLATOXIN AND OCHRATOXIN PRODUCTION (γ per g.)

Wheat	Aspergillus flavus				A. parasiticus		A. ochraceus	Penicillium viridicatum
	NRRL 3251	NRRL 3353	NRRL 3357	NRRL 3517	NRRL 2999	NRRL 3145	NRRL 3174	NRRL 3712
	Aflatoxin B-1						Ochratoxin A	
Sterilized								
Untreated	2,910[b]	0.5	230	550	510	90	3,060	510
Treated	3,050	0.5	490	680	680*	100	3,160	1,820*
Unsterilized								
Untreated	1,970	0.8	150	190	100	40	2,660	310
Treated	2,080	1.4	80	180	160*	30	2,820	690*
	Aflatoxin G-1						Ochratoxin B	
Sterilized								
Untreated	ND[c]	0.04	ND	ND	260	80	250	160
Treated	ND	0.06	ND	ND	310*	160*	140*	490
Unsterilized								
Untreated	ND	0.07	ND	ND	40	50	140	100
Treated	ND	0.27	ND	ND	70*	60*	100*	170

[a]150 g./Fernbach, 28° C., 6 days, Gump shaker, 200 r.p.m., except NRRL 3712, 20° C., 12 days.

[b]Geometric means of duplicate flasks.

[c]ND = not detected.

treated and untreated wheat were 40 to 60 γ per g. Insecticide treatment did not have a significant effect on M-1 production.

DISCUSSION

The statistically significant variations observed among insecticide-treated wheats show that treating sterilized and nonsterilized wheat with insecticides can increase or decrease formation of mycotoxins. The two insecticides we studied, phosphine and CCl_4:CS_2, appear to be strain specific.

On samples sterilized by autoclaving at about 25% moisture, more mycotoxin was produced than on the nonsterilized, both insecticide-treated and untreated, except for two. Sterilization may alter or destroy a natural inhibitor of metabolite formation in wheat. It may also cause physical and chemical changes in components of wheat that make nutrients more available for metabolite formation. The insecticide could affect either the inhibitor or the growth-producing enzymes of the mold necessary for mycotoxin production, or both. If the insecticide alters the inhibitor, more mycotoxin would be produced on insecticide-treated wheat, both sterilized and nonsterilized. Observations that support this supposition are on NRRL 3251 and NRRL 3174 with phosphine, and on NRRL 2999 and NRRL 3712 with $CC1_4$:CS_2. If the insecticide alters enzymes in the mold that produce growth, then less mycotoxin should be produced on insecticide-treated wheat than on untreated. Action of any insecticide on the growth inhibitor in wheat causing increased growth would be counteracted by its action on the enzymes of the mold. This effect was noted on NRRL 3145 with phosphine and on NRRL 3174 with CCl_4:CS_2.

Phosphine and CCl_4:CS_2 affect two of the mycotoxin-producing strains oppositely. Phosphine increased ochratoxin B production by strain NRRL 3174 and decreased aflatoxin G-1 production by NRRL 3145. $CC1_4$:CS_2, however, increased aflatoxin G-1 production by NRRL 3145 but decreased ochratoxin B production by NRRL 3174.

The effect of sterilizing wheat aside from any insecticide treatment is another aspect of our study. On all but two organisms (NRRL 3353 and NRRL 3174), the production of aflatoxin and ochratoxin was significantly reduced when nonsterilized wheat was used. Possibly despite heavy inoculation of nonsterile wheat with mycotoxin-producing strains, conditions remain favorable for the growth of certain natural-contaminating molds and bacteria. These compete with the toxin-producing molds, reduce their growth, and as a consequence of reduced mold growth, decrease mycotoxin production. A second possibility could be that the natural microbial flora of wheat either may produce substances which destroy mycotoxins as they are formed or contain inhibitors of mycotoxin synthesis. Natural microbial flora of wheat may also cause changes that affect availability of nutrients.

