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I am submitting herewith a thesis written by David L. Jones entitled "Effect of Moisture, Inoculum, and Preharvest Stress on Aflatoxin B_1 Production in Corn." I recommend that it be accepted in partial fulfillment of the requirements for a Master of Science Degree, with a major in Food Technology and Science.

J. Orvin Mundt, Major Professor

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EFFECT OF MOISTURE, INOCULUM, AND PREHARVEST STRESS ON AFLATOXIN B_1 PRODUCTION IN CORN

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

David L. Jones

March 1977

1317140

ACKNOWLEDGMENTS

The author wishes to express him most sincere appreciation to Dr. J. O. Mundt for his guidance and technical assistance in the completion of this thesis. A special acknowledgment is extended to Dr. E. A. Childs for his participation in the planning of this project. Appreciation is extended to Dr. J. T. Miles for his understanding and cooperation throughout the course of my research and to members of the graduate committee, Drs. J. L. Collins and L. M. Josephson.

A special note of gratitude is given to Teresa Webb for her invaluable assistance in identification of fungi.

Gratitude is extended to my wife and parents for their moral and financial support without which this study never could have been completed.

ABSTRACT

Experiments were undertaken to define the main effects and interactions of moisture, inoculum, and pre-harvest stress on aflatoxin production by Aspergillus parasiticus PC 5225.

The results of this study indicated that aflatoxin was readily produced in corn under the experimental conditions. Moisture and inoculum were found to be significant using a least squares analysis of variance ($P < 0.10$). Stress was not found to be significant in the experimental design ($P < 0.10$).

The interaction of moisture and inoculum was found to be most pronounced when the sample contained 23 percent moisture and had been sterilized and inoculated.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

The aflatoxins are a closely related group of fungal metabolites that are commonly produced by members of the Aspergillus flavus group. Recent work has demonstrated that other members of Aspergillus as well as some species of Penicillium (Hodges, 1964) and Rhizopus (Van Walbeek, 1968) are equally able to elaborate aflatoxins. All of these fungi are ubiquitous and are commonly found in soil, living on decaying plant and animal material, and are airborne in the form of spores. The nature of the fungi is such that they frequently invade agricultural commodities of economic interest (Christensen, 1957). The invasion of agricultural commodities and the subsequent production of aflatoxins presents a health problem of cosmopolitan dimension.

A. parasiticus has been found to be a common organism in stored grains (Christensen, 1957). In addition to the ubiquity of A. parasiticus, Hesseltine et al. (1966) demonstrated that some strain of A. parasiticus produced much greater quantities of aflatoxins than many strains of A. flavus. The ubiquity and toxigenic potential of A. parasiticus has made this organism the subject of increasing study in recent years.

I. HISTORY

The vast majority of papers concerning aflatoxins generally refer to the turkey X disease as the beginning of the association

of moldy corn and toxicity. Christensen (1957), however, points out that in the early twentieth century a group of Italian workers postulated a cause and effect relationship between the consumption of moldy corn and toxic manifestations. This investigation was carried to the point that the culpable fungus was isolated in pure culture, grown on a suitable substrate, and included in the ration of domestic animals to demonstrate its toxic factors. It was at this point that the investigation was terminated and the toxic agent was investigated no further.

It was no rare occurrence in agrarian areas for the outbreak of moldy corn disease to decimate domestic livestock. No discerning investigation was ever undertaken and for many years the cause of the moldy corn disease escaped elucidation (Burnside, 1957). In the 1950's, an extensive outbreak of moldy corn disease occurred in the south-eastern United States in which hundreds of swine became ill after foraging on harvested corn fields. A mycologic survey of the field revealed a number of fungi including A. flavus. This fungus, when grown on autoclaved corn proved rapidly fatal to pigs when included in their rations. Since the toxic agent itself had not been demonstrated, this research was largely ignored by toxicologists and mycologists.

In 1960, turkey X disease occurred in Britian. The result of this unprecedented pestilence was the death of some 100,000 young turkey poults and lesser numbers of other domestic fowl. The affected animals usually died within a week of onset, during which

time they became anorexic, lethargic, and developed weakness in the wings. A characteristic attitude of the head, neck, and wings was adopted at the time of death (Spensley, 1963). There was no evidence that the diseases were infectious so the possibility of a toxin or poison was considered. The impact of the economic loss was sufficient to instigate investigations into the causes of the disease. The feed of infected birds was examined for the presence of insecticides, inorganic poisons, and for poisonous plant parts, such as castor bean. None of these avenues proved fruitful. Eventually, the common factor was found to be associated with two lots of groundnuts that had originated in Brazil (Blount, 1961). It was the first time that any of this meal had been imported into England so the possibility that this was the cause of the mysterious disease was not remote. Feeding experiments with this groundnut meal rapidly confirmed suspicions by causing death in the experimental animals.

When the presence of a toxic factor was demonstrated in the groundnut meal, intensive studies were undertaken to define the nature of this substance. Initially, the unknown factor resisted all efforts at isolation. As increasingly sophisticated techniques were applied to the problem, the unknown factor began to yield its secrets (Asao, 1965). The substance was initially extracted from the groundnut meal with hot methanol with subsequent partitioning into chloroform. Concentration of the chloroform extracts was followed by thin layer chromatographic (TLC) analysis and examination under long wave ultra violet illumination. Under these conditions, a bright

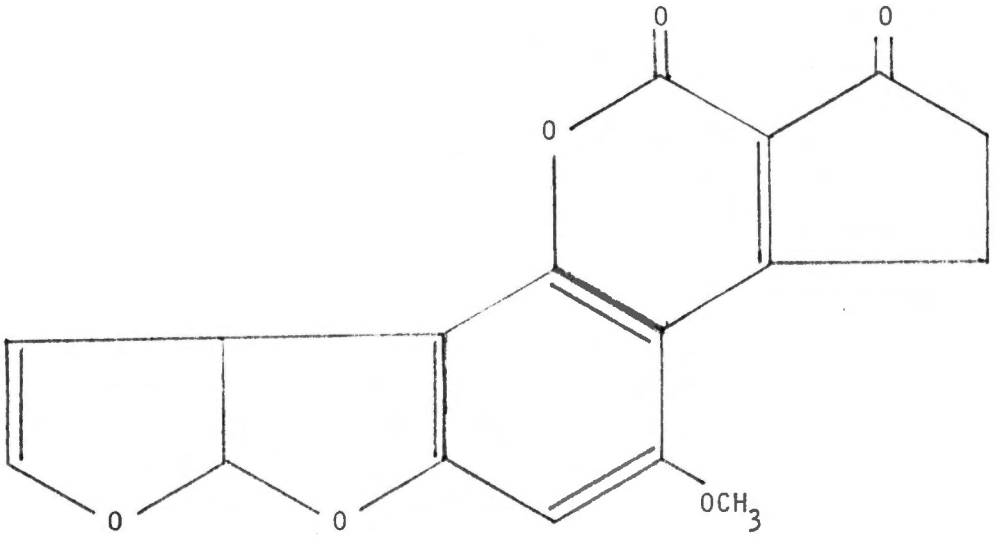
blue fluorescent compound with an R_f value of approximately 0.7 was observed. The R_f value refers to the ratio of travel distance between a specified compound and the maximum travel distance of the developing solvent (Wogan, 1966).

