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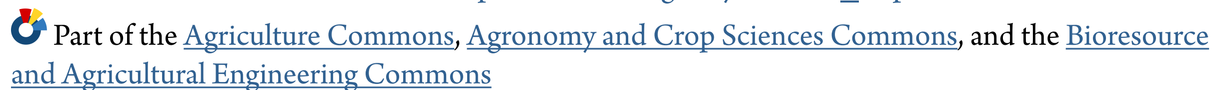
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Production of Aflatoxin on Damaged Corn Under Controlled Environmental Conditions

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ABSTRACT

AFLATOXIN B_1 production by *Aspergillus flavus* (NRRL 6432) was determined on physically damaged corn stored at 35,25 and 20°C and at relative humidities of 97 and 92%. Production at lower relative humidities of 80 and 85% were also tested at 25°C. Production lag times were significantly different for all test conditions. Lag times ranged from 46 h at 97% relative humidity and 35°C to 173. 8 h at 20°C and 92% relative humidity. Aflatoxin B_1 concentration doubled every 6 to 10 hours after a level of 10 ppb had been reached. Production rate constants were not significantly different between temperatures at 97% relative humidity; however, significant differences in the production rate constant were observed between 97 and 92% relative humidities evaluated at 25°C and between 25 and 35°C evaluated at 92% relative humidity.

INTRODUCTION

Aflatoxins are metabolites formed by toxigenic strains of two ubiquitous molds, *Aspergillus flavus* and *Aspergillus parasiticus.* These compounds are only a few of over 120 mycotoxins produced by fungi (Nichols, 1983); however, they are the most well-known and the most studied.

Aflatoxin in corn is a major problem and has caused losses in millions of dollars by yielding grain that is unmarketable and toxic to animals (Payne, 1983). If corn is stored at a high moisture content, dangerous levels of aflatoxin can develop as a result of *A. flavus* growth. Storage guidelines for shelled corn have been established to prevent reductions in grain quality which result from microbiological activity during storage. These guidelines are based on a dry matter loss (DML) of 0.5%, at which point significant quality degradation can be expected (Steele et al., 1969). Seitz et al. (1982a and 1982b) reported that extensive fungal invasion and resulting mycotoxin production can be observed in some cases prior to a 0.5% DML. These results indicate that DML guidelines may not be acceptable for storage conditions which favor mycotoxin production.

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The production characteristics of aflatoxin by *A. flavus* as described by production lag times and rate constants under different storage conditions have not been fully analyzed and compared. The purpose of this research was to evaluate the significance of temperature and relative humidity on the production characteristics of aflatoxin B_1 by one strain of *A. flavus* (NRRL 6432) on damaged corn.

METHODS AND PROCEDURES

Experimental test conditions included the optimum growth and toxin production tempratures of 35°C and 25°C, respectively for *Aspergillus flavus* (NRRL 6432) (obtained from USDA Northern Regional Research Lab, Peoria, IL) (Miller and Golding, 1949; Diener and Davis, 1969b; Schroeder and Hein, 1967). A third temperature (20°C), which is comparable to the average storage temperature in the fall after harvest, was included to make a comparison with the optimum conditions. Relative humidities (RH) of 97%,92%,85%and 80% were selected for testing because of the wide range of toxin production potential they represented and because they could be controlled with saturated salt solutions. The highest relative humidity (97%) represented the RH that gives optimum toxin production (Northolt et al., 1976; Goldblatt, 1969) while 85% level represented the estimated lower limit for aflatoxin production (Northolt et al., 1977; Diener and Davis, 1969a).

As illustrated in Table 1, the combination of temperature and relative humidity conditions formed an experimental block design. The first test block was constructed by testing all three temperatures at 92 and 97% RH. The other experimental block was formed with all four relative humidities at 25°C.

