Raffinose Content May Influence Cottonseed Susceptibility to Aflatoxin Contamination

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ABSTRACT: The effect of the cotton storage trisaccharide raffinose and cottonseed storage protein (CSP) in combination on aflatoxin production by *Aspergillus flavus* was investigated. The ability of ground whole cottonseed and water-extracted cottonseed meal to support fungal biosynthesis of aflatoxin also was assessed *in vitro*. Dose response data showed that utilization of raffinose as a sole carbon source supported growth and aflatoxin production by *A. flavus*. When raffinose was a carbon source and CSP was the sole nitrogen source, aflatoxin levels were stimulated up to fourfold above those in raffinose reference media. Results with ground whole cottonseed as a sole carbon/nitrogen source demonstrated the capacity for aflatoxin production in *A. flavus* cultures. Lipid extraction of ground whole seed severely reduced aflatoxigenesis potential; however, lipid extraction followed by water extraction of ground whole seed restored much of the aflatoxin biosynthetic potential, suggesting the presence of a water soluble inhibitory factor. Accessible carbon appeared to be the limiting resource in water-extracted meal, as a raffinose supplement stimulated aflatoxin production. Either cottonseed storage lipid or raffinose was capable of providing accessible carbon to *A. flavus*. Raffinose and CSP in developing and mature cottonseed may predispose seed to potentially high levels of aflatoxin contamination by *A. flavus* upon seed infection.

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KEY WORDS: Aflatoxin, *A. flavus*, cottonseed, cottonseed meal, cottonseed storage protein, *Gossypium hirsutum*, raffinose.

Aspergillus flavus, a toxigenic and widely distributed fungal saprophyte, is capable of opportunistic pathogenesis in plants under specific environmental conditions. As a consequence, the value of cottonseed is diminished due to infection and fungal production of the potent carcinogen aflatoxin.

Cottonseed contains several major components that are potential fungal nutrient sources: storage proteins, lipids, and saccharides. Cottonseed storage protein (CSP), a globulin, is localized in developing and mature cotyledons. Cottonseed contains 39% protein by weight with most predominantly present as globulin seed storage proteins (1). Cottonseed also contains up to 10% by weight of the storage trisaccharide raffinose (2). These seed reserves constitute substantial carbon and nitrogen resources that are potentially available during microbial seed infection.

Oilseed storage proteins can stimulate aflatoxin production by *A. flavus*. Inclusion of storage protein from corn (zein) or cottonseed as supplements in a defined culture medium stimulates aflatoxin production by six- to tenfold (3). These oilseed storage proteins in conjunction with a metabolically accessible carbon source (e.g., glucose, sucrose) also stimulate toxin production by three- to eightfold over defined medium controls (4). In addition, raffinose can also support aflatoxin production by both *A. flavus* (5) and *A. parasiticus* (6).

Since raffinose and CSP can independently support aflatoxin production by *A. flavus*, an investigation was conducted to determine the combined effects of these components on toxin production and other fungal parameters. The potential role of raffinose in susceptibility of cottonseed to aflatoxin contamination was further assessed in ground whole cottonseed (7).

EXPERIMENTAL PROCEDURES

Biological materials. *Aspergillus flavus* AF13 was isolated from soil samples collected in southern Arizona (Yuma area) and maintained on a 5% V-8 vegetable juice (Campbell Soup Co., Camden, NJ) agar medium (pH 5.2) at 30°C (8). Culture medium was seeded (200 µL/70 mL) with a conidial suspension containing 10^7 to 10^8 spores per mL. Raffinose was purchased from J.T. Baker Co. (Philadelphia, PA). CSP was extracted from defatted cottonseed flour (gift of E.J. Conkerton, Southern Regional Research Center, ARS, USDA, New Orleans, LA) according to the method of Marshall and Conkerton (9). The protein preparation was exhaustively dialyzed against deionized water and lyophilized to dryness. Acid-delinted cottonseed (*Gossypium hirsutum* 'DeltaPine 90') was obtained from the DeltaPine Seed Company (Greenville, MS).

