

To the Graduate Council:

I am submitting herewith a thesis written by David Carter Mobley entitled "The Effect of Temperature, Initial Moisture Content, and Level of Inoculum on Aflatoxin and Ochratoxin Production in Corn." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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THE EFFECT OF TEMPERATURE, INITIAL MOISTURE CONTENT, AND
LEVEL OF INOCULUM ON AFLATOXIN AND OCHRATOXIN
PRODUCTION IN CORN

A Thesis
Presented for the
Master of Science
Degree
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David Carter Mobley

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DEDICATION

This manuscript is dedicated to my parents, Mr. and Mrs. Fred W. Mobley, my sister, Lee Ann Vinson, and my grandparents, Mr. and Mrs. Cleo Hodges and Mrs. Fred W. Mobley.

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ABSTRACT

Mycotoxin contamination of grains can occur as a result of invasion by field fungi or by growth of storage fungi in improperly stored crops. Environmental factors and competing mycoflora can affect fungal growth and mycotoxin production. Since mycotoxins pose a potential health hazard to both man and animals, this study was conducted to evaluate combinations of temperature, initial moisture content (MC), and level of inoculum during storage of corn to determine optimal and limiting conditions for aflatoxin and ochratoxin production during fungal competition and growth in pure culture.

Two fungi were used in this study, Aspergillus parasiticus NRRL 3145 and A. sulphureus NRRL 4077. Fungal spores were added to sterilized corn at levels of 10^1 , 10^3 , 10^5 , and 10^7 per 50g corn at 16%, 20%, 24%, and 28% MC. Samples were incubated at 20, 24, 28, and 32C for 3 weeks. Aflatoxins and ochratoxin A were then extracted from corn samples and analyzed by HPLC and TLC.

More toxin was produced during fungal competition than when each fungus was grown alone. Maximum production of aflatoxin B₁, ochratoxin A, and total toxin was at a lower temperature (24C) in mixed culture than in pure culture. Maximum production of aflatoxin G₁ occurred at 24C in both pure and mixed culture. For aflatoxin B₁, G₁, ochratoxin A, and total toxin in mixed culture, 24C resulted in significantly more toxin production ($P < 0.05$) than 20, 28, and 32C. In pure culture, 20C resulted in significantly less aflatoxin B₁,

G₂, ochratoxin A, and total toxin production ($P < 0.05$) than 24, 28, and 32°C. Competition affected the temperature dependent relationship of aflatoxin B₁ and G₁.

In both pure and mixed culture, 16% was the minimum MC for toxin production. For pure cultures, 16% MC resulted in significantly less aflatoxin B₁, aflatoxin G₁, ochratoxin A, and total toxin production ($P < 0.05$) than 20, 24, and 28% MC. Optimum conditions for toxin production were more limited during fungal competition. Inoculum was not a significant source of variation. Only for aflatoxin G₂ and ochratoxin A in pure culture did the 10^1 inoculum level result in significantly less toxin production ($P < 0.05$) than the other three inoculum levels. No significant differences between inoculum levels were found in mixed culture.

In summary, the presence of A. parasiticus enhanced ochratoxin A production by A. sulphureus and the presence of A. sulphureus enhanced aflatoxin production by A. parasiticus. Toxin production increased probably as a means of survival. Based on the results of this experiment, recommendations for corn storage to reduce or prevent toxin formation by A. parasiticus and A. sulphureus are: a moisture content $< 16\%$; a temperature $< 20^\circ\text{C}$; and an inoculum level $< 10^1$ spores/50g since in some cases, 10 spores per 50 grams of corn resulted in toxin production.

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CHAPTER I

INTRODUCTION

Mycotoxins are secondary metabolites produced by molds. Aspergillus flavus and A. parasiticus are fungi which produce aflatoxins--mycotoxins which function as hepatotoxins, carcinogens, teratogens, and/or mutagens. Seven Aspergillus species and six Penicillium species produce ochratoxins--mycotoxins which function as nephrotoxins, hepatotoxins, and teratogens, but not as carcinogens. Cereal grains (especially corn) represent the most important feed and food source that may become contaminated with mycotoxins (3, 69). Mycotoxin contamination of corn is governed by environmental factors and occurs as a result of crop invasion by fungi in the field or by growth of the fungi in improperly stored crops. The presence of mycotoxins in corn represents a potential health hazard to man and animals. Because corn is transported around the globe, there are essentially no areas of the world that can be considered mycotoxin-free (70).

Jarvis (81) classified the environmental factors affecting growth and mycotoxin production on natural substrates into three main areas: physical factors, chemical factors, and biological factors. Of all the factors, Jarvis (81) considered temperature, relative humidity, strain of toxigenic organism, and occurrence of competitive growth as the major ones. Northolt et al. (101) reported temperature as the most important factor for mold growth. All toxigenic fungi have

maximum, minimum, and optimum temperatures for growth and mycotoxin formation (71). Production of aflatoxins B₁ and G₁ have been reported to be temperature dependent (44, 48, 49, 51, 90, 123, 126, 135). Although a fungus may grow and produce mycotoxins over a wide range of temperatures when grown as a pure culture, growth and toxin production may be more limited under natural conditions because of competition (71). Jarvis (81) reported that two or more toxigenic fungal strains growing together may produce more or even less toxicity than either might produce alone. The influence of a fungal competitor cannot be predicted but must be determined for each situation (149). Diener (51) reported that moisture content or relative humidity governed fungal growth and mycotoxin production. Northolt et al. (101) found that Aw was the most important factor for mycotoxin production. The moisture range for aflatoxin production is 16-40% (71). The moisture range for ochratoxin A production is not well defined.

Aflatoxin may be contributed to control flasks by the inoculum (125, 135). Christensen and Mirocha (34) and Hesselstine et al. (68) found fungal spores and mycelium to be sources of aflatoxin. Sharma et al. (131) and Haggblom (63) reported that smaller inoculum sizes may result in increased aflatoxin and ochratoxin A production. Many other factors such as pH, substrate, incubation time, and atmosphere, and combinations of these factors, may also affect mycotoxin production.

This study was undertaken to evaluate three environmental factors: temperature, initial moisture content, and level of inoculum during storage of corn to determine optimal and controlling conditions for aflatoxin and ochratoxin production during fungal competition as compared to pure culture.

CHAPTER II

REVIEW OF THE LITERATURE

I. JUSTIFICATION AND OBJECTIVES

Mycotoxin contamination of small grains can occur as a result of invasion by fungi in the field or by growth of fungi in improperly stored crops. Environmental factors such as moisture, temperature, atmosphere, incubation time, inoculum size, pH, substrate composition, and competition by fungi and other microorganisms can affect fungal growth and mycotoxin production. Much research has been done on the effect of environmental factors on the production of aflatoxin and ochratoxin in culture media in pure culture. However, only limited work has been done on aflatoxin and ochratoxin production by mixed cultures in corn with respect to environmental conditions (26, 95, 104, 129, 151). Therefore, this study was undertaken to evaluate combinations of temperature, initial moisture content, and level of inoculum during storage of corn to determine optimal and controlling conditions for aflatoxin and ochratoxin production during fungal competition as compared to pure culture.

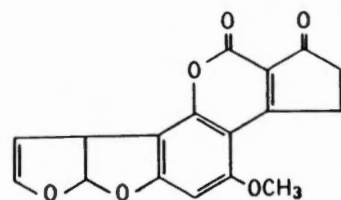
II. CHEMISTRY

Chemical Structures and Properties

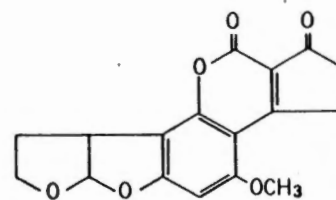
The term aflatoxin was given to the toxic compounds first isolated from the mold Aspergillus flavus (140). Aflatoxins are

secondary metabolites produced by the fungi, A. flavus and A. parasiticus (3). The two major types of aflatoxins are named B and G because they fluoresce blue and green respectively under longwave ultraviolet light (3, 148). The B and G aflatoxins were further designated as B₁, B₂, G₁, and G₂ based upon their order of separation into individual components when analyzed by thin layer chromatography (98, 140). The B aflatoxins contain a coumarin nucleus fused to a bifuran structure and a cyclopentenone ring which is replaced in the G aflatoxins by a six-membered lactone (3, 148). Aflatoxins B₂ and G₂ lack a double bond in the bifuran structure and are designated as the dihydro-derivatives of aflatoxins B₁ and G₁, respectively (3, 62). Chemical structures of the aflatoxins are presented in Figure 1 while their physical properties are listed in Table 1.

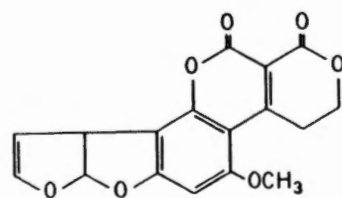
The term ochratoxin is derived from the name of the fungus from which it was first isolated, Aspergillus ochraceus (77). There are five commonly recognized members of the ochratoxin family: ochratoxin A, B, C, α , β (77), and their structures are shown in Figure 2. Ochratoxin A contains 7-carboxy-5-chloro-3,4-dihydro-8-hydroxy-3R-methylisocoumarin linked over its 7-carboxy group to L- β -phenylalanine by an amide bond (69, 139). Ochratoxin A is a colorless crystalline compound with the molecular formula C₂₀H₁₈ClNO₆ (64). Ochratoxin B (C₂₀H₁₉NO₆) and C (C₂₂H₂₂ClNO₆) are the unchlorinated analogue and ethyl ester, respectively, of ochratoxin A (64, 69). Ochratoxin A fluoresces green under UV light while ochratoxin B fluoresces blue (98, 148). Mirocha and Christensen (98) reported that ochratoxins A and B were relatively heat stable. Ochratoxin α



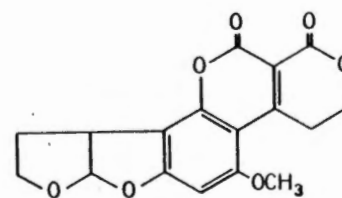
B₁



B₂



G₁

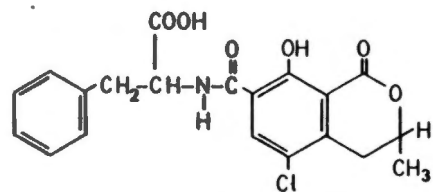


G₂

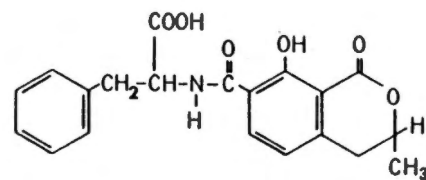
FIGURE 1. Chemical structures of the aflatoxins.

TABLE 1. Characteristics of the Aflatoxins

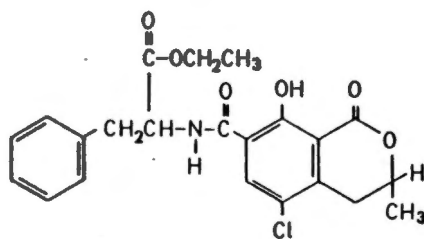
Aflatoxin	Molecular Formula	Molecular Weight	Max. Fluorescent Emission (nm)
B ₁	C ₁₇ H ₁₂ O ₆	312	425
B ₂	C ₁₇ H ₁₄ O ₆	314	425
G ₁	C ₁₇ H ₁₂ O ₇	328	450
G ₂	C ₁₇ H ₁₄ O ₇	330	450



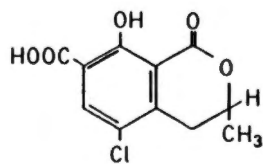
A



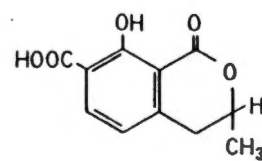
B



C



α



β

FIGURE 2. Chemical structures of the ochratoxins.

(5-chloro-7-carboxymellein) is the non-amino acid portion of ochratoxins A and C while ochratoxin β (7-carboxymellein) is the non-amino acid portion of ochratoxin B (77).

Biosynthesis

Aflatoxins are produced biosynthetically by a polyketide pathway (3, 12, 94, 147). Polyketides are formed by condensation of an acetyl unit with malonyl units, with simultaneous decarboxylation (12, 147). Polyketides possess activated methylene groups which may undergo internal aldol-type condensations to form aromatic compounds which are subject to electrophilic substitution (147). The polyketide pathway leads almost entirely to secondary metabolites and is used mainly by fungi of the Ascomycetes and Fungi Imperfecti (147). Polyketides are classified as triketides, tetraketides, pentaketides, etc., according to the number of C_2 -units which have contributed to the polyketide chain (12, 147). Aflatoxins are classified as nonaketides (12, 147).

Details of aflatoxin biosynthesis have been obtained mainly from ^{14}C and ^{13}C -labeling experiments and the use of blocked mutants (12, 13, 15, 47, 94, 134). Dichlorvos, an organophosphate insecticide, has been used as a specific aflatoxin inhibitor to also determine steps in the biosynthetic pathway (41, 134). The entire carbon skeleton of aflatoxin B_1 was found to be derived from acetate with methionine contributing the methoxy-methyl group (15, 74, 138). Bennett et al. (13) proposed that there are six intermediates involved in the biosynthesis of aflatoxin B_1 ; five are polyhydroxyanthraquinones

(norsolorinic acid, averantin, averufin, versiconal hemiacetal acetate, versicolorin A), while the sixth is a xanthone (sterigmatocystin) (12). In 1965, Thomas (143) suggested that presumed biogenetic intermediates could all be involved in a common biosynthetic pathway:

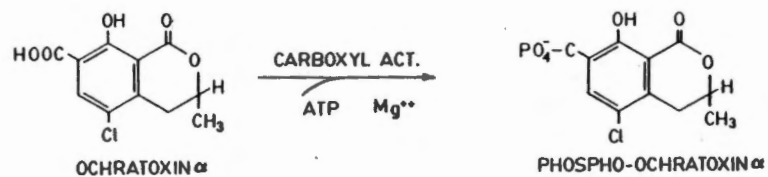
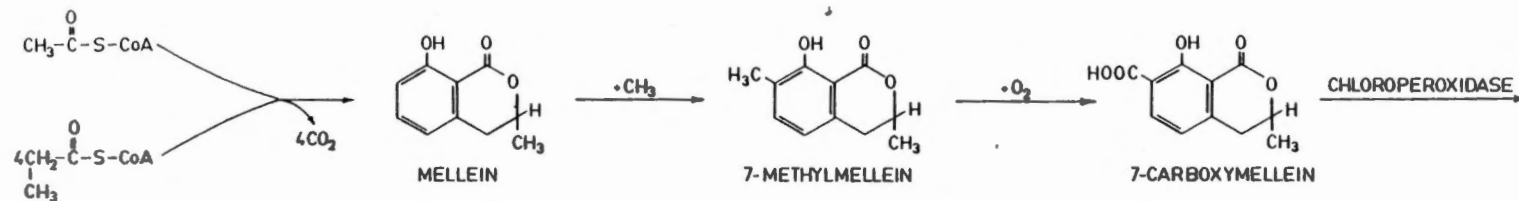
anthraquinones $\longrightarrow$ xanthenes $\longrightarrow$ coumarins.