Growth of Aspergillus flavus

Hand harvested and shelled corn kernels at 25% moisture content (wet basis) (MC) were surface sterilized with 2% sodium hypochlolrite and dried to appropriate test moisture contents. Kernels were then damaged with a commercial blender. Approximately 50 g of corn were placed in the blender and exposed to three 5-s bursts on the "chop" setting. The average percentage of damaged

TABLE 1. BLOCK DESIGN OF THE ENVIRONMENTAL PARAMETERS TESTED WITH AVERAGE SAMPLE MOISTURE CONTENT (WET BASIS) SHOWN FOR EACH CONDITION

	Relative humidity			
Temperature	97%	92%	85%	80%
35°C	22.4	18.7		
25°C	23.1	19.6	17.1	16.2
20°C	24.5	19.4		

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kernels, for all the lots tested were categorized by weight as: whole to half kernels, 73%; less than half kernels 16%; and fines (passing through a 12/64 sieve), 11%. The percentage of damaged kernels in each category did not vary greatly between the different test samples.

A. flavus spores were harvested from week-old cultures grown on acidified Potato Dextrose Agar using a phosphate buffer. To inoculate test samples one ml of a spore suspension, containing 3500 colony forming units (CFU) per ml, was placed into a plastic petri dish before adding the 20 g of damaged corn. Excess buffer was allowed to dry before adding the substrate. Each plate was shaken vigorously to distribute the spores throughout the substrate. These procedures provided optimum toxin production conditions through extensive kernel damage, limitation of microbial competition by surface sterilization, and a thorough inoculation with *A. flavus* spores.

The inoculated corn samples were placed inside controlled RH boxes (Wieman, 1984) which were in turn located inside a controlled temperature chamber. Each RH box contained 18 individual inoculated samples and 9 uninoculated samples. The uninoculated samples were used for MC determinations during the test period. Two inoculated samples were removed from each of two RH boxes, containing identical salt solutions, at each sampling.

Sampling times depended upon the appearance of mycelia or levels of aflatoxin B_1. Samples were taken at least every 24 h in all tests to allow a comparison to be made between the various treatments. Toxins were either extracted from the samples immediately after removal, or the samples were stored at -60°C for later extraction. There were no apparent differences in measured levels of aflatoxin B_1 resulting from storage in the freezer.

Extraction Procedures

Aflatoxin compounds were extracted using a combination of procedures as indicated in Fig. 1. The initial extraction was accomplished with a 80% methanol-20% water solvent. Separation of the solvent and corn residue after blending followed centrifuge techniques suggested by Seitz and Mohr (1977). After removal of proteins and other impurities with hexane, a solvent transfer was used to remove the aflatoxins from the initial extraction solvent (Seitz and Mohr, 1977). Clean-up of the samples then was conducted with Sep-Pak cartridges (Waters Assoc., Milford, MA 01757), which prepared the samples for quantitation using a high pressure liquid chromatography system (Thean et al., 1980).

Calibration tests showed that the above extraction procedures recovered an average of 51.3% of the aflatoxin added to spiked test corn samples. Therefore, aflatoxin measurements in this study can be assumed to represent approximately 51.3% of existing aflatoxin concentrations in the test samples.*