Ground whole cottonseed extraction. Whole cottonseed was ground in an analytical subsampling mill (RAS Mill, Romer Labs Inc., Analytical Instruments Division, Union, MO) and passed through a 1-mm mesh screen. After sieving, the ground whole cottonseed was extracted with a two-part procedure. First, to remove lipids, ground seed (150 g) was extracted three times with 500 mL portions of petroleum ether (30–60°C), and spread into a thin layer to air-dry at 25°C (fume hood) for 24 h. To remove water-soluble moieties, 3.5

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g of lipid-extracted meal (per replicate) was added to a 50-mL disposable conical centrifuge tube (plastic) with 30 mL of deionized water and vigorously shaken to thoroughly suspend the meal solids. The suspension was centrifuged (9000 × *g* for 10 min) and the supernatant was decanted and discarded. Then, pellets were resuspended in water and extracted a second time.

Fungal incubations. The defined fungal medium (10) contained sucrose (50 g/L) as the carbon source and sodium nitrate (3 g/L) as the nitrogen source (11) and was adjusted to pH 5.0 before sterilization. Incubations were conducted in 70 mL of medium in 250-mL flasks. For ground whole cottonseed/meal incubations, each replicate received an amount of ground material/meal (3.5 g/70 mL medium) equivalent to the carbon source present in defined medium. In some cases, 5% (wt/vol) raffinose (50 g/L) was utilized as the carbon source in reference media. Both ground whole seed and extracted ground seed incubations were conducted in the given defined medium, only lacking both carbon (sucrose or raffinose) and nitrogen ($NaNO_3$) sources. Water-extracted meal pellets were resuspended in this minimal defined medium before transfer to incubation flasks. Separate water-extracted meal treatments received 1.4% (wt/vol) raffinose (1.0 g/flask) supplements before spore inoculation. All fungal cultures were grown for 5 d at 32°C (dark) with shaking (200 rpm).

Aflatoxin analysis. Following incubation, cultures were stopped and aflatoxins solubilized with 50% (vol/vol) acetone. Four microliters of the medium/acetone solution was spotted on silica gel G plates which were developed in diethyl ether/methanol/water (96:3:1,vol/vol/vol). Aflatoxin B_1 was quantified directly on thin layer plates by fluorescence densitometry (12). Mycelial mats were separated by filtration *in vacuo*, dried at 50°C for 2 d, and weighed. Fungal weights were not determined for the ground whole cottonseed/meal incubations due to the large amount of residual material remaining when these incubations were terminated. Aflatoxin data was log transformed prior to statistical analysis in order to improve homogeneity among variances. Experiments were performed two to three times. The results reported here are representative of those experiments.

RESULTS

Raffinose was sufficient as the sole carbon source to support aflatoxin production. The yield of aflatoxin B_1 from cultures was directly correlated ($r^2 = 0.98$, $P < 0.05$) to the initial concentration of raffinose in the medium (Table 1). However, raffinose consistently supported lower toxin production than an equivalent level of sucrose. *Aspergillus flavus* biomass also increased with raffinose concentration ($r^2 = 0.66$, $P < 0.05$, Table 1), and was similar to that observed when sucrose was utilized as a carbon source. In addition, the final pH of the culture medium decreased with increasing raffinose concentration ($r^2 = -0.90$, $P < 0.05$).

With raffinose present as a carbon source, cottonseed storage protein was sufficient as the sole nitrogen source to support aflatoxin biosynthesis. Aflatoxin yield increased with CSP concentration ($r^2 = 0.95$, $P < 0.05$, Table 2). Levels of aflatoxin observed at the highest CSP concentrations tested were about fourfold greater than those observed in reference media with sodium nitrate as the nitrogen source. Biomass production also increased as a function of initial CSP concentration ($r^2 = 0.996$, $P < 0.05$, Table 2). Final culture pH generally increased with increasing initial CSP concentration ($r^2 = 0.72$, $P < 0.05$).

Aflatoxin production was compared in cultures using ground whole cottonseed or extracted cottonseed meal as a sole carbon/nitrogen source (Table 3). Aflatoxin production in lipid-extracted ground seed was reduced to 0.3% of that observed with ground whole seed. However, aflatoxin production was restored to 30% of original ground seed levels when ground seed was extracted first with petroleum ether and then with water. Supplementation (1.4% wt/vol) of the water, lipid-extracted meal with raffinose (1.0 g/flask) restored about 90% of the ground whole seed treatment levels of aflatoxin (Table 3).