A proposed pathway for the biosynthesis of aflatoxin B₁ is presented in Figure 3 (13, 134). One acetyl CoA molecule is condensed with nine malonyl CoA molecules to form a hypothetical C₂₀ polyacetate compound which is unstable and is converted into norsolorinic acid (75, 134). Bennett et al. (13) determined that norsolorinic acid was converted into averantine, and averantine into averufin. Averufin is subsequently converted by ring cleavage and other reactions to versiconal hemiacetal acetate (VHA), previously misidentified as versiconal acetate and now reported to exist as a mixture of two hemiacetal forms (12, 41, 59). Versicolorin A, which contains the unique difurano group, is formed from VHA. After a series of modifications and oxidation reactions, versicolorin A is converted into sterigmatocystin. The latter compound then undergoes an oxidative ring cleavage to yield aflatoxin B₁. Using ¹⁴C labeled aflatoxins as substrates for homogenates prepared from A. parasiticus, Maggon and Venkitasubramanian (93) found aflatoxin B₁ to be the biogenic precursor of all the other aflatoxins. Heathcote et al. (67) indicated that aflatoxin B₁ is converted into most of the other aflatoxins either by oxidation and/or hydroxylation. The conversion of aflatoxin B₁ to G₁ was proposed to involve a keto-lactonase system (93).

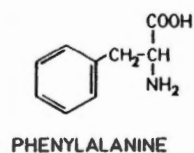
Ochratoxin A is also believed to be derived by a polyketide pathway and is classified as a pentaketide (77, 147). Labeling experiments with ^{14}C and ^{13}C labeled precursors have been useful in determining the biosynthetic scheme for ochratoxin A (40, 77, 128, 137, 147). The scheme proposed by Huff and Hamilton (77) is presented in Figure 4. Much evidence indicates that biosynthesis begins with the condensation of acetyl CoA and malonyl CoA by stepwise, head-to-tail addition of acetyl units from malonyl CoA until a pentaketide (five C_2 -units) is formed (77, 128, 137). The enzyme-bound pentaketide then cyclizes to mellein which undergoes methylation at the seventh position (its phenolic group is ortho-directing) to form 7-methylmellein, a proposed intermediate. Subsequent oxidation of the methyl group results in the formation of 7-carboxymellein (ochratoxin B) (77). The enzyme chloroperoxidase is assumed to be required for the chlorination of 7-carboxymellein to ochratoxin α (5-chloro-7-carboxymellein) (77). Chloroperoxidase has not been demonstrated in ochratoxigenic fungi, but it is commonly found in plants, animals, and microorganisms and is involved in the biosynthesis of over 200 naturally occurring halogenated compounds (133). The carboxyl group of ochratoxin α is activated by acceptance of a phosphate group from ATP in the presence of Mg^{2+} to form the enzyme bound intermediate-phospho-ochratoxin α .

The biosynthesis of phenylalanine is assumed to occur via the shikimic acid pathway although this pathway has not been actually demonstrated during ochratoxin biosynthesis (40, 88). However,

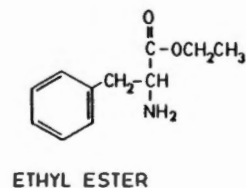
POLYKETIDE:



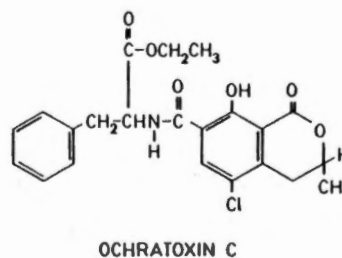
SHIKIMIC ACID PATHWAY



ESTERIFICATION



SYNTHESIS:



ESTERASE

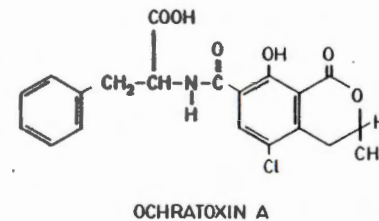


FIGURE 4. Proposed scheme for the biosynthesis of the ochratoxins.

phenylalanine is known to be incorporated totally into ochratoxin A (77, 128, 137). Huff and Hamilton (77) proposed that phenylalanine is converted to an ethyl ester via a phosphorylated intermediate.

The synthesis reaction begins with the nucleophilic attack by the free amine group of the phenylalanine ester on the carbonyl group of phospho-ochratoxin α with subsequent displacement of the phosphate group and formation of an amide bond. The product formed is ochratoxin C, the ethyl ester of ochratoxin A. Ferreira and Pitout (58) demonstrated that the joining reaction required the presence of an enzyme complex called ochratoxin synthetase. Ochratoxin C is converted by an esterase to ochratoxin A, the final terminal biosynthetic product.

III. ASPECTS OF THE PROBLEM

Natural Occurrence and Contamination

Isolates of Aspergillus flavus and A. parasiticus are known aflatoxin producers (62). Aflatoxin is found naturally in edible nuts (peanuts, Brazil nuts, pistachio nuts, almonds, walnuts), oil-seeds (cottonseed, copra), and cereal grains (corn, oats, barley, sorghum, wheat, rice) (39). Cereal grains (especially corn) represent the most important feeds and foods that may become contaminated with mycotoxins (3, 70). It is estimated that approximately 80% of the Americans' exposure to aflatoxin in the diet is due to the consumption of contaminated corn and its products (106). Aflatoxin residues have been detected in meat, in milk, and in the human liver

(3, 27, 114). The occurrence of aflatoxin in corn is a problem in the southern United States and wherever corn is grown in the warmer and more humid parts of the world (72). Because cereal grains are transported around the globe, there are essentially no areas of the world that can be considered aflatoxin-free (70). Aflatoxin contamination of corn is moderated by environmental factors and occurs either as a result of crop invasion in the field or by growth of fungi in improperly stored crops (3).

Ochratoxin A is produced by seven Aspergillus species (A. ochraceus, A. sulphureus, A. melleus, A. sclerotiorum, A. alliaceus, A. ostianus, A. petrakii) and six Penicillium species (P. viridicatum, P. palitans, P. cyclopium, P. commune, P. variabile, P. purpurescens) (46, 69). Ochratoxigenic fungi are frequent contaminants of corn, rice, barley, oats, sorghum, peanuts, pecans, Brazil nuts, and cottonseed (40, 64, 85). Levels of ochratoxin A were found to range from 100 ppb in wheat to 27,500 ppb in a mixture of moldy barley and oats (46). Ochratoxin A has been reported as a natural contaminant of grains and feeds in Bulgaria, Rumania, Yugoslavia, Sweden, Denmark, and North America (40, 85). Swine and poultry are very susceptible to ochratoxicosis and ochratoxin A residues have been detected in swine and poultry products (100, 127, 141). Like aflatoxin, ochratoxin A contamination is also controlled by environmental factors and pre-harvest and post-harvest conditions (70).

The presence of mycotoxins in foods and feeds represents a definite health hazard to man and animals (39). Thus, mycotoxins

could have an economic impact on worldwide livestock and meat production (80). It is of vital importance to investigate the entire range of environmental factors under which both aflatoxin and ochratoxin are produced so that the limiting and controlling conditions can be determined and mycotoxin production reduced or prevented.

Toxicity and Metabolism

Aflatoxins function as hepatotoxins, carcinogens, teratogens, and/or mutagens (23, 38). The liver is considered the primary target organ and bile duct proliferation is the typical pathological change (39, 141). Holzapfel et al. (73) determined the order of aflatoxin toxicity (based on the LD50 in ducklings) to be $B_1 = M_1 > G_1 > M_2 = B_2 > G_2$. Toxicity of aflatoxin B_1 was proposed to require metabolic activation by a microsomal mixed-function oxidase system to an active form, the 8,9 epoxide of aflatoxin B_1 (previously named 2,3 epoxide but renumbered according to IUPAC recommendations) (38, 76). This active isomer can form adducts with nucleic acids (RNA and DNA) and proteins, thus rendering these macromolecules biologically inactive (89). Biochemical lesions result from adduct formation and may inhibit basic metabolic processes such as protein synthesis (76).

Turkeys, ducklings, chickens, mice, rats, trout, rabbits, guinea pigs, hamsters, dogs, cats, pigs, cattle, sheep, and monkeys have all been used to study the biological effects of aflatoxin (24, 25). Economic losses can result from the ingestion of aflatoxin by livestock (80). When exposed to aflatoxin, pigs had decreased weight gain and feed conversion, beef cattle experienced lower weight

gains, and dairy cattle produced less milk (3, 65). When aflatoxin B₁ is ingested by dairy cattle, it is converted into aflatoxin M₁ which is excreted in the milk (3). There is concern about the safety of milk because aflatoxin M₁ (the milk toxin) retains the toxicity of aflatoxin B₁ even though it is less carcinogenic (3). Young animals are generally more susceptible to aflatoxicosis than older ones, and males are more prone than females (65). The order of susceptibility in mammals was determined to be: pigs (3-12 weeks old) and pregnant sows > calves > fattening pigs and mature cattle > sheep (2). Dvorak et al. (53) and Fehr and Delage (57) found that aflatoxin alters the rumenal microbial population which effects the proportions of volatile fatty acids (VFA), the major energy source of ruminants (96). However, the relative resistance of ruminants to aflatoxicosis indicates that some rumenal bacteria may be able to detoxify aflatoxin (96).

There is little direct evidence of aflatoxicosis in humans, but there is considerable indirect evidence from Africa, Southeast Asia, and India which links aflatoxin to human illness (22, 130, 152). High incidences of liver cancer have been correlated with the aflatoxin content of various foods in these areas (22). Reye's syndrome, a disease of unknown origin which causes encephalopathy and fatty degeneration of the viscera, has also been associated with aflatoxin (38, 39, 152).

Ochratoxins are nephrotoxins, hepatotoxins, and teratogens, but are not carcinogens (46, 98, 111). The main target organ is the

kidney, and ochratoxicosis usually results in renal damage (46, 98). Ochratoxin A is more toxic than ochratoxin B, but ochratoxin A is about half as toxic as aflatoxin B₁ (111). When injected into rats, ochratoxin A was metabolized to ochratoxin α and 4-hydroxy-ochratoxin A (79). However, in vitro, ochratoxin A was hydrolysed by proteolytic enzymes to ochratoxin α and phenylalanine (112). The toxic effects of the ochratoxins may result from the interaction of the phenolic hydroxyl group with proteins and enzymes (35, 64). Investigators have suggested that ochratoxin A inhibits the phosphorylase enzyme system (a complex which catalyzes the breakdown of glycogen to phosphorylated glucose), carbohydrate metabolism, and possibly protein metabolism (64, 112).

The biological effects of ochratoxin A have been studied in rats, mice, chicks, hens, ducklings, rainbow trout, guinea pigs, swine, dogs, goats, sheep, and dairy cattle (28, 64, 99, 119, 148). Ribelin et al. (119) found that cattle fed up to 100 mg/kg of ochratoxin A converted most of the toxin to ochratoxin α , a nontoxic hydrolysis product. They concluded that ochratoxicosis in adult ruminants would most likely occur only after prolonged ingestion of ochratoxin A (119). Ochratoxin A residues have been found in the meat of swine and poultry (98).

Porcine nephropathy is a mycotoxicosis caused by the possible synergistic activity of two toxins, ochratoxin A and citrinin (148). This disease was associated with the ingestion of moldy grains such as rye and barley by swine in Denmark (28). Balkan endemic nephropathy,

found only in certain areas of Bulgaria, Romania, and Yugoslavia, is a fatal renal disease in humans which is very similar to porcine nephropathy (106). Krogh and his associates (84) have suggested ochratoxin A as a possible cause of endemic Balkan nephropathy.

IV. INFLUENCE OF ENVIRONMENTAL FACTORS

Temperature

Agnihotri (1) stated that temperature was the most important environmental factor affecting metabolic activities of fungi, while other researchers agreed that environmental factors such as moisture content and relative humidity were of equal importance (90, 101, 102, 104). Fungal growth occurs within a certain range of temperature which varies with different fungi (1). Aflatoxin production was found to occur within a more limited temperature range than that allowing growth (107). Schindler et al. (123) reported that maximum growth was not correlated with maximum aflatoxin production and, at certain temperatures, growth occurred without aflatoxin production.

Panassenko (107) determined the minimum growth temperature for Aspergillus flavus to be 6-8C. Recently, Park and Bullerman (109) reported no growth of either A. flavus or A. parasiticus at 5C in a liquid medium. Other researchers observed the minimum growth temperature of A. flavus to be in the range of 10-14C (9, 49, 101). The maximum growth temperature for A. flavus was found to be between 44 and 46C (9, 107). The optimum growth temperature for A. flavus was reported as 18C (117), 25C (126), 29C (123), 30C (113), 32C (101),

33C (9), and 35C (123). Optimum growth of A. parasiticus was found between 32C and 35C (101, 132).

Many researchers have reported 24-25C as the temperature range for maximum aflatoxin production (42, 48, 49, 51, 81, 82, 102, 108, 123, 132). Northolt et al. (102) found that different strains of A. flavus produced maximum levels of aflatoxin at different temperatures, and they detected aflatoxins at temperatures of 13-16C, 10-24C, and 31C (102).

Previous research has shown that the production of aflatoxins B₁ and G₁ was temperature dependent (44, 48, 49, 51, 90, 123, 126, 135). Maximum B₁ production occurred at temperatures of 24C (51, 101, 102, 117), 30C (44), 32C (101, 102), 35C (44), 28-30C (51), 28-32C (135), and 30-35C (51). Maximum G₁ production was reported at 20C (44), 24C (123), 25C (44), 28C (135), 30C (44, 51), and 20-25C (51). Diener and Davis (48) found that aflatoxin B₁ generally increased with higher temperature, whereas aflatoxin G₁ decreased. Results of their studies showed that the decrease in total aflatoxins (B₁ and G₁) produced by A. parasiticus at 35C in comparison with that at 25 and 30C was mainly a result of a decrease in the amount of aflatoxin G₁ produced (48). Many researchers have found more G₁ production at lower temperatures and more B₁ production at higher temperatures (44, 49, 51, 90, 123, 126, 135). Schroeder and Hein (126) suggested that aflatoxin G₁ was more easily metabolized than B₁ at higher temperatures. Sorenson et al. (135) postulated that aflatoxin G₁ may be sensitive to degradative enzymes which were not

active at temperatures below 32°C. Lin et al. (90) proposed that the enzyme responsible for the B₁ to G₁ conversion was more efficient at 25°C than at 33°C. Their results also indicated that the length of time at optimal temperature, fungal strain, available nutrients, and other environmental factors all have a significant affect on total amounts and relative proportions of aflatoxins B₁ and G₁ (90).

The lower limit for aflatoxin production has been reported as 7°C (123), ≤ 7.5 (125), 12°C (44, 51), 13 ± 1 °C (49), 15°C (109), and 10-13°C (102). The upper limit for production of aflatoxin was determined to be approximately 41°C (44, 49, 51, 123, 125) while Rabie and Smalley (117) reported an upper limit of 36°C.

