Influence of Lipids with and without Other Cottonseed Reserve Materials on Aflatoxin B_1 Production by *Aspergillus flavus*

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Cottonseed storage lipids (primarily triglycerides), in either crude or refined form, were found to support growth and aflatoxin B_1 production by *Aspergillus flavus*. When lipids were removed from ground whole cottonseed by petroleum ether extraction, aflatoxin production dropped by more than 800-fold. Reconstitution of the lipid-extracted ground whole seed with a crude preparation of cottonseed lipids restored aflatoxin production to the previous levels. Fungal utilization of the three major cottonseed reserve materials, raffinose, triglycerides (refined cottonseed oil), and cottonseed storage protein, was monitored in vitro over a 7 day fermentation period. The fermentation medium contained the reserve compounds in proportions approximating those found in mature cottonseed. *A. flavus* rapidly converted raffinose to fructose and melibiose, presumably by action of invertase, and then hydrolyzed the melibiose. These simple sugars apparently supported initial growth and aflatoxin B_1 production. Raffinose and the resulting melibiose were nearly exhausted by day 2. Fungal hydrolysis of triglycerides began as exhaustion of carbohydrate approached. After day 2, rapid catabolism of the released fatty acids began and coincided with glucose regeneration through gluconeogenesis, which peaked on day 6. The fungus did not preferentially utilize specific fatty acids. *A. flavus* also produced a number of storage metabolites, including arabitol, erythritol, mannitol, and trehalose. Mannitol was produced in much higher concentrations than the other storage metabolites. Selective use of simple carbohydrates by *A. flavus* to drive aflatoxin production may suggest strategies for reducing vulnerability of cottonseed to aflatoxin contamination.

Keywords: *Aflatoxin; Aspergillus flavus; cottonseed lipids; cottonseed storage protein; gluconeogenesis; Gossypium hirsutum; raffinose*

INTRODUCTION

Aspergillus flavus, a toxigenic and widely distributed fungal saprophyte, is capable of opportunistic pathogenesis in oilseed crops under specific environmental conditions. During crop infection, *A. flavus* frequently produces potent carcinogens, the aflatoxins, and as a result, the value of cottonseed is diminished.

Cottonseed contains several major components that are potential fungal nutrient sources: lipids, saccharides, and storage proteins. Cottonseed storage protein (CSP) and storage lipids are localized in the cotyledons that comprise most of a cottonseed's volume. Cottonseed contains 39% protein by weight, and most occurs as the globulin family of seed storage proteins (Bewley and Black, 1978). In addition, this oilseed contains 33% lipid by weight (Bewley and Black, 1978), with triglycerides predominant (96−98% of total lipid) (Jones and King, 1990). Cottonseed also contains up to 10%, by weight, of the storage trisaccharide raffinose (Muller and Jacks, 1983). These seed reserves constitute substantial carbon and nitrogen resources potentially available during microbial seed infection.

Oilseed storage proteins stimulate aflatoxin production by *A. flavus*. Inclusion of storage protein from corn (zein) or cottonseed as supplements in defined culture media stimulates aflatoxin production by 6−10-fold (Mellon and Cotty, 1998b). In addition, the presence of either CSP or zein as a sole nitrogen source in conjunction with a rapidly accessible carbon source (e.g., glucose, sucrose) also stimulates aflatoxin production by 3−8-fold over chemically defined medium controls (Mellon and Cotty, 1998b). Raffinose supports aflatoxin production by both *A. flavus* (Davis et al., 1967) and *Aspergillus parasiticus* (Abdollahi and Buchanan, 1981).

In an effort to further describe utilization of oilseed substrates by *A. flavus* during aflatoxin biosynthesis, influences of cottonseed storage lipids on toxin production were investigated. In addition, the relationship between substrate utilization and aflatoxin biosynthesis was assessed in culture media containing the three major storage components in ratios similar to those *in plantae* (in seed). A preliminary report has been given (Mellon and Cotty, 1998a).

EXPERIMENTAL PROCEDURES

Biological Materials. *A. flavus* AF13 was isolated from soil samples collected in southwestern 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 (Cotty, 1989). Culture medium was seeded (200 μL/70 mL) with a conidial suspension containing 10^6 to 10^7 spores/mL. Raffinose was purchased from J.T. Baker Co. (Philadelphia, PA). Cottonseed storage protein was extracted from defatted cottonseed flour according to the method of Marshall and Conkerton (1991). The protein preparation was exhaustively dialyzed against deionized water and lyophilized to dryness. Refined cottonseed oil was produced by Lou'Ana Corp. Acid-delinted cottonseed (*Gossypium hirsutum* "DeltaPine 90") was obtained from the DeltaPine Seed Co. (Greenville, MS).