Refinement of the developing solvent provided a means of further separating the blue fluorescent compound into four discrete components (Hartley et al., 1963). These compounds were collectively assigned the name aflatoxins in view of their elaboration by A. flavus. The discrete compounds were further designated B_1, G_1, B_2, and G_2 in order of decreasing predominance. Elaborate analytical investigations utilizing mass spectrometric and nuclear magnetic resonance spectra indicated that the primary compound, aflatoxin B_1, consisted of the empirical structure $C_{17}H_{12}O_6$ (de Iongh, 1962). Exhaustive study of the aflatoxins revealed that there were in reality eight closely related compounds and possibly a ninth with the trivial name aspertoxin. All of the aflatoxins with the exception of aspertoxin consist of the same basic structure, usually differing in only one moiety on the planar carbon skeleton.

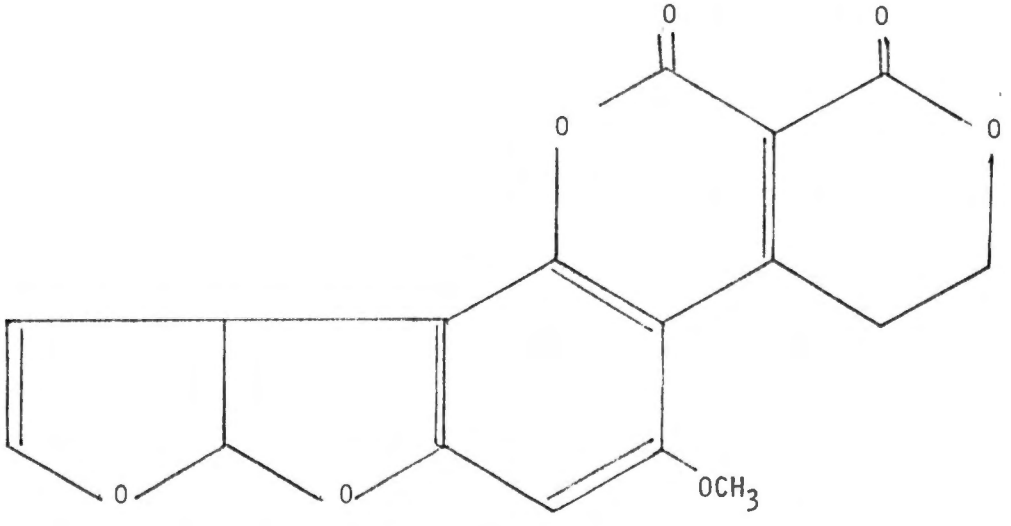
Buchi and Rae (1969) and Biollaz et al. (1970) determined that acetate is the major contributor of carbon in the biosynthesis of aflatoxin B_1. The basic structure of aflatoxins B_1 and G_1 is illustrated in Figure 1 (Asao et al., 1965).

II. BIOLOGICAL EFFECTS

The biological potential of the aflatoxins is two-fold. Since the compounds are toxic as well as carcinogenic, a number of factors



Aflatoxin B_1



Aflatoxin G_1

Figure 1. Structure of Aflatoxins B_1 and G_1.

determine the extent of the effect of the compounds. The effects may be either acute or chronic depending on the length of the feeding trials, the dosage, the method of administration, whether given orally or by injection, and the biological system being affected.

In attempts to define the effects of aflatoxins on living organisms, the toxins have been applied to a number of test systems ranging from cell free studies and microorganisms to almost every domesticated animal. The purpose of bioassay is to evaluate the relative potency of a compound and to provide a reliable estimator for that potency. Di Palma (1965) suggested that there are several principles to be considered in the biological evaluation of toxicity of the aflatoxins. These are (1) The test system should be related to object of the test. That is, if the system is designed to demonstrate the tumorogenesis of aflatoxins, the biological system should be one that is susceptible to tumor induction. (2) With regard to the bioassay system, a dose response curve should be derived indicating the relationship between the dose of toxin received and the toxic manifestations. (3) The activity of the compound in question should be compared to the activity of a chemically pure reference standard. (4) The bioassay must be conducted under a strict experimental design to insure that precision and accuracy are maintained at a maximum.

If these aspects of the bioassay system are carefully executed, the resulting data will be of much greater value to the scientific community. The degree of toxicity of aflatoxin B_1 in various test animals as a relative measure of toxicity in vivo is presented in Table 1.

TABLE 1

VALUES FROM LITERATURE FOR THE LD_{50} OF AFLATOXIN B_1 ADMINISTERED ORALLY TO VARIOUS TEST ANIMALS

Animal	LD_{50}	Duration	Reference
Rats	7.3 mg/kg	3-7 days	Butler, 1964a
Guinea pigs	1.4 mg/kg	1-4 days	Butler, 1966
Hamsters	10.2 mg/kg	7 days	Wogan, 1965
Ducklings	0.0335 mg/kg	6 days	Butler, 1966b
Dogs	1.0 mg/kg	7 days	Newberne et al.,1966
Monkeys	2.2 mg/kg	4 days	Rao and Gehring, 1971

Since the effects of aflatoxins are multifaceted, many organ systems have been studied concerning their response to varying doses of the toxin. A summary of the literature evaluation of these effects is presented in Table 2. The three major effects cited in the literature are (1) interference with nucleic acid synthesis (de Redondo *et al.*, 1966; Edwards *et al.*, 1971), (2) genetic mutations resulting in cell growth abnormalities (Goldblatt, 1969; Lancaster *et al.*, 1961; Garner, 1973), and necrosis and/or liver lesions (Manhaven and Goplan, 1965).