The optimum temperature for growth of Aspergillus ochraceus was determined to be 20°C (121), 25°C (121), and 28-32°C (110). Northolt et al. (103) reported the lower and upper limits for growth as 8°C and 37°C, respectively.

Ochratoxin A production occurred within a narrower range of water activity (Aw) and temperature compared with those for growth (104). Maximum ochratoxin A production was reported at 25°C (63, 121), 28°C (37, 51, 146), 30°C (10), and 31°C (103). Northolt et al. (103) found the lower and upper limits for ochratoxin A production to be 12°C and 37°C respectively. Ciegler (37) detected very small amounts of ochratoxin A in corn and wheat incubated at 10°C. Results from the study by Trenk et al. (146) showed very little ochratoxin A production in corn, rice, and wheat bran at 4°C and low levels in rice and wheat bran at 37°C. Haggblom (63) found no toxin production at

10C by A. ochraceus. He proposed that enzymes involved in ochratoxin A synthesis were more sensitive to low temperatures than enzymes of primary metabolism (63). Sansing et al. (121) reported no ochratoxin A production by A. ochraceus at 10, 40, and 45C. Optimal and limiting temperatures for ochratoxin A production were dependent upon the strain and species of the organism producing the ochratoxin (146).

Moisture Content and Relative Humidity

Diener (51) concluded that the moisture content (MC) of the substrate or the relative humidity (RH) surrounding the substrate was the major factor in determining growth and aflatoxin production by Aspergillus flavus and A. parasiticus. However, Northolt et al. (101) stated that temperature was the most important factor for fungal growth, while water activity (A_w) was the major factor for aflatoxin production.

Growth of storage fungi was observed at 13-18% MC (81). Gilman and Barron (61) reported growth of A. flavus at 18% MC in oats, wheat, and barley. Barley was only slightly invaded by aspergilli when stored for 24 weeks at 14% MC and 20C (92). Christensen and Mirocha (34) reported that members of the A. flavus group require a MC of 18.0-18.5% to invade starchy cereal grains such as rice, wheat, and corn. Corn is more susceptible to deterioration than wheat because it is harvested and frequently stored at higher MC (115). Corn stored at 16% MC and 15C supported no growth of A. flavus after six months of storage (115). However, at 18% MC and 25C, A. flavus grew within 4.5 months. Koehler (83) reported growth of A. flavus at a MC of 18.3% in corn.

No aflatoxin was produced by A. parasiticus in barley with a MC of $\leq 16.5\%$ (29). However, at 28% MC, all four aflatoxins (B_1 , B_2 , G_1 , G_2) reached maximum concentrations. Chang and Markakis (29) concluded that a MC of $\geq 16\%$ was inadequate for safe storage of barley at temperatures near 25C.

Northolt and Bullerman (104) stated that water activity (A_w) was a more useful expression for available water than moisture content (MC). A_w is defined as the ratio of vapor pressure of substrate to the vapor pressure of pure water at the same temperature and pressure (101). At a low A_w , water is bound by salts, sugars, proteins, and other solutes and is not available for utilization by fungi. Relative humidity (RH) is water activity expressed as a percentage.

Galloway (60) observed the minimum RH for fungal growth to be 75 to 95% depending on the species. Minimum growth of A. flavus occurred at 80% RH and 30C (8, 136). A relative humidity of $\geq 85\%$ was shown to support the growth of A. flavus (8, 18).

The minimum RH for aflatoxin production was determined to be 83-86% in peanuts (50, 51), 84% in corn (78), 85% in rice (18), and 92% in soybeans (44). In general, $83.5 \pm 0.5\%$ appeared to be the limiting RH for aflatoxin production in agricultural products depending on the substrate, fungal species, and length of storage period (44). Diener (51) determined the optimum RH range for growth and aflatoxin production in peanuts to be 89-99% at 30C and 8 weeks of storage. Boller and Schroeder (18) found 100% RH as optimum for

aflatoxin production in rice by A. parasiticus. Optimum RH for aflatoxin B₁ production was 95% and 99% depending upon the substrate (49). Sanders et al. (120) studied the effects of a combination of factors on growth and aflatoxin production by A. flavus in peanuts and found aflatoxin levels to decrease as the RH decreased from approximately 99% to 92% to 86%.

On agar media, Ayerst (9) reported the limiting Aw for A. flavus as 0.78. Northolt et al. (101) demonstrated that growth without toxin production occurred at 0.83 Aw and 0.82 Aw on malt-extract sucrose agar and malt-extract glycerine agar, respectively. Maximum aflatoxin B₁ production occurred at 0.99 Aw on both agar media (101). Ayerst (9) found the optimum Aw for A. flavus as > 0.98. Northolt et al. (101) concluded that a reduced Aw inhibits toxin production more than growth.

Northolt and Bullerman (104) observed that ochratoxin A was produced within narrower ranges of Aw and temperature than those for growth. Christensen (30, 33) found that pure cultures of A. ochraceus invaded wheat with 15-16% MC in equilibrium with a RH of approximately $\leq 80\%$ at 20-25C, while Koehler (83) reported growth in corn with 15.6% MC. The minimum Aw for mold growth was found to be 0.79-0.83 (103). On agar media containing either sucrose or glycerol for Aw adjustment, the minimum Aw for ochratoxin A production was determined to be 0.83-0.87 (103). Northolt et al. (103) reported the optimum Aw for ochratoxin A production by A. ochraceus to be 0.99 at 24C. Haggblom (63) observed maximum accumulation of ochratoxin A in barley with an initial MC of 22% after 28 days of incubation at 25C.

Inoculum Size

Sharma et al. (131) proposed that total growth of A. parasiticus NRRL 3145 in a chemically defined medium was independent of inoculum size. However, an inverse relationship between toxin production and inoculum size existed, as the inoculum size decreased, aflatoxin production gradually increased. On day 14 of incubation, as the inoculum size was reduced by 4 to 5 log cycles, the amount of total aflatoxin in the medium almost doubled. On day 28 of incubation, aflatoxin levels were similar in all cultures, suggesting that cultures with smaller inoculum size produced more aflatoxin, but also degraded it faster. When the inoculum size was $> 10^3$ spores, only biphasic toxin production occurred; monophasic production occurred with spore levels $< 10^3$. Biphasic shows two periods of peak toxin production, while monophasic exhibits only one period of maximum toxin production. Ciegler et al. (36) observed that cyclic variations in aflatoxin production were associated with inoculum size. Applegate and Chipley (4) found production of A. flavus NRRL 3145 to be monophasic in cultures exposed to gamma irradiation. Both serial dilution and irradiation were methods used by Sharma et al. (131) to reduce the inoculum size to their experimental levels. They also found a lag period of two to three days in aflatoxin production for cultures developing from smaller inoculum size in comparison to larger inoculum size. Aflatoxin was detected on day seven for the smaller inoculum size and on day four for the larger inoculum size.

Inoculum has been considered as a possible source of aflatoxin and may explain reports of aflatoxin detected in control flasks (inoculated, but not incubated) and in cultures maintained outside temperature or moisture limits (125, 135). Fungal spores and mycelia were found to be sources of aflatoxin (34, 68). Diener and Davis (48) suggested that the age of the inoculum may contribute to experimental variation. Some cultures less than 7-11 days old or older than 25 days were found to produce lower amounts of aflatoxin (48).

Hagblom (63) reported that barley inoculated with 1.4×10^5 spores of A. ochraceus (#589.68) yielded 46 μg ochratoxin A/gram, while the smaller inoculum size (1.4×10^3 spores) yielded 17 μg ochratoxin A/gram. However, for A. ochraceus SLV, the smaller inoculum size (1.4×10^3 spores) yielded slightly larger amounts of ochratoxin A than the 1.4×10^5 level (63). Hagblom (63) also observed a linear increase in production of ochratoxin A by A. ochraceus SLV with time. All the fungi in his study were reported to have approximately the same amount of growth regardless of the initial inoculum size, a phenomenon which may be explained by limited nutrients in the substrate or by the presence of nonoptimal growth conditions (63).

* Competition

Jarvis (81) reported that two or more toxigenic fungal strains growing together may produce more or even less toxin than either might produce alone. Weckbach and Marth (149) stated that the influence of a fungal competitor cannot be predicted but must be determined for each fungus.

Calvert et al. (26) proposed that A. parasiticus was less aggressive than A. flavus in competing for the same corn substrate. This may explain why A. flavus was found more commonly in contaminated corn than A. parasiticus. A. flavus was believed to be limiting aflatoxin G₁ production by A. parasiticus.

Aspergillus niger and Trichoderma viride are molds found commonly on corn at harvest (95). No aflatoxin was detected when A. flavus was paired with either A. niger or T. viride on a sterilized corn substrate (95, 151). Against T. viride, a strong antagonist, A. flavus was unable to compete, it did not grow (95). A. niger was found to be a weak antagonist of A. flavus, both were able to grow. Recently, Northolt and Bullerman (104) reported that aflatoxin production by A. flavus in competition with A. niger was less than in pure culture.

Wicklow et al. (151) suggested that successful invasion of a grain substrate by A. flavus may depend on the sequence of fungal invasion and biological properties of the co-invading species. They presented the first indirect evidence showing that the biosynthesis of kojic acid by A. flavus can occur separately from aflatoxin synthesis in a solid substrate. Also, they proposed that fungal populations sharing a common resource over evolutionary time will become ecologically specialized in response to fungal competition.

Seitz et al. (129) in a recent study inoculated corn with a mixture of A. flavus, A. niger, Penicillium brevi-compactum, and P. citrinum. In contrast to the study by Wicklow et al. (151), they found that A. niger did not prevent aflatoxin production by A. flavus. A. flavus was found growing on the same kernel as A. niger.

Wicklow and Hesselstine (150) stated that co-fungal competition would probably reduce the amount of substrate available to one of the two fungi. They also proposed that the sequence of fungal colonization controlled the ability of A. flavus to invade small grains in the field and to produce significant amounts of aflatoxin and kojic acid.

According to Christensen (32), the reason A. flavus did not predominate in rice stored under favorable conditions was probably due to competition with A. glaucus and A. candidus. Boller and Schroeder (17) inoculated rice simultaneously with A. parasiticus and A. chevalieri and found that aflatoxin production by A. parasiticus was greatly reduced in the presence of A. chevalieri. They suggested that A. chevalieri might be metabolizing some of the aflatoxin or out-competing A. parasiticus for a nutrient essential to the production of aflatoxin. They proposed that fungal competition was a factor affecting concentrations of aflatoxins in products. In a later study, Boller and Schroeder (18) found that when the number of conidia of A. parasiticus in the inoculum was equal to or less than the number of conidia of A. candidus, A. candidus became the predominant species in rice after seven days of storage.

In peanuts, both A. niger and Rhizoctonia solani limited development of A. flavus when they were allowed to grow for 48 hours prior to inoculation of A. flavus (7). Ashworth et al. (7) suggested that A. flavus always had a competitive advantage. Stutz and Krumperman (142) found that A. parasiticus did not compete well with the natural mycoflora of peanuts even when present in superior numbers.

Simultaneous inoculation of A. parasiticus and Lactobacillus casei into APT (glucose) broth resulted in less aflatoxin production by A. parasiticus and greater degradation of aflatoxin (54). El-Gendy and Marth (54) stated that the effect of a particular fungi or micro-organism in a competing situation seemed to be unpredictable. Wiseman and Marth (153) inoculated APT broth simultaneously with A. parasiticus and Streptococcus lactis and determined the relationship to be amensalism. Under certain conditions, S. lactis stimulated growth of A. parasiticus, while at other times, A. parasiticus acted as a buffering agent for S. lactis (153).

A. parasiticus was grown in the presence of Rhizopus nigricans, Saccharomyces cerevisiae, Acetobacter aceti, or Brevibacterium linens and the aflatoxin content of the YES medium was determined after 3, 5, 7, and 10 days of incubation at 28C by Weckbach and Marth (149). R. nigricans and S. cerevisiae inhibited growth and aflatoxin production by A. parasiticus. B. linens caused slight inhibition, and A. aceti stimulated growth and aflatoxin production by A. parasiticus. These results indicated unpredictable variation in aflatoxin production by A. parasiticus during competition even when the substrate was favorable for both growth and aflatoxin production.

Fanse and Christensen (56) inoculated rice (MC 15.5%) with a mixture of five fungi: A. heterocaryoticus, A. restrictus, A. candidus, A. flavus, and A. ochraceus. After eight months of storage, no cultures of A. ochraceus or A. flavus were isolated. Christensen (30) reported that A. ochraceus cannot compete well with other storage

fungi, while Fanse and Christensen (56) explained that growth of A. flavus was inhibited by the low MC of the rice. Christensen (30) inoculated wheat with a mixture of A. ochraceus, A. glaucus, A. flavus, and A. candidus and found slower invasion by A. ochraceus during competition than when present alone. A. ochraceus grew almost exclusively from the wheat embryo.

Fungal growth and aflatoxin production have been reported during competition at various temperatures. A. flavus competing against the natural mycoflora of corn grew at 19C (83). At $\geq 24C$, A. flavus produced aflatoxin when the moisture content (MC) was $> 17.5\%$ (145). At 28C, A. flavus rapidly invaded corn blends with a MC of 17-18% (122). Bottomley et al. (19) reported A. flavus as the predominant mold during storage at 30C for 12 days. Trenk and Hartman (145) found higher aflatoxin levels at 30 and 35C than at 24C in remoistened corn. At 45C, A. flavus was no longer the predominant mold species (19).

Members of the A. glaucus group out-competed A. parasiticus in rice stored at 15, 20, and 25C (18). Enhanced activity of the natural mycoflora seemed to be associated with a reduction in toxin production by A. parasiticus. At 30 or 35C, A. parasiticus in competition with the natural mycoflora of rice produced only aflatoxin B₁. However, at 25C, all four aflatoxins (B₁, B₂, G₁, G₂) were produced. In a competing situation, Boller and Schroeder (18) determined maximum aflatoxin production (B₁, B₂) at 35C, while maximum G₁ production occurred at a lower temperature of 30C.

Limited research has been done to investigate the relation of growth and ochratoxin A production in a competing situation to temperature. A. ochraceus was the predominant fungal species in corn stored for several weeks at 25C and 18.5% moisture content (MC) (33, 91). Invasion of wheat by A. ochraceus was reported at storage temperatures between 20-25C (30, 51). However, Diener (51) found that whenever A. glaucus was already established on the wheat kernels, competition by A. ochraceus was difficult.

Bottomley et al. (19, 20) reported that moisture content (MC) was one of the factors determining which species of fungi might predominate in a competitive situation. Qaseum and Christensen (115) found that members of the A. glaucus group were the only species to invade corn stored at a MC of 14%, and were usually the first fungi to invade cereal grains in storage (5). At 18% MC, A. flavus was competing with the natural mycoflora of corn (115). No invasion of corn was found by A. flavus at MC < 17.5% (91), MC < 18% (116), and MC 16-18% (115). At 24% MC, A. glaucus was usually found as the predominant fungal species in corn (20). However, at 31-32% MC, the presence of A. glaucus was greatly reduced and A. flavus represented a major part of the mycoflora (20).