Quantitation of aflatoxins with a HPLC system

The aflatoxin compounds were quantified using a

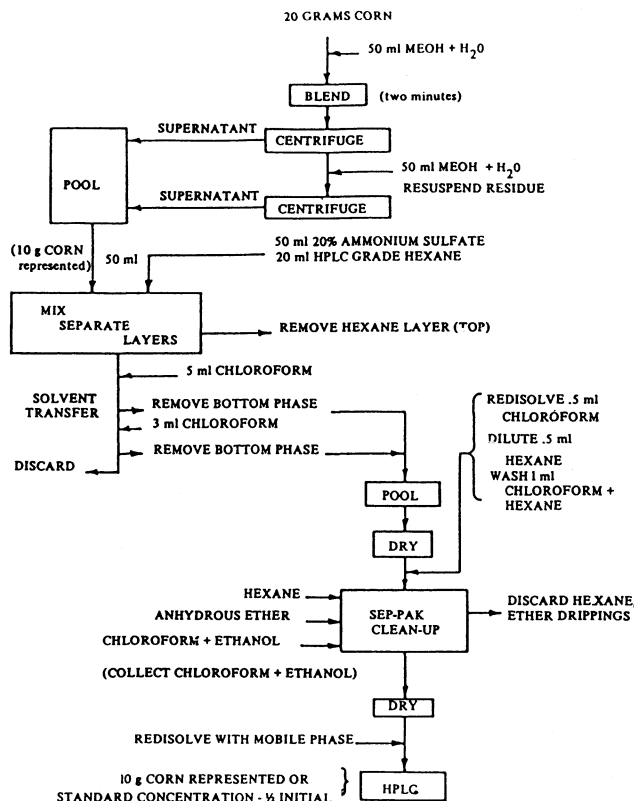

Fig. 1—Extraction flow chart.

HPLC system which included: an Aerograph Model 8500 high pressure single piston pump (Varian Instrument Division, #25 Rt. 22, Springfield, NJ 07081), Valco Sample Injection Valve with a calibrated 10 microliter sample loop (Varian Inst. Co.), Zorbax Sil microparticulate normal phase column (5um, 4.6mm i.d. × 2cm long) (distr. by Fisher Scientific Co., 5481 Creek Rd., Cincinnati, Ohio 45242). A Varian Fluorichrom fluorescence detector (Varian Instr. Co.) (365 nm excitation and 431nm emission) with a silica gel packed flowcell produced the quantitation signal response. A Model 252 servo-potentiometric type recorder (Linear Instr. Corp., 17282 Eastman Ave., Irvine, CA 92705) converted the detector output into a chromatogram. Operational settings and procedures followed recommendations of Thean et al. (1980). External standards were used to calibrate the detector response and determine unknown concentrations following procedures described by Pons and Franz (1978).

RESULTS AND DISCUSSION

Average experimental MC of the corn for each of the environmental test conditions are shown in Table 1. Moisture contents were determined using the 72 h oven method (ASAE, 1983), and they generally remained within ± 1% of the levels shown in Table 1.

Aflatoxin B_1 concentrations in parts per billion (ppb), calculated with respect to the initial corn sample weights (ca. 20 g), were determined for each sample. These values were uncorrected for extraction procedure losses. The results from these toxin determinations established a production pattern for each combination of conditions tested. Figs. 2 through 6 illustrate semilogarithmic plots of aflatoxin B_1 concentrations as a function of time for

*A later examination of extraction procedures showed that an additional 22.5% of the spiked toxin could be recovered by using an extra 3 mL of chloroform—ethanol to elute the toxin from the Sep-pak clean up column. This produced total extraction levels comparable to those reported by other investigators.

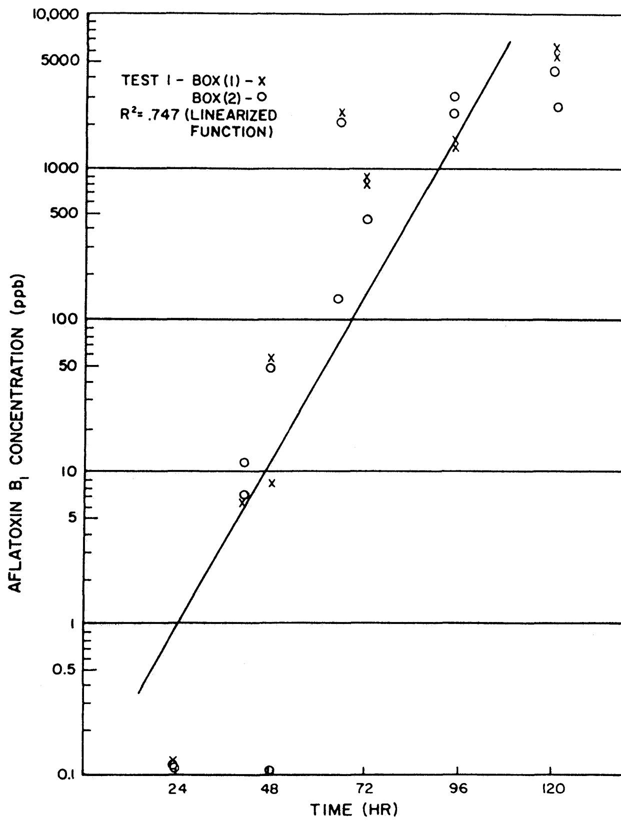

Fig. 2—Aflatoxin B_1 concentration vs. time for 35 C and 97% relative humidity with regression line shown for linearized exponential function of toxin production.

