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Effect of Temperature on Aflatoxin Production in Barley by *Aspergillus parasiticus*

Hak-Gil Chang and Pericles Markakis*
Wheat and Barley Research Institute, Office of Rural Development, Suweon 170
**Departmeut of Food Science and Human Nutrition, Michigan State University,*
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*Aspergillus parasiticus*에 依한 보리의 아플라톡신 生成에 對한 温度의 影響

張 鶴吉 · 피 마카키스*
農村振興廳 麥類研究所 · *미쉬간州立大學校 食品營養學科
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Abstract

The influence of temperature and moisture on aflatoxin production on solid substrate(barley) by *Aspergillus parasiticus* NRRL 2999 has been studied in some detail.

The optimum temperature for production of aflatoxin under the conditions employed is 25 and 30°C. No aflatoxin was detected at the moisture levels of 13%, and only traces at 16% moisture. The ratio of the production of aflatoxin B to G varied with temperature and moisture level. Aflatoxin G is elabolated at a more rapid rate than B and also metabolized at a more rapid rate. Also lower temperatures favored the production of aflatoxin G. The intensity of the yellow pigment of the chloroform extracts correlated with the concentration of aflatoxin.

Introduction

The discovery that aflatoxin is a potent hepatotoxin produced by *Asp. flavus*[1] has led to intensive studies on the presence of mycotoxins in foodstuffs [2~4]. Because this ubiquitous fungus is capable of growing over wide temperature and moisture ranges, many food materials are susceptible to aflatoxin contamination.

It is widely recognized that the most important factor in growth and aflatoxin production by *Asp. flavus* is environmental factors such as temperature, relative humidity and moisture content of the substrate[5~7]. Significantly accumulations may occur in peanuts grown in subtropical and tropical areas, where high temperatures and relative humidities favor rapid fungal growth[8,9]. Diener and Davis[10] also indicated that high carbohydrate substrates such as wheat and rice generally support larger yields of aflatoxin than oilseeds.

Since barley is harvested and frequently stored at a higher moisture content and temperature, it is important to know the moisture content and temperature limits that permit invasion of barley kernels by mycotoxin producing fungus. However, there is still relatively little information with aflatoxin production which is caused invasicn of barley by *Asp. parasiticus*.

In this paper, we report on the influence of moi-

sture content and temperature on aflatoxin production and accumulation by *Asp. parasiticus* NRRL 2999.

Materials and Methods

Materials *Aspergillus parasiticus* NRRL 2999 obtained from the culture collection of the USDA Northern Regional Research Laboratory, Peoria, ILL., was used in this study. Barley was a Coho spring barley which received from the Department of Soil and Crop Science, Michigan State University.

Spore suspension The stock culture was maintained on potato dextrose agar (Difco) and stored at 4°C. Spores of the stock culture was transferred to fresh potato dextrose agar and incubated at 25°C for 9 days before use. The spores were rinsed from the agar surface with sterile demineralized water containing 0.01% Tween 80 as a wetting agent. This conidial suspension was filtered through sterilized cheese cloth to remove mycelia debris. The filtered suspension was centrifuged and washed three times by centrifugation with sterile solution of 0.01% Tween 80. Finally the spore pellet was dispersed and diluted to the desired concentration (1×10^6 conidia per *ml*) in the Tween solution. Microscopic examination confirmed the absence of spore clumps or mycelia fragments.

Preparation of barley sample Kernels were surface-sterilized by washing for 2 minutes in a 1% sodium hypochloride solution, rinsed twice in sterile demineralized water and dried. Fifty grams of sterilized barley were placed in 4 oz square bottles, and moisture content were adjusted to 13, 16, 20 and 25% by adding sterile water and 1 *ml* of spores suspension. The bottles of barley were kept 3 days at 0°C and shaking occasionally to permit uniform distribution of the water. After 3 days, the bottles of grain were stored in desiccators at 15, 20, 25, 30 and 35°C for 10 to 30 days, on the perforated plate of desiccator containing saturated solution which maintained a relative humidity in equilibrium with each moisture content used. Saturated solution of sodium dichromate dihydrate, ammonium sulfate, ammonium dihydrogen phosphate and water were used for relative humidities of 53.3, 80.0, 92.5 and 100%, respectively[11~13]. The mouths of bottles were left loosely closed to allow free exchange of air.

