POTENTIAL ROLE FOR STORAGE PROTEINS AND SUGARS IN COTTONSEED SUSCEPTIBILITY TO AFLATOXIN CONTAMINATION

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Abstract

Cottonseed storage protein (CSP) and several other proteins (bovine serum albumin [BSA], collagen and zein) stimulated aflatoxin production when incorporated into a defined medium containing sucrose and nitrate. With protein as the sole carbon and nitrogen source, collagen, but not BSA, CSP or zein, produced aflatoxin levels comparable to defined medium controls. A dose response study using CSP as the sole carbon and nitrogen source revealed that aflatoxin (r^2=0.89, P<0.05) and biomass (r^2=0.99, P<0.05) production were correlated with protein concentration. A pronounced stimulation of aflatoxin production was observed when utilizing medium containing CSP and sucrose, but without nitrate, in a 5-day fermentation. Cottonseed storage protein induced the production and secretion of an elastinolytic protease activity in *A. flavus* cultures, when no defined carbohydrate was present in the medium. In addition, inclusion of raffinose as a sole carbon source in the growth medium supported aflatoxin production. The results suggest that seed storage protein composition and storage saccharides may be important factors influencing aflatoxin contamination in cottonseed.

Introduction

The toxigenic fungus *Aspergillus flavus* is a widely distributed saprophyte that is capable of opportunistic pathogenesis in plants. As a result, the value of oilseed crops is diminished due to infection and aflatoxin production by the fungus.

Cotton seeds contain significant levels of protein storage reserves. Cottonseed storage protein (CSP), a globulin (salt-soluble), is localized in the developing cotyledons. Cottonseed contains 39 percent protein by weight, and CSP is the major seed protein family present (Bewley and Black, 1978). In addition, CSP contains 18 percent nitrogen (Altschul *et. al.*, 1966). Cottonseed also contains considerable levels of the storage trisaccharide raffinose (Muller and Jacks, 1983). Thus, seed protein and sugar reserves represent substantial carbon and nitrogen resources available to the fungus during seed infection.

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Fungal proteases are an important hydrolytic tool, allowing the utilization of seed reserves. Soybean seed protein induces an extracellular protease from *A. flavus* (Srinivasan and Dhar, 1990). Elastinolytic protease production is strongly conserved in *Aspergillus* section *Flavi* (Mellon and Cotty, 1995). The enzyme responsible for this proteolytic activity from *A. flavus* has been purified and characterized (Mellon and Cotty, 1996b). It is capable of hydrolyzing a wide range of protein substrates, including CSP, and may play an important role in fungal utilization of seed nutrients.

Oilseed storage proteins and saccharides may contribute to seed vulnerability to aflatoxin contamination. The current study investigated effects of CSP and raffinose on aflatoxin and fungal protease production in liquid culture. A preliminary report has been given (Mellon and Cotty, 1996a).

Materials and Methods

Biological Materials. *Aspergillus flavus* AF13 was isolated from Southern Arizona and maintained on a 5% V-8 (vegetable juice, Campbell Soup Co., Camden, NJ) medium at 30◦C (Cotty, 1989). Culture medium was seeded (200 µl/70 mL) with a conidial suspension containing 10^7 to 10^8 spores per mL. Collagen (Type I), bovine serum albumin (BSA), raffinose, and zein were purchased from the Sigma Chemical Company (St. Louis, MO). Cottonseed storage protein (CSP) was prepared as previously described (Mellon and Cotty, 1996b).

Fungal Incubations. The defined fungal medium (Adye and Mateles, 1964) contained sucrose (50 g/L) as a carbon source and sodium nitrate (3 g/L) as a nitrogen source (Cotty, 1988). Different treatments contained varying amounts of the five test components: CSP, zein, BSA, collagen and raffinose. In some cases, the fungal medium lacked either the defined carbon source (sucrose) or the defined nitrogen source ($NaNO_3$) or both, but did contain the other components of the defined medium. Fungal cultures were grown for 5 days at 30◦C (dark).

