Reducing Sugars and Minerals from Lint of Unopened Cotton Bolls as a Substrate for Aflatoxin and Kojic Acid Synthesis by *Aspergillus flavus*

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We thank T. J. Jacks for freeze-dried cotton bolls, B. Piccolo for elemental measurements, and F. W. Parrish for total carbohydrate analyses.

Accepted for publication 12 January 1983.

ABSTRACT

Lee, L. S., Conkerton, E. J., Ehrlich, K. C., and Ciegler, A. 1983. Reducing sugars and minerals from lint of unopened cotton bolls as a substrate for aflatoxin and kojic acid synthesis by *Aspergillus flavus*. Phytopathology 73:734-736.

Invasion of developing cotton (*Gossypium hirsutum*) bolls by *Aspergillus flavus* and subsequent formation of aflatoxins in cottonseed within such bolls is predicated on growth of the fungus on the lint. Unopened cotton bolls in the field and a water extract made from lint of unopened cotton bolls were evaluated as substrates for mycelial growth and synthesis of kojic acid and aflatoxin by *A. flavus*. Solids from the water extract were 47.3% carbohydrate and 6.96% protein that was partly peroxidase. Reducing sugars, which comprised 76% of the carbohydrate fraction, were a mixture of equal amounts of glucose and fructose. These reducing sugars on lint in unopened cotton bolls decreased from 15% 20 days after flowering to 0.1% 40 days after flowering, but the ratio of glucose to fructose remained constant. All minerals detected on the lint were also detected in the water extract, but the levels were lower in the extract than in the lint. The water extract was an excellent substrate for fungal growth and production of kojic acid and aflatoxin. In contrast, even though the fungus grew well in field-inoculated cotton bolls and kojic acid was produced, no toxin formed on the lint. Toxins were detected only in the seed. The possible involvement of zinc and enzymes present on lint in living cotton bolls in secondary metabolism of *A. flavus* is hypothesized.

Infection of field cotton (*Gossypium hirsutum* L.) following invasion by *Aspergillus flavus* (Lk. ex Fr.) during favorable climatic conditions often results in accumulation of aflatoxin in the seed. Infection by *A. flavus* has been correlated with pink boll worm infestation (2) and can accompany secondary insect invasion (16). *A. flavus* infection is demonstrated by the formation of a characteristic bright greenish yellow (BGY) fluorescence on the lint or fuzz (11). Marsh et al (13) established that BGY fluorescence results from the action of host peroxidase on kojic acid, a secondary metabolite of many *Aspergilli*. In their study on seed from intact loculi, Lee and Russell (8) reported aflatoxin in a few individual seeds with BGY fluorescent lint, but they found no toxin on the BGY lint surrounding these seeds even though fungi cultured from the lint produced toxins. Similar results were reported by Marsh et al (12). Lee and Russell concluded that lint in unopened bolls is an excellent substrate for the elaboration of kojic acid, the precursor of the compound responsible for BGY fluorescent lint, but not for aflatoxin formation. Lack of production of a specific group of secondary metabolites such as aflatoxins by toxigenic fungal strains could be due to the presence of an inhibitor in lint from unopened cotton bolls. In search of such an inhibitor, we compared extracts from unopened loculi (locks) with intact locks in unopened bolls in the field with respect to chemical composition and as substrates for kojic acid and aflatoxin synthesis by *A. flavus*. The sugar and mineral rich extract from cotton locks and fractions made from it alone and as additives to two chemically defined media were similarly compared.

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MATERIALS AND METHODS

Cotton plants (*G. hirsutum* L. 'Deltapine-61') were field grown near New Orleans during the summer of 1981.

Inoculated bolls. Holes, 3–4 mm in diameter, were drilled in the carpel walls of bolls 15, 20, 25, 30, 35, and 40 days after flowering. Spores of *A. flavus* (SRRC 1000) were dusted into each hole with a small brush; then bolls were left to grow for an additional 10 days. At harvest, bolls were dried in paper bags, and the dried lint was removed from the seed. A 1-g subsample of lint from pooled bolls of each age was extracted with methanol, and the fluorescence of the BGY compound was measured at 495 nm in an Aminco Bowman spectrofluorometer. A second 1-g subsample was assayed for kojic acid (13), and a third for aflatoxin B_1 content, as described by Pons et al (15). Excised seeds were assayed individually for aflatoxin B_1 by the method used by Lee and Russell (8).