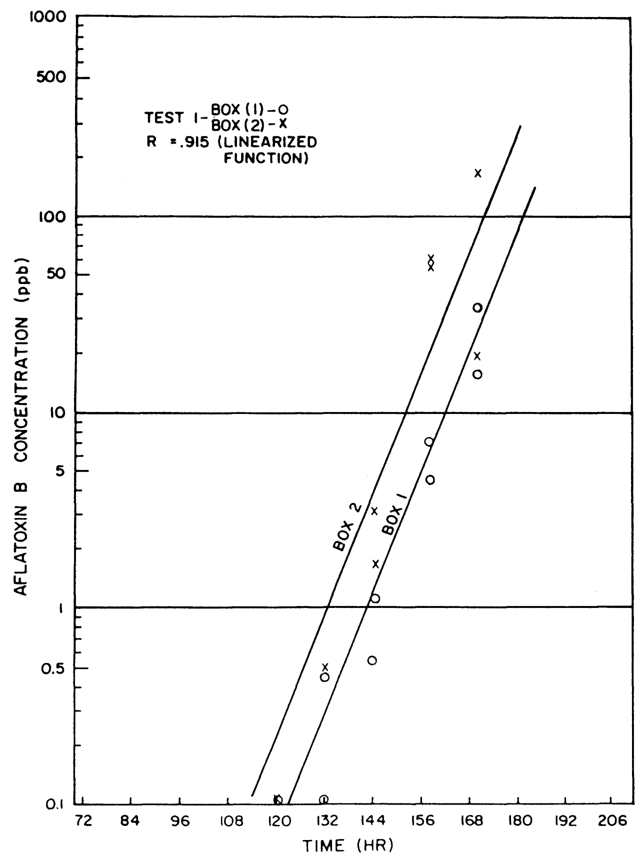

Fig. 4—Aflatoxin B_1 concentration vs. time for 20 C and 97% relative humidity with regession line shown for linearized exponential function of toxin production.

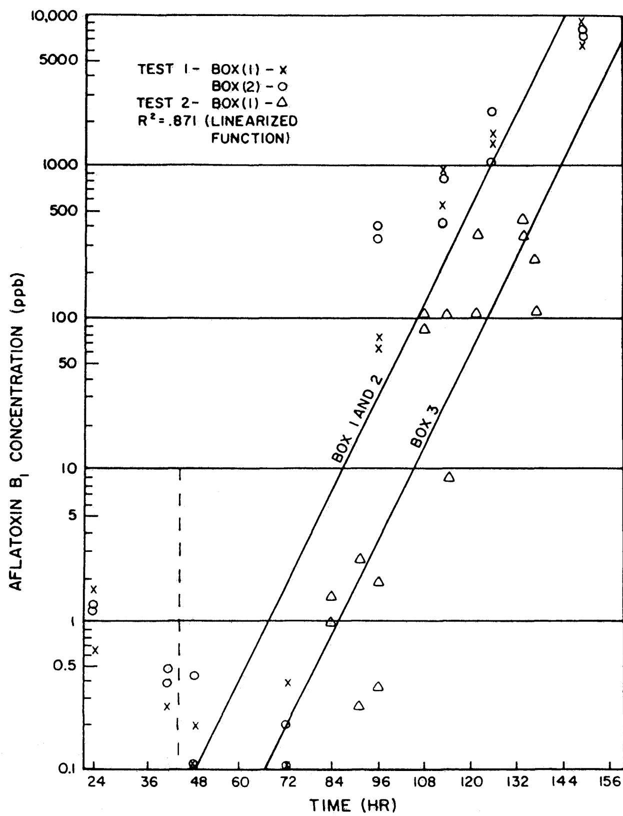

Fig. 3—Aflatoxin B_1 concentration vs. time for 25 C and 97% relative humidity with regression lines shown for linearized exponential function of toxin production. Only points to the right of the dashed line were used in the regressions.