That these solution actually did maintain the relative humidities they were supposed to is indicated by the fact that in all cases the moisture contents of the samples when tested during storage agreed, within the range of experimental error (plus or minus 0.3%). Moisture content were determined by the two-stage air oven method specified by the AACC approved methods[14].

Determination of aflatoxin Assays for aflatoxin were made on each substrate at the time of incubation was terminated. Molded barley samples (50 *g*) were grounded and extracted with 25 *ml* of water, 25 *g* of diatomaceous earth and 250 *ml* of chloroform on a reciprocate shaker for 30 min to determine the aflatoxin using the AOAC method[15].

Aflatoxin was determined quantitatively in each extract by spotting samples onto thin-layer glass plates (precoated Silica Gel-HR 25; Brinkman Ins., Inc.). The plates were developed with ether-methanol-water (96 : 3 : 1, v/v) at 23 to 25°C until aflatoxins reach Rf 0.3 to 0.7. Amounts of aflatoxin present were determined by densitometric analysis. A double beam scanning-recording-integrating spectrodensitometer SD 3000-4 (Schoeffel Ins.) and Density computer (Schoeffel SDC 300) was used for quantifying the TLC plates.

To confirm the presence of aflatoxin, standard and extracts were spotted and developed with the upper phase of a benzene-ethyl alcohol-water(46 : 35 : 19, v/v) solvent system[16]. The identify of aflatoxin B_1 and G_1 were also confirmed in representative samples by two-dimensional chromatography[17,18]. Extracts were applied to TLC plates and trifluoroacetic acids (2 μl) was superimposed on spots. After drying and cooling, TLC plates were developed with the water-acetone-chloroform solvent system. Aflatoxin reference standards (Aldrich Chemical Co.) were prepared according to the AOAC method and contained 0.515 $\mu g/ml$ of B_1, 0.197 $\mu g/ml$ of B_2, 0.538 $\mu g/ml$ of G_1 and 0.197 $\mu g/ml$ of G_2. All standards were stored at −18°C in 4-dram glass teflon-lined screw-cap vials.

Results and Discussion

The aflatoxin concentration of barley at low moisture levels is shown in Table 1. No aflatoxin was found in barley at 13% moisture content at any temperature and incubation period. Little aflatoxin was found in barley held at 16% moisture content. The moisture content of 16% in the barley grains was a critical factors for promoting toxin production.

It is apparent that below 13% moisture level in the grain, the fungus could not grow and hence biogenesis of aflatoxin was not detectable even in the delibrately infected barley.

The importance of humidity (or moisture) factors

Table 1. Effect of low level moisture on aflatoxin production in barley by *Asp. parasiticus* NRRL 2999

Moisture content (%)	Temperature (°C)	Time (Days)	Aflatoxin						
			B_1	B_2	G_1	G_2	Ratio (B_1=1.00)		
			($\mu g/kg$)				B_1	G_1	G_2
13	15~35	10~30	ND*	ND	ND	ND			
16	15~20	10~30	ND	ND	ND	ND			
16	25	10~20	ND	ND	ND	ND			
16	25	30	3.12	—	2.49	—	—	0.80	—
16	30	10	1.27	—	0.67	—	—	0.54	—
16	30	20	5.30	—	12.42	0.76	—	2.34	0.14
16	30	30	7.21	0.81	12.43	1.20	0.11	1.72	0.17
16	35	10	1.35	—	1.81	—	—	1.34	—
16	35	20	2.21	0.23	3.27	0.54	0.10	1.48	0.24
16	35	30	4.35	0.56	5.91	0.91	0.13	1.36	0.21

*Not detected

Data obtained from the average of 3 replications.

Table 2. Aflatoxin production by *Asp. parasiticus* NRRL 2999 on barley at 20% moisture level

Temperature (°C)	Time (days)	Aflatoxin						
		B_1	B_2	G_1	G_2	Ratio (B_1=1.00)		
		($\mu g/kg$)				B_1	G_1	G_2
15	10	ND*	ND	ND	ND			
15	20	0.58	—	0.81	—	—	1.40	—
15	30	1.75	—	2.92	0.61	—	1.67	0.35
20	10	ND	ND	ND	ND			
20	20	1.67	0.30	2.20	0.19	0.18	1.32	0.11
20	30	30.78	6.03	89.61	21.36	0.20	2.91	0.69
25	10	0.47	—	1.42	—	—	3.02	—
25	20	7.73	0.83	30.88	4.03	0.11	4.35	0.52
25	30	163.58	19.26	368.28	52.92	0.12	2.25	0.32
30	10	23.59	3.09	65.14	8.27	0.13	2.76	0.35
30	20	258.83	49.05	349.80	56.41	0.19	1.35	0.22
30	30	637.84	140.96	777.37	127.29	0.22	1.22	0.20
35	10	25.32	3.22	14.09	2.74	0.13	0.56	0.11
35	30	129.90	25.70	66.02	13.22	0.20	0.51	0.10
35	30	306.56	72.56	145.29	25.56	0.24	0.47	0.08