Aflatoxin Analysis. Following incubation, each flask was diluted to 50% (v/v) acetone and allowed to soak for 18 h. 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). Aflatoxin B_1 was quantitated by a standard plate densitometry method (Stoloff and Scott, 1984). Mycelial mats were separated by filtration *in vacuo* and dried at 50◦C for 2 days.

Elastase Assays. Culture filtrates were analyzed for protease activity by means of a radial diffusion assay which employed elastin as the protein substrate (Mellon and Cotty, 1995). Culture filtrates were first treated with a reversed phase C_{18} cartridge (Sep-Pak, Millipore, Milford, MA) to remove aflatoxin. The C_{18} cartridge was charged with 3 mL of methanol, followed by a wash with 5 mL of deionized

water. Aflatoxin bound to the cartridge matrix and the effluent was tested directly for protease activity (3-mL sample aliquots).

Results

Relatively low levels of supplementary proteins resulted in substantial changes in cultures grown in AM (Adye & Mateles) defined medium. When AM medium was supplemented with 5 g per liter of either CSP, zein, collagen or BSA, there was a stimulation of both aflatoxin (Table 1) and biomass (dry weight) production. Cultures containing CSP or zein showed a 6- to 10-fold stimulation of aflatoxin over AM medium not supplemented. Cultures containing only BSA or CSP as both carbon and nitrogen source (no sucrose or $NaNO_3$) produced little or no aflatoxin. Cultures containing collagen (0.5%) as the sole C and N source produced aflatoxin levels similar to AM (5% sucrose, Table 1). Utilization of starch as a carbon source (1-20 g/L) instead of sucrose in the fungal medium resulted in a linear increase in aflatoxin production (40-470 µg/g dry wt.).

Dose response experiments utilizing CSP as the sole C and N source were conducted. Aflatoxin production increased with increased protein concentration. A positive correlation (linear) was observed between aflatoxin production and CSP concentration (r^2=0.96, P<0.05)(Table 2). In addition, there was a good correlation (linear response) between protein concentration and biomass (dry weight) production (r^2=0.99, P<0.05) (Table 2).

A stimulation of aflatoxin production was also observed in 5-day fermentations using CSP-supplemented (1-20 g/L) AM medium without nitrate (Table 3) when compared to the standard defined medium. Aflatoxin production was highly correlated with CSP concentration (r^2 = 0.94, P<0.05).

Culture medium containing raffinose (1-10%) as a carbon source produced aflatoxin levels comparable to AM control medium during a 5-day fermentation period (data not shown).

Based on a radial diffusion assay, cultures which contained sucrose, with or without supplementary protein, produced little or no protease activity in the culture medium. However, when CSP was supplied as the sole C and N source, increased levels of elastinolytic protease activity were secreted into the medium. Elastase activity was not quantitatively correlated with protein concentration within the range tested.

Discussion

Stimulation of aflatoxin production by relatively low levels of protein has interesting implications for fungal contamination of oilseed crops. Stimulation of toxin production occurred with additions of all tested proteins (BSA, collagen, CSP, zein) to the defined medium, although there were quantitative differences expressed by different protein types (Table 1). Of the tested proteins, the storage proteins of corn and cottonseed (zein, CSP) caused the greatest toxin stimulation. Thus, the protein composition of seeds may partly determine crop vulnerability to contamination.

Where protein comprised the sole C and N source of the medium, there was considerably less toxin production than in AM controls. The fungus presumably has a small reserve of protease which is secreted into the surrounding medium in order to digest the available substrate. However, considering the protein substrate available (0.5%) was one-tenth the level of carbon substrate (5% sucrose) available to AM control cultures, reduced toxin production would seem an expected result. In addition, protein is a complex substrate, compared to sucrose, and would require more time to release carbon into the primary metabolic pools. Only then could the fungus enter into a growth phase with its many concomitant requirements (enzymes, cell wall synthesis, etc.) before carbon could enter into the secondary carbon pools to produce aflatoxin. In addition, a high metabolic priority for the fungus would be the production and secretion of additional protease molecules to appropriate additional C and N resources. The collagen addendum (-C/N) was the exception to this trend, producing aflatoxin levels equivalent to AM controls. The mechanism by which collagen stimulates toxin production is not clear. Biomass production (cell dry weight) for the collagen cultures (-C/N) was not greatly different from -C/N cultures with other proteins. Although, in the case of zein and CSP, toxin production was reduced, it was positively correlated with protein concentration. Considering that cottonseed can contain up to 30% CSP by weight, protein resources alone may be sufficient to drive very high levels of aflatoxin production.