Noninoculated bolls. Bolls harvested at 5-day intervals starting at 15 days after anthesis and continuing until near dehiscence were dehulled, and the intercarpellary membrane was removed. Loculi from each set were frozen, then dried in vacuo. Seeds were separated from the lint, and the lint was weighed. Reducing sugars were determined on an aliquot of a water extract of weighed dried lint by the method reported by Pettersson and Porath (14).

Minerals were quantitated on a second subsample of dried lint, using an Ortec energy dispersing x-ray fluorescence spectrometer. Additional intact locks including seed were weighed and oven-dried for moisture determination. No attempt was made to determine the moisture content of seeds within the loculi.

Liquid media and water extract. Loculi from many unopened bolls of varying ages were pooled, soaked overnight in deionized water, and filtered, and the filtered extract was freeze-dried. Because an enzyme or enzyme-mineral complex could have been responsible for the inhibitory effect observed by Lee and Russell (8), a fractionation was made to separate large molecules. The freeze-dried material termed WSCL (water-solubles cotton locks) was redissolved in deionized water and dialyzed overnight at 20 C against water (dialysis membrane, 3,500 molecular weight cutoff point). Both the dialysate (D, inside bag) and the dialysis fluid (DF, outside bag) were freeze-dried. On the WSCL, DF, and D fractions, total carbohydrates were measured by the method of Dubois et al (7), and total protein was measured as described by Lowry et al (10). A qualitative peroxidase test was made on a water solution of each freeze-dried material using a saturated water solution of guaiacol and 0.1% hydrogen peroxide (19). Dark red coloration in the solution was used as evidence of the presence of peroxidase. Reducing sugars were determined on WSCL and DF by the method used on the extract of dried cotton lint (14).

High pressure liquid chromatography (HPLC) with a Waters instrument fitted with an anion exchange carbohydrate column (Bio-Rad Aminex-HP X-87) and a refractive index detector was used to determine the ratio of reducing sugars in WSCL and DF. The analyses were done at 64 C with deionized HPLC water as the elution solvent at a flow rate of 0.04 ml/min.

WSCL, DF, and D were compared directly and as additives to two chemically defined synthetic media, Adyes and Mateles (A&M) (1) and Shih and Marth (S&M) (17), as substrates for fungal growth and for aflatoxin and kojic acid production. Both media contain glucose as the carbon source, have similar mineral contents, and have salts of ammonia, potassium, magnesium, sodium, molybdenum, iron, copper, manganese, and zinc. S&M also contains glycine and glutamic acid. Based on the sugar content of WSCL and DF, varying amounts of these freeze-dried materials were weighed so that 10 ml of media contained 0.5 g of reducing sugars, an amount equal to the sugar content of 10 ml of the two synthetic media. Solutions of WSCL, DF, and D were filtered through a series of Millipore filters from 8 to 0.6 μ, followed by sterile filtration through a 0.45 Nalgene filter unit. Ten milliliters of each medium contained in 50-ml Erlenmeyer flasks was inoculated with spores from cultures of *A. flavus* SRRC 1000, an aflatoxin B_1 and B_2 producer. Triplicate flasks inoculated for each treatment were incubated at 27 C for 7 days with continuous shaking on A&M media or no shaking on S&M media. The technique reported by Bennett et al (4) was used to extract and quantitate aflatoxins both from mycelia and the medium from two flasks. Kojic acid was measured on a 1-ml aliquot of media removed from two flasks of each media 3 days after inoculation (13). The third flask from each regimen was used for a dry weight determination of the mycelial mat.

