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Substrate Utilization by *Aspergillus flavus* in Inoculated Whole Corn Kernels and Isolated Tissues

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Utilization of the major corn (*Zea mays*) reserve materials (free saccharides, starch, triglycerides, and zein) was monitored during infection of detached kernels by *Aspergillus flavus* (*A. flavus*) over a 12-day period. Inoculated whole kernels were compared to noninoculated kernels. Concentrations of sucrose and raffinose in inoculated seed decreased to nearly zero at 6 days, whereas concentrations of these saccharides in noninoculated seed dropped at a considerably slower rate, and significant levels remained at the end of the incubation period. Triglyceride concentrations remained unchanged in the noninoculated seed but dropped continuously after 2 days in the inoculated seed. Starch and zein concentrations did not change during the 12-day incubation period. Aflatoxin B_1 was first detected after 2 days and increased to about 20 μg/g (20,000 ppb) after 12 days. Very low aflatoxin concentrations were detected in the noninoculated seed. Significant concentrations of erythritol, arabitol, and mannitol were produced during infection, with peak concentrations occurring at 8 days. Whole seed and germ tissue appeared to support good fungal growth and aflatoxin production, whereas ground tissues and endosperm did not. *A. flavus* preferentially utilized saccharides as initial carbon substrates followed by triglycerides. When invading nonwounded corn kernels, the fungus selectively targets the germ tissue where these materials are localized in the highest concentrations.

KEYWORDS: **Aflatoxin; *Aspergillus flavus*; cornstarch; raffinose; sucrose; sugar alcohols; triglycerides; *Zea mays*; zein**

INTRODUCTION

Corn (*Zea mays*) is one of several oilseed commodities (e.g., cotton, peanuts, tree nuts) that are subject to infection by the saprophytic fungus *Aspergillus flavus* (*A. flavus*) under certain environmental conditions. Fungal infection can lead to contamination with the potent carcinogenic mycotoxin aflatoxin, resulting in diminished crop values.

Starch, lipids, and zein, a member of the prolamin class of storage proteins, comprise the principal reserve materials of corn. Corn reserve materials are localized in the endosperm and seed germ. Corn contains 70−75% starch, 5% lipids (triglycerides), and 11% protein by weight (*1*). Zein comprises 50% of the total protein component in the mature seed. In addition, the germ contains significant concentrations of free sugars, principally in the form of sucrose. Corn kernel reserves represent significant carbon and nitrogen resources potentially available during seed infection by *A. flavus*.

In culture, *A. flavus* selectively uses saccharides for initial biomass and aflatoxin production when available. This phenomenon occurs in media containing the major reserve materials of cotton in ratios similar to those found in mature cottonseed where raffinose is selectively consumed (*2*). However, in complex nutrient mixtures, aflatoxin production is not solely driven by carbon source. Oilseed storage proteins are known to stimulate aflatoxin production by *A. flavus*. Presence of zein as a supplement in chemically defined culture media stimulates aflatoxin production up to 10-fold and its presence as a sole nitrogen source, together with a rapidly accessible carbon source, stimulates aflatoxin production by 8-fold over reference cultures grown on a chemically defined medium (*3*). Similarly, the presence of raffinose as a carbon source and cottonseed storage protein as a sole nitrogen source stimulates aflatoxin production 4-fold over chemically defined growth media (*4*). A hexose transport gene is associated with the aflatoxin biosynthesis cluster (*5*). Activation of this gene by a suitable saccharide may be required to up-regulate aflatoxin production. Proteinaceous nitrogen sources can also serve to stimulate aflatoxin production (*3, 6*).

If saccharide substrates are not available, then *A. flavus* is not as selective in utilization of complex substrates. The fungus concurrently uses both starch and triglycerides in a medium simulating corn kernels until glucose concentrations attain

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sufficiently high levels (*7*). Triglyceride hydrolysis then declines until glucose concentrations return to a relatively low level. Thus, *A. flavus* appears to be metabolically versatile when confronted with complex nutrient mixtures.

To investigate the utilization of corn reserve materials by the fungus *in planta* during infection and to assess the value of model media studies in predicting the course of fungal infection, a timed study of *A. flavus*-inoculated whole corn kernels was undertaken. Since model media work indicated that available saccharides are critical for rapid fungal growth and aflatoxin biosynthesis and removal of lipids from ground cottonseed dramatically lowers the ability of that substrate to support aflatoxin production (*2*), particular interest was focused on fungal utilization of these potential substrates in the whole-kernel system. This seed disease system (detached organ) was used to assess the sequence of substrate utilization with respect to biosynthesis of aflatoxin and other fungus-specific metabolites. The ability of specific corn kernel tissues to support aflatoxin production was also investigated.