Ashworth et al. (7) stated that aflatoxin production by A. flavus was limited by competition which was controlled by the moisture content of the substrate. A. flavus grew and produced large quantities of aflatoxin in peanuts with 23-34% MC. At 30C, as the relative humidity (RH) increased, invasion of rice by

A. parasiticus increased, more aflatoxin was produced, and more fungal competition occurred (18). Invasion of barley by various aspergilli was limited at a MC of 14% and 20C (92). Rice with a MC of 15.0-17.0% was rapidly invaded by members of the A. ochraceus group and a wide variety of other storage fungi (31). Wheat was slowly invaded by A. ochraceus at 15.0-15.5% MC, while rapid invasion occurred at $\geq 16.0\%$ MC (30). Christensen (30) found that a difference of $< 1\%$ MC in the wheat substrate had a great influence on invasion by A. ochraceus.

pH

Hydrogen and hydroxyl ions are present in natural substrates which commonly support the growth of fungi. Agnihotri (1) stated that substrate pH can effect growth, sporulation, pigmentation, and many other fungal processes. Numerous studies have been conducted on the effect of pH on mycotoxin production in artificial media; however, pH effects on mycotoxin production in natural substrates have not been reported in the literature.

Davis et al. (42) observed that the initial pH of YES medium (20% sucrose and 2% yeast extract) did not significantly affect either aflatoxin production or growth of Aspergillus flavus. They found that a final pH of 4.0 was generally obtained regardless of the initial pH of the medium. Other investigators have reported changes in the initial pH of the medium during aflatoxin production (50, 66). In contrast to Davis et al. (42), Joffe and Lisker (82) reported that the initial pH did have an effect on aflatoxin production, with

higher aflatoxin yields being obtained at an initial pH of 4.0 than at an initial pH of 7.4 in Czapek's medium. Optimal aflatoxin production in SL medium was detected at a pH of 4.5 by Reddy et al. (118). Buchanan and Ayres (21) reported maximal growth and aflatoxin production by A. parasiticus at initial pH levels of 5.0 and 6.0, respectively. Furthermore, initial pH levels < 6.0 favored production of B toxins, while pH levels > 6.0 favored G toxin production. They concluded that the influence of pH depends on the composition of the medium, and that for each medium, the initial pH for optimal aflatoxin production must be determined. Diener and Davis (50) stated that the pH effect on aflatoxin production was probably dependent on the composition of the medium and the strain of fungus.

Davis et al. (45) found peak ochratoxin A production by Aspergillus ochraceus to occur in a medium consisting of 4% sucrose and 2% yeast extract. The final pH of the medium and the sucrose concentration were inversely related. At a sucrose concentration of 4% or higher, the final medium pH was < 7.0. Aspergillus sulphureus NRRL 4077 produced maximum amounts of ochratoxin A at pH 6.0 to 6.3 in a glucose-inorganic salt medium incubated for 8 days at 22 to 27C (86). Sansing et al. (121) reported that high pH values of 7.6-8.2 coincided with high ochratoxin production by A. ochraceus at 25C with 8-14 days incubation in a medium consisting of 4% sucrose and 2% yeast extract. The high pH was probably due to ammonia accumulation in the medium as a result of deamination of amino acids from yeast extract. Paster and Chet (110) found the

pH of their xylose-inorganic salt medium to decrease during the growth period of A. ochraceus. They proposed that acidic metabolites excreted into the medium lowered the pH during growth and that pH changes are related to the composition of the medium.

Substrate

Substrate was an important factor affecting fungal growth and aflatoxin production. The extent of fungal growth and aflatoxin production varied with the different substrates. Both natural substrates and artificial media have been used by various researchers. Yields of aflatoxin on synthetic and semisynthetic media were reported to be small in comparison to those obtained on natural substrates (51, 81). Davis et al. (42, 43) found YES (a semisynthetic medium containing 2% yeast extract and 20% sucrose) to support better growth and aflatoxin production than any chemically defined medium. Yeast extract was probably supplying the proper combination of amino acids, minerals, vitamins, and other growth factors necessary for good fungal growth and toxin production. Shih and Marth (132) stated that artificial media was commonly used by researchers to eliminate the variability in natural substrates.

Natural substrates high in carbohydrates (rice, wheat, corn) were found to generally support more aflatoxin production than oilseeds (peanuts, cottonseed, soybeans) both in the laboratory and in the field (51). In a recent study by Park and Bullerman (109), they reported that high protein, low carbohydrate feeds do not support high levels of aflatoxin production by A. parasiticus, even though

extensive fungal growth and sporulation occurred. However, small amounts of carbohydrate were utilized by A. flavus to produce substantial amounts of aflatoxin. The amount of utilizable carbohydrate in a food substrate was determined to be an important factor in the production of aflatoxin.

Diener (51) reported that substrate moisture determined growth and aflatoxin production by A. flavus or A. parasiticus. Galloway (60) showed that atmospheric moisture was more important to fungi than the moisture in the substrate. In opposition, Block (16) reported that substrate moisture was of utmost importance for fungal activities. He reported that the minimum humidity for mold growth was related to the moisture content of each substrate at each humidity. The greater the quantity of water absorbed by the substrate, the more water that was available to the mold. Block (16) proposed that fungi were incapable of obtaining moisture directly from the atmosphere but obtained it from the substrate which derived its moisture from the atmosphere. Davis and Diener (44) reported that the limiting relative humidity (RH) for agricultural commodities depended upon the type of substrate and the length of storage. Fungal growth and embryo damage in barley appeared to be related to the available moisture in the substrate (5).

Northolt et al. (102) explained the variation in optimum relative humidity and temperature values reported by other investigators to be due to different substrates and fungal strains. They also found substrate and fungal strain to have a greater effect at a higher

temperature. In contrast, Schroeder and Hein (126) reported that the influence of substrate on toxin production decreased as the temperature increased. They suggested that the amount of hull material and the time required for the fungus to penetrate the hull material were related to the substrate effect. Diener and Davis (49) reported that broken mature peanut kernels appeared to be more rapidly invaded by A. flavus than sound mature, immature, or unshelled Early Runner peanuts. They concluded that an intact outer hull was only a temporary barrier to invasion by A. flavus. Structural differences in kernels were found by Calvert et al. (26) to influence fungal development and aflatoxin production.

Northolt and Bullerman (104) found both substrate and fungal strain to influence the production of ochratoxin A. Trenk et al. (146) reported substrate as the major factor in determining the optimal time for ochratoxin A production.

Ochratoxin A has been produced on both natural and artificial media (121). Selection of substrate (natural or artificial) has been known to simplify the isolation procedure for toxins and to increase toxin yields (45). Lai et al. (86) preferred the use of a synthetic medium (liquid) to a natural (solid) medium because of the ease of toxin isolation. Corn and peanuts (natural substrates) were found to be frequently contaminated with one or more pigments that interfered with the isolation of the ochratoxins (45). A semi-synthetic medium, YES (Yeast Extract Sucrose) containing 2% yeast extract and 4% sucrose, was found to produce maximum amounts of ochratoxin A (45).

According to Bottomley et al. (19), sterilized grain supported better fungal growth than viable seed due to elimination of natural competition and inactivation of seed enzymes. Haggblom (63) showed that autoclaved barley was a good substrate for ochratoxin A production by A. ochraceus. Chopped corn was found to be the best substrate for ochratoxin A production at 28C in comparison with bleached flour, wheat bran, and polished rice (146). Hesseltine et al. (69) reported rice as a poor substrate for ochratoxin production, while wheat was determined as a better substrate than corn. A. ochraceus was found by Diener (51) to frequently produce both ochratoxin A and penicillic acid on natural substrates.

Storage

The length of the storage period, light, and oxygen concentration can affect fungal growth and toxin production. Most studies on aflatoxin production have been concerned with determining toxin levels in cultures grown for a period of 5-10 days (6). Diener and Davis (48) reported maximum production of aflatoxin B₁ by A. flavus within a 7-9 day incubation period. Davis et al. (42) found maximum aflatoxin production by A. flavus in YES medium within 5-12 days. Shih and Marth (132) determined maximum toxin formation at five days with toxin levels decreasing as the incubation period increased. The levels of aflatoxins B and G were found to vary with the time of incubation at any given temperature (135). At 15C, there was approximately five times more B₁ than G₁ after 8 days of incubation. However, after 14-21 days, there was more G₁ present than B₁. Sorenson et al.

(135) also found that at temperatures $\geq 28^{\circ}\text{C}$, aflatoxin G_1 decreased with the incubation time.

Arseculeratne and Bandunatha (6) reported significant cyclic variation (biphasic) in aflatoxin production by A. flavus on grated coconut with increasing length of storage. In YES broth, cyclic variation was observed by Park and Bullerman (108). The first peak occurred at day 10 and the second peak at day 21. They proposed that cyclic variation was due to degradation of aflatoxin during certain growth phases. Goldblatt (62) found only monophasic aflatoxin production with maximum levels at 5-10 days of incubation. Arseculeratne and Bandanatha (6) concluded that because of the cyclic variations in aflatoxin production, determination of toxin levels at a single interval of incubation would not provide realistic estimates of the toxigenic capacity of the fungus unless determinations were made at the time of maximum aflatoxin production.

Bennett et al. (11) observed that fungi vary in their response to light. They classified A. flavus and A. parasiticus as intermediate in light requirement; they produce conidiospores in the dark but produce even more conidiospores in the light. In their studies, they found no light inhibition of aflatoxin. Bennett et al. (14) reported the influence of light and darkness in relation to temperature on aflatoxin production. At 30°C , maximum aflatoxin production occurred in the dark while light was only slightly inhibitory on aflatoxin production. No light inhibition of aflatoxin production was observed at 20°C and 25°C . Joffe and Lisker (82) reported that

A. flavus produced large amounts of aflatoxin at 24C in the dark, while in the light, aflatoxin production was reduced. Bennett et al. (14) observed complete inhibition of growth by light at 15C. Olutiola (105) reported light inhibition on both growth and sporulation of A. flavus.

Shih and Marth (132) suggested that the degree of aeration of a culture was an important factor in controlling the biosynthesis of aflatoxin. Landers et al. (87) found a reduction in the oxygen content of the atmosphere generally decreased aflatoxin production. In contrast, Shih and Marth (132) found that aspergilli produced less aflatoxin and lipid when aeration was increased during storage. Low oxygen conditions (anaerobic) favored glucose catabolism via the Embden-Meyerhof pathway and enhanced aflatoxin formation. Aerobic conditions favored the hexose monophosphate pathway. Optimal synthesis of aflatoxin and lipid by A. parasiticus was found to occur when conditions bordered on anaerobiosis. Decreasing the oxygen concentration of air to 10% reduced aflatoxin production, but only at O₂ levels less than 1% were both growth and aflatoxin production completely inhibited (104). However, some researchers have reported mold growth even when O₂ levels were less than 1% (20, 97). Davis and Diener (44) determined the largest decreases in aflatoxin production to occur when O₂ levels were decreased from 5 to 1% and from 1 to 0.1%.

Low water activity (Aw), refrigeration, preservatives, vacuum-packaging, and high CO₂ atmospheres were suggested as means of preventing mold growth on finished products (104). Combinations

of these preventive measures were found to be more efficient than when used alone. Sanders et al. (120) prevented aflatoxin production in peanuts by using a combination of 20% CO₂, 17C, and 86% RH while Landers et al. (87) reported similar results using 1% O₂, 19% N₂, and 80% CO₂. High concentrations of CO₂ were proposed by the latter to be the main source of inhibition rather than low O₂ concentrations when the fungus was grown at optimum temperature and moisture content. However, high CO₂ concentrations ($\geq$ 80%) resulted in peanuts with terrible odors and off-flavors (44, 87).

Optimal incubation time for ochratoxin A production was determined to vary from 7-14 days at 28C (146). At 25C, maximum ochratoxin A production by A. ochraceus was reported to occur after 10 and 12 days incubation on YES agar (2% yeast extract, 4% sucrose), but at 30C, ochratoxin A production declined rapidly with each 2-day period of incubation after the first 8 days (121). Haggblom (63) found maximum ochratoxin A production after 28 days on barley while Schindler and Nesheim (124) reported 19-21 days as the optimum time for maximum ochratoxin A production on shredded wheat. In a synthetic medium, A. sulphureus NRRL 4077 produced maximum amounts of ochratoxin A within 8-10 days (86). When the cultures were incubated for up to 20 days, only slight reductions in ochratoxin A levels were detected.

Paster and Chet (110) observed abundant production of conidial heads by A. ochraceus in the dark while fewer heads were produced in the light.

Ciegler (37) stated that because A. ochraceus was able to grow and produce ochratoxin A at low temperatures: simple refrigeration as the sole method of preservation during storage was not recommended. Trenk et al. (146) detected very low levels of ochratoxin A at 4C after 4 weeks of incubation. They stated that if grain was stored at low temperatures for a longer period of time, increased production of ochratoxin A was possible. Ciegler (37) recommended the use of refrigeration in combination with a gaseous atmosphere, such as CO₂, to inhibit mold growth and toxin production.

CHAPTER III

MATERIALS AND METHODS

I. TEST ORGANISMS

The test organisms, Aspergillus parasiticus NRRL 3145 and A. sulphureus NRRL 4077, were obtained from the Northern Regional Research Laboratory, Agricultural Research Service, Peoria, IL. Stock cultures were grown on YES slants at 24C for 3 weeks and stored at 4C.

II. GROWTH MEDIUM

The fungi were grown on yeast extract sucrose agar (YES) consisting of 2% yeast extract, 1.5% agar, and an appropriate sucrose level. The medium was dispensed in 10 ml amounts into screw-capped tubes and autoclaved at 121C for 15 minutes. A. parasiticus cultures were grown on YES slants containing 20% sucrose while A. sulphureus cultures were grown on YES slants containing 4% sucrose.

III. TEST MEDIUM

Pioneer Brand Commercial Hybrid Corn 3184 was obtained in the dried form from Dr. Dennis West, Department of Plant and Soil Science, The University of Tennessee, Knoxville. This corn was previously analyzed and found to contain less than 2 ppb aflatoxin. Foreign matter and debris were removed from the corn using a wire-screen hand

shaker. Moisture content of the corn was determined by the vacuum oven method. Samples (2g) of ground corn (in triplicate) were dried at 57-60C for 24 hours under vacuum, and an average moisture content determined. Corn was placed into 500 ml wide mouth Erlenmeyer flasks, and distilled water was added to bring the moisture content up to the desired level. The total weight of corn and water in a flask was 50 grams. The flasks were plugged with nonabsorbant cotton, covered with aluminum foil, and autoclaved at 121C for 15 minutes. The flasks were allowed to cool to room temperature before the addition of inoculum.

IV. INOCULUM PREPARATION

Five ml of a 0.005% sterile Triton X-100 solution was added to a YES slant, and the spores were suspended by agitation. Fungal spores were counted using a Petroff-Hausser and Helber counting chamber and a Bausch and Lomb Balplan microscope. The spores were harvested into sterile tubes containing 0.005% sterile Triton X-100 solution and diluted to obtain the desired inoculum levels of 10^7 , 10^5 , 10^3 , and 10^1 spores/ml.

