Aflatoxin and Sclerotial Production by *Aspergillus flavus*: Influence of pH

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ABSTRACT

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Sodium nitrate and ammonium sulfate have different influences on aflatoxin and sclerotial production when used as sole nitrogen sources in solid media. The effects are attributable, at least in part, to pH. Ammonium-based media became acidified during culture, while aflatoxin production increased, and sclerotial production was totally inhibited. Sclerotia were produced on buffered ammonium media. Sclerotial production increased with increased pH on buffered ammonium media while aflatoxin production declined; aflatoxin was not produced after sclerotial maturation (5 days of incubation). Aflatoxin B_1 concentrations declined in acidic ammonium media, whereas aflatoxin B_2 remained stable. Nitrate-based media pH increased with fungal growth and production of large quantities of sclerotia and low concentrations of aflatoxin. Lowering nitrate media pH during culture with exogenous HCl totally inhibited sclerotial production and increased aflatoxin production 10-fold. Sclerotia produced on nitrate media contained less aflatoxin than those produced on ammonium media. Results suggest a pH-mediated, interrelated regulation of sclerotial morphogenesis, and aflatoxin biosynthesis.

Aflatoxins are toxic, carcinogenic/mutagenic agents that frequently contaminate agricultural commodities and pose a serious threat to human and domestic animal health (5). Production of aflatoxins is restricted to fungal species *Aspergillus flavus* and *A. parasiticus*. More than 90% of corn and cottonseed aflatoxin contaminations are caused by *A. flavus* (8).

Detailed studies on nutritional requirements for aflatoxin production in fermentation examined nitrogen-source influences on toxin production; ammonium supported greater aflatoxin production than nitrate (3,7,13). Studies using agar media, however, demonstrated that sclerotia were produced by *A. flavus* on nitrate media but not on ammonium media (2). Nitrogen-source influences were repeatedly attributed to specific effects of nitrate or ammonium and not to pH effects even though ammonium media become acidic, while nitrate media become alkaline during fungal growth (4,11).

The current study examined influences of pH on sclerotial and aflatoxin production by *A. flavus* on agar media containing either ammonium or nitrate as sole nitrogen sources.

MATERIALS AND METHODS

Cultures and media. *A. flavus* isolate AFMC 5A86 from cottonseed collected in Maricopa County, AZ, in 1986 was used in all studies. The isolate was transferred by single spores twice serially and demonstrated to be highly virulent on *Gossypium hirsutum* cultivar Deltapine Acala 90 as described (12). Active cultures were maintained in the dark at 30 C on 5% V-8 juice, 2% agar. For long-term storage, plugs of sporulating cultures were refrigerated (8 C) in 4-dram vials containing 5 ml of distilled water.

The defined medium of Mateles and Adye (13) was solidified with 2% agar and used as the basal medium; it consists of 50 g of sucrose, 10 g of KH_2PO_4, 2.0 g of $MgSO_4 \cdot 7H_2O$, 0.7 mg of $Na_2B_4O_7 \cdot 10H_2O$, 0.5 mg of $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$, 10 mg of $Fe_2(SO_4)_2 \cdot 6H_2O$, 17.6 mg of $ZnSO_4 \cdot 7H_2O$, 0.3 mg of $CuSO_4$, and 0.11 mg of $MnSO_4 \cdot H_2O$ per liter of H_2O. Sodium nitrate and ammonium sulfate (0.04 M) were utilized for nitrogen sources and either 0.02 M citric acid, 0.04 M succinic acid, or 0.05 M 2-(*N*-morpholino)ethanesulfonic acid (MES) were used as buffers. In all experiments cultures were grown at 30 C in an unilluminated incubator. Treatments were replicated 3–6 times and randomized. Experiments were performed twice.

Sclerotial production. Nine-centimeter petri dishes with 20 ml of medium were inoculated in the center with 10 μl of distilled water

containing about 1,000 spores. After 7–9 days of incubation, colony diameters were measured, plates were flooded with 0.01% Triton X-100, and sclerotia dislodged with a rubber policeman. Sclerotia were collected by vacuum filtration on preweighed 5-cm-square pieces of Miracloth (Behring Diagnostics, La Jolla, CA), dried at 60 C for 48 hr, and weighed. To determine final pH, entire plate contents were blended in 50 ml of distilled water for 2 min at high speed in a commercial blender, and the pH of the resulting suspension was measured.