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Ground Whole Cottonseed Preparation. 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 lipids were removed by extracting the ground seed (150 g) three times with 500 mL portions of petroleum ether. After extraction, the ground seed was spread into a thin layer to air-dry in a fume hood at room temperature for 24 h. Lipid extracts were pooled, and the petroleum ether was removed (rotary evaporator). This crude cottonseed oil preparation was stored at 4 °C until later use.

Fungal Incubations. The chemically defined culture medium (Adye and Mateles, 1964) contained raffinose (50 g/L) as the carbon source and sodium nitrate (3 g/L) as the nitrogen source (Cotty, 1988) and was adjusted to pH 5.0 before sterilization. Incubations were conducted in 70 mL of medium in 250 mL flasks. The defined medium was modified by replacing both raffinose and nitrate with either ground whole seed or extracted ground seed at levels of 3.5 g/70 mL of medium. All fungal fermentations were performed in the dark at 31 °C with shaking (200 rpm). The standard 5 day incubation period was used, except for the timed study (0−7 days).

In an effort to understand various interactions among major seed storage components during aflatoxin biosynthesis, a study was initiated with a chemically defined medium lacking carbon and nitrogen sources. This medium was supplemented with raffinose (0.3 g/70 mL), CSP (1.2 g/70 mL), and lipids (1.2 g/70 mL) in proportions (1:4:4, respectively) similar to those in mature cottonseed. Fermentations were monitored for 7 days for pH, and concentrations of total reducing sugar, aflatoxin B_1, specific saccharides, specific sugar alcohols, triglycerides, and specific fatty acids. Trends in biomass production were estimated on the basis of the mass and nitrogen content of solids present at each time point (see below).

Reducing Sugar Assay. After each culture was removed from the shaker, pH was measured and a 1 mL portion of growth medium was removed. These samples were treated with a reversed-phase C_{18} cartridge (SepPak, Millipore, Milford, MA) to remove aflatoxin. The C_{18} cartridge was charged with 2 mL of acetonitrile, followed by a wash with 5 mL of deionized water. Aflatoxin quantitatively bound to the cartridge matrix, and the effluent was free of aflatoxin (<1 ng/mL). To determine reducing sugar concentrations, 3 μL of each detoxified sample was diluted with 247 μL of deionized water and assayed by the *p*-hydroxybenzoic acid hydrazide method (Lever, 1972). Raffinose yielded a response that was about 5% of an equivalent concentration of glucose.

Aflatoxin Analysis. In the conditions used in these fermentations, *A. flavus* AF13 produced only aflatoxin B_1. Following removal of the reducing sugar sample, cultures were stopped and aflatoxins solubilized with 50% (v/v) acetone. The medium acetone solution was spotted beside aflatoxin standards (B_1, B_2, G_1, G_2) on silica gel G plates. After development in diethyl ether−methanol−water (96:3:1), aflatoxin B_1 was quantified directly on thin layer plates by fluorescence densitometry (Stoloff and Scott, 1984) with a densitometer (Shimadzu CS-9301PC, Kyoto, Japan).

Lipid/Saccharide Analysis. Following aflatoxin analysis, each culture flask received 30 mL of ethyl acetate and was vigorously swirled (about 1 min) to emulsify the contents. A 2 mL sample of the emulsion was added to a Reacti-Vial (Pierce Chemical Co., Rockford, IL); acetone/ethyl acetate was removed by purging the emulsion with a stream of nitrogen. Samples were stored at −20 °C prior to lyophilization. Frozen samples were lyophilized to remove water and derivatized for gas chromatography by addition of 1 mL of pyridine, 1 mL of hexamethyldisilazane (Pierce Chemical Co.), and 0.1 mL of trifluoroacetic acid. Cholesterol methyl ether (Sigma Chemical Co., St. Louis, MO) was used as an internal standard. Samples were heated at 60 °C for 45 min with periodic shaking and then cooled before chromatography. The gas chromatograph (Hewlett-Packard 5890 Series 2 Plus, Palo Alto, CA) was fitted with a DB-5ht fused silica column (15 m × 25 μm i.d. film thickness; J&W Scientific, Folsom, CA) and used helium as the carrier gas (about 1 mL/min). The instrument operated in constant flow mode with a split injector (340 °C, 1:50 split) and flame ionization detector (340 °C). The silyl-derivatized samples were subjected to the following column temperature program: 100 °C for 3 min; 10 °C/min to 150 °C; 5 °C/min to 250 °C; 10 °C/min to 360 °C, which was held constant for 15 min. Sample components were identified by comparing the retention times of unknown peaks with the retention times of known standards and by mass spectroscopy with a mass detector (Hewlett-Packard Model 5972). Quantitative analysis was performed by internal standardization. Experiments were conducted with three replicates a total of three times. The results reported herein are representative of those experiments.

