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Effects of Trace Metals on the Production of Aflatoxins by *Aspergillus parasiticus*

PAUL B. MARSH,* MARION E. SIMPSON, AND MARY W. TRUCKSESS[1]

Nutrition Institute, Agricultural Research Service, Beltsville, Maryland 20705, and Woodard Research Corporation, Herndon, Virginia 22070

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Certain metals added as salts to a defined basal culture medium influenced the level of aflatoxin production by *Aspergillus parasiticus* in the low micrograms-per-milliliter range of the added metal. In many cases no change or a relatively small change in mat weight and final pH of the medium accompanied this effect. With zinc at added levels of 0 to 10 μg/ml in the medium, aflatoxin increased 30- to 1,000-fold with increasing of zinc, whereas mat weight increased less than threefold. At 25 μg of added zinc per ml, aflatoxin decreased, but mat weight did not. At an added level of 25 μg or less of the metal per ml, salts of iron, manganese, copper, cadmium, trivalent chromium, silver, and mercury partly or completely inhibited aflatoxin production, without influencing mat weight.

Cottonseeds, peanuts, corn, and other agricultural commodities important in the food and feed industries may become contaminated with aflatoxins, but the basic factors that influence aflatoxin production have not been completely studied (4). The toxin is a metabolite of *Aspergillus flavus* and of the very closely related species *A. parasiticus*.

Effects of growth media on aflatoxin production by *A. flavus* have been studied mostly in comparisons during culture on various seeds and other natural substrates (4). More limited work has involved defined media containing various sources of carbon and nitrogen (3).

Mateles and Adye (10) deleted molybdenum, boron, copper, iron, zinc and manganese, singly and in combination, from their growth medium and concluded that "... zinc is required for the production of aflatoxin. The slightly reduced yields obtained when iron or manganese were deleted were due to a visible reduction of growth, which was not observed when zinc was deleted." Omission of copper, molybdenum, and boron did not reduce aflatoxin production. Lee et al. (5) also reported that zinc was specifically required for production of aflatoxins by *A. flavus*. An approximate maximum in mat weight was obtained with 0.4 μg of zinc per ml, whereas aflatoxin increased from a low level at this concentration to a maximum at about 2.0 μg of zinc per ml.

Davis et al. (2) concluded, "The influence of minerals on aflatoxin synthesis may be indirect through their essentiality for growth or more directly on the process of toxin synthesis per se. The influence of zinc and iron appeared to be indirect, whereas that of molybdenum appeared direct." Zinc and iron, at 0.5 to 1 μg of the sulfate per ml, stimulated both aflatoxin production and growth, i.e., mat weight; they also inhibited aflatoxin production, but not growth, at 10 μg/ml. A minimum of 0.5 g of $MgSO_4$ per liter appeared essential for maximum production whereas a fifth of this amount was sufficient for maximum growth.

The purpose of this work was to determine in synthetic culture whether salts of certain metals at low parts-per-million levels could influence the production of aflatoxins by *A. parasiticus* and, if so, whether this effect would be accompanied by changes in the overall growth of the fungus as measured by mat weight or in the final pH of the growth medium.

MATERIALS AND METHODS

Organism. The isolate used was *A. parasiticus* NRRL 2999, from the USDA Northern Regional Research Laboratory, Peoria, Ill.

Media and culture methods. The medium for maintenance of the fungus in test tubes contained the major mineral salts listed immediately below plus: glucose, 5 g/liter; peptone, 1 g/liter; yeast extract, 0.25 g/liter; and agar, 20 g/liter. The major components of the basal experimental medium were: glycerol, 20 g/liter; NH_4NO_3, 1 g/liter; KH_2PO_4, 0.9 g/liter; K_2HPO_4, 0.7 g/liter; and $MgSO_4 \cdot 7\,H_2O$, 0.75 g/liter.