Aflatoxin production. Fifteen-centimeter glass culture tubes with 5 ml of medium were inoculated with 100 μl of distilled water containing about 10,000 spores. After 5 days of growth the quantity of aflatoxin produced was determined (6). Six milliliters of acetone was added to each tube, the culture was crushed with a glass rod, solvent was decanted into a beaker, and the agar was extracted three times with 10 ml of methylene chloride. The methylene chloride and acetone fractions were combined, filtered through 25 g of anhydrous sodium sulfate, and concentrated to dryness at room temperature. Residues were solubilized in methylene chloride for thin-layer chromatography (TLC). Extracts and aflatoxin standards were separated on TLC plates (silica gel 60, 250 μm thick) by development with diethyl ether-methanol-water (96:3:1, v/v/v) (17) and quantified using a scanning densitometer (model cs-930, Shimadzu Scientific Instruments, Inc., Tokyo) (15).

Quantities of aflatoxin in buffered and unbuffered ammonium media were also estimated by measurement of fluorescence of the agar 5 mm beneath the mycelial mat (6). Agar fluorescence is directly proportional to the quantity of aflatoxin contained in the medium (6).

Acidification of nitrate medium. To reduce nitrate medium pH during culture, 10 μl of concentrated HCl was added to each of four wells (3 mm diameter) oriented around the perimeter of inoculated culture plates. This was done daily for the first 3 days of culture. Six days after inoculation three agar plugs (16 mm diameter) per plate were removed from adjusted and unadjusted

cultures, pH was measured with a flat electrode, plugs were placed in 15-mm culture tubes, extracted by the above procedure and their aflatoxin content quantified. Sclerotial production was determined on parallel plates.

Aflatoxin content of sclerotia. Dried sclerotia (300–400 mg) were weighed and placed in 15-cm culture tubes. Five milliliters of acetone was added to each tube (Teflon-lined caps), and tubes were vigorously shaken for 2 min. After incubation for 20 hr at room temperature, 10 ml of methylene chloride was added to each tube with subsequent shaking. After 4 hr the solvent was filtered into beakers, concentrated to dryness, and the residues solubilized in methylene chloride for aflatoxin analysis.

RESULTS

Sclerotial production by *A. flavus* was profuse on nitrate and buffered ammonium media but did not occur on unbuffered ammonium media (Table 1). Buffering ammonium media with 0.02 M citric acid prevented agar pH from dropping below 4.4, whereas unbuffered ammonium media had a final pH below 3.0 (Table 1). Total aflatoxin production on unbuffered ammonium media was more than two orders of magnitude greater than on nitrate media (Table 1). Buffering with citric acid reduced ($P = 0.05$) aflatoxin production on ammonium media (Table 1).

Influences of pH on aflatoxin concentrations were complex. Concentrations of aflatoxins B_1 and B_2 produced in citrate-buffered ammonium media were inversely related to initial pH (Table 2). Concentrations of aflatoxin B_2 in unbuffered ammonium media exceeded those in buffered media after both 5 and 9 days (Table 2). However, differences ($P = 0.05$) among concentrations of aflatoxin B_1 in buffered and unbuffered treatments occurred less frequently (Table 2). Aflatoxin B_1 concentrations of all treatments declined after 5 days of incubation. Quantities of aflatoxin B_2 were not significantly different after 5 and 9 days in buffered treatments, but in unbuffered media B_2 concentrations after 9 days were greater ($P = 0.05$) than after 5 days (Table 2). Aflatoxin B_1 hemiacetal was repeatedly detected in extracts of ammonium but not in extracts of nitrate media. The hemiacetal was not quantified because of its instability (14,19).

MES and succinic acid influenced both sclerotial and aflatoxin production in a manner similar to citrate (Table 3). Treatments that maintained media pH above 3.0 during a 5-day incubation stimulated sclerotial formation. The quantity of sclerotia produced was proportional to the final pH (Table 3). Aflatoxin concentration was inversely related to the quantity of sclerotia within the pH range 2.7–6.3 (Table 3). In separate tests, addition of HCl to nitrate media during culture resulted in a final pH of 2.64 ± 0.04 (three observations), total inhibition of sclerotial production, and an aflatoxin increase ($P = 0.01$) from 2.4 ± 1.5 μg/g to 31.5 ± 9.7 μg/g.