RESULTS AND DISCUSSION

Sugar and moisture content of noninoculated bolls decreased with boll maturity although the rates of decrease were not the same. Moisture content decreased from 81.8% 15 days after flowering to 53.9% 40 days after flowering, with the greatest change between 30 and 35 days. Total reducing sugars decreased from 15% of the dried lint from bolls harvested 15 days after flowering to 0.1% on 40-day bolls. As with moisture, the greatest change in sugars occurred between 30 and 35 days from flowering. Sugars on lint were 20 times less on bolls harvested 35 days after flowering (0.2%) than on those harvested 30 days after flowering (4.3%). HPLC determination of sugars from water extracts of lint from all bolls showed that reducing sugars were glucose and fructose in equal amounts. No other sugars were detected. This ratio of glucose to fructose was the same irrespective of when locks were harvested.

Results of our study differ from those of Conner et al (5) who reported glucose, fructose, and sucrose in acetone extracts of cotton locks. The water treatment we used extracted substances only from the outer surface of locks, whereas the acetone extraction of Conner et al would have dissolved some constituents close to the seed and perhaps from the seed coats.

Unlike moisture and sugar content, not all minerals decreased with boll maturity. Our results were similar to those of Leffler and Turbertini (9). Magnesium, calcium, aluminum, phosphorus, silicon, and potassium were determined in milligrams per gram of lint (range, 13.0 for potassium to 0.3 for phosphorus), and manganese, copper, iron, nickel, and zinc in micrograms per gram (range, 287.8 for iron to 0.9 for nickel). Minerals measured in parts per thousand (mg/g) generally decreased with boll maturity, but no such decrease was observed in those measured in parts per million (μg/g). Zinc levels were highest (33.8 μg/g) in locks harvested 15 days after flowering and lowest (15.3 μg/g) in locks harvested 25 days after flowering. The average zinc level was 22.7 μg/g of dried lint.

Because minerals were comparable in locks from bolls harvested at all ages after flowering and because the ratio of glucose to fructose was constant in extracts of locks of all ages, the water extract made to test laboratory production of kojic acid, BGY fluorescent material, and aflatoxin was made from locks harvested at all ages from flowering and pooled. Nearly 15 g of the WSCL material was recovered from the freeze-dried water extract from 700 g of freshly harvested cotton locks. Results of total carbohydrate and protein analyses were: WSCL, 47.3% carbohydrate and 6.96% protein; DF, 55.6% carbohydrate and 7.60% protein; and D, 22.8% carbohydrate and 4.52% protein. Reducing sugars comprised 82% of the carbohydrate from DF and 76% of WSCL. D contained high molecular weight compounds, some of which were protein. Surprisingly, the percent protein in D was not greater than that of WSCL from which it was separated. However, the test (10) depends on phenolic hydroxyl groups, and the results probably reflect protein plus nonprotein phenolic compounds. The high protein values in WSCL and DF could be due to interference by such phenolics. Peroxidase activity was detected in WSCL and D but not in DF.

Aflatoxins were detected in the mycelial mat and also in the media of all substrates except D, which showed no fungal growth (Table 1). Since D was largely protein or other large molecular weight material, there were insufficient sugars and minerals present to support growth and secondary metabolism. Mycelial growth

TABLE 1. Aflatoxin and kojic acid production by *Aspergillus flavus* on cotton lint extract, fractions of cotton lint extract, Adye & Mateles (1) medium (A&M), Shih and Marth (17) medium (S&M), and lint extract and fractions of lint extract added to A&M and S&M

Substrate	Mycelial dry wt. (g)	Aflatoxin		Kojic acid media (ng/10 ml)[a]
		Mycelia (ng/g)[a]	Media (ng/10 ml)[a]	
WSCL[b]	0.38	80	120	620
DF[c]	0.32	60	130	760
D[d]	0.04	ND[e]	ND	ND
S&M	0.24	80	90	260
S&M + WSCL	0.41	60	100	520
S&M + DF	0.36	40	100	510
S&M + D[f]	0.21	40	60	200
A&M	0.36	75	90	260
A&M + WSCL	0.32	70	80	530
A&M + DF	0.31	70	80	550
A&M + D[f]	0.28	50	60	210