MATERIALS AND METHODS

Biological Materials. *A. flavus* AF13 (ATCC 96044) was isolated from soil in southwestern Arizona (Yuma area) and maintained on a 5% V8 vegetable juice (Campbell Soup Co., Camden, NJ) agar medium at 31 °C (*8*). Corn seed was a commercial No. 2 yellow dent hybrid produced in Southern Michigan in the summer of 1998 and was stored dry (9% moisture) in a climate-controlled room (8 °C) until used.

Whole-Seed Incubations. Corn seed (850 g) was divided into two lots. Initial moisture content of this seed was 5.2%, as determined with a moisture balance (Ohaus MB200, Florham Park, NJ). Corn seed was immersed in 70% (v/v) ethanol and shaken vigorously for 1 min to surface-sterilize the kernels. The ethanol was removed, and the seed was allowed to air-dry. Seed was then transferred to sterile one-liter plastic bottles. Sufficient sterile deionized water was added to the control lot to adjust the moisture content to 33%. A conidial suspension of *A. flavus* (6×10^5 spores/mL) was added to the inoculated lot to bring the moisture content of this seed to 33%. The seed was slowly rolled (1.5 rpm) for 4.5 h to allow for uniform distribution of water and fungal conidia. Seed lots were divided into replicates (about 20 g/replicate) and transferred to sterile 250-mL flasks covered with a stopper capable of gas exchange (Bug Stoppers, Whatman, Clifton, NJ). Seed-containing flasks were incubated at 31 °C and sampled at 0, 2, 4, 6, 8, and 12 days (3 replicates per sample period). Fungal growth was terminated at the appropriate time by transferring flasks to a 60 °C forced-air drying oven for 24−48 h. After being cooled to room temperature, seed from each replicate was ground in an analytical mill (RAS Mill, Romer Labs, Inc., Analytical Instruments Division, Union, MO), weighed, and stored in a plastic bag with a desiccant (silica gel).

Kernel Tissue Incubations. Endosperm was isolated by manually dissecting and removing the germ with a scalpel. In some cases, the procedure was performed on dry seed; in others, seed was soaked in cold water (5 °C) for an hour before dissection. Pericarp was not removed from the endosperm tissue before incubation. Germ isolation was accomplished by dissecting dry seed or by a steeping procedure. This steeping procedure consisted of a seed-in-water soaking step, followed by milling steps (blender) to release the germ (*9*). Steep-isolated germ tissue was dried at 50 °C for 24 h.

For each tissue treatment (except ethanol-sterilized seed), 80 g was transferred to a 250-mL Pyrex bottle and subjected to two 20-min autoclave cycles. After cooling, moisture contents of tissues were determined with the moisture balance. Inoculated treatments received 10 mL of an *A. flavus* conidial suspension (1.6×10^6 spores/mL) and additional sterile deionized water to adjust the moisture to 25%. Whole-seed controls received only sterile water. After water addition, corn tissues were rolled (1.5 rpm) for 12 h to evenly distribute water and fungal inoculum. Tissue lots were divided into four replicates and incubated at 31 °C for 5 days. Tissue treatments were dried at 60 °C for 24−48 h following incubation and ground as described above.

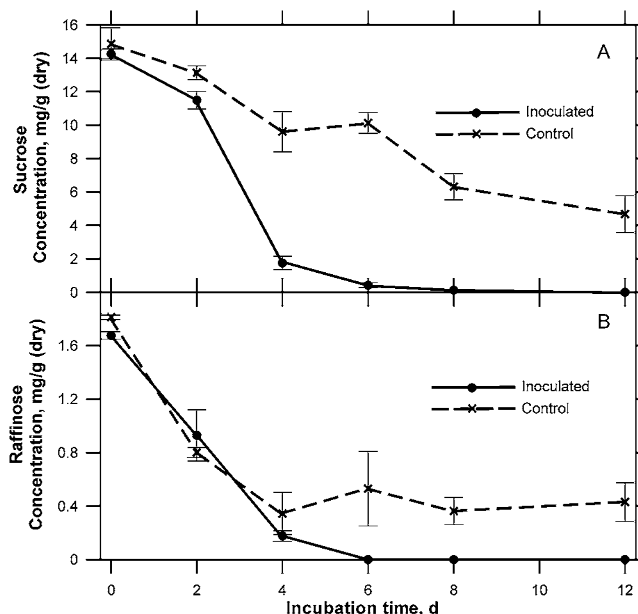

Figure 1. Sucrose (**A**) and raffinose (**B**) concentration profiles of noninoculated and inoculated whole corn kernels. Concentrations are expressed in mg/g seed (dry) ($n = 3$).