III. *IN VITRO* EFFECTS

With the intensive interest in the effects of aflatoxins on the total animal system, it is only reasonable to expect that studies would also be carried out on an empirical basis. This implies that the individual organ systems would also come under consideration.

In vitro studies can often be of great benefit in evaluating the effect of a toxin on the entire animal system. The usual vehicle for this type of study is cell culture or the study of the effects of chemicals on the embryonic stage of animal development. Legator (1969) noted that as little as 0.01 parts per million (ppm) aflatoxin B_1 inhibits mitosis and produced chromosomal aberations in human embryonic lung cells and leukocytes. Aflatoxin may have the ability to bind to nucleic acids and proteins (Maher and Summers, 1970; Gurtoo and Dahms, 1974).

TABLE 2

LITERATURE SUMMARY OF TOXIC EFFECTS OF AFLATOXIN B_1 IN THE LABORATORY RAT

Organ	Effect	Dose	Reference
Liver	Hepatoma	0.015 ppm	Wogan and Newberne, 1967
Liver	Inhibition of parenchymal cell mitosis	3 mg/kg	Rogers and Newberne, 1967
Liver	Lesions	4-5 ppm	Butler, 1966b
Kidney	Tubular necrosis	7-7.5 ppm	Butler, 1966b
Adrenals	Hemorrhage		
Spleen	Dimunition of white pulp		
Pancreas	Changes in inlet		

The *in vitro* binding of aflatoxins to nucleic acids is of particular interest since this binding might explain in part the carcinogenic effects of the aflatoxins. It was reported by Edwards *et al.* (1970) that aflatoxin exhibited a weak affinity for DNA *in vitro*. To further study this avenue, Childs (1971) investigated the interaction of aflatoxin with histone. He demonstrated that aflatoxins did not alter the ability of histone to bind to such molecules as RNA polymerase and DNA. This indicated that there is no disruption of the normal function of histone and hence the binding of aflatoxin to nucleic acid is without significance.

Many carcinogens require activation via the liver microsomal system before manifestation of inductive capabilities. It may thus be concluded that the metabolites of the aflatoxins exhibit biological activity (Clifford and Rees, 1967).

The mechanism of tumor induction is still a matter of conjecture but there are three generally accepted hypotheses. The first hypothesis states that the carcinogen acts at the stem cell level. According to this mechanism, there is an abortive attempt at differentiation which results in the abnormal development of the cell. This abnormal growth may result in the production of a cancerous mass of cells. The second of these postulations is that the DNA of the cell is altered to the point that the cell is no longer capable of carrying out its normal functions. The final theory is that mature cells are affected by the carcinogen in such a way that the cells undergo de-differentiation. It may well be that none of the proposed mechanisms is the true route to tumor formation.

IV. *IN VIVO* EFFECTS

The toxic effects of the aflatoxins on farm animals have been well documented (Allcroft, 1969). It has also been demonstrated through feeding trials that susceptibility of the farm animal varies according to the specie. The first indication that an animal has consumed toxic amounts of aflatoxins is the appearance of anorexia and weight loss. In the later stages of the disease, the animals may become ataxic and death soon follows. Upon necropsy, the most prominent pathological finding is liver damage. The extent of the damage may vary from microscopic lesions to macroscopic lesions accompanyed by pallor and edema. A summary of the hepatic effects of aflatoxins on farm animals is presented in Table 3.

In addition to the hepatotoxicity of the aflatoxins, there is a broad range of presenting pathologies. In swine, the carcass may become jaundiced and there may be gross hemorrhage throughout the body. In poultry, the symptoms of aflatoxicosis may include weakness in the wings and dermatologic hemorrhage. Mabee and Chiple (1973) demonstrated through the use of radioactively labeled aflatoxin B_1 that the toxin is distributed in many organs of the avian body. Their conclusion was that the toxin may be degraded in the liver and converted to other biologically active compounds such as hormones. Calvert *et al.* (1966) reported the death of suckling calves when their dams were maintained on a diet containing naturally contaminated groundnut meal.

TABLE 3

COMPARATIVE PATHOLOGIES OF ANIMAL DIETS
CONTAINING UP TO 20 PERCENT TOXIC GROUNDNUT MEAL

Pathology	Cattle	Pig	Sheep	Duckling	Turkey Poult	Chicken
Liver Lesions: Acute Necrosis and Hemorrhage	-	-	-	+	+	-
Chronic Fibrosis	+	+	-	-	-	-
Regeneration Nodules	+	+	-	-	+	-
Bile Duct Proliferation	+	+	-	+	+	+
Veno-occlusive Lesions	+	-	-	-	-	-
Hepatic Cells						
Enlarged Cells (Megalocytosis)	+	+	-	+	+	-
Enlarged Nuclei	+	+	-	+	+	+
Inflamatory Cell Infiltration	-	-	-	-	+	+
Liver Tumors	0	0	0	+	0	0

+ = positive
- = negative
0 = no information available

(Allcroft, 1969)

V. PRESENCE OF AFLATOXIN IN AGRICULTURAL PRODUCTS

The ubiquity of the organisms capable of elaborating substances that are potentially toxic or carcinogenic to man or animal places them in the forefront of the problems confronting the food industry (Goldblatt, 1965). In the period that preceded the elucidation of aflatoxins, fungal spoilage of agricultural products was treated as a nuisance rather than a potential health hazard (Feuell, 1969). As a temple to the ignorance that surrounded the implications of fungal spoilage, some societies prefer molded staple on a flavor basis. As Scott (1965) pointed out, it is really rather remarkable that "no systematic thought was given before 1960 to the possible existence of fungal metabolites toxic to higher organisms." This thought is especially surprising in light of the widespread knowlege of the antibiotics of fungal origin such as penicillin.

Aflatoxins have been isolated from a wide variety of agricultural commodities (Golumbic and Kulik, 1969). It would appear that the incidence of aflatoxin is higher in stored products when compared to the same product freshly harvested. This might be due to the fact that some storage practices are conducive to fungal growth. It is known that fungal attack results in a loss of viability which renders seed valueless for planting or malting. Golumbic (1965) indicated that fungal spoilage of cereal grains reduced milling quality of the grain and resulted in increased refining losses of processed oilseeds.

