

Conditions for Production of Ochratoxin A by *Aspergillus* Species in a Synthetic Medium¹

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Trace elements were required by *Aspergillus melleus* and *A. ochraceus*, but not by *A. sulphureus*, to grow and to elaborate ochratoxin A. The composition of the medium affected the synthesis of the toxin more than the growth of the mycelium.

Aspergillus ochraceus, *A. sulphureus*, and *A. melleus* are three species of the *A. ochraceus* group (4) thus far reported to produce ochratoxins (M. Lai et al., *Phytopathology* 58:1056, 1968). Because a synthetic medium is preferred over solid media for the isolation of toxins, the effect of the composition of the medium on ochratoxin A production, principally by *A. sulphureus*, was examined. Ferreira (3) and Davis et al. (2) made a comparable study with *A. ochraceus*.

Cultures used in the study (*A. ochraceus* NRRL 3174, *A. sulphureus* NRRL 4077, *A. melleus* NRRL 3519, and *A. melleus* NRRL 3520) were toxic to chicks and mice (G. Semeniuk et al., Proc. 1st U.S.-Japan. Conf. Toxic Microorganisms, *in press*) and produced ochratoxin A. Strain NRRL 3174 was the original ochratoxin producer (6); strain NRRL 4077 was a soil isolate from Mysore, India, received in 1953 from E. Yuill; NRRL 3519 (formerly A-2306) was received in 1948 from Juana Winitzky, Argentina; and NRRL 3520 (formerly A-13,653) was received in 1965 from Philip Mislivec (his number 108), Terre Haute, Ind. Synthetic medium contained (per liter of deionized water): 3.0 g of NH_4NO_3 , 50 g of glucose, 0.15 M phosphate or 0.1 M citrate buffer for pH control, 1.0 g of KCl, and 1.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; trace elements were 0.7 mg of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.5 mg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 10.0 mg of $\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$, 0.3 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.11 mg of $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$, and 17.6 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, as given by Ayde and Mateles (1). The medium was distributed in

50-ml amounts into 300-ml Erlenmeyer flasks, closed with cotton plugs, sterilized for 15 min at 121 C, inoculated with 1 ml of a heavy aqueous spore suspension from a 1-week-old culture grown on potato dextrose agar slants, and incubated in a stationary condition at 22 to 27 C in a room with diffuse light. At the end of the incubation period (8 days for *A. sulphureus*; 12 days for *A. ochraceus* and *A. melleus*), mycelial mats were separated from the medium by tared nylon-mesh discs on a Buchner funnel and dried to constant weight. Culture filtrates were collected and their content of ochratoxin A was determined in 10-ml samples. The samples were extracted three times in a separatory funnel with 5 ml of chloroform, and the chloroform extract was cleared of water and of denatured protein with anhydrous Na_2SO_4 . Graded amounts of a standard chloroform solution of ochratoxin A (supplied by I. F. H. Purchase, National Nutritional Research Institute, Pretoria, South Africa) were applied to chromatographic plates (layer of silica gel DS, 0.3 mm in thickness) and developed with toluene-ethyl acetate-formic acid (5:4:1, v/v). Amounts of ochratoxin A in spots were estimated from the standard under long ultraviolet light illumination (Black ray UVL 22, San Gabriel, Calif.). Formation of ochratoxin A by the four *Aspergillus* strains was confirmed in the chloroform extracts of culture filtrates, which yielded spots identical to that of authentic ochratoxin A developed in 10 different solvent mixtures. Ochratoxin B was produced in trace amounts; ochratoxin C was not produced.

In the synthetic medium, *A. sulphureus* NRRL 4077 produced maximum amounts of ochratoxin A within 8 to 10 days, and these amounts were reduced only slightly by incubating cultures for as long as 20 days. Within the range of pH 3.0 to 7.8, maximum toxin production was attained at pH 6.0 to 6.3 (Fig. 1). With different sugars (5% concentration, medium buffered at pH 7.0), most

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toxin was produced from glucose and sucrose (200 to 210 mg/liter) and decreasing amounts (mg/liter) from maltose (140), mannose (135), galactose (80), xylose (70), and arabinose (12). The fungus did not grow on lactose, and mycelial mats grown on glucose or galactose failed to produce ochratoxin when subsequently washed and incubated with lactose. With different salt concentrations (medium buffered at pH 6.3), maximum toxin production was obtained in the presence of 100 mg of K^+ per liter and 25 mg of P^{5+} per liter, whereas maximum mycelial yields were attained when their respective concentrations were increased three and four times. A 300-mg amount of $MgSO_4$ per liter was sufficient as the sole source of Mg^{2+} and S^{6-} for maximum toxin yield. For sulfur (26 mg of S/liter), SO_4^{2-} , SO_3^{2-} ,

$S_2O_4^{2-}$, methionine, and cystine were better sources than $S_2O_3^{2-}$, or S^{2-} .