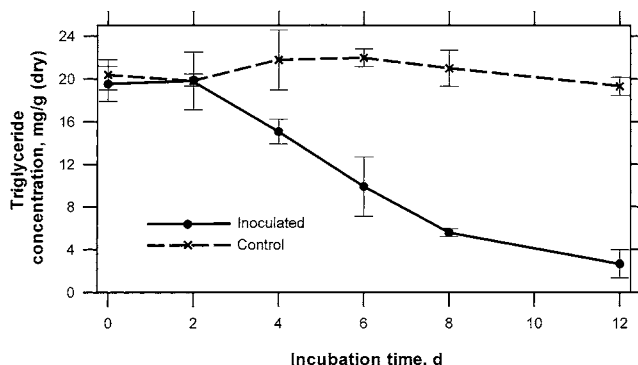

Figure 2. Triglyceride concentration profiles of noninoculated and inoculated whole corn kernels. Concentrations are expressed in mg/g seed (dry) ($n = 3$).

Aflatoxin Analysis. One gram of dried, ground corn seed material was thoroughly shaken in 10 mL of methylene chloride for 1 min, allowed to steep for 5 min, and the process was repeated. Solid material was removed by filtration (gravity), and the extract was transferred to a vial. Sample extracts were spotted beside aflatoxin standards (B_1, B_2, G_1, G_2) on silica gel plates. After development in diethyl ether/methanol/water (96:3:1), aflatoxin B_1 was quantified directly on thin-layer plates by fluorescence densitometry (*10*) (Shimadzu CS-9301PC densitometer, Kyoto, Japan). Samples were concentrated or diluted, as required, to facilitate accurate densitometry.

Lipid/Saccharide Analysis. Saccharide analysis was initiated by adding 0.2 g of ground corn sample to 2.0 mL of deionized water, allowing the mixture to sit for 5 min, and then vortex mixing for 1 min. This soak/mix cycle was repeated twice, and the sample was centrifuged at $1500 \times g$ for 5 min. The supernatant was removed and filtered through a 0.22-μm filter (Millex-GV, Millipore Corp., Bedford, MA). One milliliter of this filtrate was transferred to a Reacti-Vial (Pierce Chemical Co., Rockford, IL) and stored at −20 °C before lyophilization to remove water. The remainder of the water extract was stored at 4 °C for use in the reducing sugar assay (below).

Lipid analysis was initiated by placing 0.5 g of ground corn sample in 2.0 mL of methanol/chloroform (1:1), vortex mixing for 1 min, and then allowing it to sit for 9 min. This mix/soak cycle was repeated twice, and the sample mixture was centrifuged at $1500 \times g$ for 5 min. The supernatant was removed, filtered through a 0.45-μm filter (Millex-

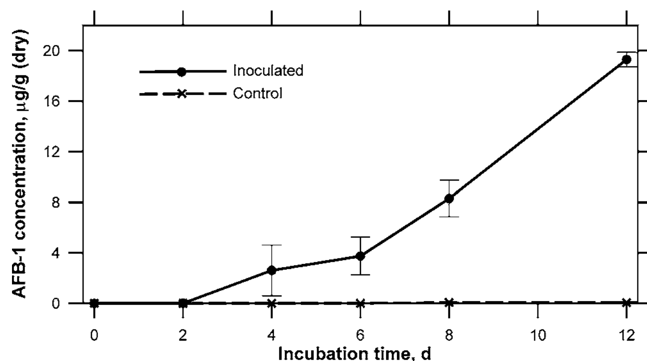

Figure 3. Aflatoxin B_1 concentration profiles of noninoculated and inoculated whole corn kernels. Concentrations are expressed in μg/g seed (dry) ($n = 3$).

HV), and placed in a Reacti-Vial. Then, the pellet (solid sample material) was re-extracted with a 2.0-mL aliquot of methanol/chloroform using the above procedure. The lipid extracts (MeOH/$CHCl_3$) were pooled in the Reacti-Vial, and the solvent was removed under a stream of nitrogen. Samples were stored at −20 °C until analysis.