The microflora associated with grain may be divided into two classifications: field fungi and storage fungi (Christensen, 1957). The field fungi are those that are predominant in grain at the time of harvest. The nature of the field fungi is generally saprophytic or parasitic. The major genera of field fungi include Alternaria, Helminthosporium, Fusarium, Cladosporium, Diploidia, Chaetomium, Rhizopus, and Absidia. It may be noted that the field fungi require high moisture levels for growth and development. The required moisture range is on the order of 20-25 percent (wet weight basis).

The storage fungi are those that predominate in seeds and grains that have been stored. At the time of harvest, they are present in marginal numbers due to competition offered by the field fungi. The two major genera that comprise the storage fungi are Aspergillus and Penicillium. These fungi are active at moisture levels of 18-25 percent. Most of the degradative activity of the storage fungi is directed toward the germ or embryo of the seeds causing discoloration and eventual decay.

An important characteristic of rapid fungal growth is heating and the production of hot spots in the grain bulk. Hot spots are the result of metabolic heat produced by the spoilage organisms. Gilman and Barron (1930) showed that under laboratory conditions several species of Aspergillus were capable of heating grain to as much as 26°C above the initial temperature. These findings were later confirmed by Christensen (1957). The rise in temperature can be even greater if the bulk of grain contains large quantities of damaged

kernels. Christensen (1957) has said that the heating of grain is the final effect of fungal deterioration and not the beginning of mold invasion.

VI. CONTROL OF FUNGAL SPOILAGE

The best method of mitigating contamination of agricultural commodities with aflatoxin is to prevent the growth of toxigenic fungi. To prevent the colonization of agricultural products by toxigenic fungi, conditions must be created that are unfavorable for fungal growth. The most convenient and economical preventative measure is the reduction of the moisture content of the grains. It is generally accepted that fungi will not invade grains and oilseeds when the moisture content of these commodities is in equilibrium with 70 percent relative humidity. The equilibrium moisture content of corn at this humidity is 16.5 percent (Coleman and Fellows, 1925). It is of extreme importance that the moisture content of the grain be ascertained accurately since a deviation in moisture of as little as one percent may determine whether grain is attacked or spared by fungi. One might notice a quandry in this method of fungal control. Sampling which is not uniform and representative of the entire grain bulk has obvious implications. Insect infestation has been implicated in grain heating. Often, insect infestation of grain is also accompanied by increases in the moisture level of the grain bulk. To preclude the possibility of insect or fungal invasion of grain, there are several measures that may be instituted. Initially, a

thorough cleaning of the grain will remove dockage and other foreign matter which may carry a heavier load of fungal spores than grain. The second preventative measure that may be employed is the aeration of the grain prior to and during storage. Aeration is intended to cool and dry the grain and to reduce the possibility of redistribution of moisture in the grain bulk. It must be noted, however, that aeration is of little value in climates where the ambient humidity is sufficiently high to be in equilibrium with dangerous moisture levels. A third measure that might be applied in attempts to lessen fungal invasion is the use of effective insecticides. Several workers have demonstrated that some grain infesting insects are capable of inoculating grain with storage fungi. Examples of insects that invade stored grains are Rhyzopertha dominica and other Coleoptera (Bailey, 1965), Plodia interpunctella (Abdel-Rashad et al., 1969), and Sitophilus granarius (Agrawal et al., 1957).

Wilson and Jay (1975) studied the effect of modifed atmospheres on aflatoxin production in corn. In their study, none of their experimental systems showed significant development of aflatoxins. Bailey (1965) demonstrated that the role which insects play in the inoculation of stored grain could be greatly reduced when the grain is stored under atmospheres high in carbon dioxide. High carbon dioxide content is rapidly attained under hermetic conditions due to the respiration of high moisture grain in addition to that of contaminating microflora and insects.

Temperature can be an effective deterrent to the production of aflatoxins by fungi. Northolt (1976) demonstrated that slight growth

of A. parasiticus was possible at low temperatures but there was no accompanying production of aflatoxins. Northolt further stated that the range of temperatures within which aflatoxins can be produced is 13-32°C. Trenk and Hartman (1970) indicated that the optimum temperature at which aflatoxins are produced is 24°C. In the control of aflatoxins in agricultural products, moisture is the most important consideration in view of efficiency and economy.

VII. AFLATOXIGENIC POTENTIAL OF A. PARASITICUS

Hesseltine et al. (1966) report A. flavus and A. parasiticus are capable of producing a variety of toxins beside aflatoxins. Among the substances liberated by the toxigenic Aspergilli are kojic acid, b-nitropropionic acid, penicillin F (flavicidin, flavicin), flavacol, aspergillic acid, hydroxy-aspergillic acid, and grangegillin. Hesseltine went on to indicate that A. parasiticus possesses a higher propensity for aflatoxin production than A. flavus. The work of Hesseltine verifies an earlier work by Codner et al. (1963) in which strains of A. flavus and A. parasiticus were examined for their ability to elaborate aflatoxins. Codner et al. (1963) found that A. parasiticus (C.M.I. 15957) greatly out-produced strains of A. flavus.

VII. LITERATURE SUMMARY

A summary of the literature concerning aflatoxin and its implications will indicate that (1) A. flavus and A. parasiticus strains are but a few of the fungi capable of producing aflatoxins. (2) The

ubiquity of the toxigenic molds in grains and oilseeds is uncontested. (3) Toxicity studies have indicated that the aflatoxins may be the most potent hepatocarcinogens known. (4) Corn as well as other grains and oilseeds readily support the growth of aflatoxigenic fungi.

The literature observations have emphasized the magnitude and urgency of the health hazards posed by the consumption of aflatoxins. The extent to which aflatoxin contaminates agricultural commodities places it among the leading causes of economic and product loss. Since corn is widely grown throughout the United States and on-the-farm storage is a common practice, it is possible that the hazard of aflatoxin in corn may exceed that in peanuts, rice and cottonseed.

VIII. RESEARCH GOALS

The present study was undertaken to investigate the following questions:

1. What is the effect of recycled freezing and thawing temperatures on the germination and toxigenic capabilities of A. parasiticus on corn?
2. What is the role of competing microflora on the ability of A. parasiticus to elaborate aflatoxins?
3. What part does the ERH (equilibrium relative humidity) play in mold growth and aflatoxin production?
4. With what reliability may the presence of BYGF (bright yellow-green fluorescence) be indicative of the presence of aflatoxins in corn?

CHAPTER II

MATERIALS AND METHODS

I. OBJECTIVE

The objective of this experiment was to determine the capacity of Aspergillus parasiticus PC 5225 to produce aflatoxins in corn under varying conditions. Included in the conditions was a stressing factor which was designed to test the survivability of the organism and the retention of the systems necessary to produce aflatoxins. The experimental factors considered in this experiment were moisture content of the corn, inoculum, and recycled freeze-thaw conditions.