*Not detected

Data obtained from the average of 3 replications

Table 3. Aflatoxin production by *Asp. parasiticus* NRRL 2999 on barley at 25% moisture level

Temperature (°C)	Time (days)	Aflatoxin						
		B_1	B_2	G_1	G_2	Ratio (B_1=1.00)		
		($\mu g/kg$)				B_2	G_1	G_2
15	10	1.81	0.21	2.63	0.19	0.12	1.45	0.11
15	20	1.70	0.39	5.50	0.65	0.14	2.04	0.24
15	30	12.22	1.84	25.65	2.60	0.15	2.10	0.30
20	10	2.01	0.32	3.99	0.42	0.16	1.99	0.21
20	20	13.43	2.69	18.33	3.37	0.10	1.37	0.25
20	30	226.10	85.63	1007.34	99.94	0.38	4.46	0.44
25	10	167.57	19.75	655.09	89.59	0.12	3.91	0.54
25	20	506.93	66.15	1253.40	254.33	0.13	2.47	0.50
25	30	708.06	97.78	1713.85	348.38	0.14	2.56	0.49
30	10	60.32	6.31	163.38	20.82	0.11	2.71	0.35
30	20	300.45	46.10	806.96	95.56	0.15	2.69	0.32
30	30	685.44	106.59	463.26	85.52	0.16	0.68	0.13
35	10	25.10	2.42	29.85	4.91	0.10	1.19	0.20
35	20	17.25	2.37	14.32	3.00	0.14	0.83	0.17
35	30	8.38	2.31	6.31	—	0.28	0.75	—

Data obtained from the average of 3 replications.

has also been emphasized by earlier authors[19,20]. Lopez and Christensen[21], and Trenk and Hartman[2] reported moisture contents of 17.5~18% as the minimal levels for the growthof *Asp. flavus* in corn.

Maximal total production of aflatoxin was obtained when *Asp. parasiticus* was grown at a temperature of 25 or 30°C (Table 2 and 3, Fig. 1 and 2)

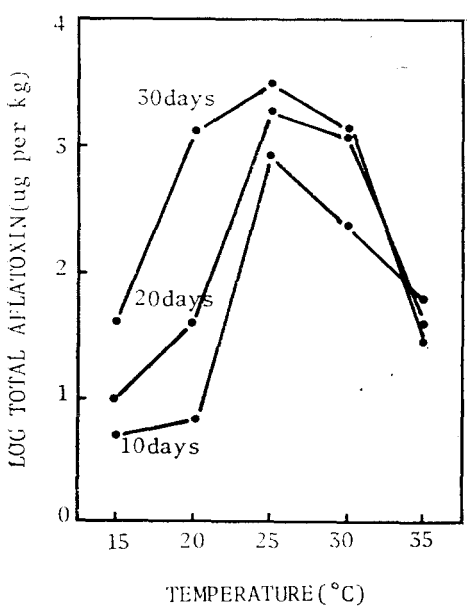

Fig. 1. Effect of incubation temperature on formation of total aflatoxin at 25% moisture content

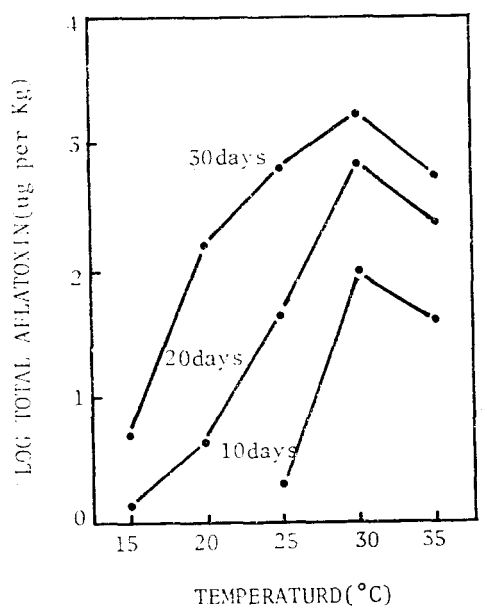

Fig. 2. Effect of incubation temperature on formation of total aflatoxin at 20% moisture content

Total aflatoxin concentrations up to 2868.07 μg per kg of samples were formed in 30 days. Panassenko[22] reported that the minimum and maximum temperatures for growth of *Asp. flavus* are 6 to 8°C and 44 to 66°C, respectively. The temperature range within which aflatoxin is produced, however, is more restricted.