Dried samples were deriviatized for analysis by gas chromatography. Trimethylsilyl derivatives of sugar and lipid extracts were made by reaction with hexamethyldisilazane (Pierce Chemical Co.) and trifluoroacetic acid in pyridine (*2*). To complete the reactions, samples were heated for 45 min at 60 °C and then allowed to cool. Chromatography was accomplished with a Hewlett-Packard 5890 Series 2 plus chromatograph (Palo Alto, CA) with a DB-5ht silica capillary column (15 m × 25 mm i.d. × 0.1 μm film thickness). Helium was used as the carrier gas with a flow rate of 1 mL/min. The instrument was operated in constant-flow mode with a split detector (1:50 split ratio) and flame ionization detector. The temperature program was: 100 °C for 3 min, 10 °C/min to 150 °C, 5 °C/min to 250 °C, 10 °C/min to 360 °C, and 360 °C for 10 min. Components were identified by comparing the retention times of standards with those of sample peaks; quantification was performed by internal standardization with cholesterol methyl ether. Response factors were measured for all of the detected sugars, sugar alcohols, and fatty acids. Because the procedure separates triglycerides by carbon number (i.e., sum of acyl chain lengths), the quantity of triglycerides was estimated from response factors developed for standards with fixed carbon numbers. Experiments were conducted twice with three replicates. The results reported here are representative of those experiments.

Reducing Sugar Assay. Water extracts remaining after saccharide analysis were analyzed for reducing sugars by the *p*-hydroxybenzoic acid hydrazide method (*11*). Glucose was used as a standard.

Starch Analysis. Corn starch analysis was conducted by polarimetry using the official method (A-20) of the Corn Refiners Association (*12*).

Zein Analysis. Ground corn material (3 g) was added to 10 mL of deionized water, vortex mixed for 1 min, steeped for 5 min, and again vortex mixed for 1 min. This slurry was centrifuged at 1500 × *g* for 5 min. The supernatant was discarded and the pellet was resuspended

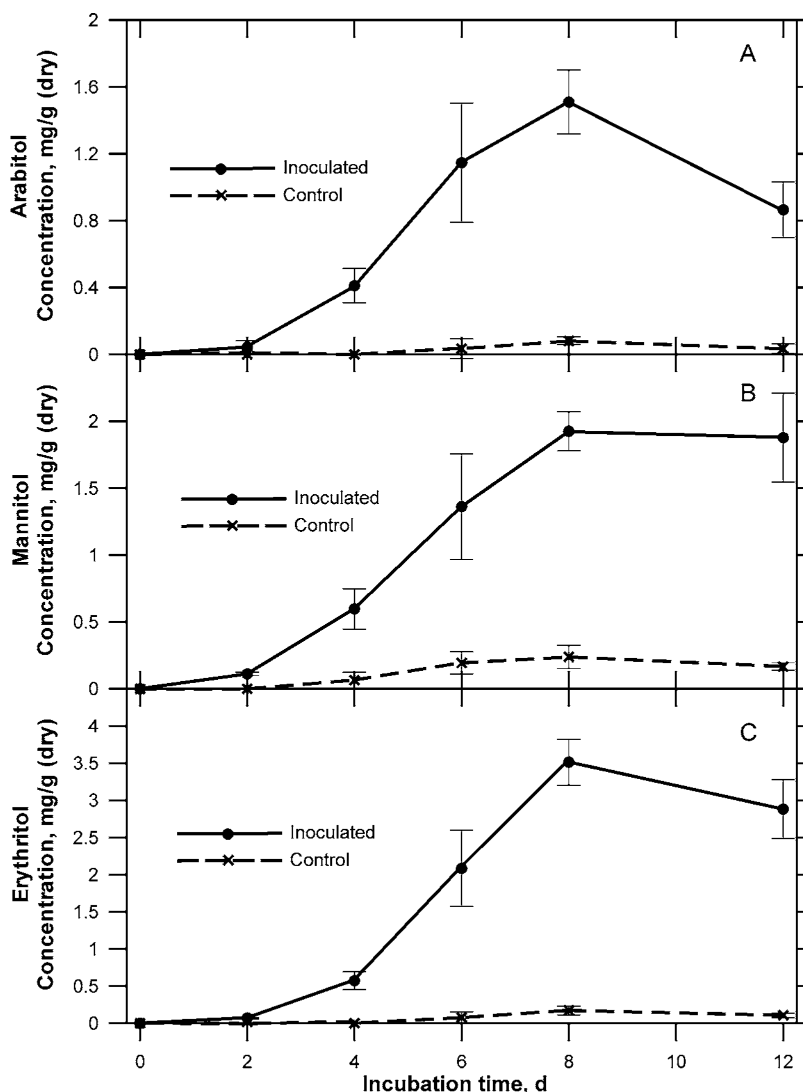

Figure 4. Arabitol (**A**), mannitol (**B**), and erythritol (**C**) concentration profiles of noninoculated and inoculated whole corn kernels. Concentrations are expressed in mg/g seed (dry) ($n = 3$).