II. SAMPLE PREPARATION

The corn used in this study was Pioneer Brand 3147 yellow hybrid and was grown on the University of Tennessee Blount County Farm. The corn was harvested when the moisture content was approximately 26 percent and was stored in a cooler at 2° C until the study was begun. To facilitate adjustment of moisture content, the bulk of corn was divided into three 30-pound lots. Initial moisture was determined by placing six 20-gram samples from each lot into a forced air oven at 120° C for 24 hours. The difference in weight was used to calculate the moisture content. Each of the 30-pound lots was placed in a drying oven at 45° C until the desired moisture content was achieved. Then, each 30-pound lot was further divided into two 15-pound lots. One 15-pound lot was placed in an airtight plastic bag and stored in the

cooler at 2° C until needed. The remaining 15-pound lots were placed in desiccators with 10 ml of propylene oxide. The purpose of the propylene oxide treatment was to sterilize the corn without the use of heat. After exposure to propylene oxide, a darkening of the kernels was noted. The efficiency of the sterilizing procedure was evaluated by removing several kernels from the desiccators and culturing on malt extract agar supplemented with sucrose (MES). The test cultures were incubated for a minimum of seven days. At the end of the incubation period, the plates were examined under magnification to determine the presence of fungal growth. The results of this examination indicated that no fungal growth had occurred.

III. ASPERGILLUS PARASITICUS PC5225

The strain of A. parasiticus employed in the study was originally isolated from pecans. The acceptability of this organism for experimental purposes in terms of aflatoxin production was evaluated. Sterilized high moisture shredded wheat was inoculated with spores and incubated for five days at 22°C. At the end of the incubation period, the shredded wheat was extracted with chloroform, the extract was filtered and the concentrated filtrate analyzed through TLC. The TLC plates were developed in chloroform: acetone (90:10) and examined under longwave ultra violet light. The presence of a bright blue fluorescent spot at R_f 0.7 confirmed the production of aflatoxins.

IV. SPORE COLLECTION

Spores of A. parasiticus were dusted into Erlenmeyer flasks containing 15 ml of MES medium. The flasks were closed with cotton plugs and incubated seven days at 32°C. Northolt (1975) noted that maximum vegetative growth occurred in the range of 30-32°C. At the end of the incubation period, the spores were harvested using "harvest vials." The vials were small screw top vials containing 10 ml of 0.1 percent Tween 80 solution and three small glass beads. The harvest vials were autoclaved for fifteen minutes at fifteen pounds pressure per square inch gage (psig). To harvest the spores, the contents of one harvest vial was poured into the culture flask and the flask was then swirled for several minutes. The resulting spore suspension was transferred into a sterile bottle. Concentration of the spore suspension was adjusted to 1,200,000 spores per milliliter with a Neubauer type hemocytometer.

V. EXPERIMENTAL DESIGN

Pilot studies on aflatoxin production by A. parasiticus had indicated that great variation existed in the amount of toxin produced between replicates. In view of the variation inherent in aflatoxin production, the number of parameters investigated was reduced and the number of replicates increased to maximize precision. The parameters investigated were moisture, inoculum, and pre-harvest stress. The moistures employed were 23, 18, and 13 percent measured on a wet weight basis. The inoculum treatments consisted of sterile,

inoculated corn; non-sterile, inoculated corn; and non-sterile, non-inoculated corn. Pre-harvest stress treatments were recycled freezing (-18° C) and thawing (2° C) temperatures alternated at twelve hour intervals. Table 4 indicates the combinations of experimental treatments.

VI. SAMPLE ASSEMBLY

All glassware into which the samples were weighed was sterilized by autoclaving. Transfers were made in a hood to exclude airborne contamination. The hood was rendered sterile by swabbing the interior with household bleach and then illuminated for one hour with a germicidal ultraviolet lamp. Samples were inoculated by delivering 0.1 ml of the spore suspension. This inoculum resulted in a concentration of spores equivalent to 1200 spores per gram of sample. In all samples, 100 grams (dry matter) of corn was used.

The prepared samples were placed into growth chambers. The chambers were eighty-three liter plastic receptacles fitted with a thirty-eight centimeter disc of perforated fiber board. The disc was supported from below with three quart-size Mason jars. Once the samples had been stacked within the chambers, a solution of sulfuric acid in water was introduced into the space below the perforated disc. The concentration of the acid solutions was varied to achieve the desired equilibrium humidities (Wilson, 1921). Table 5 presents the concentration and density of the solutions used to control humidity.

TABLE 4

COMBINATIONS OF EXPERIMENTAL TREATMENTS

Treatment	Moisture %	Inoculum	Stress
1	23	Non-Sterile, Non-Inoculated	Freeze-Thaw
2	23	Non-Sterile, Non-Inoculated	None
3	23	Sterile, Inoculated	Freeze-Thaw
4	23	Sterile, Inoculated	None
5	23	Non-Sterile, Inoculated	Freeze-Thaw
6	23	Non-Sterile, Inoculated	None
7	18	Non-Sterile, Non-Inoculated	None
8	18	Non-Sterile, Non-Inoculated	Freeze-Thaw
9	18	Sterile, Inoculated	None
10	18	Sterile, Inoculated	Freeze-Thaw
11	18	Non-Sterile, Inoculated	None
12	18	Non-Sterile, Inoculated	Freeze-Thaw
13	13	Non-Sterile, Non-Inoculated	None
14	13	Non-Sterile, Non-Inoculated	Freeze-Thaw
15	13	Sterile, Inoculated	None
16	13	Sterile, Inoculated	Freeze-Thaw
17	13	Non-Sterile, Inoculated	None
18	13	Non-Sterile, Inoculated	Freeze-Thaw

TABLE 5

DENSITY AND CONCENTRATION OF SULFURIC ACID SOLUTIONS USED TO CONTROL HUMIDITY

Corn Moisture %	Relative Humidity %	Acid Concentration %	Solution Density
23	90	18	1.125
18	77	28	1.200
13	50	43	1.330

The necessary humidities were obtained from literature sources (Coleman and Fellows, 1925). The density of the solutions was determined through the use of a hydrometer.