V. TREATMENTS

The moisture content of the corn was adjusted initially to 16%, 20%, 24%, and 28%. For pure cultures of A. parasiticus and A. sulphureus, fungal spores in 1 ml aliquots were added separately to flasks at levels of 10^7 , 10^5 , 10^3 , and 10^1 per 50 g sample. For

mixed cultures, 0.5 ml of A. parasiticus and A. sulphureus spore suspensions were added to flasks to give total levels of 10^7 , 10^5 , 10^3 , and 10^1 spores per 50 g sample. Samples were incubated at 20, 24, 28, and 32C for 3 weeks. Two replications of the entire experiment were conducted.

VI. TOXIN ASSAY

Extraction

Aflatoxin and ochratoxin were extracted from ground corn samples by the AOAC procedure for aflatoxin. Corn samples (50 g) were ground for 30 seconds using a Waring Blender. Methanol (250 ml) was added, and the samples were shaken for 30 minutes on a Burrell wrist-action shaker. Extracts (100 ml) were filtered through Whatman 18.5 cm #1 filter paper and placed in separatory funnels. Two hundred mls of 20% aqueous ammonium sulfate and 100 ml of hexane were added to each extract, and the mixture was shaken for 1 minute. After separation, the top layer was discarded and detoxified. To the bottom layer, 100 ml of chloroform was added, and the solution shaken for 1 minute. The bottom layer was drawn off through anhydrous sodium sulfate into a 500 ml round-bottomed boiling flask. The remaining top layer was then reextracted with chloroform (100 ml). Extracts were concentrated to dryness with a Buchi Rotavapor-R flash evaporator at 65C. Aflatoxin extracts from pure cultures of A. parasiticus were reconstituted with 5 ml of acetonitrile and filtered from HPLC analysis through a Millex-SR 0.5 μ m filter. Ochratoxin A extracts from pure cultures

of A. sulphureus were reconstituted with 5 ml of chloroform but not filtered since ochratoxin A extracts were analyzed by TLC. For mixed cultures, aflatoxin and ochratoxin were extracted simultaneously, and the final extract reconstituted with 5 ml of chloroform. Extracts were then filtered through a Millex-SR 0.5 μ m filter prior to HPLC analysis. All extracts were stored in the dark in glass screw-capped vials at -15C.

Detoxification

All toxic wastes and contaminated solvents resulting from chemical extraction were treated with full-strength sodium hypochlorite (Chlorox). It should be noted that when one detoxifies wastes with sodium hypochlorite, adequate ventilation is required due to the production of chlorine gas. Detoxified wastes were held in double-lined biohazard bags and in metal drums until disposed of by the University of Tennessee Toxic Waste Procurement Department. All contaminated laboratory glassware was soaked in a sodium hypochlorite/soap/water solution for at least eight hours, washed in a strong soap and water solution, rinsed, and allowed to air dry. In case of an accidental spill or human exposure, all areas were thoroughly cleaned with sodium hypochlorite and then washed with soap and water.

Standards

Table 2 shows the aflatoxin and ochratoxin standards obtained from Sigma Chemical Company, St. Louis, MO.

TABLE 2. Aflatoxin and Ochratoxin Standards

Standard	Solvent	Concentration ($\mu\text{g/ml}$)
Aflatoxin B and G Mixture*		
B ₁	Acetonitrile	5.0
B ₂	Acetonitrile	1.5
G ₁	Acetonitrile	5.0
G ₂	Acetonitrile	1.5
Aflatoxin B ₁	Acetonitrile	1000.0
Ochratoxin A**	Chloroform	333.3
Aflatoxin B ₁ **	Chloroform	333.3
Aflatoxin G ₁ **	Chloroform	333.3

*100 μl of the original aflatoxin B and G mixture (benzene: acetonitrile 98:2 v/v) was transferred to a small vial, evaporated to dryness using air, and reconstituted with 100 μl of acetonitrile.

**100 μl of each standard were mixed together to form 300 μl of a mixed standard solution.

High-Performance Liquid Chromatography

High-performance liquid chromatographic separations were conducted with a Waters Liquid Chromatograph (Waters Associates Inc., Milford, MA.) equipped with a Model 420C and 420E Fluorescence Detector fitted with a 360 nm excitation filter and a 440 nm emission filter, M6000 pump, and a U6K septumless injector. Injection was by means of a Hamilton 25 μ l syringe. Chromatograms were recorded on an Omniscribe (Houston Instrument) two-pen recorder with a chart speed of 0.2 inch/minute. Two different HPLC systems were used: one for pure cultures of A. parasiticus and another for mixed cultures of A. parasiticus and A. sulphureus. For pure cultures of A. parasiticus, aflatoxins B₁, G₁, and G₂ were resolved using a μ -Porasil (Waters Associates Inc.) column and a solvent system of water-saturated methylene chloride containing 0.5% methanol. This solvent system was prepared by shaking 500 ml of methylene chloride with 200 ml of water in a 1 liter separatory funnel. After layer separation, the methylene chloride (bottom layer) was filtered through a Millipore FHVP filter. Methylene chloride treated in this manner is referred to as "water-saturated." Five ml of methanol was added to a 1 liter volumetric flask and water-saturated methylene chloride added to volume. Aflatoxins B₁, G₁, and G₂ had retention time of 11:00, 12:30, and 16:40 minutes, respectively, at a flow rate of 1.5 ml/min. The HPLC method of Engstrom et al. (55) was used to detect and resolve aflatoxin B₁, aflatoxin G₁, and ochratoxin A in corn samples inoculated with a mixture of both A. parasiticus and A. sulphureus. The system

included a μ -Bondapak/C18 column (Waters Associates Inc.), an acetonitrile-water-glacial acetic acid (55:45:2 v/v/v) solvent system, and a Model 420C and 420E Fluorescence Detector, fitted with a 360 nm excitation filter and a 440 emission filter. The retention times of aflatoxin G₁, aflatoxin B₁, and ochratoxin A were 6:30, 8:20, and 13:10 minutes, respectively, at a flow rate of 0.6 ml/min.

Thin Layer Chromatography

Ochratoxin A from pure cultures of A. sulphureus was quantitated by thin layer chromatography using Whatman TLC plates (20 x 20 cm plate coated with a 250 μ m layer of Linear K Silica Gel) which were heat activated prior to use for 30 minutes at 115C. The activated plates were then spotted with 10 μ l of sample and developed for 30-45 minutes in a saturated chromatographic tank containing benzene-acetic acid-methanol (90:10:5 v/v/v) (144). The ochratoxin A standard was diluted by transferring 10 μ l of standard into 90 μ l of chloroform and performing consecutive dilutions in chloroform to obtain a series of standards with decreasing concentrations. The following amounts of the known standard were spotted on a TLC plate: 1.0, 0.6, 0.3, 0.1, 0.06, 0.03, 0.01, 0.006, and 0.002 μ g.

Calculations

Aflatoxins B₁, G₁, G₂ (from pure culture) and aflatoxins B₁, G₁, and ochratoxin A (from mixed culture) were quantitated by using chromatographic peak height and peak area measurements in the following formula:

$$\text{Toxin } (\mu\text{g}/50 \text{ g}) = \frac{(C_{\text{STD}}) (X_{\text{SAM}}) (F_{\text{STD}}) (V_{i\text{STD}}) (V_{\text{LTOT}})}{(X_{\text{STD}}) (F_{\text{SAM}}) (V_{i\text{SAM}})}$$

where:

C_{STD} = concentration of the standard solution in $\mu\text{g}/\text{ml}$

X = peak height in mm or peak area in mm^2

F = gain of fluorescence detector

V_i = volume injected in μl

V_{LTOT} = total volume in ml

Peak height measurements were used in the calculation of aflatoxins B_1 , G_1 , and G_2 from pure cultures of A. parasiticus, while peak area calculations were used in the quantitation of aflatoxins B_1 , G_1 , and ochratoxin A from mixed cultures of A. parasiticus and A. sulphureus. Peak area (PA) was calculated from the following formula:

$$\text{PA} = (H) (W_{.5H})$$

where:

PA = peak area in mm^2

H = peak height in mm

$W_{.5H}$ = width of peak at 1/2 peak height in mm

All toxin values were divided by 50 so that all amounts would be expressed as $\mu\text{g}/\text{g}$ of corn (ppm). Total toxin was determined by summation and all toxin levels were expressed as $\mu\text{g}/\text{g}$ of corn (ppm).

$$\text{Total toxin (pure)} = (B_1 + G_1 + G_2 + \text{ochratoxin A})$$

$$\text{Total toxin (mixed)} = (B_1 + G_1 + \text{ochratoxin A})$$

During chemical extraction, only 100 ml of the original 250 ml methanol extract was used. To account for the total amount of toxin in the

sample, all toxin values were multiplied by a factor of 2.5 (250 ml of the original methanol extract divided by 100 ml of the filtered extract).

Quantitation of ochratoxin A in the sample (pure culture of A. sulphureus only) was determined under UV light by visual comparison with the fluorescent intensity of the known standards (45, 69, 86, 121, 124). The visual comparison of each sample was performed by two different persons (52), and an average toxin level determined. The amount of toxin present in the sample was determined by multiplying the average toxin level (in μg) by a factor of 500 (final volume of extract '5000 μl ' divided by the amount of extract spotted '10 μl '). Toxin levels were divided by 50 so that amounts would be expressed as $\mu\text{g/g}$ corn (ppm).

VII. EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The experiment was designed with 4 temperatures: 20, 24, 28, and 32°C; 4 initial moisture contents: 16, 20, 24, and 28%, 4 levels of inoculum: 10^1 , 10^3 , 10^5 , and 10^7 spores/50 g corn; and 2 organisms: Aspergillus parasiticus and A. sulphureus. The randomized block experimental design was used with a total of 8 blocks for each organism and 8 blocks for the mixture of two organisms. Each block consisted of 8 treatments-1 temperature, 4 inoculum levels, and 2 moisture levels, a total of 64 treatment combinations per organism and for the mixture of the two organisms. The experiment was replicated twice. Data was analyzed using an analysis of variance

(ANOVA) to detect significant differences in main effects and interactions, while Duncan's Multiple Range test was used to separate means when significant differences were found.

VIII. PRELIMINARY TESTS

Both a μ -Porasil column with a solvent system of water-saturated methylene chloride containing 0.3% methanol and a μ -Bondapak/C18 column with an acetonitrile-water-glacial acetic acid (55:45:2 v/v/v) solvent system were tested to determine the resolution of aflatoxin and ochratoxin A standards.

Standard curves were constructed by injecting various amounts of a mixed standard solution (aflatoxin B₁, G₁, and ochratoxin A) at concentrations of 0.333, 0.033, and 0.003 $\mu\text{g}/\mu\text{l}$ to determine the linearity of the relationship between peak height or peak area and the amount of standard (in μg).

A study was conducted to determine the changes in moisture content of uninoculated corn within flasks over time under experimental conditions. Corn was placed into flasks, the moisture content (MC) was adjusted to 16, 20, 24, and 28%, and then autoclaved. After the flasks had cooled, 1 ml of a sterile 0.005% Triton X-100 solution (representing the amount of inoculum) was added to each flask, and the flasks incubated at 32C for 3 weeks. Moisture content was determined at intervals of 0, 1, 2, and 3 weeks.

Flasks containing sterile corn (adjusted initially to 24% MC) were set up to determine if the inoculum was contributing aflatoxin

or ochratoxin A to the corn. Fungal spores (pure inoculum in 1 ml amounts) of A. parasiticus and A. sulphureus were added separately to flasks at levels of 10^7 , 10^5 , 10^3 , and 10^1 per 50 g corn. For the mixed inoculum, both fungal spores of A. parasiticus (0.5 ml) and A. sulphureus (0.5 ml) were added to flasks at levels of 10^7 , 10^5 , 10^3 , and 10^1 per 50 g corn. After allowing 30 minutes for inoculum absorption, the corn was extracted, the extracts reconstituted in chloroform and filtered prior to HPLC analysis. The HPLC system (55) consisted of a μ -Bondapak/C18 column, an acetonitrile-water-glacial acetic acid (55:45:2 v/v/v) solvent system, and a fluorescence detector.

Reproducibility of the HPLC system using a μ -Bondapak/C18 column and a mobile phase of acetonitrile-water-glacial acetic acid (55:45:2 v/v/v) at a flowrate of 2 ml/minute was evaluated by taking the mean of peak heights from six injections (3 μ l each) of an aflatoxin B₁ standard with a concentration of 1 mg/ml, and calculating the coefficient of variation (C.V.). $C.V. = (\pm \text{standard deviation} / \text{mean}) \times 100$.

A sample of sterilized corn (50 g) which was used in the study was extracted using the AOAC procedure for aflatoxin and analyzed by HPLC (55) to ensure that the corn did not contain more than 2 ppb aflatoxin.

CHAPTER IV

RESULTS AND DISCUSSION

I. PRELIMINARY TESTS

Initially, a μ -Porasil column with a mobile phase of water-saturated methylene chloride containing 0.3% methanol was tested. When the solvent polarity was increased to 0.5% methanol, the retention time of the aflatoxins decreased without reducing peak separation. Thus, the μ -Porasil/0.5% methanol mobile phase system was used to resolve aflatoxins B₁, G₁, and G₂ produced by pure cultures of A. parasiticus. Aflatoxin B₂ was not quantitated because it could not be detected in corn samples inoculated with pure cultures of A. parasiticus.

Because of a delay in shipment of a new μ -Porasil column and an approaching abstract deadline, thin layer chromatography (TLC) was used instead of HPLC to quantitate ochratoxin A from pure cultures of A. sulphureus. Ideally, HPLC should have been used.

The μ -Bondapak/C18 column with a mobile phase of acetonitrile-water-glacial acetic acid was used to separate aflatoxin B₁, aflatoxin G₁, and ochratoxin A produced by mixed fungal cultures of A. parasiticus and A. sulphureus. This column/mobile phase system did not resolve aflatoxins B₂ and G₂ from aflatoxins B₁ and G₁, respectively. Engstrom et al. (55) also reported no resolution of aflatoxins B₂ and G₂ from aflatoxins B₁ and G₁ using a similar HPLC system. An overestimate

in the quantitation of aflatoxin B₁ and G₁ could occur due to the incorporation of aflatoxin B₂ and G₂ into the peaks of B₁ and G₁, respectively, but the overestimate would be accounted for in the amount of total toxin.