TABLE 1
Effect of Raffinose Concentration on Aflatoxin Production, Culture pH, and Biomass Production in *Aspergillus flavus*

Treatment	Aflatoxin[a]	Biomass[b]	pH[c]
Ref. medium A[d]	742 ± 61 a	0.63 ± 0.006 a	5.46 ± 0.1 d
Raffinose, 10 g/L	18.9 ± 20 d	n.d.[e] d	6.11 ± 0.12 b
Raffinose, 20 g/L	88.2 ± 49 c	0.31 ± 0.015 c	6.31 ± 0.02 a
Raffinose, 40 g/L	174 ± 29 b,c	0.57 ± 0.065 b	5.73 ± 0.02 c
Raffinose, 80 g/L	301 ± 152 a,b	0.60 ± 0.023 a,b	5.24 ± 0.12 e

[a]Mean aflatoxin B_1 is expressed in µg per culture ($n = 3$). Values in the same column followed by the same letter are not significantly different ($P = 0.05$) by Fisher's LSD.
[b]Mean dry weight of mycelium is expressed in g per culture ($n = 3$).
[c]Mean final pH of culture medium ($n = 3$).
[d]Reference medium A contained sucrose (50 g/L) as a sole carbon source.
[e]n.d., Not determined. Mass was less than 0.1 g and was not recovered.

TABLE 2
Effect of Raffinose and Cottonseed Storage Protein (CSP) Concentration on Aflatoxin Production, pH, and Biomass Production in *A. flavus*

Treatment	Aflatoxin[a]	Biomass[b]	pH[c]
Ref. medium A[d]	546 ± 204 c	0.59 ± 0.03 c,d	4.97 ± 0.07 b
Ref. medium B[e]	449 ± 116 c,d	0.61 ± 0.04 c,d	5.66 ± 0.04 a
Raff./CSP (1 g/L)[f]	362 ± 142 c,d	0.31 ± 0.05 e	4.14 ± 0.15 d
Raff./CSP (2 g/L)	296 ± 128 d	0.53 ± 0.05 d,e	3.75 ± 0.08 e
Raff./CSP (5 g/L)	307 ± 22 c,d	0.84 ± 0.08 c	3.74 ± 0.05 e
Raff./CSP (10 g/L)	949 ± 265 b	1.32 ± 0.06 b	4.40 ± 0.14 c
Raff./CSP (20 g/L)	1944 ± 585 a	2.31 ± 0.38 a	4.76 ± 0.27 b

[a]Mean aflatoxin B_1 is expressed in µg/culture ($n = 3$). Values in the same column followed by the same letter are not significantly different ($P = 0.05$) by Fisher's LSD.
[b]Mean dry weight is expressed in g/culture ($n = 3$).
[c]Mean final pH of culture medium ($n = 3$).
[d]Reference medium A contained sucrose (50 g/L) as a sole carbon source.
[e]Reference medium B contained raffinose (50 g/L) as a sole carbon source.
[f]Treatments contained raffinose (80 g/L) as a carbon source and varying CSP concentrations as a sole nitrogen source. For abbreviation see Table 1.

TABLE 3
Effect of Ground Whole Cottonseed and Lipid-/Water-Extracted Ground Whole Cottonseed on Aflatoxin Production in *A. flavus*

Treatment	Aflatoxin[a]	pH[b]
Ref. medium B[c]	371 ± 148 a	5.61 ± 0.38 c
Ground whole seed[d]	75.6 ± 17.8 b	5.39 ± 0.03 c
Meal, lipid-extracted[e]	0.27 ± 0.07 d	6.70 ± 0.06 a
Lipid-, water-extracted meal[f]	22.8 ± 7.1 c	6.29 ± 0.25 b
Lipid-, water-extracted meal + raff.[g]	68.3 ± 45.3 b	5.69 ± 0.15 c

[a]Mean aflatoxin B_1 is expressed in μg/culture ($n = 4$). Values in the same column followed by the same letter are not significantly different ($P = 0.05$) by Fisher's LSD.
[b]Mean final pH of culture medium ($n = 4$).
[c]Reference medium B contained raffinose (50 g/L) as a sole carbon source and $NaNO_3$ (3 g/L) as a sole nitrogen source.
[d]All ground cottonseed/meal treatments were incubated in minimal defined medium (no sucrose or $NaNO_3$).
[e]Ground whole cottonseed was extracted three times with petroleum ether and dried.
[f]Lipid-extracted meal was extracted with water (2×).
[g]Lipid-, water-extracted meal supplemented with 1.4% (wt/vol) raffinose. For abbreviation see Table 1.