It was determined that five liters of the acid would be sufficient to provide the desired humidity for the volume of the growth chamber. Moisture content of the corn was monitored indirectly by determining the density of the acid solutions with a hydrometer. As the solution became increasingly dense, the relative humidity of the atmosphere within the chamber was declining. Deionized water was added to the acid solutions to maintain the proper humidity as determined by density.

The chambers were divided into two groups according to the experimental design. Stressed samples were placed in a freezer at -18° C for twenty-four hours and then removed to a cooler at 2° C for twenty-four hours. The freeze-thaw cycle was repeated through fourteen days. At the end of the stress period, the chambers were placed at 22° C for fourteen days. Non-stressed samples were placed immediately into incubation at 22° C for fourteen days. After incubation, the growth chambers were frozen at -18° C until the analysis for aflatoxin could be conducted.

VII. EXTRACTION OF AFLATOXIN

Many schemes for the extraction of aflatoxins from agricultural commodities have been proposed. Prior to the initiation of the experiment, several methods were evaluated for their efficiency.

The methods that were evaluated were those of Eppley (1966) and Shannon and Shotwell (1975). Both methods were found to be unsatisfactory and were not used.

The best method was found to be that proposed by Pons (1966) for the quantification of aflatoxins in cotton and cottonseed. In this procedure, the initial extraction is with 70 percent acetone in water. The advantage to an initial extraction with aqueous acetone is that it yields extracts essentially free of interfering lipids which might complicate extract purification. The initial extraction is followed by precipitation of gossypol pigments with a neutral solution of lead acetate (20 percent w/w in water). Following precipitation of gossypol pigments, aflatoxins in the initial extract are partitioned into chloroform. A silica gel column is suggested to remove lipids and fluorescent interferences. The purified eluate was concentrated and spotted on TLC plates. The TLC plates are developed in a mixture of chloroform:methanol (97:3).

The extraction method employed in the experiment was modified slightly from that of Pons (1966). The 100 gm sample of corn was first examined for the presence of BYGF. This examination was conducted by examination of the sample under longwave ultra violet light. The sample was then placed into a 500 ml Erlenmeyer flask to which 250 ml of 70 percent acetone were added. The flasks were then shaken for thirty five minutes on a Burrell Wrist Action Shaker. The contents of the flask was then filtered through Whatman's No. 4 filter paper. To the filtrate was added 20 ml of lead acetate and 60 ml of water.

The volume of the solution was then reduced to approximately 250 ml on a hot plate. Ten grams of Celite filter aid were added and the resulting suspension was filtered under vacuum through Whatman's No. 1 filter paper. The filter cake was washed with 60 ml of 20 percent acetone. The filtrate was transferred to a 500 ml separatory funnel and aflatoxins partitioned into two 50 ml washes of chloroform. Each wash was filtered through 10 grams of anhydrous sodium sulfate. The volume of the filtrate was reduced to approximately 3 ml and then quantitatively transferred to a 13 x 100 mm test tube.

VIII. QUANTIFICATION OF AFLATOXIN

The chloroform filtrate was reduced to incipient dryness in warm (60° C) water. The dried residue was dissolved by vortexing with 0.1 ml of chloroform.

Brinkman precoated (0.25 mm silica gel with gypsum) TLC plates were heat activated at 120° C for a minimum of four hours prior to use.

Ten µl aliquots of the sample extract were spotted on the plate using an Eppendorf pipette. As references, 4.0 and 0.4 ug of aflatoxin B_1 standards were spotted on the plate.

The TLC plates were placed in a tank covered with aluminum foil and developed in chloroform:acetone (90:10). The plates were removed when the solvent had traveled approximately 12.5 cm. Aflatoxins were quantitated using a Schoffel Spectrodensitometer (SD 3000). The function of the instrument was adjusted so that it operated in a single

beam fluorescence mode. The equation used for the calculation of aflatoxins is µg aflatoxins =

$$\frac{\frac{\text{Area of sample peak} - 5.00}{3.45}}{\text{Area of standard peak}} \times 0.4\ \mu g/\mu l \times 10\ \mu l \times \frac{100\ \mu l}{10\ \mu l}$$

The corrective factors applied to the area of the sample peak were derived from data supplied by the standard curve.

IX. VALIDITY OF ANALYTICAL METHOD

Standard Curve

A standard curve of the fluorescent properties of the aflatoxins at various concentrations was derived by spotting TLC plates with 2, 4, 6, 8, and 10 µg of aflatoxin B_1. The mean peak areas of two replicates were used to plot the standard curve and a regression analysis was performed to define the regression equation. Figure 2 is the standard curve obtained from the data. The correlation coefficient is 0.960 and the regression equation is

$$Y = 5.00 + 3.45\ (X)$$

where X represents µg of aflatoxin B_1 and Y represents the area under the curve.

Percent Recovery

The percent of added aflatoxin B_1 recovered from 50 grams corn samples was determined by the addition of 4, 8, and 12 µg of aflatoxin B_1 followed by extraction and quantification as described earlier. Percent aflatoxin B_1 recovered was calculated as:

$$\% \text{ recovered} = \frac{\mu g \text{ aflatoxin recoverd}}{\mu g \text{ aflatoxin added}} \times 100$$