Table 1. Corn Substrate and Fungal Metabolite Concentrations in *A. flavus*-inoculated Kernel Tissues

treatment	[AFB$_1$][a]	[sucrose][b]	[triglyceride][b]	[fatty acids][b]	[erythritol][b]
			Whole Seed		
noninoc. (0 d)[c]	0.00	14.9 ± 1.0	20.4 ± 1.4	2.18 ± 0.08	0.00
noninoc. (5 d)	0.00	2.39 ± 0.13	21.1 ± 0.28	0.94 ± 0.051	0.009 ± 0
inoculated (5 d)[d]	22.0 ± 4.55	0.00	4.62 ± 0.28	3.47 ± 0.62	1.69 ± 0.15
EtOH, inoc. (5 d)[e]	27.4 ± 10.6	0.00	4.60 ± 0.63	4.62 ± 1.11	1.07 ± 0.06
ground, inoc. (5 d)[d]	2.06 ± 0.35	0.00	0.802± 0.25	1.42 ± 0.15	0.702 ± 0.19
			Endosperm		
whole, inoc. (5 d)[d]	1.28 ± 0.22	0.00	0.169 ± 0.042	0.757 ± 0.068	0.418 ± 0.049
ground, noninoc.(0 d)[c]	ND[f]	3.22 ± 0.03	0.378 ± 0.266	1.43 ± 0.16	0.045 ± 0.004
ground, inoc. (5 d)[d]	0.019 ± 0.005	0.00	0.011 ± 0.02	0.412 ± 0.037	0.168 ± 0.065
			Germ[g]		
noninoc. (0 d)[c]	ND[f]	0.366 ± 0.036	241 ± 10.5	2.44 ± 0.07	0.00
inoculated (5 d)[d]	0.642 ± 0.24	0.00	143 ± 12.2	148 ± 8.83	0.095 ± 0.007
			Germ + Endosperm[h]		
ground, inoc. (5 d)[d]	0.051 ± 0.017	0.00	1.23 ± 0.14	1.64 ± 0.29	0.331 ± 0.076

[a] Aflatoxin B$_1$ concentrations after 5 days of incubation are expressed as μg/g of seed ($n = 4$) (limit of detection = 1 ng/g). [b] Component concentrations are expressed as mg/g of seed/tissue ($n = 4$). [c] Component concentrations are derived from pre-inoculated tissues (0 time). [d] Seed/tissue was heat sterilized, inoculated with *A. flavus*, and incubated for 5 days at 31 °C. [e] Seed was surface-sterilized with 70% ethanol, inoculated with *A. flavus*, and incubated for 5 days. [f] Not determined. [g] Germ was isolated by steeping seed with water and milling to release tissue. [h] This treatment consisted of 6% germ and 94% endosperm by weight (similar to mature seed); designed to simulate the ratio of these tissues in mature seed.

in water and extracted using the above method. The resultant pellet was suspended in 100 mL of 60% (v/v) aqueous 2-propanol and heated at 60 °C for 30 min with frequent shaking. Solids were removed by gravity filtration, and the aqueous 2-propanol was removed from the resulting zein extracts with a rotary evaporator. Sample residues were dried at 50 °C for 24−48 h and weighed.

RESULTS

A. flavus demonstrated clear substrate preferences in the inoculated corn kernel study. Reducing sugar profiles differed between inoculated and control kernels. Reducing sugar concentrations in inoculated seed rose at a rapid rate over the first 2 days of seed infection (peak of 17 mg glu equiv/g) and then decreased linearly for the remainder of the test (data not shown). Reducing sugar concentrations in noninoculated control seed rose over a period of 8 days before decreasing. Sucrose concentrations rapidly declined in inoculated seed until near depletion after 6 days (**Figure 1A**). Sucrose concentrations decreased over the entire 12-day test in the noninoculated controls (**Figure 1A**). However, significant levels of sucrose remained at the end of the incubation. Raffinose concentration profiles of both inoculated and noninoculated seed demonstrated a significant decrease over the first 4 days of incubation (**Figure 1B**). However, after this point, raffinose levels remained essentially constant in the noninoculated seed but continued a steep decline in the inoculated seed until complete depletion after 6 days (**Figure 1B**). Glucose concentrations varied between 2 and 3 mg/g over the 12-day test period; there were no significant differences between inoculated and noninoculated kernels (data not shown). Triglyceride levels in inoculated and control seed were equivalent until 2 days after inoculation (**Figure 2**). From day 2 to day 8, triglyceride levels in inoculated kernels declined at about 3 mg/g per day. This declining trend continued at a more gradual rate during the remainder of the test until the triglyceride content of infected kernels was only 10% that in noninoculated controls. Triglyceride concentrations remained essentially constant in the noninoculated seed (**Figure 2**).