The CSP dose response study provided some insight into parameters controlling aflatoxin production. In the case where CSP was used as an addendum to medium containing a defined C source (5% sucrose) but no additional N source, toxin production was stimulated (4-fold) above AM controls (Table 3). These results suggest the protein is preferred over nitrate as a nitrogen source for aflatoxin production. The results are consistent with observed amino acid influences (Adye and Mateles, 1964) This may also explain the toxin production elevation observed in AM cultures supplemented with low levels (5 g/L) of various proteins.

The fungal protease activity induced by CSP was the metalloprotease previously purified from *A. flavus* (Mellon and Cotty, 1996b). This protease can hydrolyze a wide variety of protein substrates and apparently is the predominant protease activity secreted upon introduction of seed proteins. However, enzyme production was very sensitive to carbohydrate substrate levels. When sucrose was present in the medium, little or no protease activity was

observed. Similar observations have been reported by Srinivasan and Dhar (1990) for an uncharacterized protease from *A. flavus*. They also report that cultures promptly cease producing and secreting protease upon addition of a carbohydrate source. Apparently, carbohydrates repress protease production. Cottonseed storage protein stimulated medium protease levels to the highest observed values. This result is in agreement with Srinivasan and Dhar (1990) who report that of the 10 proteins tested for protease production, cottonseed protein produces the highest levels. Proteases probably play an active role during aflatoxin contamination of cottonseed.

The results may lead to insight into characteristics of cottonseed resulting in the crop's vulnerability to aflatoxin contamination. In cottonseed, CSP is present in the storage protein bodies of the cotyledons (30% of seed by weight). Mature cottonseed also contains significant amounts of raffinose (Muller and Jacks, 1983). This storage trisaccharide may serve as an accessible carbon source utilized by the fungus to produce aflatoxin. The close proximity of storage proteins to raffinose may explain copious production of aflatoxin in developing cottonseed under certain conditions. Altering CSP composition may provide an avenue for reducing cottonseed susceptibility to aflatoxin contamination.

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Table 1. Effect of supplementary protein substrates on aflatoxin production.

	Total Aflatoxin		
Treatment	AM	-C	-C/N
Control	160	0	0
Bovine serum albumin	224	0	1.4
Collagen	363	141	314
Cottonseed storage prot.	1487	1.6	1.5
Zein	948	19.9	55

Mean aflatoxin is expressed in µg per flask (n=3).
AM = defined medium of Adye & Mateles (1964); -C = AM without sucrose; -C/N = AM without sucrose or $NaNO_3$.
All protein supplements present at 5 g/L.

Table 2. Effect of CSP concentration on aflatoxin and biomass production.

Treatment	Total Aflatoxin	Dry Wgt (g)
Control	0	0.02
CSP, 1 g/L	2.77	0.095
CSP, 2 g/L	7.17	0.134
CSP, 5 g/L	14.2	0.252
CSP, 10 g/L	10.2	0.41
CSP, 20 g/L	41.1	0.96

Aflatoxin expressed as µg per flask (n=3); r^2= 0.96, P<0.05.
All incubations used AM medium without sucrose or $NaNO_3$.

Table 3. Effect of CSP concentration on aflatoxin production.

	Total Aflatoxin		
Treatment	AM	-N	-C/N
Control	415	ND	ND
CSP, 1 g/L	ND	218	0.101
CSP, 2 g/L	ND	173	0.078
CSP, 5 g/L	ND	538	0.317
CSP, 10 g/L	ND	1055	7.46
CSP, 20 g/L	ND	1408	19.9

Mean aflatoxin is expressed in µg per flask (n=3); r^2=0.94, P<0.05 (-N); r^2=0.96, P<0.05 (-C/N).
AM = defined medium of Adye & Mateles (1964); -N = AM without $NaNO_3$; -C/N = AM without sucrose or $NaNO_3$; ND = Not Determined.