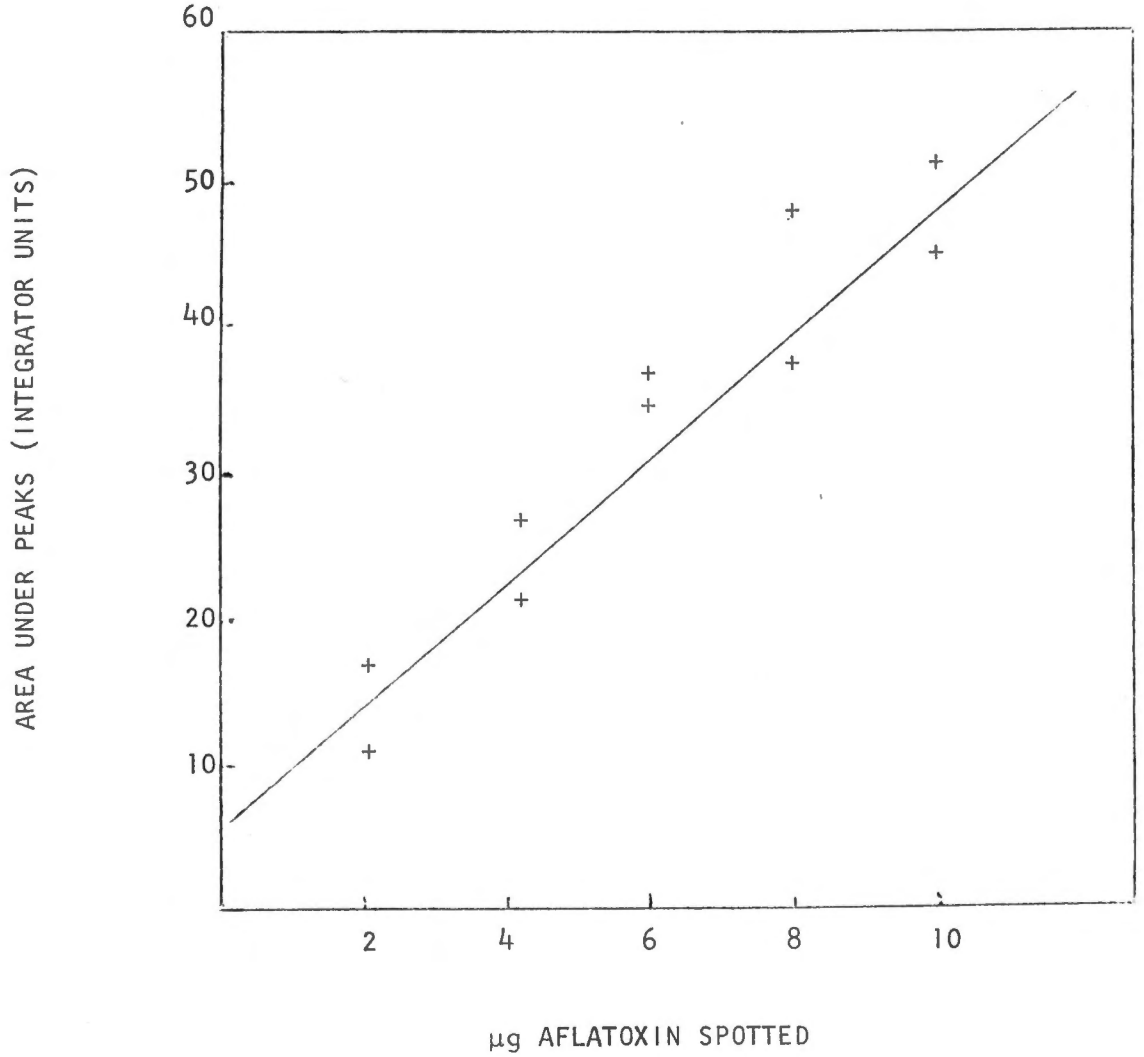


Figure 2. Spectrodensitometer standard curve obtained from the mean peak areas of two replicates containing 2, 4, 6, 8, and 10 µg aflatoxin B_1.

The recovery data are presented in Table 6. The recovery of more aflatoxin than is added is noted by Shotwell and Shannon (1975) and verified in this study. The most probable explanation for the recovery of inordinate amounts of aflatoxin is the presence of interferences that chromatograph at the same R_f as authentic aflatoxin.

Sensitivity Of Analytical Method

The sensitivity of the analytical method was determined by the addition of diminishing quantities of aflatoxin B_1 to 100 grams corn samples. The samples were analyzed as previously described and the least aflatoxin detected was considered to be the sensitivity limit of the method. The data are presented in Table 7. The data indicate that the analytical method as modified from Pons (1966) easily detected the smallest quantity of added aflatoxin.

TABLE 6

PERCENT RECOVERY OF ADDED AFLATOXIN B_1

Aflatoxin B_1 Added	Replicate 1	Replicate 2	Mean	Percent
		µg		
4	3.93	3.64	3.79	94.75
8	8.91	8.44	8.68	108.44
12	10.91	12.23	11.57	96.42

TABLE 7

SENSITIVITY LIMIT FOR ANALYTICAL PROCEDURE

µg Aflatoxin B_1 Added	µg Aflatoxin B_1 Recovered	Mean Percent
2.0	1.720	86.16
1.0	0.949	94.90
0.8	0.799	99.89
0.4	0.599	149.83
0.2	0.424	212.27

CHAPTER III

RESULTS AND DISCUSSION

After the samples had been assayed for aflatoxins, the data were grouped according to moisture level and compared by mean aflatoxin production. There was essentially no growth or toxin formation at the 13 percent moisture level. This was not unexpected since this low moisture is well within the accepted safety range. Aflatoxin B_1 was detected and quantified in the 18 and 23 percent moisture levels. The quantities of aflatoxin B_1 present in the intermediate and high moisture levels are presented in Table 8. A quantitative spore count was conducted to determine the load of spores present in each experimental combination. The spore count data are presented in Table 9. A significant increase in the load of spores per gram of sample was noted only in the high moisture samples. This indicates that a relatively high water activity (A_w) may be required for spore formation but not for toxin formation. The absence of significant increases in spore load and the presence of aflatoxin in the intermediate moisture samples indicates that toxins may be produced at moisture levels below those required for sporulation. The effect of moisture level of the samples was analyzed by a least squares analysis of variance and found to be significant at $P < 0.10$ (Table 10).

TABLE 8

MEAN AFLATOXIN YIELD PER MOISTURE LEVEL[1]

Moisture %	Inoculum[2]	Stress[3]	µg Aflatoxin	Standard Deviation µg
23	N-N	F-T	0.179	0.272
23	N-N	N	0.139	0.124
23	S-I	F-T	8.594	4.395
23	S-I	N	8.046	2.478
23	N-I	F-T	0.185	0.177
23	N-I	N	0.195	0.118
18	N-N	N	0.057	0.114
18	N-N	F-T	0.040	0.079
18	S-I	N	0.299	0.685
18	S-I	F-T	0.025	0.036
18	N-I	N	0.006	0.010
18	N-I	F-T	0.005	0.009

[1]Moisture level thirteen was not included due to absence of quantifiable amounts of aflatoxin B_1.