Trace elements Fe^{3+} , Zn^{2+} , Cu^{2+} , B^{3+} , and Mo^{6+} were not needed for good growth and ochratoxin production by *A. sulphureus* (Table 1). They were required, however, in some combination with one another (probably with Zn^{2+}) by *A. ochraceus* NRRL 3174 and *A. melleus* NRRL 3519, whereas only Zn^{2+} was beneficial to *A. melleus* NRRL 3520. In other tests, Cu^{2+} in single combination with Zn^{2+} sharply reduced ochratoxin production by *A. melleus* NRRL 3520 to the level obtained when all elements were present together but mycelial yields were not reduced. Fifty- and 500- μg amounts of Zn^{2+} per liter were threshold quantities for maximal growth and ochratoxin production, respectively, by this fungus.

In agreement with Ferriera's observation (3) and with the data of Davis et al. (2), most of our results suggest that ochratoxin A production by *A. sulphureus* depends more on the nutritional conditions under which the mycelium grows than on the amount of mycelium formed. Our data indicate that small and large amounts of the toxin elaborated depended on the carbon and sulfur sources and on the near-optimal quantities of sulfur, magnesium, potassium, and phosphorus contained in the growth media. A similar dependence was suggested for *A. ochraceus* and for the two strains of *A. melleus* subjected to a restricted supply of trace elements in the medium. The dependence of yield on mycelium quantity with *A. sulphureus*, as shown in the pH control test (Fig. 1), in turn may relate to the composition of the medium.

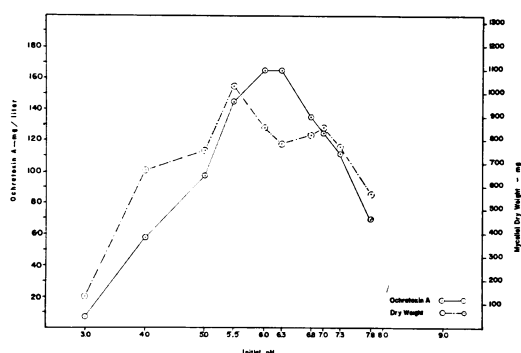


FIG. 1. Effect of pH on ochratoxin A production and growth of *Aspergillus sulphureus* NRRL 4077 in a synthetic medium for 8 days at 22 to 27 C. Medium was buffered with 0.15 M sodium phosphate (pH 5.0 to 7.8), or with 0.1 M citric acid-sodium citrate (pH 3.0 and 4.0) plus 1.5 g of Na_2HPO_4 per liter.

TABLE 1. Effect of trace elements on the yield of mycelium and of ochratoxin A from four aspergilli in stationary culture on a synthetic medium^a

Element ^b (mg/liter)	<i>Aspergillus ochraceus</i> (NRRL 3174)		<i>Aspergillus sulphureus</i> (NRRL 4077)		<i>Aspergillus melleus</i> (NRRL 3520)		<i>Aspergillus melleus</i> (NRRL 3529)	
	Mycelial weight (mg)	Ochratoxin A (mg/liter)	Mycelial weight (mg)	Ochratoxin A (mg/liter)	Mycelial weight (mg)	Ochratoxin A (mg/liter)	Mycelial weight (mg)	Ochratoxin A (mg/liter)
0.08 Cu	667	0	187	101	710	3	487	0
0.08 B	817	0	274	111	523	2	387	0
2.20 Fe	772	0	237	98	490	2	311	0
0.02 Mn	529	0	205	105	424	1	362	0
0.27 Mo	746	0	238	111	472	1	334	0
3.94 Zn	759	Trace	219	102	913	60	683	29
None	774	0	221	105	497	1	539	0
All elements	1,006	98	197	109	654	11	1,021	131

^a Determinations were made on the 8th day (*A. sulphureus*) and on the 12th day (other aspergilli).

^b Sources were $CuCl_2 \cdot 2H_2O$, $Na_2B_4O_7 \cdot 10H_2O$, $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $(NH_4)_6 Mo_7 O_{24} \cdot 4H_2O$, and $Zn(NO_3)_2 \cdot 6H_2O$. Sulfur was supplied as 1.0 g of $MgSO_4 \cdot 7H_2O$ /liter.

LITERATURE CITED

1. Ayde, J., and R. I. Mateles. 1965. Incorporation of labelled compounds into aflatoxin. *Biochem. Biophys. Acta* 86: 418-420.
2. Davis, N. D., J. W. Searcy, and U. L. Diener. 1969. Production of ochratoxin A by *Aspergillus ochraceus* in a semisynthetic medium. *Appl. Microbiol.* 17:742-744.
3. Ferreira, N. P. 1967. Recent advances in research on ochratoxin. Part 2. Microbiological aspects, p. 157-168. In R. I. Mateles and G. N. Wogan (ed.), *Biochemistry of some food-borne microbial toxins*. M.I.T. Press, Cambridge, Mass.
4. Raper, K. B., and D. I. Fennell. 1965. The genus *Aspergillus*. The Williams & Wilkins Co., Baltimore.
5. van der Merwe, K. J., P. S. Steyn, and L. Fourie. 1965. Mycotoxins. Part II. The constitution of ochratoxin A, B, C, metabolites of *Aspergillus ochraceus* Wilh. *J. Chem. Soc.*, p. 7083-7088.