The trace element composition of the basal medium was as indicated in the tables (see Tables 1-4). To make stock solutions of the standard trace metal

[1] Present address: U.S. Food and Drug Administration, Washington, D.C. 20204.

salts, we dissolved $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4$, and $Ca(NO_3)_2 \cdot 4H_2O$ in water. Zinc glycerophosphate was used to avoid zinc precipitation. Thus, 90 mg of this salt was dissolved in a minimum amount of glacial acetic acid and water, the solution was adjusted to pH 5.2, and the volume was made up to 25 ml. One ml of the zinc glycerophosphate solution in 1 liter of basal medium provided a final concentration of 1 μg of zinc per ml. Cadmium, chromium, silver, cobalt, nickel, lead, and aluminum were added as nitrates, mercury as mercuric chloride, and ruthenium as the chloride. The complete salt medium was adjusted to pH 5.2 with glacial acetic acid. All water was passed through an ion-exchange column.

Thirty milliliters of medium was dispensed into each of several 250-ml flasks. The flasks, previously acid washed, were stoppered with cotton plugs and autoclaved 15 min at 15 lb. of steam pressure. After cooling, they were inoculated by needle with spores of the fungus and incubated at 30 C without shaking for time periods as specified in table headings (see Tables 1-4). The treatments were replicated six times in individual flasks, except that Cr^{3+} and Ag^{+} treatments were replicated five times.

Assays. After incubation, the final pH of the filtrate was measured, and the culture fluid was filtered through Whatman no. 1 paper. The mycelial mats were washed with 4 ml of distilled water, and the wash water was combined with the filtrate. The mycelial mats were dried for 3 days at 40 C and weighed. Aflatoxin analyses were performed on both filtrates and mats.

A 25-ml portion of the pooled filtrates was extracted with 20 ml of $CHCl_3$ for 30 min in a 50-ml glass-stoppered centrifuge tube. Then it was centrifuged for 10 min at 2,000 rpm, and the aqueous phase was removed. The $CHCl_3$ phase was passed through a column of anhydrous sodium sulfate, and the column was rinsed with an additional 20 ml of $CHCl_3$. The chloroform extracts were evaporated to dryness, and the residues were saved for thin-layer chromatography (TLC).

The combined mycelial mats from each treatment were placed into a blender cup and blended for 5 min at full speed with 50 ml of acetone-water (70:30). A 10-ml portion was transferred to a 50-ml glass-stoppered centrifuge tube, and 2 ml of lead acetate plus 14 ml of water was added to the tube. The precipitate that formed was sedimented by centrifugation for 10 min at 2,000 rpm. The supernatant was extracted with 20 ml of $CHCl_3$, phase separation was aided by centrifugation, and the aqueous phase was discarded. Then the $CHCl_3$ phase was extracted with 2 ml of 0.1 N NaOH, shaking for 1 min. The sodium hydroxide was removed after centrifugation, and the $CHCl_3$ phase was passed through a column of anhydrous sodium sulfate. The column was rinsed with 20 ml of $CHCl_3$, the combined $CHCl_3$ effluents were evaporated to dryness, and the residue was saved for TLC.

The residues were dissolved in 200 ml of $CHCl_3$, and aliquots were applied to silica gel-prepared TLC plates (Schleicher and Schuell no. 1500). The plates were developed in unlined, unequilibrated tanks with 150 ml of acetone-chloroform (1:9). Developing time was about 45 min for a solvent movement of 12 cm. Quantitation was by comparison with reference standards of aflatoxins on the same TLC plate. Aflatoxin production was calculated as follows:

$$\text{Nanograms per flask} = \frac{\text{Nanograms aflatoxin in medium (= total milliliters of medium} \times \text{ng/ml)}}{\text{number of replicates}} + \frac{\text{Nanograms aflatoxin in mats (= total wet weight in grams} \times \text{ng/ml}}{\text{number of replicates}}$$