Utilization of starch and zein during seed infection was not observed during the live kernel study. Starch concentrations (70−72 wt%) did not differ between the noninoculated and inoculated corn seed during the 12-day study (data not shown). Recovered zein concentrations varied from 30 to 45 mg/g of seed sampled in this study. No significant differences between zein levels recovered from noninoculated and inoculated seed were observed (data not shown).

Several fungus-specific metabolites were produced in *A. flavus*-inoculated whole kernels. Aflatoxin B$_1$ was detected at very low levels (<0.06 μg/g) in the noninoculated seed, whereas inoculated seed had significant aflatoxin concentrations after 2 days. Aflatoxin levels continued to increase in the inoculated corn throughout the remainder of the incubation period (**Figure 3**). Several fungal-specific sugar alcohols were produced in inoculated corn kernels. Erythritol (C_4) was produced in the greatest concentrations (**Figure 4C**), but mannitol (C_6) and arabitol (C_5) were also produced in significant concentrations (**Figure 4B** and **4A**). The sugar alcohol levels all peaked at 8 days of incubation. Low levels of these same fungal metabolites were produced in the noninoculated corn kernels and they also peaked at 8 days (**Figure 4**). Kojic acid was also produced in the inoculated seed, though at levels considerably below (peak of 0.1 mg/g at 6 days) the sugar alcohols (data not shown).

The corn kernel tissue study substantiated *A. flavus* substrate preferences demonstrated in the whole-seed data. There was no sucrose remaining in any of the *A. flavus*-inoculated kernel tissues after 5 days of incubation (**Table 1**). Triglyceride concentrations were also considerably decreased in the inoculated kernels compared to whole-seed controls. In germ tissue, where most of the storage lipids are localized, triglyceride concentrations were considerably reduced in inoculated tissues compared to pre-inoculated tissue (zero time control). Total fatty acid concentrations were elevated in the fungal-inoculated seed tissues, especially in the germ (**Table 1**). In general, relatively poor fungal growth (visible) was observed on all treatments comprised of ground seed tissues (e.g., ground whole seed, ground endosperm).

Fungal metabolite concentrations varied considerably in the different corn tissues tested. Maximum aflatoxin B$_1$ levels were observed in the whole-seed treatments (heat and ethanol sterilized, **Table 1**). Lower aflatoxin concentrations were observed in the ground whole seed, whole endosperm, and germ treatments. Very low aflatoxin concentrations were observed

Table 2. Comparison of Cut Germ to Steeped Germ for Initial Component Concentrations and Aflatoxin Production

component	steeped germ[a]	cut germ[b]
A. Noninoculated Tissue		
sucrose concentration[c]	0.201 ± 0.032	75.1 ± 1.96
raffinose concentration[c]	0.06 ± 0.002	7.49 ± 0.48
triglyceride concentration[c]	241 ± 10.5	172 ± 8.26
fatty acid concentration[c]	2.44 ± 0.07	4.21 ± 0.20
B. Inoculated Tissue		
Aflatoxin B_1 concentration[d]	3.48 ± 1.33	36.9 ± 19

[a] Germ was isolated by steeping seed with water and milling to release tissue. [b] Germ was isolated by manually cutting tissue from whole seed. [c] Component concentrations are expressed as mg/g and were determined in noninoculated tissue (zero time control) ($n = 4$). [d] Aflatoxin B_1 concentrations are expressed in μg/g of tissue (dry wt) after 5 days of incubation with *A. flavus* ($n = 4$).

in the ground endosperm and ground germ/endosperm treatments (**Table 1**). Erythritol concentrations were up to 190-fold higher in inoculated corn kernels/tissues compared to noninoculated controls (**Table 1**). Trehalose was also present in inoculated kernels and tissues at levels of up to 0.6 mg/g (whole kernels), depending on how well the fungus grew in a given treatment (data not shown).

In a re-examination of corn germ tissue, germ that had been dissected from dry seed was compared to germ isolated from steeped seed. In comparing concentrations of sucrose and raffinose, noninoculated cut germ tissue contained considerably higher levels of both of these metabolites than steeped germ tissue (**Table 2**). In addition, cut germ tissue, when inoculated with *A. flavus*, supported 10-fold more aflatoxin production when compared to inoculated steeped germ tissue (**Table 2**).