During an 11-day time-course experiment, aflatoxin B_1 concentrations increased for 5 days in unbuffered ammonium media

TABLE 1. Influence of nitrogen source and pH on production of sclerotia and aflatoxins by *Aspergillus flavus*

Nitrogen[v] source	Buffer	Final[w] pH	Sclerotia[x] (mg/cm^2)	Aflatoxin[y] (μg)
Nitrate	None	5.89 ± 0.02 a[z]	220 ± 20 b	0.38 ± 0.2 c
Ammonium	None	2.83 ± 0.04 c	0 c	91.36 ± 16.1 a
Ammonium	Citrate	4.44 ± 0.11 b	310 ± 40 a	23.41 ± 7.3 b

[v] NaNO$_3$ 0.04 M and (NH$_4$)$_2$ SO$_4$ 0.04 M.

[w] Media were adjusted to pH 5.5 before autoclaving.

[x] Weight of sclerotia produced on 20 ml of medium in 9-cm plastic petri dishes during 9 days' growth.

[y] Total quantity of aflatoxin B_1 plus aflatoxin B_2 produced in 5 ml of medium in culture tubes during 5 days' growth.

[z] Values in the same column followed by the same letter are not significantly different ($P = 0.05$) by Fisher's LSD.

TABLE 2. Influence of pH on formation of sclerotia and aflatoxins B_1 and B_2 by *Aspergillus flavus* on ammonium-based media

Buffer	Initial pH	Days	Final[w] pH	Sclerotia[x] (mg/cm^2)	Aflatoxin B1[y] (μg)	Aflatoxin B2[y] (μg)
Citrate	5.50	5	ND[z]	ND	28.4 ± 7.4 d	0.9 ± 0.1 e
		9	4.3 ± 0.1	46 ± 12	17.9 ± 2.5 e	1.0 ± 0.2 e
	5.00	5	ND	ND	46.7 ± 19.8 bc	1.8 ± 0.6 d
		9	3.7 ± 0.1	32 ± 5	30.4 ± 5.1 cd	1.7 ± 0.4 d
	4.50	5	ND	ND	81.6 ± 33.9 a	3.8 ± 1.6 c
		9	3.2 ± 0.1	21 ± 4	50.1 ± 12.1 b	3.4 ± 0.8 c
None	5.00	5	ND	ND	88.4 ± 11.4 a	7.9 ± 0.8 b
		9	2.4 ± 0	0	36.4 ± 7.8 bc	12.0 ± 0.6 a

[w] Values are significantly different ($p = 0.05$) by Fisher's LSD.

[x] Weight of sclerotia produced on 20 ml of medium in 9-cm plastic petri dishes during 9 days' growth. Values are significantly different ($P = 0.05$) by Fisher's LSD.

[y] Quantity of aflatoxin B_1 or B_2 produced in 5 ml of medium in culture tubes. Values in the same column followed by the same letter are not significantly different ($p = 0.05$) by Fisher's LSD.

[z] ND = not determined.

TABLE 3. Production of sclerotia and aflatoxins B_1 and B_2 by *Aspergillus flavus* on ammonium media containing various buffers

Buffer[v]	Initial[v] pH	Final[w] pH	Sclerotia[x] (mg/cm^2)	Aflatoxin B_1[y] (μg)	Aflatoxin B_2[y] (μg)
MES	5.5	2.8 ± 0.1 f[z]	0 c	47.9 ± 11.6 a	4.2 ± 1.2 a
	6.5	5.2 ± 0.2 c	7.1 ± 2.1 a	12.3 ± 1.5 c	0.4 ± 0.1 d
Succinate	5.5	3.8 ± 0.2 e	3.5 ± 0.6 b	44.3 ± 6.3 a	2.7 ± 0.6 b
	6.5	5.8 ± 0.2 b	8.0 ± 2.9 a	11.1 ± 5.5 c	0.3 ± 0.1 d
Citrate	5.5	4.9 ± 0.2 d	7.4 ± 1.6 a	27.5 ± 4.8 b	1.0 ± 0.2 c
	6.5	6.3 ± 0.1 a	9.2 ± 1.0 a	8.9 ± 1.8 c	0.2 ± 0.1 e
Control	5.5	2.7 ± 0.1 f	0 c	29.6 ± 2.9 b	3.3 ± 0.4 ab
	6.5	2.7 ± 0.1 f	0 c	33.3 ± 5.7 b	3.7 ± 0.9 ab