A test showed that the trace elements used in the culture flasks did not interfere with aflatoxin analyses. To each of a series of test tubes, a 10-ml sample of control medium with 240 ng of aflatoxin B_1 per ml was transferred. Then each tube was spiked with a trace element at a concentration of 5 μg/ml. The trace elements used were iron, copper, zinc, manganese, cadmium, cobalt, lead, mercury, aluminum, and nickel. After extraction of the aflatoxin as described above, 10-μl amounts from each tube were spotted on TLC plates next to 10-μl aliquots of extract from the control medium with aflatoxin and no added trace elements. The plates were developed as described above. The intensities of the fluorescent spots from the control medium extract could not be distinguished from those from the spiked media extracts.

RESULTS

Table 1 shows results with progressive additions of zinc, manganese, iron, and copper salts to the basal medium with a constant level of each of the remaining elements tested.

At a zero-added level of zinc (Table 1), aflatoxin production was very low, mat weight was low, and final pH of the medium was low. At zinc levels of 1 to 25 μg/ml, aflatoxin production was much higher, with a moderate depression at 25 μg/ml, whereas both mat weight and final pH were higher and nearly constant. The fact that at the 1 μg/ml level of added zinc the aflatoxin production was increased to about 1,000 times that with no zinc whereas mat weight was increased only 2.5 times suggests, in accord with previous reports (5, 6, 10), that zinc has a stimulating influence on aflatoxin production apart from its essentiality for overall growth. The ratio of aflatoxins in the mat to aflatoxins in the medium is not recorded here, because it did not vary consistently in this or other experiments. Thus, we used only the mat-plus-medium summation figures in all tables. In general, aflatoxin $B_1 > G_1 > B_2 > G_2$ throughout all zinc concentrations, a relationship also seen in later experiments with most other trace elements.

With added manganese from 0 to 25 μg/ml (Table 1), aflatoxin production increased to its highest level at the 5 and 10 μg/ml levels and

TABLE 1. *Effect of addition of salts of zinc, manganese, iron, and copper to a basal medium on the production of aflatoxins, mat dry weight, and final pH of medium after 7-day incubation with A. parasiticus 2999*

Expt	Trace metals in basal medium[a]	Added trace metal	Levels of added trace metal (μg/ml)	Aflatoxins[b] (μg)					Mat wt (mg)	Final pH
				B_1	B_2	G_1	G_2	Total		
1A	Fe, Cu, Mn, Ca	Zn^{2+}	0.0	0.04	0.005	ND	ND	0.04	96	3.0
			1.0	46.7	0.38	7.6	0.003	54.7	236	7.0
			5.0	46.7	0.24	10.8	0.04	57.8	241	7.0
			10.0	37.1	0.24	33.6	0.10	71.0	248	7.0
			25.0	25.1	0.04	4.6	0.003	29.7	252	7.0
2	Fe, Cu, Zn, Ca	Mn^{2+}	0.0	2.1	0.03	0.21	0.003	2.3	249	6.5
			1.0	4.2	0.07	0.15	0.003	4.4	241	6.7
			5.0	13.1	0.27	0.41	0.005	13.8	244	6.7
			10.0	11.5	0.30	0.74	0.005	12.5	247	6.6
			25.0	7.4	0.20	0.35	0.003	8.0	251	6.6
3	Zn, Cu, Mn Ca	Fe^{2+}	0.0	19.3	1.7	2.9	0.02	23.9	167	4.5
			1.0	21.1	1.0	2.0	0.02	24.1	239	6.9
			5.0	12.2	0.36	1.6	0.02	14.2	237	6.9
			10.0	10.5	0.36	1.6	0.02	12.5	242	7.0
			25.0	1.9	0.03	0.2	0.003	2.1	241	7.1
4	Fe, Mn, Zn, Ca	Cu^{2+}	0.0	24.6	0.24	1.7	0.01	26.6	223	6.5
			1.0	13.8	0.11	0.85	0.01	14.8	224	6.6
			5.0	0.14	ND	ND	ND	0.14	215	6.7
			10.0	ND	ND	ND	ND	ND	210	6.7
			25.0	ND	ND	ND	ND	ND	219	6.8

[a] Ca at 10 μg/ml; all others at 1 μg/ml.
[b] Aflatoxin per flask; ND, not detected.

then decreased somewhat again at 25 μg/ml whereas mat weight and final pH were essentially constant throughout. Manganese present as an impurity in the basal medium was obviously adequate for growth.

