EFFECT OF TEMPERATURE ON PRODUCTION OF AFLATOXIN ON RICE BY ASPERGILLUS FLAVUS

by

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(with 1 fig.)

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INTRODUCTION

During the past 5 years, production of toxic metabolites by fungi has become widely recognized as a potentially serious problem. A group of compounds produced mainly by species of *Aspergillus*, and referred to as aflatoxins, have received special attention initially because of the socalled Turkey "X" disease which was traced to these compounds (for a review see SPENSLEY, 1963).

Aspergillus flavus LINK ex FRIES has long been known to be one of the most abundant of the Aspergilli, being cosmopolitan in distribution and occurring on a wide variety of substrates (RAPER & FENNELL, 1965). Although not in such abundance in the atmosphere as such other genera as *Cladosporium, Alternaria, Fusarium* and *Penicillium*, spores of *Aspergillus* species constitute a considerable part of the airborne fungus biota (KRAMER et al., 1963). According to SEMENIUK (1954), *A. flavus* has been found on various cereal grains and grain products and is common on corn. Its cardinal growth temperatures have been reported by PANASSENKO (1941).

Our study was undertaken to determine the effect of temperature on aflatoxin production by *A. flavus* when grown on a cereal grain substrate. Previous work at the Northern Laboratory showed that rice is an excellent substrate for aflatoxin production (HESSELTINE et al., 1966; SHOTWELL et al., 1966). RABIE (1965) studied the influence of temperature on aflatoxin production in a semisynthetic medium, but he presented no quantitative data.

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Materials and Methods

Culture and inoculum

Aspergillus flavus NRRL 2999 was used throughout these experiments. Inoculum was grown on potato dextrose agar (Haynes et al., 1955) and incubated at 28° C for at least 7 days before use. Cultures older than 21 days were not used. Spore suspensions were prepared by adding 3 ml of 0.005 % Triton X—100[2]) per slant, gently scraping the surface of the agar with a sterile loop and then thoroughly shaking the slant. Several such suspensions were pooled to make a common one for any given series of flasks. The detergent aided in wetting the spores.

Fermentation

The substrate for these experiments was polished long-grain rice (Sunnyfield Brand) used at the rate of 50 g per 300 ml Erlenmeyer flask. The rice was allowed to stand 2 hours with 25 ml tap water and autoclaved for 15 minutes at 15 psi. After cooling, the rice was inoculated with 0.5 ml of spore suspensions per flask. The flasks, except for the controls, were incubated in a New Brunswick Psychrotherm shaker-incubator for various periods of time at temperatures ranging from 8—37° C. The shaker has a 2-inch orbit and was operated at 188 rpm. Sterile tap water (5 ml per flask) was added at 48 hours. An equal amount of water was added to a flask at 24 hours only if the substrate appeared excessively dry. Each flask was vigorously shaken by hand daily to prevent clumping. After incubation, the flasks were autoclaved for 1 to 2 minutes at 15 psi and stored in a Deepfreeze until assayed for aflatoxin content.

Assay procedure

Aflatoxins were extracted by mixing the fermented substrate (50 g) with methanol : water (55:45 v/v) (250 ml) in a Waring Blendor 2 minutes (Nesheim et al., 1964). The resulting slurry was centrifuged 15 minutes at 5,000 rpm and filtered. The residue was washed with 50 ml methanol : water (55:45 v/v) and the filtrate plus wash was extracted with 100 ml hexane in a separatory funnel to remove lipids. The hexane layer was washed once with the methanol : water mixture (50 ml). The methanol layer and wash were combined and concentrated *in vacuo* to remove methanol. The concentrated aqueous solution was extracted once with 75 ml of chloroform and twice with 50-ml portions of chloroform. Combined extracts were concentrated almost to dryness *in vacuo* and transferred quantitatively with chloroform to 10-ml volumetric flasks in which the volume was made up to 10 ml with chloroform for thin-

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layer chromatography. Extracts were placed in tightly sealed test tubes (11 × 125 mm) that were covered with aluminum foil and stored in a Deepfreeze.

Thin-layer chromatoplates were run by the method of PONS & GOLDBLATT (1965). Plates were coated with silica gel G-HR (Brinkmann Instruments, Inc., Westbury, New York) to a thickness of 0.25 mm, developed with 3 % methanol in chloroform (v/v), and inspected under ultraviolet light (366 mμ) in a Chromato-Viewer (Ultra-Violet Products, Inc., San Gabriel, California). The spots corresponding to the four aflatoxins were then compared with those obtained with standard solutions for quantitative estimation of aflatoxin content.

Aflatoxin content of spores

Since aflatoxins are insoluble in hexane, this liquid was used to remove spores from the potato dextrose agar slants. Hexane spore suspensions were concentrated by centrifugation and evaporated to dryness on a steam bath. The centrifuge tubes used were stored in a desiccator over anhydrous $CaCl_2$ for a minimum of 16 hours before weighing to determine the weight of the spore pellet.