DISCUSSION

The reducing sugar profiles observed during this study may be valid indicators of the different metabolic environments for noninoculated and inoculated seed. The inoculated-seed profile demonstrates an initial increased mobility of saccharide reserves when compared to noninoculated seed. This observation is consistent with increased metabolic activity during microbial stress (*13*). Reduced sugar levels declined after day 2 as fungal utilization took place. The noninoculated seed profile shows a general upward trend in reducing sugar concentrations. These data may reflect increased mobility of sugar reserves taking place in viable seed as pregermination processes occur. It could also reflect the effects of slowly developing internal contamination. The data set does not allow easy discrimination between these two hypotheses. This study utilized ethanol-sterilized seed instead of heat-sterilized seed in order to observe an in vivo plant−fungal interaction.

Fungal substrate preferences observed during the whole-corn-kernel study are consistent with those observed in model media. Saccharide utilization drives both initial biomass production and aflatoxin biosynthesis. In this study, both sucrose and raffinose reserves were essentially exhausted after 6 days of incubation (**Figure 1**). A similar phenomenon is observed in media simulating cottonseed in which raffinose is completely depleted prior to fungal utilization of other carbon sources (*2*). A wide range of saccharides is known to support aflatoxin biosynthesis in defined media (*14*). In the kernel tissue study, sucrose concentrations after 5 days of incubation were zero (**Table 1**). Once available saccharide reserves have been exhausted, the fungus utilizes host lipid reserves. Triglyceride levels remained unchanged in the noninoculated seed but dropped in the inoculated seed from 20 to 3 mg/g from day 2 to day 12 (**Figure 2**). Again, an analogous situation occurs in media simulating cottonseed where triglycerides are utilized by *A. flavus* after raffinose depletion (*2*). Apparently, media formulated to simulate crop plants provide useful models to study resource utilization and aflatoxin biosynthesis in vitro. In the kernel tissue study, triglyceride levels were reduced in inoculated tissues but not entirely depleted (**Table 1**). *A. flavus* does not appear to favor the use of storage proteins as a carbon substrate. In the current study, fungal inoculation did not lead to reductions in zein levels. In media simulating either cottonseed or corn, the storage protein component was the last to be utilized by the fungus (*2, 7*). Indeed, when *A. flavus* is forced to use storage proteins as a sole carbon source, it does not grow well and aflatoxin production is low (*3*).

Ground corn seed or tissues support reduced growth and aflatoxin biosynthesis by *A. flavus* compared to whole kernels. This is contrary to what was expected on the basis of increased fungal access to the major carbohydrate reserve material, starch. The fungus did not grow as well on ground whole seed, whole endosperm tissue, or ground endosperm as on germ and whole seed. It is possible that the hygroscopic nature of starch granules impedes water acquisition by the fungus, thus slowing growth. In addition, homogenization of corn kernel tissues by grinding may expose the fungus to inhibitory materials that are normally compartmentalized or not encountered during the seed invasion process. Corn seed is known to contain a number of fungal and aflatoxin biosynthetic inhibitory materials (*15, 16*).

Fungal metabolic activities occur at a slower rate in the whole-seed and tissue systems compared to model media systems. An incubation period of 2 days was required for initiation of active acquisition of carbon resources (e.g., sucrose, triglycerides) in whole kernels. Presumably, this period is required by the fungus to breach mechanical defense barriers in the seed, including the pericarp and a cutin layer, or for migration through the tip cap. Production of fungus-specific metabolites, including aflatoxin, was also delayed in whole kernels as compared to media simulating cottonseed or corn. This is probably attributable to time required for sufficient seed penetration and fungal growth to support biosynthetic processes.

A number of differences occurred in the fungal-specific metabolite profiles of inoculated whole corn as compared with model media. In media simulating either cottonseed or corn, aflatoxin concentrations peaked at 4−5 days of incubation (*2, 7*), whereas in inoculated whole corn kernels, aflatoxin concentrations were still increasing after 12 days (**Figure 3**). In the model media systems, mannitol was the sugar alcohol produced in considerably greater concentrations than arabitol or erythritol, whereas in inoculated whole corn, erythritol concentrations were double those of mannitol or arabitol. Thus, all these sugar alcohols appear to have some importance in *A. flavus* biosynthetic metabolism. These fungal metabolites may function as osmoregulatory agents (*17*) or as protectants from both oxidative and osmotic stress (*18*). These sugar alcohols could be viewed as diagnostic indicators of fungal growth. Levels of all of these sugar alcohols were zero in sterile corn seed (zero time control). Their occurrence at low levels in the noninoculated seed at later incubation periods would suggest low-level internal infection of kernels surface-disinfected with ethanol. Indeed, some replicates of noninoculated seed did develop visible growth of *A. flavus* toward the end of the incubation period (8−12 days).