Table 1. Effect of Cottonseed Lipids in Ground Whole Cottonseed on Aflatoxin B_1 Production and Culture pH in *A. flavus*

treatment	aflatoxin B_1[a]	pH[b]
reference medium[c]	107 ± 32.2	5.13 ± 0.14
ground whole seed[d]	11.6 ± 7.8	5.47 ± 0.11
lipid-extracted ground seed[e]	0.014 ± 0.008	6.00 ± 0.13
lipid-extracted ground seed + lipid[f]	13.0 ± 9.5	5.20 ± 0.06

[a] Mean aflatoxin B_1 is expressed in micrograms per culture (n = 4). [b] Mean final pH of culture medium (n = 4). [c] Reference cultures contained raffinose (50 g/L) as a sole carbon source. [d] Cultures contained 3.5 g/70 mL of ground whole cottonseed with no additional C or N source. [e] Cultures contained 3.5 g/70 mL of ground seed which had been extracted with petroleum ether. [f] Cultures contained 2.34 g of lipid-extracted ground seed plus 1.16 g of crude cottonseed lipid per 70 mL of medium.

Biomass/CSP Estimation. Following lipid/saccharide sampling, the liquid portion of each culture was removed by filtration in vacuo. The solid residue, which consisted of fungal biomass (mycelia) and residual CSP, was collected on filter paper, dried at 50 °C for 24 h, and weighed. After weighing, residues were powdered with a mortar and pestle (25 °C). A portion of each residue was then dried at 135 °C for 2 h to determine moisture content and to calculate dry matter. Nitrogen analysis was conducted on the sample residues using a standard combustion method (American Association of Cereal Chemistry Method 46-30) with a nitrogen analyzer (LECO Model FP-428, St. Joseph, MO). Using this method, *A. flavus* mycelial wall material (grown with a water-soluble N source) contained 4.0% nitrogen and pure CSP contained 17.8% nitrogen. These values are consistent with previous reports (Barbosa and Kemmelmeier, 1993; Altschul et al., 1966). Residue proportions consisting of cell wall and protein were estimated algebraically from the measured nitrogen content under the assumption that the residue consisted of only mycelium (4%N) and CSP (18%N). Constituent masses were calculated by multiplying the proportion times the residue mass.

RESULTS

A. flavus AF13 successfully converted cottonseed triglycerides into biomass and aflatoxin B_1 when these lipids were present as the sole carbon source with sodium nitrate as the sole nitrogen source. Both a crude cottonseed lipid preparation (AFB_1, 534 ng/mL) and refined cottonseed oil (AFB_1, 704 ng/mL) supported aflatoxin production in the defined fungal medium, though at levels about 1/10 those of the raffinose reference cultures (AFB_1, 7490 ng/mL). However, crude cottonseed lipids (dry weight, 36.4 mg/mL) and refined cottonseed oil (dry weight, 36 mg/mL) supported greater biomass production than observed in raffinose reference medium (dry weight, 13.1 mg/mL). The ability of ground whole cottonseed to support aflatoxin production was reduced by more than 800-fold when cottonseed lipids were removed by petroleum ether extraction (Table 1).

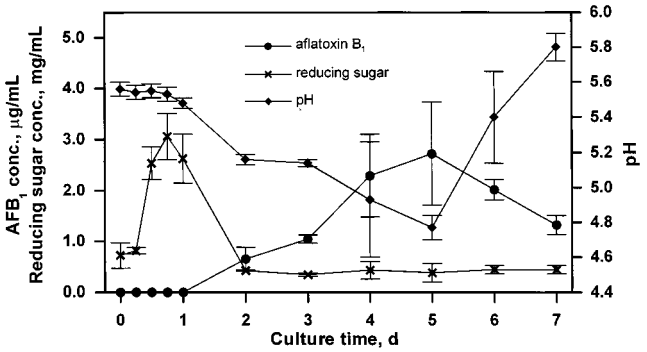

Figure 1. Effect of culture age on aflatoxin B_1 production, reducing sugar concentration, and culture pH in a medium containing raffinose, triglycerides, and CSP. The mean aflatoxin B_1 concentration ($n = 3$) is expressed in μg/mL of culture medium. The mean reducing sugar level ($n = 3$) is expressed in mg of glucose equivalents/mL of culture fluid.