Aflatoxins were extracted from the spores with two separate portions of 70 % aqueous acetone. The acetone extracts were placed in a separatory funnel and extracted with two separate portions of chloroform. The chloroform extracts were subsequently drawn off through a bed of anhydrous sodium sulfate, evaporated to dryness over steam, transferred quantitatively to 10 ml chloroform, and spotted on thin-layer chromatoplates as described above.

RESULTS AND DISCUSSION

Aflatoxins were detected in all flasks including the control flasks which were inoculated but not incubated. The control flasks contained less than 14 ppb of aflatoxin B_1. Evidently some aflatoxin was carried over with the inoculum. HESSELTINE et al. (1966) reported that spores, as well as mycelium, contain aflatoxin. Although aflatoxins were found in all of 10 spore preparations tested, results varied and only small amounts were detected (B_1 and B_2). Therefore, aflatoxin content within the spores does not account for the carry-over observed. However, hyphal fragments, as well as spores, are present in inoculum preparations. Spore suspensions, prepared with hexane to determine aflatoxin content of spores, were examined microscopically for hyphal fragments. None were observed in any of the samples examined.

Aflatoxin production at various incubation times and temperatures is summarized in Table I. PANASSENKO (1941) reported that the minimum and maximum temperatures for growth of *A. flavus* are 6—8° and 44—46° C, respectively. The temperature range within which aflatoxin is produced, however, is more restricted.

TABLE I

Effect of temperature on aflatoxin production on rice by Aspergillus flavus NRRL 2999)*

Temperature °C	Time (days)	Aflatoxin						
		B_1	B_2	G_1	G_2	Ratio (B_1=1.00)		
		μg/g				B_2	G_1	G_2
Control**)		< 0.01	ND***)	< 0.01	ND			
8	21	< 0.01	ND	< 0.01	ND			
11	14	< 0.01	ND	< 0.01	ND			
11	21	0.1	< 0.01	0.09	< 0.01	<0.10	0.90	<0.10
15	8	0.3	0.02	0.06	< 0.01	0.07	0.20	<0.03
15	14	0.9	0.1	1	< 0.1	0.11	1.11	<0.11
15	21	2	0.2	3	0.3	0.10	1.50	0.15
18	6	34	4	37	3	0.12	1.09	0.09
18	9	124	19	160	12	0.15	1.29	0.10
25	4	304	53	198	25	0.17	0.65	0.08
25	5	507	80	256	31	0.16	0.50	0.06
25	7	449	85	235	32	0.19	0.52	0.07
28	2	184	20	64	10	0.11	0.35	0.05
28	4	760	167	458	56	0.22	0.60	0.07
28	7	760	111	180	25	0.15	0.24	0.03
32	3	633	166	71	11	0.26	0.11	0.02
32	5	760	133	64	10	0.18	0.08	0.01
32	7	760	125	46	5	0.16	0.06	0.01
34	3	29	0.8	0.6	< 0.01	0.03	0.02	<0.01
34	5	36	3	0.2	< 0.01	0.08	<0.01	<0.01
34	7	18	2	< 0.2	< 0.01	0.11	<0.01	<0.01
37	3	0.7	ND	0.01	ND			
37	5	0.4	ND	ND	ND			
37	7	0.3	ND	ND	ND			

*) Incubated on a New Brunswick rotary shaker, 188 rpm.
**) Inoculated but not incubated.
***) Not detected.

Although there was little change in appearance of the substrate at 8° and 11° C, growth at 34° and 37° C appeared normal. Characteristically, chloroform extracts of this strain on rice contain a deep yellow pigment, which is indicative of heavy growth. Extracts from cultures grown at 37° C exhibited typical pigmentation. The optimum temperature range for aflatoxin formation on rice under these conditions is 28—32° C for aflatoxin B and 28° C for aflatoxin G (Fig. 1). Considerably less aflatoxin was formed at 34° C, and very little was detected at 37° C.

Although the levels encountered were low, production of aflatoxin at 11° C (51.8° F) indicates the potential danger of aflatoxin formation in agricultural commodities during storage or in the field before harvest. At 15° C (59° F), considerably greater quantities of aflatoxin were detected. Such commodities as wheat, corn and sorghum, which have already been shown to support aflatoxin pro-

duction (HESSELTINE et al., 1966), might conceivably contain significant quantities of aflatoxin if invaded by suitable strains of *A. flavus* under favorable conditions of moisture and temperature. As the temperature was increased above 11° C, greater yields were obtained and less time was required for aflatoxin formation. Accord-