[2]N-N = Non-Sterile, Non-Inoculated
S-I = Sterile, Inoculated
N-I = Non-Sterile, Inoculated

[3]F-T = Freeze Thaw Cycle
None = Not subjected to stress

TABLE 9

QUANTITATIVE SPORE COUNT

Moisture %	Inoculum[1]	Stress[2]	Initial Inoculum	Load After Incubation Spores/gram
23	N-N	F-T	0.00	3.50×10^4
23	N-N	N	0.00	9.48×10^5
23	S-I	F-T	1.18×10^3	1.98×10^7
23	S-I	N	1.18×10^3	1.69×10^7
23	N-I	F-T	1.18×10^3	8.64×10^5
23	N-I	N	1.18×10^3	6.56×10^5
18	N-N	N	0.00	4.77×10^4
18	N-N	F-T	0.00	6.65×10^4
18	S-I	N	1.18×10^3	0.00
18	S-I	F-T	1.18×10^3	0.00
18	N-I	N	1.18×10^3	5.10×10^4
18	N-I	F-T	1.18×10^3	5.05×10^4
13	N-N	N	0.00	3.73×10^5
13	N-N	F-T	0.00	5.25×10^4
13	S-I	N	1.18×10^3	4.80×10^4
13	S-I	F-T	1.18×10^3	0.00
13	N-I	N	1.18×10^3	5.53×10^4
13	N-I	F-T	1.18×10^3	4.32×10^4

[1] N-N = Non-Sterile, Non-Inoculated
S-I = Sterile, Inoculated
N-I = Non-Sterile, Inoculated

[2] F-T = Freeze Thaw Cycle
None = Not subjected to stress

TABLE 10

SUMMARY OF LEAST SQUARES ANALYSIS OF VARIANCE OF STRESS VERSUS MOISTURE IN RELATION TO AFLATOXIN B_1 PRODUCTION

Source of Variation	Degrees of Freedom	Partial Sum of Squares	F Ratio
Replicate	10	11.206	1.8474[2]
Stress	1	0.163	0.2690[2]
Moisture	1	2.159	3.5595[3]
Inoculum	2	61.192	1.1370[3]
Moisture x Stress	1	0.119	0.6659[2]
Residual	10	2.966	

[1]Thirteen percent moisture level was deleted from the analysis.

[2]F ratios not significant at $P < 0.10$.

[3]F ratio significant at $P < 0.10$.

The stressing factor was found not significant ($P < 0.10$) in the least squares analysis of variance. The lack of significance of the stressing factor indicated that A. parasiticus retains viability and toxigenic capability after being subjected to recycled low temperature stress.

Inoculum was found to be significant in the production of aflatoxins ($P < 0.10$). Samples that had been sterilized and inoculated with spores of A. parasiticus showed the most dramatic aflatoxin production. Samples which were not sterilized and inoculated or not sterilized and not inoculated did not produce aflatoxin in amounts commensurate with sterilized and inoculated samples. The variation in toxin production indicates that A. parasiticus may be elaborate copius quantities of toxin when placed in a competitive environment. The ability of A. parasiticus to thrive in a competitive environment was not examined. It was noted in pilot studies that although A. parasiticus was able to grow in nutrient broth containing Penicillium notatum no aflatoxin was produced. The data representing aflatoxin production as a function of inoculum are presented in Table 11.

BYGF was evaluated for its reliability in indicating the presence of aflatoxin. The data indicate that the presence of BYGF is not a reliable indicator for the presence of aflatoxins except at high aflatoxin concentrations (Table 12).

A mycologic survey of the corn used in the study revealed that the microflora consisted of species of Alternaria, Penicillium, Rhizopus, and Cladosporium. A Pseudomonas specie was also found in the corn. No Aspergillus species were found in the grain bulk when it arrived from the farm.

TABLE 11

AFLATOXIN PRODUCTION AS A
FUNCTION OF INOCULUM

Inoculum	Mean Production of Alfatoxin, µg
Sterile, Inoculated	6.960
Non-Sterile, Inoculated	0.263
Non-Sterile, Non-Inoculated	0.105

TABLE 13

RELIABILITY OF BYGF AS AN
INDICATOR OF AFLATOXIN

Moisture %	Inoculum[1]	Stress[2]	Percent Positive[3]	Percent Negative[4]
23	N-N	F-T	18.20	45.50
23	N-N	N	100.00	0.00
23	S-I	F-T	27.30	63.60
23	S-I	N	100.00	0.00
23	N-I	F-T	36.40	27.30
23	N-I	N	27.30	36.40
18	N-N	N	27.00	36.50
18	N-N	F-T	0.00	45.50
18	S-I	N	0.00	72.70
18	S-I	F-T	0.00	54.60
18	N-I	N	0.00	72.70
18	N-I	F-T	0.09	18.20

[1] N-N = Non-Sterile, Non-Inoculated
S-I = Sterile, Inoculated
N-I = Non-Sterile, Inoculated

[2] F-T = Freeze Thaw Cycle
None = Not subjected to stress

[3] Positive was defined as samples containing both BYGF and aflatoxin.

[4] Negative was defined as samples which contained BYGF without aflatoxin or samples which contained aflatoxin without BYGF.

Large variances are noted in all of the experimental treatments. Such inconsistencies (Table 8) would indicate the complexity of the factors influencing the production of aflatoxins *in vitro*. This variation may have been due to (1) inherent variation in the ability of the organisms to produce aflatoxins. (2) Uniform differences in the preparation of samples although a completely randomized block design was used. (3) Variations in the extraction procedure although every precaution was taken to standardize the method among replicates.

It must be noted that the use of propylene oxide for sterilization of corn is not completely effective. Subsequent to the exposure of the corn to the propylene oxide vapors, a *Penicillium* specie grew out after approximately 15 days. The inefficiency of propylene oxide in the sterilization of grain was also noted by Pixton and Warburton (1973).

CHAPTER IV

SUMMARY

The acceptability of corn as a substrate for aflatoxin production by A. parasiticus was demonstrated in this study. The vast quantities of corn produced in the United States make possible aflatoxin contamination a severe problem.

This study indicated that in the context of this experimental work, a moisture content of at least 18 percent (77 percent relative humidity) in corn is necessary for aflatoxin production. Corn stored on-the-farm in the Southeastern United States is in constant danger of becoming contaminated with aflatoxin due to high prevailing relative humidities (average above 65 percent).

Based on the results of this study the following conclusions were made.

1. Moisture content of stored corn combined with the nature of the inoculum is highly influentual in the production of aflatoxins by A. parasiticus. High moisture levels and a non-competitive environment were found to be exceedingly conducive to toxin production.
2. Pre-incubation stress did not affect the ability of A. parasiticus to grow and elaborate aflatoxins.
3. Growth of A. parasiticus on sterilized high moisture corn produced significantly higher amounts of aflatoxin when compared to the other treatment combinations ($P < 0.10$).

4. BYGF was found to be non-significant in indicating the presence of aflatoxins except when the toxins were present in large quantity.

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