Occurrence of some of the other fungal-specific metabolites was consistent with observations in model media systems. Trehalose was produced in both inoculated whole kernels and tissues at similar levels to those observed in model media (*2, 7*). Trehalose levels were highly correlated with growth of the fungus in a given treatment. Kojic acid was produced in inoculated whole kernels (timed study), though at levels about one-tenth those observed in model corn media (*7*). Thus, with respect to these fungal metabolites, model media appear to simulate conditions in *A. flavus*-infected oilseed crops.

A comparison of the cut germ tissue to the germ tissue isolated by a steeping procedure revealed the latter to be an artifact of isolation. The germ tissue initially utilized in the tissue experiments was steep-isolated and resulted in good fungal growth but poor aflatoxin production. Subsequently, analysis of cut germ tissue derived from noninoculated seed revealed saccharide concentrations (sucrose, raffinose) over a 100-fold higher than present in steeped germ tissue (**Table 2**). In addition, aflatoxin production was 10-fold higher in cut germ tissue than in steeped germ tissue (**Table 2**). Known metabolic phenomena in *A. flavus* would explain these results. While a triglyceride carbon substrate supports good biomass production, aflatoxin production is roughly one-tenth that of cultures provided with an equivalent mass of saccharide (sucrose, glucose, etc.) (*2*). Thus, steep-derived germ tissue supported good fungal growth but reduced aflatoxin production due to high triglyceride and low saccharide concentrations. Cut germ tissue supported increased aflatoxin production due to the increased initial saccharide concentrations. *A. flavus* appears to require a readily accessible saccharide to initiate rapid, maximum aflatoxin biosynthesis.

The observation that aflatoxigenic fungi selectively invade the germ tissue of corn kernels has been made in other systems. A study that focused on resistance mechanisms in maize kernels found that the viability of the embryo (in germ) is more important to seed resistance than fungal access to the endosperm via cracks (*19*). Another system utilized colored mutants of aflatoxigenic fungi to localize infection in kernel tissues. These mutants, presumed to retain wild-type infection mechanisms, selectively infected the embryo (germ) and aleurone, but not the endosperm (*20*), in mature, nongerminating seed. However, in germinating corn seed, the aflatoxigenic mutant fungi targeted the endosperm and not the embryo (*20*). These results are consistent with what has been observed regarding *A. flavus* substrate preferences in model media systems, as well as the current whole-kernel study. Since free saccharides and reserve lipids are localized in the germ (embryonic tissues), the fungus actively seeks these tissues in mature seed for easily accessible carbon substrates. Likewise, in the whole-kernel study, *A. flavus* selectively utilized sucrose and raffinose reserves first before switching to lipid reserves. Thus, in the case of mature corn kernels (nongerminating), *A. flavus* appears to selectively seek the germ tissue due to the presence of high concentrations of free saccharides and triglycerides. In the case of germinating corn seed, endogenous host α-amylase is hydrolyzing starch reserves to release glucose, thus providing an accessible saccharide (glucose) within the endosperm that is targeted by the fungus.

A body of work suggests that fungal α-amylase-catalyzed hydrolysis of starch in the endosperm is a major mechanism for *A. flavus* infection and aflatoxin production in corn (*21*, *22*). In addition, genetically modifying corn to express an inhibitor of fungal α-amylase has been suggested as a viable aflatoxin contamination control strategy (*23*). The data obtained from model media studies, the previously cited maize kernel work, and the current whole-kernel study do not support this hypothesis. This whole-kernel study employed nonwounded kernels at 25−30% moisture levels. Results indicate that *A. flavus* selectively utilized available saccharide reserves, followed by triglyeride reserves in the germ tissue. In addition, starch concentrations in both inoculated and noninoculated kernels remained at initial levels throughout the study period. Thus, *A. flavus* did not appear to use starch reserves in the endosperm at all. Glucose levels of both inoculated and noninoculated corn kernels were not significantly different (2−3 mg/g) throughout the test period, substantiating the observation that starch was not being used as a carbon substrate by the fungus. Of course, if saccharides are not readily available and easy access to starch exists (e.g. wounded kernels) along with ample moisture levels, *A. flavus* will certainly produce α-amylase to hydrolyze starch (*7*). However, given that field conditions generally are closer to conditions used in this whole-kernel study, an aflatoxin contamination control strategy based on inhibition of fungal α-amylase digestion of endosperm starch resources would not appear to be entirely productive.

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