At 15°C, considerably greater quantities of aflatoxin were detected. As the temperature was increased above 20°C, greater yields were obtained and less time was required for aflatoxin production. Considerably less aflatoxin was formed at 35°C.

In this study, it appears that the most important single factor contributing to their growth is the moisture content although seed moisture relationships are, of course, thereby makiug interpretation of moisture data difficult.

Noteworthy is the pattern of aflatoxin accumulation in relative amounts of the B and G components among the temperatures. At 25°C and below, the relative proportion of the principal aflatoxins in relation to incubation period showed that aflatoxin G was elaborated at a significantly higher rate than the B, however, it was also metabolized rapidly as the temperatures increased. Several investigators have shown that the ratio to one another of the four major aflatoxins produced is temperature dependent; aflatoxin B tends to predominate at higher temperature, but the preportion of G increases as incubation temperature is lowed[23~25].

From this data, the shift in relative amounts of the aflatoxin B_1 and G_1 as the temperature was changed is note worthy. The experimental data also indicate that aflatoxin G_1 and G_2 may be less stable than B_1 and B_2. As the incubation period was increased the B and G continuously in creased at same temperature. However, it was found that the G_1 and G_2 to B_1 ratio was continuously decreased when *Asp. parasiticus* was grown at 30 and 35°C without regard to moisture content. This phenomenon illustrates that lower temperatures favored the production of aflatoxin G_1 and G_2.

Spectrophotometric determinations on chloroform extracts of 27 randomly selected samples at same temperature treatment confirmed preliminary positive (r=0.966**) TLC results Fig. 3. Maximum

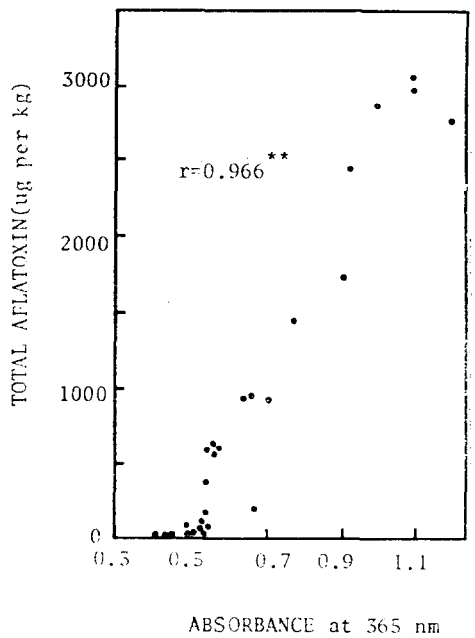

Fig. 3. Relationship between total aflatoxin and absorbance

absorption typical of aflatoxins was obtained on a DU-2 spectrophotometer at 365 nm from samples. *Asp. parasiticus* NRRL 2999, we found that this observation generally pertained but that pigment production increased with temperature.

Several investigators[23,26] have noted that chloroform extracts of *Asp. flavus* characteristically contain aflatoxin and yellow pigment which appears to be synthesized concurrent with aflatoxin. The biochemical significance of this correlation between color and production of aflatoxins by *Asp. parasiticus* has not been investigated.

要 約

Aspergillus parasiticus NRRL 2999에 依한 보리의 aflatoxin生成에 對한 溫度와 水分의 影響을 檢討하였다.

Aflatoxin生成 最適溫度는 25 및 30°C였다. 水分含量이 13%일 때는 aflatoxin이 檢出되지 않았고, 水分含量 16%에서는 적은 量이 生成되었다.

Aflatoxin B와 G의 生成比率은 溫度와 水分含量에 따라 差異를 보였다. 즉 Aflatoxin G는 B보다 初期에 生成이 促進되었으나 退化도 빨랐다. 또한 G는 낮은

溫度에서 生成이 많았다.

Chloroform抽出物의 黃色程度와 aflatoxin含量과는 正의 相關(r=0.966**)이 있었다.

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