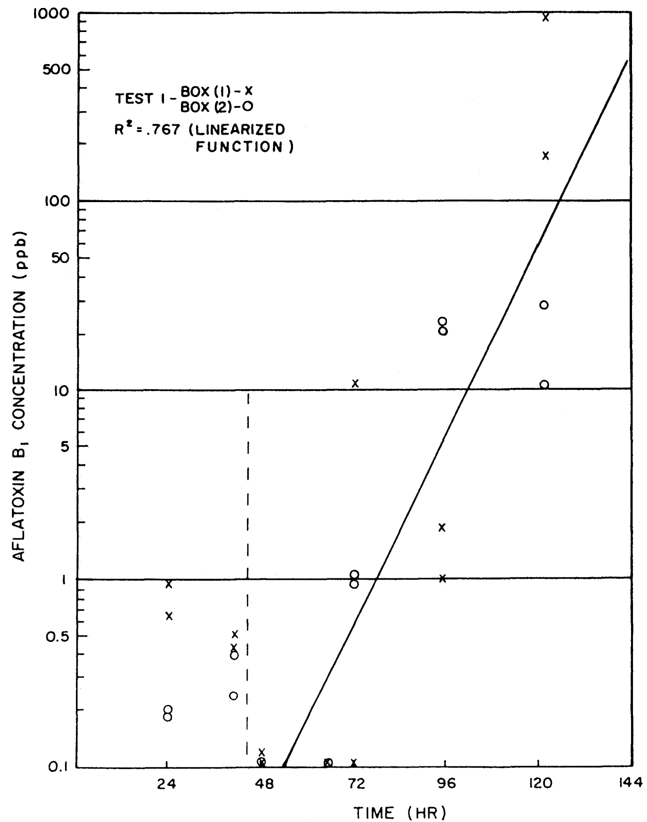

Fig. 5—Aflatoxin B_1 concentration vs. time for 35 C and 92% relative humidity with regression line shown for lineraized exponential function of toxin production. Only points to the right of the dashed line were used in the regession.

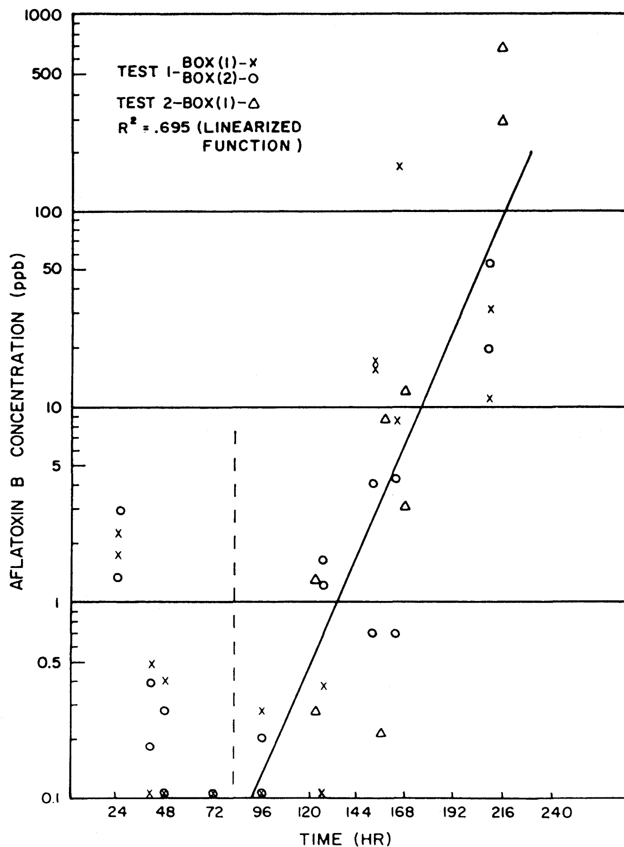

Fig. 6—Aflatoxin B_1 concentration vs. time for 25 C and 92% relative humidity with regression line shown for linearized exponential of toxin production. Only points to the right of the dashed line were used in the regression.

the five environments which produced aflatoxin.