[v] MES [2-(*N*-morpholino) ethanesulfonic acid] 0.05 M, pK = 6.15; succinic acid 0.04 M, pK = 4.19, 5.57; citric acid 0.02 M, pK = 3.06, 4.74, 5.40.
[w] pH of 20 ml of medium seeded with *A. flavus* and incubated 9 days.
[x] Weight of sclerotia produced on 20 ml of medium during 9 days' growth.
[y] Quantity of aflatoxin B_1 or B_2 produced in 5 ml of medium in culture tubes during 5 days' growth.
[z] Values in the same column followed by the same letter are not significantly different ($p = 0.05$) by Fisher's LSD.
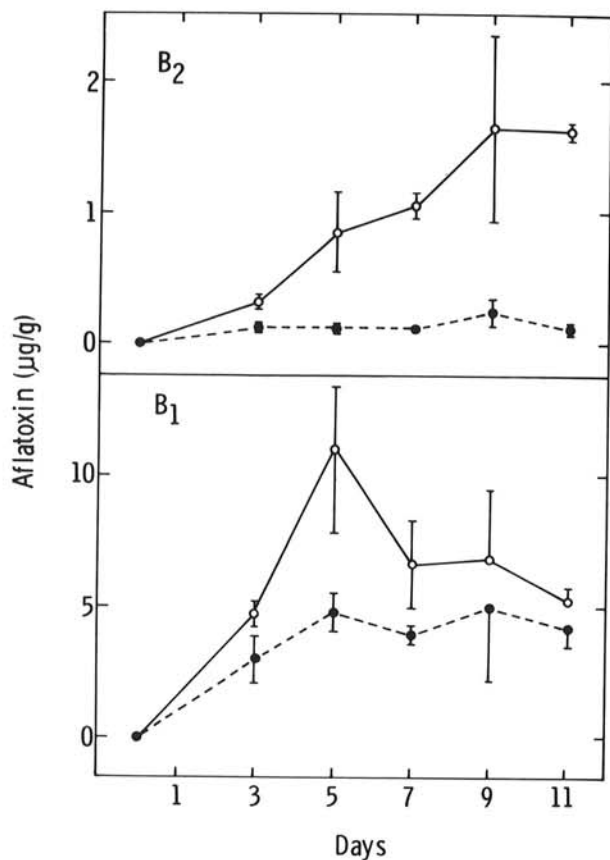

Fig. 1. Production of aflatoxin B_1 and aflatoxin B_2 by *Aspergillus flavus* on citrate buffered (- - -) and unbuffered (—) medium containing ammonium as the sole nitrogen source. Cultures were incubated at 30 C for 3–11 days. Mature sclerotia were present in the buffered but not in the unbuffered treatment within 5 days.
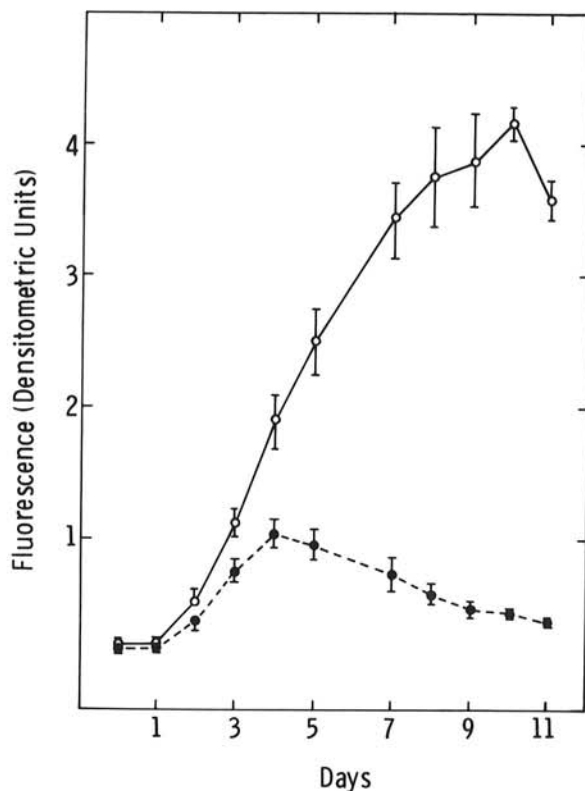

Fig. 2. Agar fluorescence of *Aspergillus flavus* cultures incubated on citrate buffered (- - -) and unbuffered (—) ammonium media at 30 C for 1–11 days. The fluorescence is directly correlated with the concentration of aflatoxin contained in the agar.

and then declined; concentrations of aflatoxin B_2 increased for 9 days (Fig. 1). In citrate-buffered ammonium media, aflatoxin B_1 concentrations increased for 5 days and then remained stable (Fig. 1). Aflatoxin B_2 concentrations varied little in buffered ammonium media during 3–11 days of incubation (Fig. 1). Sclerotial initials were evident in the buffered media after 3 days, and mature sclerotia were present after 5 days. Agar fluorescence increased through day 10 in unbuffered ammonium media but declined after day 4 in the buffered media (Fig. 2). The decline in agar fluorescence coincided with sclerotial maturation.