The relationship between mean peak height and µg of toxin standard was linear for aflatoxins B₁ and G₁ produced by pure cultures of A. parasiticus. Correlation coefficients for aflatoxin B₁ and G₁ were 0.9947 and 0.9914, respectively. Linear regression equations describing the standard curves were determined to be:

$$\hat{Y} = 2565.31X - 111.34 \text{ for aflatoxin B}_1$$

$$\hat{Y} = 312.93X - 14.42 \text{ for aflatoxin G}_1$$

where

X = toxin standard (µg)

$\hat{Y}$ = estimated mean peak height (mm)

The relationship between mean peak area and µg of toxin standard for toxins produced by mixed cultures of A. parasiticus and A. sulphureus was linear for aflatoxin B₁ and aflatoxin G₁, but not for ochratoxin A. Correlation coefficients for aflatoxins B₁, G₁, and ochratoxin A were 0.9977, 0.9986, and 0.8008, respectively. Linear regression equations describing the standard curves were determined to be:

$$\hat{Y} = 13,436.22X - 493.27 \text{ for aflatoxin B}_1$$

$$\hat{Y} = 784.16X - 23.79 \text{ for aflatoxin G}_1$$

$$\hat{Y} = 8223.54X - 217.52 \text{ for ochratoxin A}$$

where

X = toxin standard (µg)

$\hat{Y}$ = estimated mean peak area (mm²)

The reason ochratoxin A had a low correlation coefficient (0.8008) might be due to the formation of an interfering artifact with a retention time approximately the same as ochratoxin A. Sometimes, the artifact peak was distinctly separated from the ochratoxin A peak, while at other times, it seemed that the artifact peak was being incorporated into the ochratoxin A peak. Incorporation of the artifact peak with the ochratoxin A peak would result in inaccurate quantitation of ochratoxin A and would affect the linearity of the relationship between peak area and the ochratoxin A standard.

The changes in moisture content of uninoculated corn within flasks over time under experimental conditions is presented in Figure 5. All 4 initial moisture content levels decreased during the 3 week storage period. The uninoculated corn was losing moisture to the atmosphere by evaporation. However, when the corn was inoculated with A. parasiticus, A. sulphureus, or a mixture of the two, the moisture content within each flask increased. Moisture was visible on the inside walls of the flasks. Fungal respiration and metabolism were probably the reasons for the visible moisture.

Aflatoxin and ochratoxin A were not found to be contributed by the inoculum. However, interfering compound(s) were contributed by the corn. The interfering compound(s) had the exact same retention time as aflatoxin G₁ in both pure and mixed culture. The interfering compound(s) would result in an overestimate of the amount of aflatoxin G₁ present. To compensate for this overestimate, the amount of interfering compound(s) detected at the 10⁷, 10⁵, 10³, and 10¹ inoculum levels was averaged, and this average subtracted from all

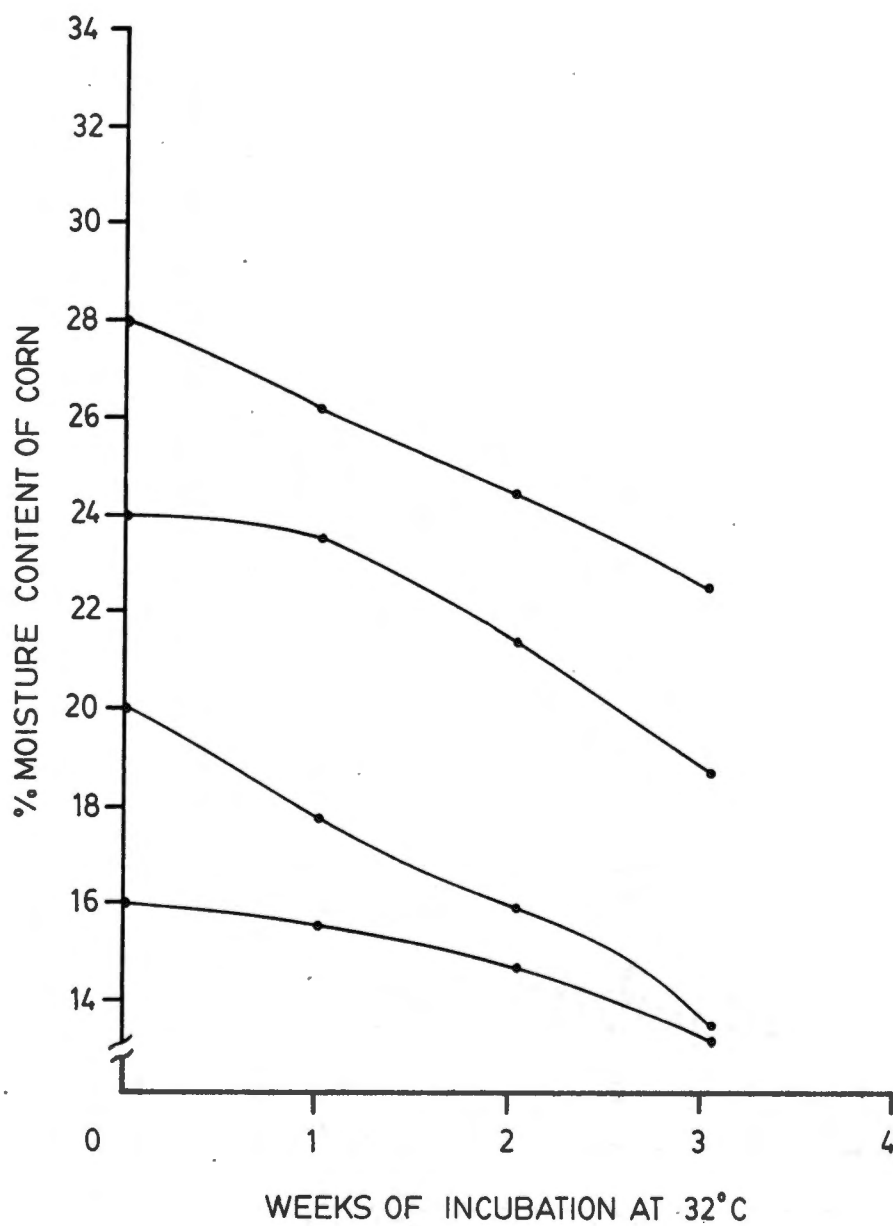


FIGURE 5. Changes in moisture content of uninoculated corn within flasks over time under experimental conditions.

G_1 values to correct for the presence of the interfering compound(s). The mean 8.53 $\mu\text{g/g}$ was subtracted from all G_1 values for pure cultures of A. parasiticus while the mean 9.46 $\mu\text{g/g}$ was subtracted from all G_1 values for mixed cultures of A. parasiticus and A. sulphureus.

An artifact was found only in one flask containing 10^7 spores/50 g corn of the mixed culture at the approximate retention time of aflatoxin B_1 . Since the artifact was found in only one flask and was not at the exact retention time for aflatoxin B_1 , it was not subtracted from B_1 toxin values.

Results from the HPLC reproducibility study were mean peak height = 31.5 mm; standard deviation from the mean = 5.50; and coefficient of variation (C.V.) = 17.46. The lower the C.V., the more precise (reproducible) the system. The system was reproducible but there was more deviation from the mean than expected. Using the same instrument, Tong (144) found his HPLC system (μ -Porasil column with a mobile phase of benzene-acetic acid-methanol 90:10:5) to be reproducible with coefficients of variation ranging from 2.38 to 5.99. I feel that the HPLC was not operating properly at all times, and should be thoroughly inspected by a Waters service representative.

The test medium, Pioneer Brand Commercial Hybrid Corn 3184, was found to contain less than 2 ppb aflatoxin.

II. EFFECT OF TEMPERATURE, INITIAL MOISTURE CONTENT, AND LEVEL OF INOCULUM ON MYCOTOXIN PRODUCTION

Aspergillus parasiticus produced maximum amounts of aflatoxin B_1 at 32C/20% moisture content (MC) and no aflatoxin B_1 at 20C/16% MC

and 20C/20% MC (Figure 6). Northolt et al. (101) using a different strain of A. parasiticus found maximum B₁ production at 32C and 0.99 Aw on malt extract-sucrose agar. Other researchers have reported maximum B₁ production at temperatures of 24C (51, 101, 102, 117), 30C (44), 35C (44), 28-30C (51), 28-32C (135), and 30-35C (51). Chang and Markakis (29) found no aflatoxin production by A. parasiticus NRRL 2999 in barley with a MC of $\leq$ 16.5%. Hesseltine (71) reported 16% as the lower limit of moisture for aflatoxin production by A. flavus in corn. Optimum toxin production occurred in the range of 24-32C and 20-28% MC. Most researchers agree that the optimum temperature range for maximum aflatoxin production is 24-25C (42, 48, 49, 51, 81, 82, 102, 108, 123, 132). All 4 inoculum levels (10^7 , 10^5 , 10^3 , 10^1) were not significantly different from each other ($P>0.05$). Different levels of inoculum are shown in Figure 6 to show variation and are not meant to show differences since inoculum levels were found to be not significantly different.

When A. parasiticus was in competition with A. sulphureus for the same corn substrate, maximum aflatoxin B₁ production occurred at 24C/24% MC and minimum production at 32C/16% MC (Figure 7). Trenk and Hartman (145) reported higher aflatoxin production by A. flavus at 30 and 35C than at 24C during competition in remoistened corn. They also found 17.5% as the minimal moisture content for growth of A. flavus (145). Optimum B₁ production occurred at 24C and 20-28% MC. Inoculum levels were not significantly different ($P>0.05$). Different levels of inoculum are shown in Figure 7 to show variation

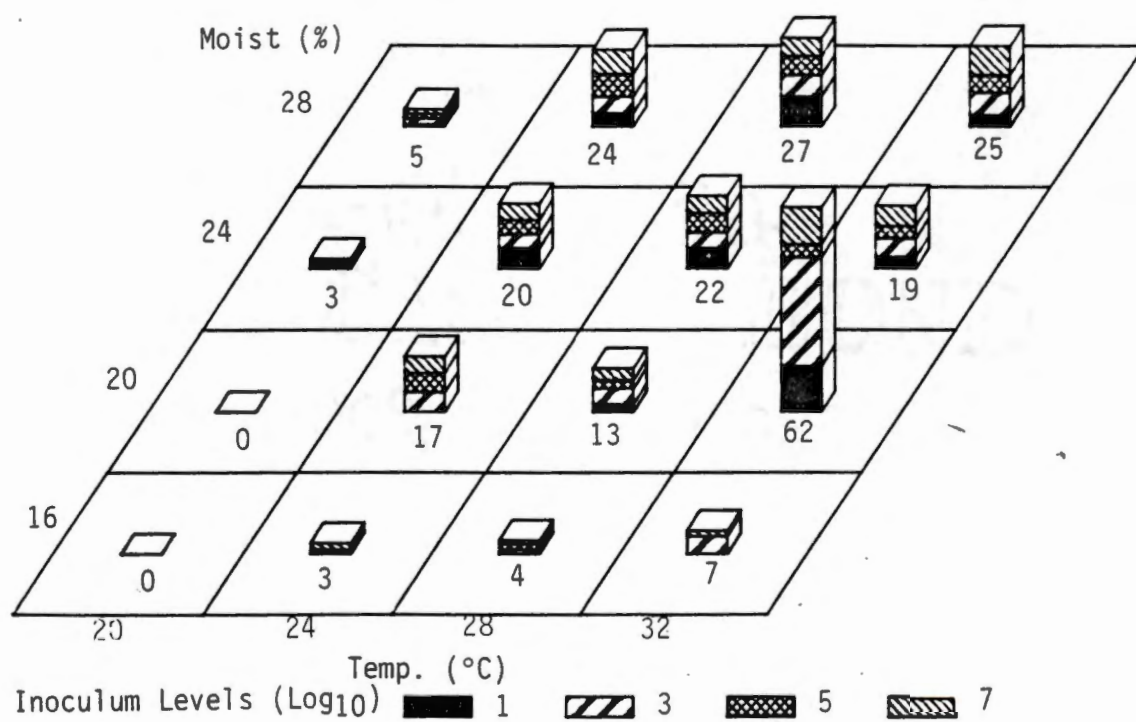


FIGURE 6. Mean level ($\mu\text{g/g}$) of aflatoxin B_1 produced by pure cultures of *A. parasiticus* as a function of temperature and moisture content. Inoculum levels were not significantly different ($P>0.05$).

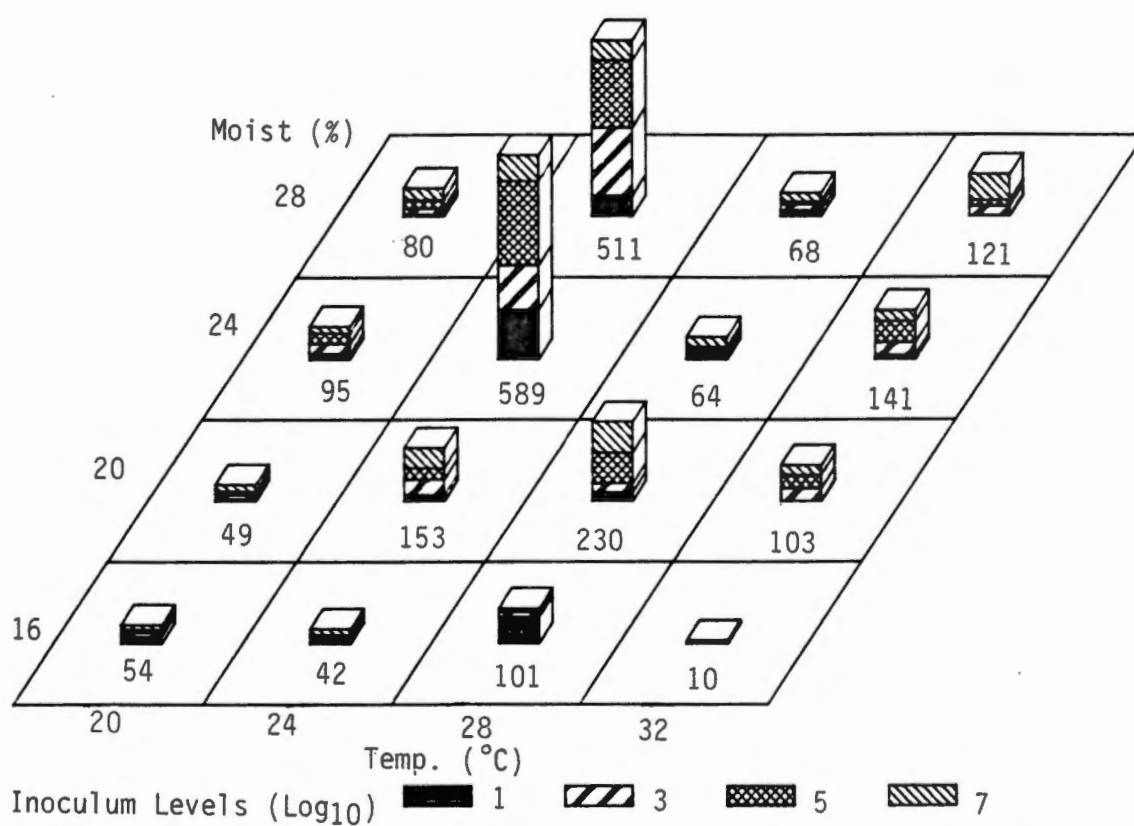


FIGURE 7. Mean level ($\mu\text{g/g}$) of aflatoxin B₁ produced by *A. parasiticus* during fungal competition as a function of temperature and moisture content. Inoculum levels were not significantly different ($P > 0.05$).

and are not meant to show differences since inoculum levels were found to be not significantly different.