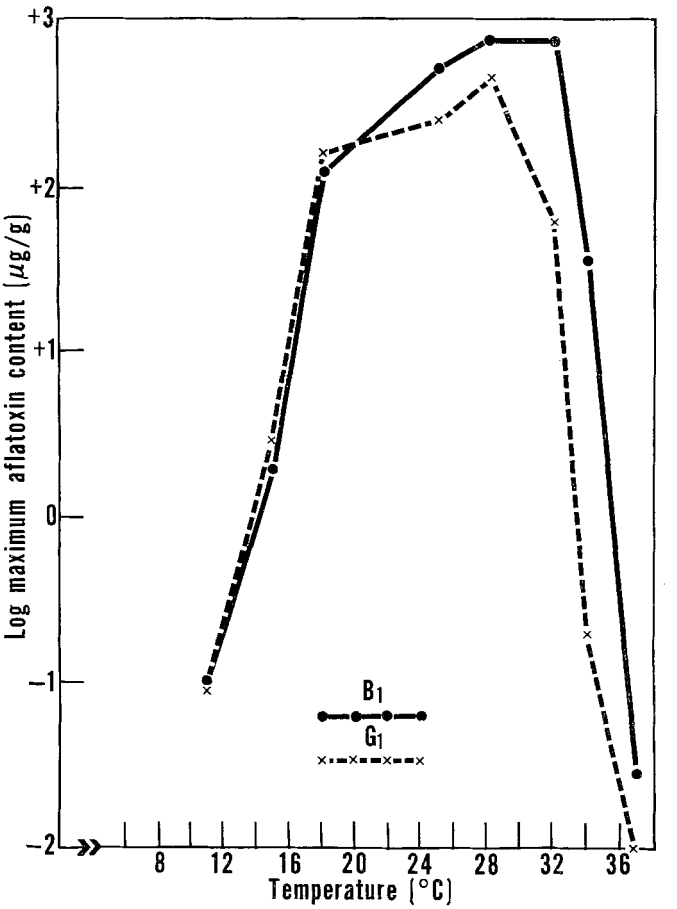

Fig. 1. Effect of incubation temperature on formation of aflatoxins B_1 and G_1.

ing to the classification scheme of COOMES et al. (1965) for aflatoxin B_1, levels reported in our paper at 11° C (21 days) would be classified as medium; those at 15° C (7 and 14 days) and 37° C would be high; and those at 15° C (21 days) and all temperatures from 18° to 34° C as high or very high. In their scheme, any sample containing less than 0.05 μg/g of aflatoxin B_1 is classified as low or zero aflatoxin B_1 levels.

Noteworthy is the shift in relative amounts of the four components as temperature was changed. Aflatoxins B_1 and G_1 were produced in essentially equal amounts at the lower temperatures although B_1 accumulated sooner than G_1. As the temperature was increased, there was a shift in relative content of the two major components until, at 28°, 32° and 34° C, aflatoxin B_1 represented 70—90 % of the total product. Indeed, although relatively little aflatoxin was formed at 37° C, B_1 constituted more than 98 % of the total product.

There were also changes in relative content with time of incubation at any given temperature. For example, although B_1 and G_1 continued to accumulate at 15° C, after 8 days there was nearly five times as much B_1 as G_1. At 14 days, there was more G_1 than B_1 and by 21 days there was twice as much G_1 as B_1. At 28° C and above, G_1 decreased with incubation time. On the other hand, B_1 generally increased up to 5 days and then leveled off (28° and 32° C), decreased after 5 days (34° C), or continuously decreased after 3 days (37° C). Possibly degradation enzymes not active at lower temperatures are activated at the higher temperatures. If this supposition is true, aflatoxin G_1 is particularly sensitive to their action above 32° C.

In the analytical procedure fluorescing zones were compared with known quantities of standard aflatoxins spotted on the same plate. The assay is accurate within 20 %. For this reason, absolute numerical values must be compared with some caution. However, there is unquestionably a great difference in yield as temperature is varied.

Summary

The effect of temperature on formation of aflatoxin on solid substrate (rice) by *Aspergillus flavus* NRRL 2999 has been studied in some detail. The optimum temperature for production of both aflatoxin B_1 and G_1 under the conditions employed is 28° C. Comparable yields of B_1 were obtained at 32° C, but considerably less G_1 was produced at this temperature. Both B_1 and G_1 were found in lesser amounts at temperatures above 32° C, and the aflatoxin content of rice incubated at 37° C was low (300—700 ppb) even though growth was good.

Reducing the temperature from 28° to 15° C resulted in progressively less aflatoxin, but 100 ppb of B_1 was detected in cultures incubated 3 weeks at 11° C. No aflatoxin was produced at 8° C.

The ratio of the four aflatoxins is affected by temperature. At the lower temperatures, essentially equal amounts of aflatoxin B_1 and G_1 were produced, whereas at 28° C, approximately four times as much B_1 was detected as G_1. At the higher temperatures, relatively less G was formed, until at 37° C, less than 10 ppb was detected.

Acknowledgement

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