With iron at a zero-added level (Table 1), aflatoxin production was relatively high, mat weight was somewhat lower than at higher iron concentrations, and the final pH was 4.5. With iron in the range of 1 to 25 μg/ml, aflatoxin production decreased progressively with increasing iron concentration while mat weight was constant around 240 mg and final pH was nearly constant around 7.0.

At all levels of added copper from 1 to 25 μg/ml (Table 1), a depression of aflatoxin production occurred, even though mat weights and the final pH were unaffected. Thus, zinc, manganese, iron, and copper all depressed aflatoxin production at 25 μg/ml or at some lower level, but copper depressed it most.

The aflatoxin-producing response of *A. parasiticus* to zinc (Table 1, experiment 1A) was verified in two similar experiments (Table 2, experiments 1B and 1C). In them, the zinc-aflatoxin relation was much like that in experiment 1A; the response of the fungus to successive increments of zinc in the levels of 0 to 10 μg/ml was much greater in aflatoxin production than in growth, and the optimum zinc concentration for aflatoxin production was higher than that for growth. In the three experiments, zinc caused 30- to 1,000-fold increases in aflatoxin levels with increases of less than threefold in growth. The decrease in level of aflatoxin between 7 and 14 days (Table 2), was in accord with the literature (1, 4).

An extra confirmatory experiment on effects of zinc was run with half-strength amounts of each of four major salts as the only deviation from experiment 1A (Table 1). In this experiment, the total aflatoxin produced in micrograms at 0, 1, 5, 10, and 25 μg of zinc per ml was 0.47, 30.5, 126.2, 155.6, and 23.5 with mat weights of 100, 221, 223, 211, and 208 mg, and final pH values of 3.1, 2.8, 3.0, 3.4, and 6.0, respectively. These results, although marked by low pH, continued to indicate that zinc stimulates aflatoxin production more than mat weight. That is, between 0 and 5 μg of zinc per

ml, the total aflatoxins increased about 268-fold, whereas mat weight only about doubled. As in the experiments reported above, the level of zinc for maximum aflatoxin production was higher than that for maximum growth. The maximum aflatoxin level was at 10 μg of zinc per ml.

Cadmium at all levels added from 1 to 25 μg/ml depressed production of aflatoxin greatly, but without influencing mat weight or final pH (Table 3). Thus, its effects were similar quantitatively to those of copper.

Trivalent chromium-depressed aflatoxin production at metal levels of 1 to 10 μg/ml, but was without major effect on mat weight or final pH (Table 3).

TABLE 2. *Effect of addition of zinc glycerophosphate to a basal medium on the production of aflatoxins, mat dry weight, and final pH of medium after 7- and 14-day incubation with A. parasiticus 2999*[a]

Expt	Level of added zinc (μg/ml)	7-Day incubation			14-Day incubation		
		Total aflatoxins (μg)	Mat wt (mg)	Final pH	Total aflatoxins (μg)	Mat wt (mg)	Final pH
1B	0	0.7	107	3.4	0.2	93	3.3
	1	80.8	254	6.2	62.8	237	5.7
	5	222.5	263	6.1	106.9	240	5.8
	10	246.7	270	6.2	83.3	246	6.0
	25	41.1	269	6.4	9.6	244	6.2
1C	0	4.7	149	7.5	10.1	151	7.3
	1	3.3	153	7.6	10.9	152	7.3
	5	57.8	170	7.7	47.6	154	7.4
	10	152.0	171	7.8	105.1	155	7.4
	25	47.7	165	7.6	98.6	159	7.4

[a] Conditions for these experiments were the same as those for experiment 1A, except that in experiment 1C the medium was supplemented with 1% asparagine.