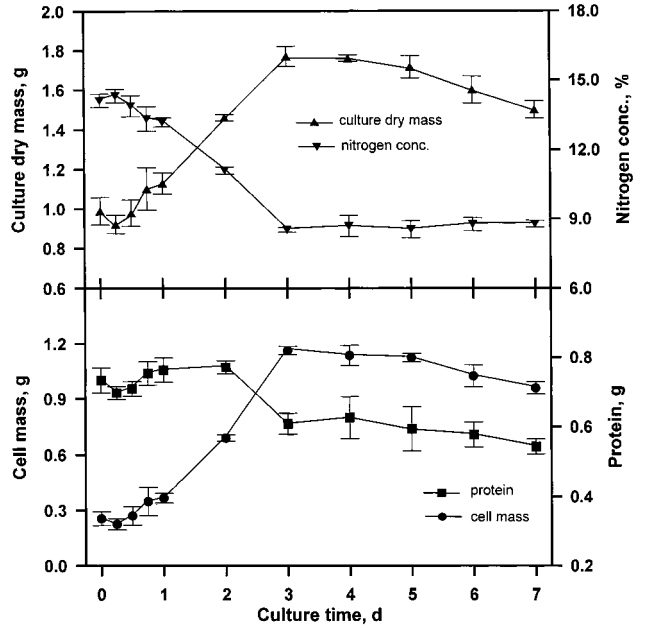

Figure 2. (A, top) Effect of culture age on culture dry mass and associated nitrogen concentrations. (B, bottom) Biomass and CSP profiles calculated from dry mass/nitrogen concentration data.

When the lipid-extracted ground seed was reconstituted with a crude lipid fraction to concentrations present in whole seed, toxin production was restored to the levels observed in ground whole seed (Table 1).

The timed study utilizing cottonseed storage materials in a chemically defined basal medium demonstrated a number of metabolic phenomena. Aflatoxin B_1 concentrations increased from day 1 and peaked at day 5, followed by a decrease through the remainder of the 7 day incubation period (Figure 1). The reducing sugar concentration rapidly increased from 6 h through 18 h, followed by a rapid decrease until day 2, after which it remained stationary during the remainder of the fermentation period (Figure 1). The pH of the culture medium gradually decreased until day 5 and then began a rapid, linear increase (Figure 1). Rapid biomass production was observed from day 1 to day 3, followed by a stationary phase (Figure 2). Cottonseed storage protein concentrations gradually decreased throughout the fermentation period.

Saccharide levels varied considerably, depending upon the compound in question. Raffinose concentrations rapidly decreased from time 0 until day 1 and disappeared entirely by day 2 (Figure 3). Maximum melibiose concentrations occurred on day 1 and dropped to essentially zero over the next 2 days (Figure 3). Low concentrations of fructose were detected up to day 1, but disappeared by day 2. Glucose concentrations increased until peaking at day 6 (Figure 3). Galactose concentrations remained low throughout the fermentation period (data not shown).

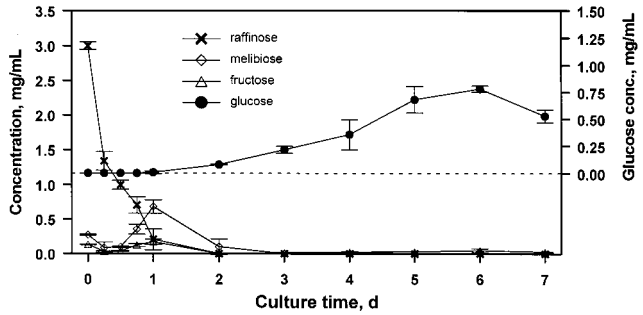

Figure 3. Carbohydrate concentration profiles of *A. flavus* cultures grown for 7 days in a medium containing raffinose, triglycerides, and CSP. Concentrations are expressed in mg/mL of culture medium.