[a] Average of determinations from duplicate flasks. Variation in aflatoxin content ± 20%; kojic acid ± 25%.
[b] Water solubles from cotton locks.
[c] Dialysis fluid from WSCL.
[d] Dialysate from WSCL.
[e] None detected.
[f] 0.05 g of D added.

appeared greater on DF and WSCL than on S&M, yet toxin production was comparable. Kojic acid production was greater on WSCL and DF than on either of the two synthetic media. Zinc in WSCL was twice that in A&M. Barham and Smits (3) reported that zinc enhances kojic acid production. This difference in zinc content could have been responsible for the high levels of kojic acid produced on WSCL and DF and on synthetic media with added WSCL and D. Toxin and kojic acid production were slightly reduced when D was added to both A&M and S&M. The large molecular weight substances present in D could have been enzymes or a complex that had not been deactivated by the extraction and freeze-drying process. This complex could have been inhibitory to secondary metabolism by *A. flavus*.

None of the BGY fluorescent compound was detected in mycelia or in the media from the inoculated liquid media. Peroxide is necessary for conversion of kojic acid to the BGY compound. Marsh et al (13) found that on plates where kojic acid had been separated, spraying with peroxidase-peroxide converted kojic acid to the BGY fluorescent compound. The complex necessary for production of peroxide may not have been extracted from locks by water. This could explain why kojic acid was detected in cultures of WSCL or DF and was not converted to the BGY fluorescing compound.

In sharp contrast to the results of experiments on liquid media, this BGY fluorescent compound was detected on lint from bolls inoculated 20, 25, 30, 35, and 40 days after anthesis. A numerical value could not be assigned because a standard is not available; relative fluorescent intensities were compared on equal weights of lint. Comparable amounts of the BGY fluorescent compound were formed on locks inoculated 20, 25, and 30 days after flowering; there was less of the BGY fluorescent compound in locks from bolls inoculated 35 and 40 days after flowering, and bolls inoculated after 15 days fell from the plant without further development.

All minerals detected on lint were also detected in the water extract from the lint. Zinc levels were higher (23 ppm) on lint than in liquid media (WSCL, 8 ppm; A&M, 4 ppm). Zinc concentration might well be a factor limiting the production of toxin on cotton lint. All levels of zinc detected in lint were well above 10 ppm. Davis et al (6) reported an increase in fungal growth and a decrease in toxin productions when levels of zinc were increased from 2 to 4 ppm. The level of zinc in cotton locks could then be inhibitory to toxin formation and concomitantly promote fungal growth and synthesis of kojic acid. Zinc could be complexed, possibly with an enzyme, so that solubility in water was difficult. Measurements of zinc on dried lint from cotton locks following water extraction indicated that not all zinc was water extracted. Although zinc is not necessary for conversion of kojic acid to the BGY fluorescent compound (13), a zinc complex could catalyze the conversion. No kojic acid per se could be detected on cotton lint. In sharp contrast to the results on liquid media, kojic acid in the boll is apparently converted immediately to the BGY fluorescent compound by the action of the peroxidase-peroxide from the lint.

Another significant difference between field and laboratory experiments is in toxin formation. In experiments in which WSCL was inoculated and incubated in the laboratory, aflatoxin and kojic acid were produced in the same media (Table 1). In field-inoculated cotton bolls, no kojic acid or toxin were detected. Kojic acid produced by the fungus is converted to the BGY fluorescent compound, and toxin formation does not occur until the fungus invades the seed. Our results parallel those of Sun et al (18) who reported optimum production of toxin in seed of bolls inoculated 30 days after flowering. Reducing sugars in such bolls comprised 4.3% of the lint, moisture content was 80%, and zinc was 21.3 μg/g.

Our study indicates that substances inhibitory to aflatoxins synthesis were not water extracted from the lint from unopened cotton bolls. The cotton boll must contain, rather than a specific inhibitor, a milieu in which many components interact to support fungal growth and kojic acid production by *A. flavus*. Some of these components interact to convert kojic acid to the BGY fluorescent compound, and other (or the same) components apparently suppress aflatoxin synthesis. Zinc could be an integral part of these systems.

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