DISCUSSION

Aspergillus flavus normally requires a carbon source (e.g., glucose, sucrose) which can be readily utilized by the primary metabolic pathways (e.g., glycolysis) in order to support good growth and aflatoxin production (13). Since developing and mature cottonseed contains considerable amounts (up to 10% by weight) of the storage trisaccharide raffinose, the question arises as to whether this sugar is adequately metabolized to support fungal growth and aflatoxin biosynthesis. The current study shows that raffinose supports high levels of aflatoxigenesis at concentrations available in developing (14) and mature (2) cottonseed. Furthermore, CSP, the nitrogen source most readily available in cottonseed, can support aflatoxin production as the sole nitrogen source, with raffinose acting as the sole carbon source.

When *A. flavus* cultures utilized raffinose as a carbon source and increasing CSP levels as the sole nitrogen source, aflatoxin production was stimulated up to fourfold. These results were similar to those obtained with sucrose as a carbon source and either CSP or zein (4) as a sole nitrogen source. Oilseed storage proteins appear to stimulate aflatoxin production in *A. flavus* when present with a metabolically accessible carbon source. Indeed, it has been shown that *A. flavus* produces maximum aflatoxin levels only in the presence of a proteinaceous nitrogen source (5). Since cottonseed contains significant levels of both raffinose and globulin storage proteins, it seems likely that these are important factors governing the susceptibility of cottonseed to aflatoxin contamination.

Final culture pH increased with initial protein concentration when CSP was the sole nitrogen source and raffinose was the carbon source. Increased pH generally decreases aflatoxin production in *A. flavus* (11) and *A. parasiticus* (15). This suggests a greater potential for stimulation of aflatoxin biosynthesis by oilseed storage proteins than is observed *in vitro*. Under conditions where seed tissue homeostasis resists pH increases, storage proteins may stimulate even greater aflatoxin production.

The observed aflatoxin levels in the incubations with ground whole cottonseed were lower than those found in reference media, suggesting an inhibition of toxin biosynthesis. The cotton tetraterpene gossypol inhibits aflatoxin biosynthesis (16). This seed component may explain the lower aflatoxin levels observed with these incubations. Lipid extraction of ground whole cottonseed considerably reduced aflatoxin production. Thus, cottonseed lipids may be an accessible carbon source that can drive aflatoxin production. However, ground seed extracted first with petroleum ether and then with water restored aflatoxin production to 30% of ground whole seed levels. This stimulation of aflatoxigenesis by water extraction may indicate the presence of a water-soluble inhibitor of aflatoxin biosynthesis such as the xylan inhibitor localized in cotton seedcoats (17). When water-extracted cottonseed meal was supplemented with raffinose, aflatoxin production was stimulated to 90% of ground whole seed levels. This result indicates that readily metabolized carbon is limiting in this meal fraction and raffinose can function as this carbon source.

The ground whole cottonseed/meal incubation system is more complex than the defined liquid medium system. In the latter, a limited number of definable components can be modulated individually to determine effects on a given metabolic product or product pathway. In the ground whole seed system, all of the various seed components are presented to the growing fungus simultaneously, which may lead to unexpected phenomena resulting from unpredicted component interactions. Of course, in the actual fungal-cottonseed interaction, the various seed components are not available to the fungus uniformly. Some seed components are not as accessible to the fungus (e.g., lignin) as others. Normally, the fungus would utilize rapidly metabolized resources initially and turn to other resources later. In addition, seed storage components such as CSP and lipids are compartmentalized (protein bodies; lipid bodies) and would not be available to the fungus until the storage body membranes had been lysed. Despite these impediments to the fungus, the potential for accessible carbon and nitrogen sources nonetheless exists and may explain the elevated production of aflatoxin in developing/mature cottonseed under certain conditions.

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