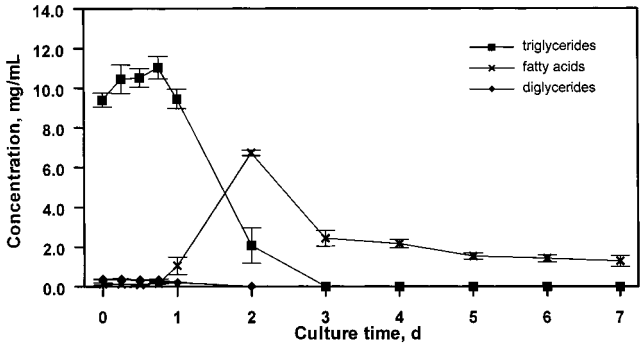

Figure 4. Lipid concentration profiles of *A. flavus* cultures grown for 7 days in a medium containing raffinose, triglycerides, and CSP. Concentrations are expressed in mg/mL of culture medium.

Triglyceride concentrations remained relatively stable for the first 18 h of fermentation and then rapidly decreased to zero by day 3 (Figure 4). Free fatty acid concentrations rose after 18 h to peak at day 2 and then substantially decreased (Figure 4). The distribution of individual fatty acids (linoleic, oleic, palmitic, and stearic acids) was essentially the same as observed in the general cottonseed triglyceride population. The dynamics of the concentration profiles were identical for all of the fatty acids released during triglyceride hydrolysis (Figure 5).

Levels of the sugar alcohols erythritol (4 carbon) and arabitol (5 carbon) gradually rose and declined, but never exceeded 0.5 mg/mL (Figure 6). Mannitol concentrations, on the other hand, rapidly rose to a peak at day 3 and then fell during the remainder of the fermentation period (Figure 6). The trehalose concentration profile closely paralleled those of erythritol and arabitol (Figure 6).

DISCUSSION

Cottonseed storage lipids (either crude or refined) clearly can support fungal growth and aflatoxin production, when present as the sole carbon source. However, aflatoxin production was considerably lower (1/10) in the

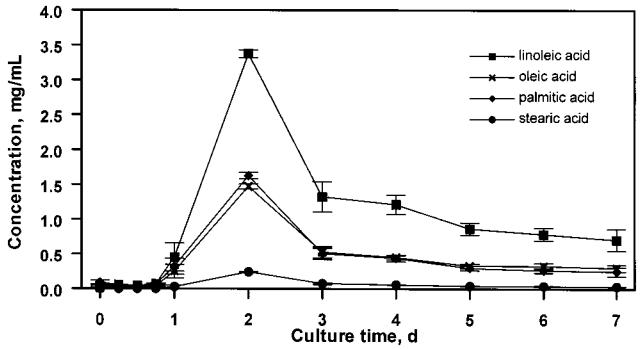

Figure 5. Fatty acid concentration profiles of *A. flavus* cultures grown for 7 days in a medium containing raffinose, triglycerides, and CSP. Concentrations are expressed in mg/mL of culture medium.

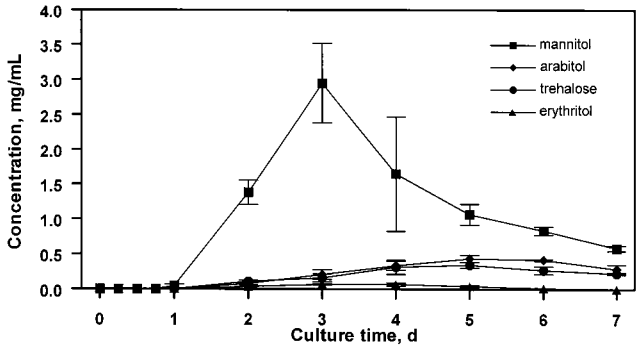

Figure 6. Sugar alcohol concentration profiles of *A. flavus* cultures grown for 7 days in a medium containing raffinose, triglycerides, and CSP. Concentrations are expressed in mg/mL of culture medium.

lipid media than in the reference medium containing raffinose as a carbon source. Removal of lipids from ground whole cottonseed significantly reduced that substrate's potential for aflatoxin production (Table 1). Further, reconstitution of the lipid-extracted ground whole seed with the crude cottonseed lipid preparation restored the ability of that substrate to support toxin production to its original levels (Table 1). It, therefore, seems likely that cottonseed lipids partially support aflatoxin biosynthesis during cottonseed infection by *A. flavus*.