A lineraized exponential function,

$$\ln C = Bt + \ln A$$

where C = aflatoxin B_1 concentration (ppb); B = slope; t = time after inoculation in hours, and ln A = intercept, was regressed through each set of data to determine the production rate constant (which corresponds to the slope of the linearized exponential function) and the lag time (time required after inoculation for 10 ppb aflatoxin B_1 to be produced). The pattern described by this function is not considered to be representative of aflatoxin B_1 production by all strains of *A. flavus*.

The scatter of the experimental points about the regression lines in Figs. 2 to 6 appears to be reasonable when one considers the fact that considerable variability in aflatoxin measurements can normally be expected as a result of large variations in sampling, sub-sampling, and measurement techniques (Dickens and Whitaker, 1983). The data points represent, in general, samples taken from two independent boxes at the indicated temperatures and relative humidities. Tests at 25°C for relative humidities of 97 and 92% were repeated using an additional RH box.

Only values which were definitely associated with the aflatoxin production phase (values to the right of vertical dashed line in the figures) were included in fitting the linearized exponential function to the data. In many of the test runs (25°C and 97%, 35°C and 92% and 25°C and 92% RH) low levels of aflatoxin were detected at early stages into the tests (as seen in Figs. 3, 5 and 6). These low levels were followed by a decrease in the toxin concentration. This activity was assumed to be independent of exponential production and therefore omitted from the regression fit. In some instances the initial corn lots were found to contain low levels of toxin (less than 3 ppb) before inoculation. This toxin was attributed to low levels of contamination in storage or the field and was not included in the data analysis.

TABLE 2. PRODUCTION RATE CONSTANTS WITH STANDARD ERRORS FOR THE VARIOUS ENVIRONMENTAL CONDITIONS TESTED

	Relative humidity			
Temperature	97%	92%	85%	80%
20°C	0.1224 ± 0.0103	No aflatoxin detected		
25°C	0.1209 ± 0.0115	0.0550 ± 0.0087	No aflatoxin detected	No aflatoxin detected
35°C	0.1043 ± 0.0166	0.0970 ± 0.0169		

Production rate constants are exparessed in h^{-1}.

Table 2 shows the production rate constants and standard errors for each of the test conditions. These constants were determined for each RH box tested at each temperature level. The constants were tested for homogeneity. No differences were found among RH boxes (p<0.05) within each environmental condition using procedures of Sokal and Rohlf (1981, Chp. 14) for data of more than one dependent variable for each independent variable. The data was then pooled for the determination of the values shown in Table 2.

An F-test was performed to determine if there were significant differences between production rate constants for 92% and 97% RH at 25°C and between production rate constants for 25°C and 35°C at 92% RH (p< 0.05). No significant difference was found between the production rate constants at 35°C because of the large standard errors at these conditions. Although the production rate constants were not significantly different, a check was made to determine if they were dependent on temperature at 97%, the highest RH tested. An Arrhenius relationship can be used to describe many enzyme-catalyzed reactions within a limited temperature range (Bailey and Ollis, 1977). The mean production rate constant was plotted as a function of the inverse absolute test temperature and a line regressed through the points. A linear relationship having a coefficient of determination of 0.963 was found for the three data points which were available for analysis of this relationship. This suggests that a relationship between temperature and aflatoxin production could exist at high RH.

The intercepts of the linearized exponential function for each RH box at a given environmental condition were tested for homogeneity using analysis of covariance (Sokal and Rohlf, 1981, Chp. 14). Differences were found between RH boxes at the 97% RH level at 20°C and 25°C (p<0.05). The data for the RH boxes that had homogeneous intercepts were pooled before estimating lag times.