TABLE 3. *Effect of addition of salts of cadmium, chromium, and silver to a basal medium on the production of aflatoxins, mat dry weight, and final pH of medium after 7-day incubation with A. parasiticus 2999*

Expt	Added trace metal[a]	Level of added trace metal (μg/ml)	Aflatoxins[b] (μg)				Mat wt (mg)	Final pH
			B_1	B_2	G_1	Total		
5	Cd^{2+}	0.0	26.5	0.25	0.83	27.6	249	6.5
		1.0	2.5	0.12	0.29	2.9	252	6.6
		5.0	0.03	0.009	0.02	0.06	2456	6.8
		10.0	0.01	0.01	0.01	0.03	2363	6.9
		25.0	ND	ND	ND	ND	248	6.7
6	Cr^{3+}	0.0	15.0	0.21	2.74	18.0	263	6.7
		0.5	18.5	0.36	3.08	21.9	265	6.6
		1.0	6.8	0.15	0.63	7.6	259	6.6
		3.0	4.1	0.10	0.62	4.8	260	6.6
		5.0	2.3	0.06	0.34	2.7	263	6.6
		10.0	4.1	0.10	4.8	9.0	255	6.6
7	Ag^{+}	0.0	12.4	0.15	2.7	15.2	269	6.6
		0.2	3.1	0.08	1.0	4.2	289	6.4
		0.4	6.3	0.14	1.9	8.3	278	6.6
		0.6	6.4	0.20	2.0	8.6	257	6.5
		0.8	4.1	0.09	1.2	5.4	236	6.5
		1.0	3.2	0.10	1.0	4.3	248	6.7
		5.0	ND	ND	ND	ND	31	5.5

[a] Fe, Cu, Zn, and Mn were all added at 1 μg/ml to the basal medium, and Ca was added at 10 μg/ml.
[b] Aflatoxin per flask; no aflatoxin G_2 was detected in any experiment; ND, not detected.

Silver up to 1 μg/ml depressed aflatoxin production without affecting mat weight or final pH (Table 3). At 5 μg/ml, it reduced aflatoxin to nondetectability and depressed mat weight greatly. Thus, at unusually low levels it effectively depressed aflatoxin production and growth.

The effects of various metals at 5 μg/ml on aflatoxin production are shown in Table 4. Mercury depressed aflatoxin level greatly without decreasing mat weight or lowering the final pH, while aluminum and ruthenium also depressed aflatoxin production somewhat. Nickel, cobalt, and lead had little or no effect on any of the three measured test results.

DISCUSSION

The results here described agree with the conclusion of previous workers that zinc stimulates aflatoxin production (2, 5, 6, 10). Also they agree with earlier findings that some trace metals at low concentrations may inhibit aflatoxin production (2). No certain explanation, however, can be offered for the mechanism of the effect shown here by the various metallic ions on the production of aflatoxins.

Four of the more potent metals in the inhibition of aflatoxin production in the present work, i.e., copper, cadmium, silver, and mercury, are also fungicidal at higher concentrations. Each of these four is known for its reaction with sulfhydryl groups, and such reactions have been suggested as a cause of their fungicial action (9). In data similar to those reported here for the aflatoxins, Takao (11) has reported inhibition of riboflavin synthesis by copper, mercury, and silver.