A. flavus clearly has substrate preferences when grown in media with multiple carbon sources. This was evident in media containing the three major cottonseed storage components (raffinose, triglycerides, and CSP). Initial, rapid disappearance of raffinose from the medium indicates selective utilization of carbohydrate as a carbon source (Figure 3). Invertase apparently initially removed fructose from raffinose (Boddy et al., 1993), as shown by initial (day 1) low fructose concentrations and much higher melibiose concentrations. Melibiose was then rapidly assimilated (Figure 3). One breakdown product of melibiose, galactose, was never detected at high concentrations. It was apparently rapidly converted to fungal products. Since the analysis procedures utilized included extraction with acetone, fungal membranes were ruptured. Thus, the metabolite concentrations reported in this study represent a summation of intra- and extracellular levels.

Selective utilization of carbohydrate substrates appears to drive initial biomass production, as well as aflatoxin production (Figures 1−3). Maximum biomass production occurred from day 1 to day 3 of the fungal fermentation. Hydrolysis of triglycerides was not observed until the initial supply of raffinose was nearly exhausted. Fatty acid release from triglycerides peaked during the early period of aflatoxin production, with fatty acid catabolism occurring only after initiation of aflatoxin production. The concentration profiles of all observed released fatty acids paralleled each other, suggesting that the fungus did not preferentially utilize any specific fatty acid as a carbon source (Figure 5). Increases in glucose concentrations from day 2 through day 6 suggest *de novo* generation of glucose. Gluconeogenesis is most likely being driven by fatty acid catabolism (Figures 3 and 4). Carbohydrate is required for cell wall synthesis, and this metabolic requirement is a major sink for the glucose derived from gluconeogenesis. A role for gluconeogenesis in converting fatty acids to aflatoxins has not been previously suggested. Therefore, gluconeogenesis may be required for production of aflatoxin from fatty acids. Interestingly, a hexose transport gene is contained within the aflatoxin biosynthesis gene cluster (Yu et al., 2000). Such a requirement may contribute to the relatively low yields of aflatoxins from triglycerides.

Arabitol and trehalose are known fungal storage metabolites (Carlile and Watkinson, 1994). The concentration profiles of these two products were similar to each other, but markedly different from that of mannitol. The mannitol concentration (increasing phase) closely paralleled biomass production until day 3 (Figure 6). Thus, the role mannitol plays during growth and aflatoxin production may differ from that of the fungal storage metabolites. Mannitol concentrations drop sharply after the cessation of rapid growth, while the glucose pool is still increasing. Thus, fungal requirements for mannitol may be temporary. Mannitol may be involved in osmoregulation or other water relations (Carlile and Watkinson, 1994), and it has recently been implicated as a protectant from both oxidative and osmotic stress (Shen et al., 1997). The protectant properties of mannitol may facilitate successful crop infection and maintenance of cellular integrity during aflatoxin biosynthesis.

The pH profile throughout the 7 day fermentation period appears to reflect a number of metabolic turn-of-events being experienced by the fungus. The initial pH drop corresponds generally with the period of active aflatoxin production. This phenomenon is consistent with a pH/aflatoxin production relationship which has previously been observed in *A. flavus* (Cotty, 1988) and *A. parasiticus* (Keller et al., 1997). After 5 days, the medium pH began a steep rise. This change occurred after the carbohydrates and triglycerides had been hydrolyzed and fatty acid catabolism was essentially finished. At this point, the only seed reserve material remaining was CSP. Past studies have demonstrated that *A. flavus* utilization of CSP as a sole carbon and nitrogen source results in a concomitant rise in pH (Mellon and Cotty, 1998b), presumably due to the generation of ammonia (Carlile and Watkinson, 1994).

The biomass estimation yielded only qualitative data because of several assumptions implicit in the analysis. The major problem is the assumption of complete separation of cottonseed lipids and CSP, which is difficult to achieve. In addition, it is likely that cell wall nitrogen content is, to a degree, variable, especially since the composition of the growth media changes over the course of the fermentation. However, because of the

large difference in nitrogen content between the two components, this method was clearly able to distinguish the major fungal growth phase (Figure 2B).

The selective use of raffinose by the fungus to drive initial biomass and aflatoxin production suggests a possible strategy for partial control of aflatoxin contamination of cottonseed. Reduction of raffinose levels in cottonseed, by either traditional breeding or genetic engineering techniques, may reduce the susceptibility of cottonseed to aflatoxin contamination. Given that cottonseed storage lipids can support aflatoxin production, albeit at considerably lower levels than carbohydrate substrates, this strategy may not represent the total answer, but it could be an integral component of a practical solution. Whether reduced levels of raffinose might affect other quality parameters of cottonseed products would have to be determined by further investigation.

ABBREVIATIONS USED

CSP, cottonseed storage protein.

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