More aflatoxin B₁ was produced in a competitive situation than in pure culture. A. sulphureus stimulated aflatoxin B₁ production by A. parasiticus. Jarvis (81) reported that two toxigenic fungal strains growing together may produce more toxins than either might produce alone. Weckbach and Marth (149) found that Acetobacter aceti stimulated growth and aflatoxin production by A. parasiticus in YES medium. Maximum aflatoxin B₁ production occurred at a lower temperature (24C) during fungal competition, and aflatoxin B₁ was produced at all conditions of temperature and moisture content. The optimum conditions were more limited during competition. For both pure and mixed cultures, the inoculum levels were not significantly different ($P>0.05$).

Maximum production of aflatoxin G₁ by A. parasiticus was at 24C/28% MC and no aflatoxin G₁ was detected at 20C/16% MC (Figure 8). Schindler et al. (123) reported maximum aflatoxin G₁ production by A. flavus at 24C on Wort media. However, other researchers have reported maximum production at 20C (44), 24C (44), 28C (135), 30C (44, 51), and 20-25C (51). Optimum conditions for toxin production were 24-28C and 20-28% MC. Inoculum levels were not significantly different ($P>0.05$). Again, different levels of inoculum are shown in Figure 8 to show variation and are not meant to show differences since inoculum levels were not significantly different.

Maximum aflatoxin G₁ production occurred at 24C/24% MC and minimum production at 20C/16% MC and 32C/16% MC during fungal

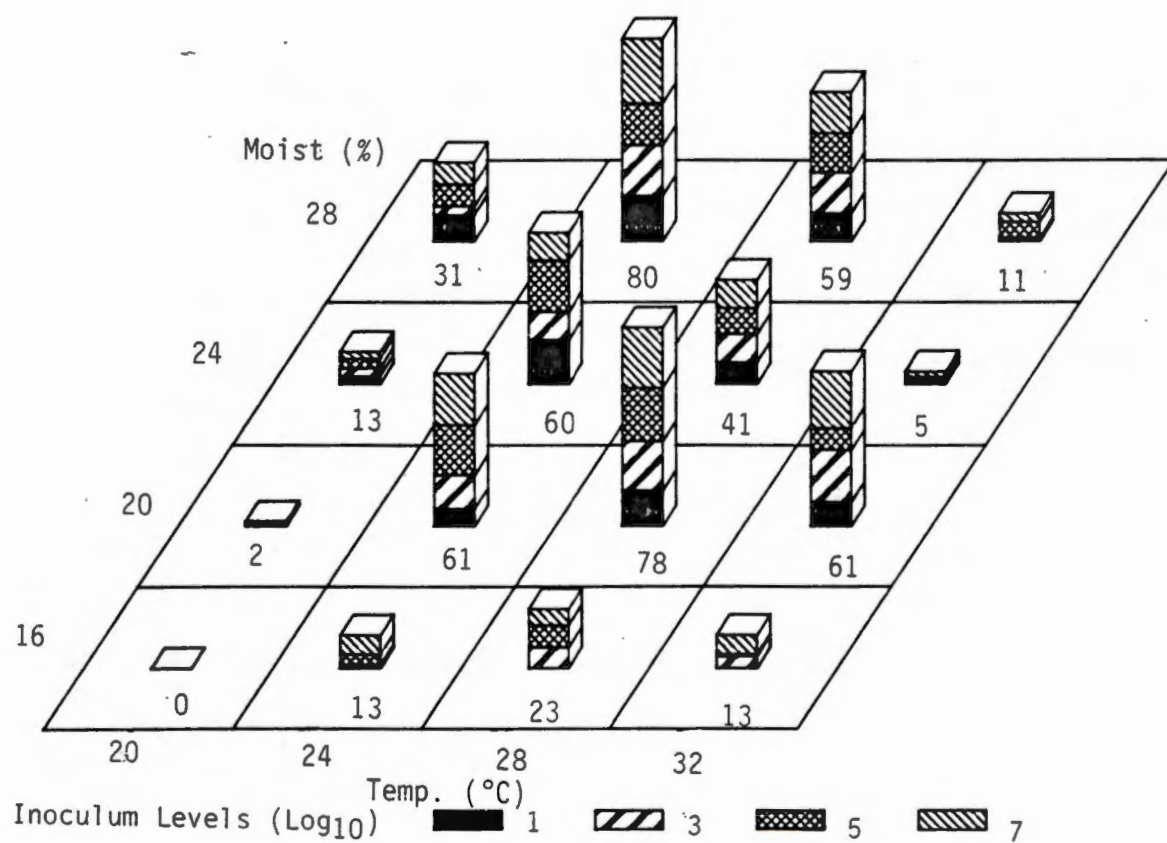


FIGURE 8. Mean level ($\mu\text{g/g}$) of aflatoxin G_1 produced by pure cultures of *A. parasiticus* as a function of temperature and moisture content. Inoculum levels were not significantly different ($P > 0.05$).

competition (Figure 9). Boller and Schroeder (18) reported maximum aflatoxin G₁ production by A. parasiticus at 30C when competing on a rice substrate. The optimal moisture content was 20-28% and 24C was the best temperature for toxin production. Inoculum levels were found to be not significantly different ($P>0.05$).

In general, more aflatoxin G₁ was produced in a competitive environment than in pure culture. However, 24-32C/16% MC resulted in slightly more aflatoxin G₁ production in pure culture than in mixed culture. Toxin production by A. parasiticus was stimulated by the presence of A. sulphureus. Both mixed and pure culture had maximum toxin production at 24C and minimum production at 20C/16% MC.

Hesseltine (71) reported that below 16% MC, A. flavus would not grow, so no aflatoxin would be produced. The range for optimum toxin production was narrower during fungal competition. For both pure and mixed culture, inoculum levels were not significantly different ($P>0.05$).

Mean toxin production by pure and mixed fungal cultures as a function of temperature is shown in Table 3 and Table 4, respectively. Values at each temperature were averaged across all 4 inoculum levels and all 4 moisture contents. For pure cultures (Table 3), more aflatoxin G₁ was produced at 20, 24, and 28C than aflatoxin B₁, while more aflatoxin B₁ was produced at 32C. In general, as the temperature increased, the quantity of aflatoxin B₁ also increased, and the amount of aflatoxin G₁ decreased. The same temperature dependent relationship for aflatoxin B₁ and G₁ was reported by Diener and Davis (48).

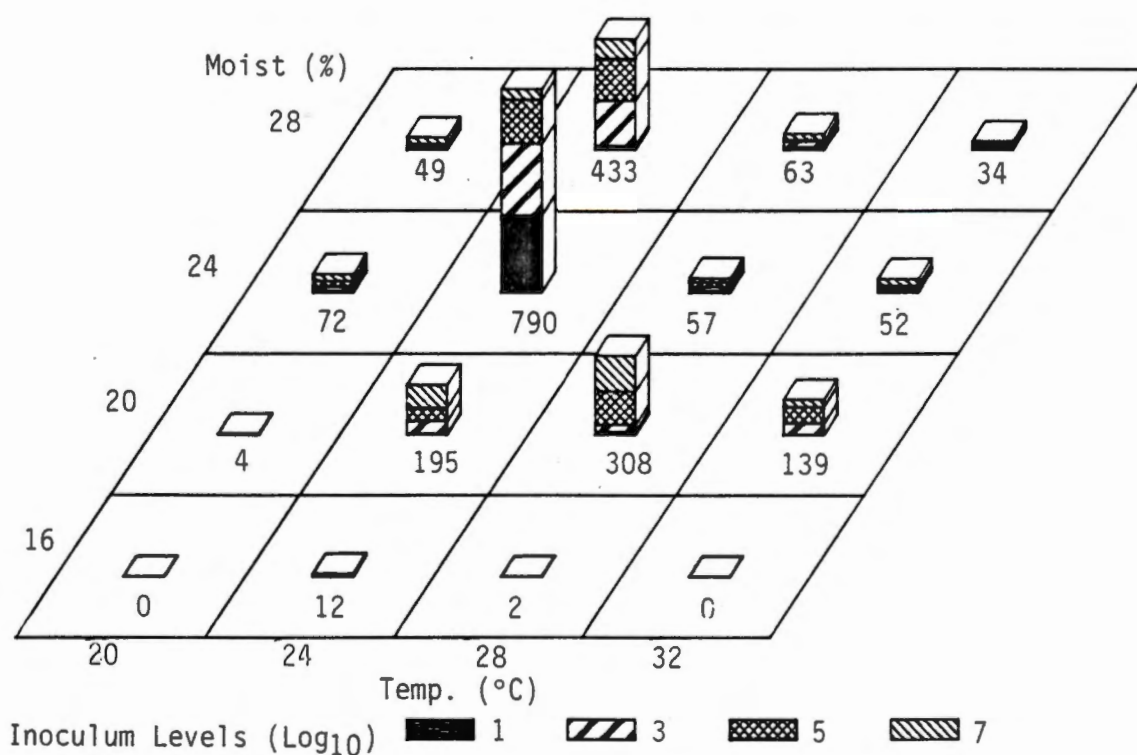


FIGURE 9.. Mean level ($\mu\text{g/g}$) of aflatoxin G₁ produced by A. parasiticus during fungal competition as a function of temperature and moisture content. Inoculum levels were not significantly different ($P > 0.05$).

TABLE 3. Toxin Production as a Function of Temperature for Pure Cultures of A. parasiticus and A. sulphureus*

Temp (C)	Toxin ($\mu\text{g/g}$)**				Total
	B ₁	G ₁	G ₂	OA	
20	2 b	11 b	1 b	2 b	16 b
24	16 a	53 a	5 a	6 a	80 a
28	17 a	50 a	4 a	7 a	78 a
32	28 a	22 b	4 a	6 a	60 a

*Values were averaged across inoculum and moisture levels.

**Means (within columns) with the same letter are not significantly different ($P>0.05$).

TABLE 4. Toxin Production as a Function of Temperature for Mixed Cultures of A. parasiticus and A. sulphureus*

Temp (C)	Toxin ($\mu\text{g/g}$)**				Total
	B ₁	G ₁	OA		
20	69 b	31 b	12 b		112 b
24	324 a	357 a	68 a		749 a
28	116 b	107 b	28 b		251 b
32	94 b	56 b	9 b		159 b

*Values were averaged across inoculum and moisture levels.

**Means (within columns) with the same letter are not significantly different ($P>0.05$).

Many other researchers have reported more G_1 production at lower temperatures and more B_1 production at higher temperatures (44, 49, 51, 90, 123, 126, 135). Schroeder and Hein (126) suggested that aflatoxin G_1 was more easily metabolized than B_1 at higher temperatures. Sorenson et al. (135) postulated that aflatoxin G_1 may be sensitive to degradation enzymes which were not active at lower temperatures, but were active above 32C. This may explain why more aflatoxin B_1 was produced at 32C than aflatoxin g_1 . Another possible explanation is that the enzyme responsible for the B_1 to G_1 conversion was less efficient at 33C than at 25C (90). For aflatoxins B_1 to G_1 conversion was less efficient at 33C than at 25C (90). For aflatoxins B_1 , G_2 , ochratoxin A, and total toxin, 20C resulted in significantly less toxin production ($P < 0.05$) than 24, 28, and 32C. Significantly more toxin ($P < 0.05$) was produced at 24 and 28C than at 20 and 32C for aflatoxin G_1 .

For mixed cultures (Table 4), the temperature dependent relationship for aflatoxin B_1 and G_1 was different. More aflatoxin G_1 was produced at 24C, while more B_1 was produced at 20, 28, and 32C. The reason for the change in the temperature dependent aflatoxin relationship is not known, but competition apparently had a major influence. For aflatoxins B_1 , G_1 , ochratoxin A, and total toxin, 24C resulted in significantly more toxin production ($P < 0.05$) than 20, 28, and 32C.

Mean toxin levels produced by pure and mixed fungal cultures as a function of moisture content (MC) are shown in Table 5 and Table 6,

TABLE 5. Toxin Production as a Function of Moisture Content (MC) for Pure Cultures of A. parasiticus and A. sulphureus*

MC %	Toxin ($\mu\text{g/g}$)**				Total
	B ₁	G ₁	G ₂	OA	
16	4 b	12 c	2 b	2 b	20 c
20	23 a	50 a	6 a	7 a	86 a
24	16 a	30 b	2 b	6 a	54 b
28	20 a	45 a	3 b	7 a	75 ab

*Values were averaged across inoculum and temperature levels.

**Means (within columns) with the same letter are not significantly different ($P>0.05$).

TABLE 6. Toxin Production as a Function of Moisture Content (MC) for Mixed Cultures of A. parasiticus and A. sulphureus*

MC %	Toxin ($\mu\text{g/g}$)**				Total
	B ₁	G ₁	OA		
16	52 b	3 b	15 b		70 b
20	134 ab	161 ab	37 ab		332 ab
24	222 a	243 a	16 b		481 a
28	195 a	145 ab	50 a		390 a

*Values were averaged across inoculum and temperature levels.

**Means (within columns) with the same letter are not significantly different ($P>0.05$).

respectively. Values at each MC were averaged across all 4 inoculum levels and all 4 temperatures. For pure cultures (Table 5), 16% MC resulted in significantly less aflatoxin B₁, aflatoxin G₁, ochratoxin A, and total toxin production ($P < 0.05$) than 20, 24, and 28% MC. Significantly more aflatoxin G₂ was produced ($P < 0.05$) at 20% MC than at 16, 24, and 28% MC.

For mixed cultures (Table 6), 16% MC was significantly different ($P < 0.05$) than 24 and 28% MC for both aflatoxin B₁ and total toxin. Significantly less aflatoxin G₁ ($P < 0.05$) was produced at 16% MC than at 24% MC, while for ochratoxin A, significantly more toxin was produced at 28% MC than at either 16% or 24% MC.

Aflatoxin G₂ was detected only in samples inoculated with a pure culture of A. parasiticus and analyzed by HPLC using a μ -Porasil column and a mobile phase of water-saturated methylene chloride containing 0.5% methanol. Aflatoxin G₂ was not detected in samples inoculated with a mixed culture because the HPLC method of Engstrom et al. (55) does not resolve aflatoxin G₂ from aflatoxin G₁. Maximum and minimum production occurred at 32C/20% MC and 20C/16% MC, respectively (Figure 10). Optimum ranges were 24-32C with 20% as the optimum moisture level. The 10^1 inoculum level produced significantly less aflatoxin G₂ than the 10^7 , 10^5 , or 10^3 levels ($P < 0.05$).