Lag times estimated from the linearized exponential function are given in Table 3. A value of 10 ppb was selected to represent the point at which exponential aflatoxin production could be assumed to exist. This value is below the FDA marketing guideline of 20 ppb, and above the low levels which were observed in some tests prior to exponential production. Standard errors for

TABLE 3. LAG TIMES (HOURS) REQUIRED FOR AFLATOXIN B_1 TO REACH 10 ppb CONCENTRATION AT VARIOUS CONDITIONS*

		Relative humidity			
Temp.	R.H. box	97%	92%	85%	80%
20°C	1	162.3 ± 13.2	No aflatoxin detected		
	2	152.4 ± 12.4			
25°C	1	85.3	179.5	No aflatoxin detected	No aflatoxin detected
	2	87.5	168.6		
		†86.4 ± 5.8			
	3	104.7 ± 6.9	173.4		
			†173.8 ± 23.3		
35°C	1	48.4	101.5		
	2	43.6	100.4		
		†46.0 ± 6.5	†101.0 ± 13.9		

*Standard errors indicated for the different test conditions.
†Pooled means of data with homogenous intercepts.

these values were estimated (Snedecor and Cochran, 1980, p.170). Statistical differences between the various lag times were determined using a two-tailed t-test. All the lag times were found to be significantly different ($p<0.05$), suggesting a dependence on both temperature and relative humidity.

As previously mentioned the extraction procedures used only recovered an average of 51.3% of the aflatoxin in a corn sample. Lag time values presented in Table 3 are based on these recovery rates and do not take into account the loss of toxin during extraction. Table 4 shows the change in lag times which would result if a 100% extraction recovery rate occurred. The average difference between the adjusted lag times and the actual data was found to be 9.0 h.

No aflatoxin B_1 production was detected at several test combinations of temperature and humidity over the two week test period. These test conditions are shown on Tables 2 and 3.

Aflatoxin B_2, which was only observed after B_1 levels were 50 ppb or greater, was measured for all tests conditions, with the exception of 35°C to 92% RH. Aflatoxin B_1 has been implicated as a precursor to the other toxins (Maggon et al., 1977). This theory was supported in this study as the concentration of B_2 was found to be consistently 3 to 4% of that of B_1 for each sample examined.

CONCLUSIONS

Aflatoxin production exhibited an exponential growth pattern under the conditions tested. Temperature and RH affected both rate of aflatoxin production and the lag time prior to exponential production. Relative humidity and temperature influenced the toxin production rate as indicated by the significant differences found in the production rate constants at 25°C and 35°C temperatures.

Production rate constants were not found to be significantly different for all the temperatures tested at 97% relative humidity. However, an Arrhenius plot of the data suggested that a relationship between the temperature and aflatoxin B_1 production rate may exist at this relative humidity.

Toxin concentrations doubled every 6 to 10 h after the lag time for all of the environmental conditions tested in which toxin production was observed. Statistical analysis showed lag times to be influenced by both temperature and relative humidity. The lag time should be considered an important limiting value in estimating allowable storage times under conditions which favor aflatoxin production.

The substrate used in this research provided optimum toxin production conditions through extensive damage and limited microbiological competition. Thus, a worst case situation was produced. The results in this paper can be used to estimate potential aflatoxin production under similar conditions.

TABLE 4. COMPARISON OF ACTUAL DATA LAG TIMES TO THOSE PROJECTED FOR 100% RECOVERY DURING EXTRACTION

		Time required to reach 10 ppb, h	
Test condition temp. - R.H.	R.H. box	Actual data*	Adjusted data†
35°C - 97%	1, 2	46.0	39.6
25°C - 97%	1, 2	86.4	80.9
	3	104.7	99.2
20°C - 97%	1	162.3	156.9
	2	152.4	146.9
35°C - 92%	1, 2	101.0	94.1
25°C - 92%	1, 2, 3	173.8	162.0

*Average recovery during extraction estimated to be 51.3%.
†Actual data adjusted to reflect 100% recovery rate.

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