Sclerotia contained very high levels of aflatoxin, although sclerotia produced on nitrate media contained significantly less aflatoxin than those produced on ammonium media (3.2 μg/g vs. 65.0 μg/g). Aflatoxin concentrations of sclerotia produced on ammonium media, however, were not significantly ($P = 0.05$)

affected by pH levels that altered total aflatoxin levels (Table 2). In these experiments, sclerotia contained 40 ± 3, 46 ± 13, and 42 ± 13 μg of aflatoxin per gram when produced on ammonium media buffered at pH 5.5, pH 5.0, and pH 4.5, respectively.

DISCUSSION

Matales and Adye (13) noted a pH drop from 4.4 to 2.3 in *A. flavus* submerged fermentations in ammonium medium and reductions in aflatoxin yield when nitrate replaced ammonium. However, variation in aflatoxin production was not attributed to pH effects. Similarly, studies investigating ammonium influences on aflatoxin precursors (3,11) did not relate toxin yields to alterations in pH. Although effects of ammonium and nitrate on aflatoxin production observed in the current study closely correspond to those observed in previous studies (3,11,13) where pH was not implicated, the results identify an association between increased aflatoxin and reduced pH. This is suggested by the

inverse relationship between aflatoxin production and pH on buffered ammonium media and elevated aflatoxin production on acidified nitrate media.

Interpretation of pH effects on aflatoxin production is complicated by transformation of aflatoxin B_1 into unstable aflatoxin B_1 hemiacetal in acidic, aqueous environments (10,14,19). Aflatoxin B_1 hemiacetal was detected in culture extracts and, therefore, this transformation probably caused the aflatoxin B_1 reductions observed after 5 days' culture in this study. Aflatoxin B_2 is stable in acidic, aqueous environments.

Agar fluorescence is proportional to total aflatoxin content (6); in acidic cultures of A. flavus this includes aflatoxin B_1, aflatoxin B_2, and aflatoxin B_1 hemiacetal. During the timecourse experiment, therefore, fluorescence of unbuffered ammonium media did not decline after 5 days as did the concentration of aflatoxin B_1. Fluorescence of buffered ammonium media, however, declined after 4 days, even though concentrations of aflatoxins B_1 and B_2 remained stable. This may indicate transport of aflatoxin from mycelia embedded in the agar matrix to the agar surface and the sclerotia.

Sclerotial production by A. flavus is inhibited on unbuffered media containing ammonium as the sole nitrogen source (2), but this inhibition can be reversed by controlling the pH with buffers. Similar inhibition occurs on nitrate media adjusted to pH levels typically obtained in unbuffered ammonium media during culture. Sclerotial development is thus influenced by pH and not by inorganic nitrogen source as previously suggested (2). Prior studies (9,16,18) demonstrated only slight pH influences on sclerotium formation by A. flavus with pH 7 to 8 optimal. Levels below pH 5.0, however, were not tested. In the current study, sclerotial production was completely inhibited below pH 3.0 and inhibited at least 50% below pH 4.0 (Tables 1 and 2).

Tests with solid media permit simultaneous evaluation of aflatoxin and sclerotial production and their interrelations. Bennett et al (2) anticipated that similar growth conditions should favor both aflatoxin and sclerotium production because "both fungal differentiation and secondary-metabolite formation occur after the period of rapid vegetative growth has ceased." In buffered ammonium media, maximum aflatoxin concentrations coincided with sclerotial maturation and concentrations remained stable after maturation (5 days of incubation). A similar coincidence of sclerotial maturation and termination of aflatoxin biosynthesis was observed in media held at various pH levels for 5 and 9 days (Table 2).

Sclerotial morphogenesis and aflatoxin biosynthesis are apparently interrelated. This is suggested by: association of pH-mediated increases in aflatoxin production with inhibition of sclerotial development, coincidence of sclerotial maturation with cessation of aflatoxin production, and high aflatoxin content in sclerotia.

The high aflatoxin levels found in A. flavus sclerotia in this and other studies indicate aflatoxins may have a function related to sclerotia (20,21). Association of sclerotial morphogenesis and aflatoxin biosynthesis supports this contention. Activities of aflatoxins against soil microorganisms (1) and insects (22) suggest possible roles for aflatoxins as protectant compounds (20). Aflatoxins, however, are produced by A. flavus strains that do not produce sclerotia in vitro and, therefore, aflatoxins may have functions not related to sclerotia (2).

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