Aflatoxin contamination of the seeds at harvest appears to be very rare in the U.S. cotton crop and is localized especially in very limited areas. *A. flavus*, it might be reasoned by some individuals, is a ubiquitous fungus which infects cotton seeds very nearly universally before harvest but produces aflatoxins only in the presence of suitable amounts of trace metals. Actually, temperature seems to us to be a factor of dominant importance and mineral supply to the cotton plant of uncertain significance. Infection of the seeds at harvest with *A. flavus* is of very limited occurrence across the cotton belt and geographical localization of the boll rot seems most strongly associated with very high temperatures in the affected areas and with the fact that *A. flavus* is a fungus adapted to growth at high temperatures (7, 8).

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LITERATURE CITED

1. Arseculeratne, S. N., and C. H. S. R. Bandunatha. 1972. Variation of aflatoxin content of cultures of *Aspergillus flavus* with duration of incubation and its relation to studies on aflatoxin production. J. Appl. Bacteriol. **35**:43-52.
2. Davis, N. D., U. L. Diener, and V. P. Agnihotri. 1967. Production of aflatoxins B_1 and G_1 in chemically defined medium. Mycopathol. Mycol. Appl. **31**:251-256.
3. Detroy, R. W., E. B. Lillehoj, and A. Ciegler. 1971. Aflatoxin and related compounds, p. 3-178. *In* A. Ciegler, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 6. Academic Press Inc., New York.
4. Goldblatt, L. A. 1969. Aflatoxin: scientific background, control, and implications. Academic Press Inc., New York.
5. Lee, E. G. H., P. M. Townsley, and C. C. Walden. 1966. Effect of bivalent metals on the production of aflatoxins in submerged cultures. J. Food Sci. **31**:432-436.
6. Lillehoj, E. B., W. J. Garcia, and M. Lambrow. 1974.

TABLE 4. *Effect of addition of salts of several metals at 5 μg of metal per ml to a basal medium on the production of aflatoxins, mat dry weight, and final pH of medium after 7-day incubation with A. parasiticus 2999*

Expt	Added trace metal[a]	Aflatoxins[b] (μg)					Mat wt (mg)	Final pH
		B_1	B_2	G_1	G_2	Total		
8A	None	11.7	0.35	0.82	ND	12.9	289	6.6
	Ni	10.5	0.32	0.35	ND	11.2	272	6.8
	Co	9.2	0.25	0.70	ND	10.2	298	6.7
	Pb	7.0	0.32	0.59	ND	7.9	296	6.7
	Al	2.0	0.32	0.42	ND	2.7	281	6.7
	Hg	1.5	0.19	0.12	ND	1.8	284	6.7
8B	None	30.8	0.60	3.34	0.05	34.8	256	6.3
	Ru	13.1	0.22	2.42	0.02	15.8	256	6.5

[a] Fe, Cu, Zn, and Mn all present at 1 μg/ml in basal medium, also Ca at 10 μg/ml.
[b] Aflatoxin per flask; ND, not detected.

Aspergillus flavus infection and aflatoxin production in corn: influence of trace elements. Appl. Microbiol. **28**:763–767.

7. Marsh, P. B., M. E. Simpson, G. O. Craig, J. Donoso, and H. H. Ramey, Jr. 1973. Occurrence of aflatoxins in cotton seeds at harvest in relation to location of growth and field temperatures. J. Environ. Qual. **2**:276–281.
8. Marsh, P. B., M. E. Simpson, and G. V. Merola. 1974. Noninterference of *Pseudomonas* in the bright greenish-yellow (BGY) fiber-fluorescence test for *Aspergillus flavus* boll rot. Phytophathology **64**:326–328.
9. Martin, H. 1969. Inorganics, p. 101–117. *In* D. C. Torgeson (ed.), Fungicides: an advanced treatise, vol. 2. Academic Press Inc., New York.
10. Mateles, R. I., and J. C. Adye. 1965. Production of aflatoxins in submerged culture. Appl. Microbiol. **13**:208–211.
11. Takao, S. 1964. Riboflavin biosynthesis by *Candida robusta*. III. Effect of metal ion and necessity of $CaCO_3$. Agric. Biol. Chem. **28**:765–769.