The relationship between mycotoxin production and insecticide treatment of wheat appears to be strain dependent, as well as insecticide dependent. While the effect of insecticide treatment on some strains is significant, they may not be of practical value except in instances where there were two- to threefold increases or decreases in mycotoxin production. Additional work is needed to explain the wide variation in effects of insecticide treatment of wheat on mycotoxin production. We are investigating other insecticides commonly used on stored grain for their effects on mycotoxin production.

Acknowledgments

We thank O. L. Brekke and A. C. Stringfellow for treating the wheat, and W. F. Kwolek (Biometrician, Biometrical Services, U.S. Department of Agriculture, stationed at the Northern Laboratory) for statistical analysis of the data.

Literature Cited

1. WENSLEY, R. N. Microbiological studies of the action of some selected soil fumigants. Can. J. Bot. 31: 277 (1953).
2. BOLLEN, W. B. Interactions between pesticides and soil microorganisms. Amer. Rev. Microbiol. 15: 69 (1961).
3. SMITH, N. R., and WENZEL, M. E. Soil microorganisms are affected by some of the new insecticides. Soil Sci. Soc. Amer. Proc. 12: 277 (1948).
4. MARTIN, J. P., HARDING, R. B., CANNEL, G. H., and ANDERSON, L. D. Influence of five annual field applications of organic insecticides on soil biological and physical properties. Soil Sci. 87: 334 (1959).
5. BOLLEN, W. B., MORRISON, H. E., and CROWELL, H. H. Effect of field and laboratory treatments with BHC and DDT on nitrogen transformations and soil respiration. J. Econ. Entomol. 47: 307 (1954).
6. RICHARDSON, L. T. Effects of atrazine on growth response of soil fungi. Can. J. Plant Sci. 50: 594 (1970).
7. LEE, W. H., and RIEMANN, H. Destruction of toxic fungi with low concentrations of methyl bromide. Appl. Microbiol. 20: 845 (1970).
8. WALES, P., and SOMERS, E. Susceptibility of aflatoxin-producing strains of *Aspergillus flavus* to a range of fungicides. Can. J. Plant Sci. 48: 377 (1968).
9. BARNES, G. L. Results of advanced bioassay of promising fungitoxic compounds for control of mold fungi on peanut pods. J. Amer. Peanut Res. Ed. Ass. 1: 75 (1969).
10. JACKSON, C. R. Laboratory evaluation of fungicides for control of some fungi found on peanuts. Plant Dis. Rep. 49: 928 (1965).
11. BEAN, G. A., KLARMAN, W. L., RAMBO, G. W., and SANFORD, J. B. Dimethyl sulfoxide inhibition of aflatoxin synthesis by *Aspergillus flavus*. Phytopathology 61: 380 (1971).
12. BELL, D. K., and DOUPNIK, B., Jr. Efficacy of chemicals for control of aflatoxins in peanut pods. J. Amer. Peanut Res. Ed. Ass. 2: 35 (1970).
13. 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).
14. HESSELTINE, C. W., SHOTWELL, O. L., SMITH, M., ELLIS, J. J., VANDEGRAFT, E., and SHANNON, G. 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).
15. HESSELTINE, C. W., VANDEGRAFT, E. E., FENNELL, D. I., SMITH, M. L., and SHOTWELL, O. L. Aspergilli as ochratoxin producers. Mycologia 64: 539 (1972).
16. STUBBLEFIELD, R. D., SHOTWELL, O. L., HESSELTINE, C. W., SMITH, M. L., and HALL, H. H. Production of aflatoxin on wheat and oats: Measurement with a recording densitometer. Appl. Microbiol. 15: 186 (1967).
17. LEE, W. V. Quantitative determination of aflatoxin in groundnut products. Analyst 90: 305 (1965).
18. 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).
19. STUBBLEFIELD, R. D., SHANNON, G. M., and SHOTWELL, O. L. Aflatoxins M_1 and M_2: Preparation and purification. J. Amer. Oil Chem. Soc. 47: 389 (1970).

[Received July 18, 1972. Accepted November 30, 1972]