A. sulphureus in pure culture produced maximum amounts of ochratoxin A at 28C/20% MC and minimum amounts at 32C/16% MC (Figure 11). Ciegler (37) reported ochratoxin A synthesis by A. sulphureus NRRL 4077 in YES medium at 28C. Trenk et al. (146) and Diener (51) found

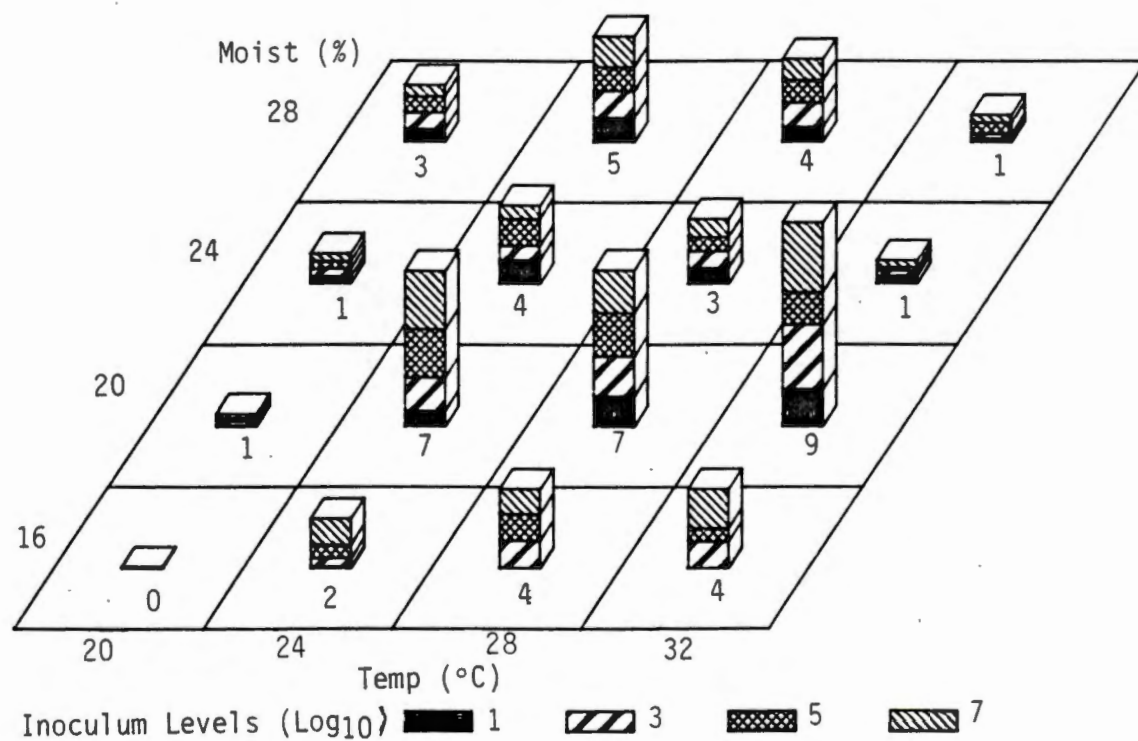


FIGURE 10. Mean level ($\mu\text{g/g}$) of aflatoxin G_2 produced by pure cultures of *A. parasiticus* as a function of temperature and moisture content. The 10^1 inoculum level was significantly different from the 10^3 , 10^5 , and 10^7 levels ($P < 0.05$).

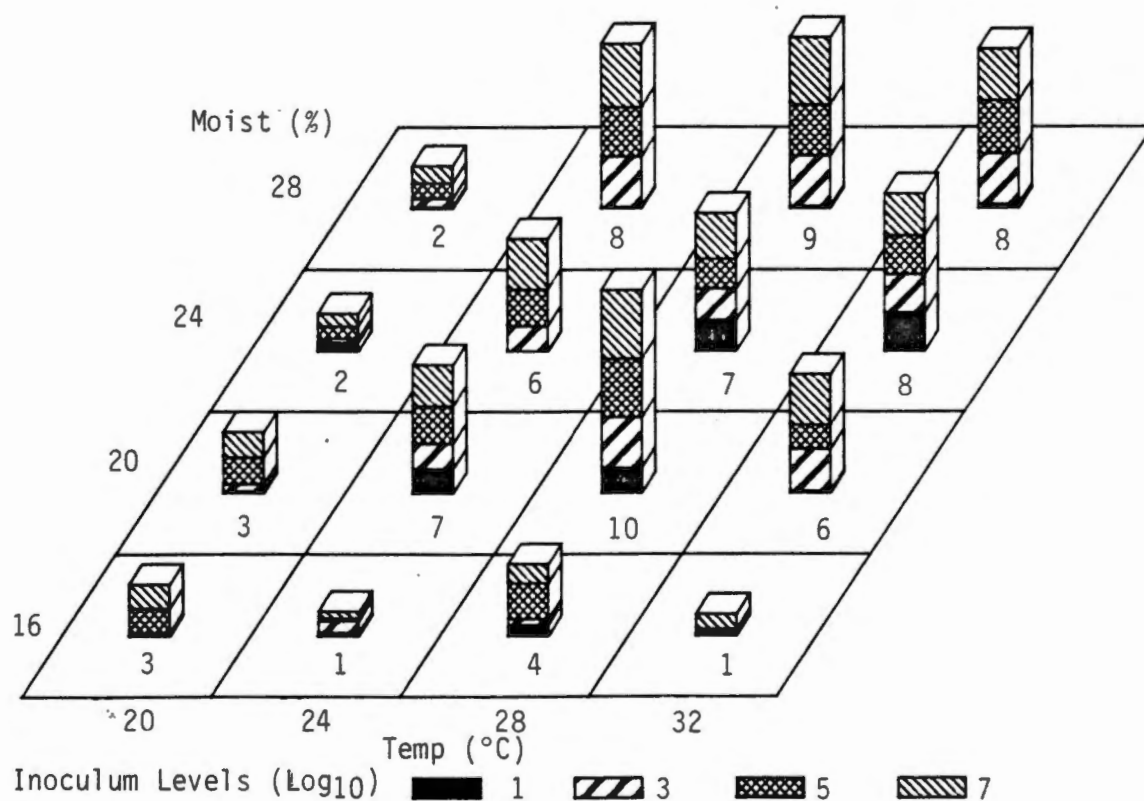


FIGURE 11. Mean level ($\mu\text{g/g}$) of ochratoxin A produced by pure cultures of *A. sulphureus* as a function of temperature and moisture content. The 10^1 inoculum level was significantly different from the 10^3 , 10^5 , and 10^7 levels ($P < 0.05$).

A. ochraceus to produce maximum amounts of ochratoxin A at 28C in corn. A. ochraceus in pure culture was reported by Christensen (33) to invade grains with 15-16% MC at 20-25C. Koehler (83) observed growth of A. ochraceus in corn with 15.6% MC. Optimum toxin production ranged from 24-32C and 20-28% MC. The level of inoculum and ochratoxin A synthesis were found to be related; as the level of inoculum increased, the amount of ochratoxin A also increased. In contrast, Haggblom (63) found that sometimes lower inoculum levels yield slightly higher levels of ochratoxin A in barley. The 10^1 inoculum level supported significantly less ochratoxin A production than the 10^3 , 10^5 , and 10^7 levels ($P < 0.05$).

When A. sulphureus competed with A. parasiticus, maximum and minimum production of ochratoxin A occurred at 24C/28% MC and 20C/16% MC, respectively (Figure 12). Toxin production was optimum at 24C and 20-28% MC. Christensen (30) observed rapid invasion of wheat by A. ochraceus when the moisture content was $\geq 16.0\%$. Inoculum levels were not significantly different ($P > 0.05$). Different levels of inoculum are shown in Figure 12 to show variation and are not meant to show significant differences.

Ochratoxin A synthesis was greater during competition than in pure culture. The presence of A. parasiticus stimulated ochratoxin A synthesis by A. sulphureus. Christensen (30) found slower invasion of wheat by A. ochraceus during competition with A. glaucus, A. flavus, and A. candidus than when present alone. Christensen (30) also reported that A. ochraceus was a poor competitor with other storage

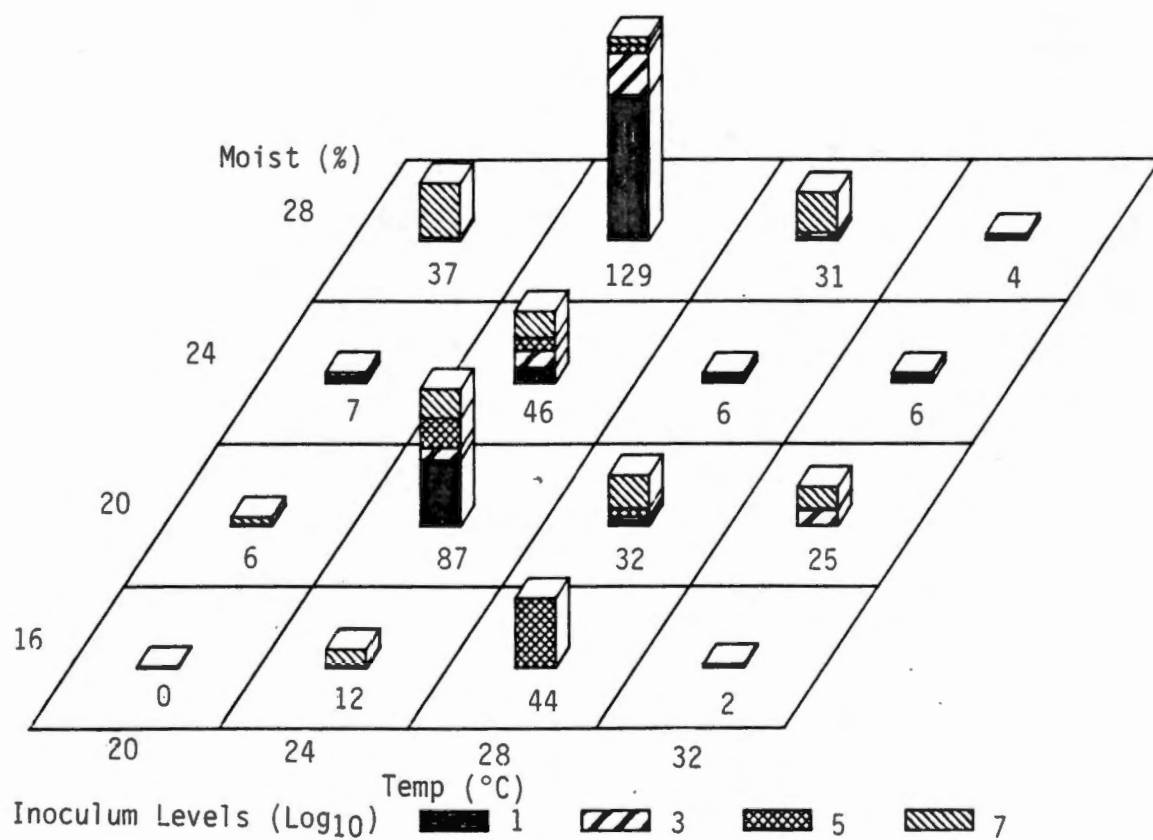


FIGURE 12. Mean level ($\mu\text{g/g}$) of ochratoxin A produced by *A. sulphureus* during fungal competition as a function of temperature and moisture content. Inoculum levels were not significantly different ($P>0.05$).

fungi. Maximum and minimum production of ochratoxin A occurred at a lower temperature in mixed culture than in pure culture. Competition also restricted the range of optimum toxin production. Only in the non-competitive situation did the 10^1 inoculum level support significantly less ochratoxin A synthesis.

Total toxin (pure culture) was determined by summing amounts of aflatoxin B₁, G₁, and G₂ produced by pure cultures of A. parasiticus with the amount of ochratoxin A produced by pure cultures of A. sulphureus. Total toxin production was maximum at 32C/20% MC and minimum at 20C/16% MC (Figure 13). Optimum conditions ranged from 24-32C and 20-28% MC. Inoculum levels were not significantly different ($P>0.05$).

Summation of aflatoxin B₁, aflatoxin G₁, and ochratoxin A was used to determine the amount of total toxin produced during fungal competition. Maximum production occurred at 24C/24% MC and minimum production at 32C/16% MC (Figure 14). Conditions for toxin production were optimum at 24C and 20-28% MC. Inoculum levels were found to be not significantly different ($P>0.05$).

The total amount of toxin produced during fungal competition was greater than when each fungus was grown alone. Maximum total toxin production for mixed cultures occurred at a lower temperature (24C) than pure (32C), and minimum production occurred at 16% MC for both mixed and pure cultures. Competition restricted optimum toxin producing conditions. Hesseltine (71) reported that although a fungus may grow and produce mycotoxins over a wide range of temperature when

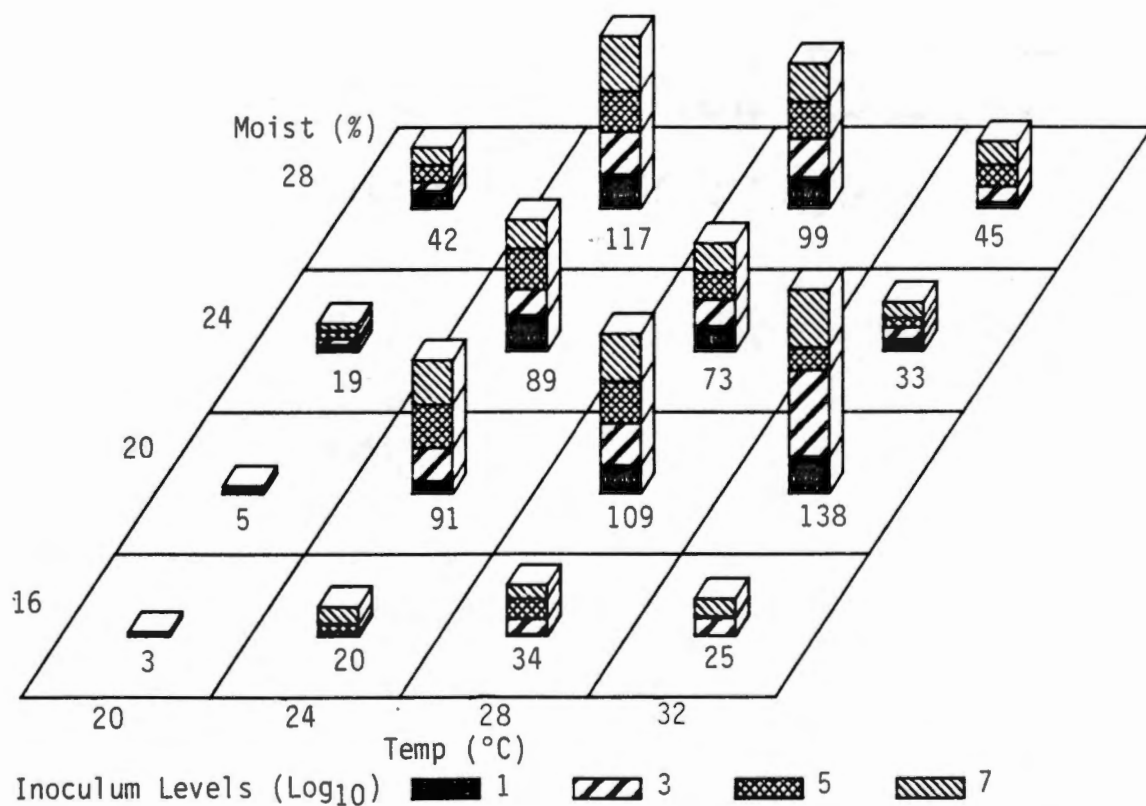


FIGURE 13. Mean level ($\mu\text{g/g}$) of total toxin (aflatoxins $B_1 + G_1 + G_2$ + ochratoxin A) produced by pure cultures of *A. parasiticus* and *A. sulphureus* as a function of temperature and moisture content. Inoculum levels were not significantly different ($P > 0.05$).

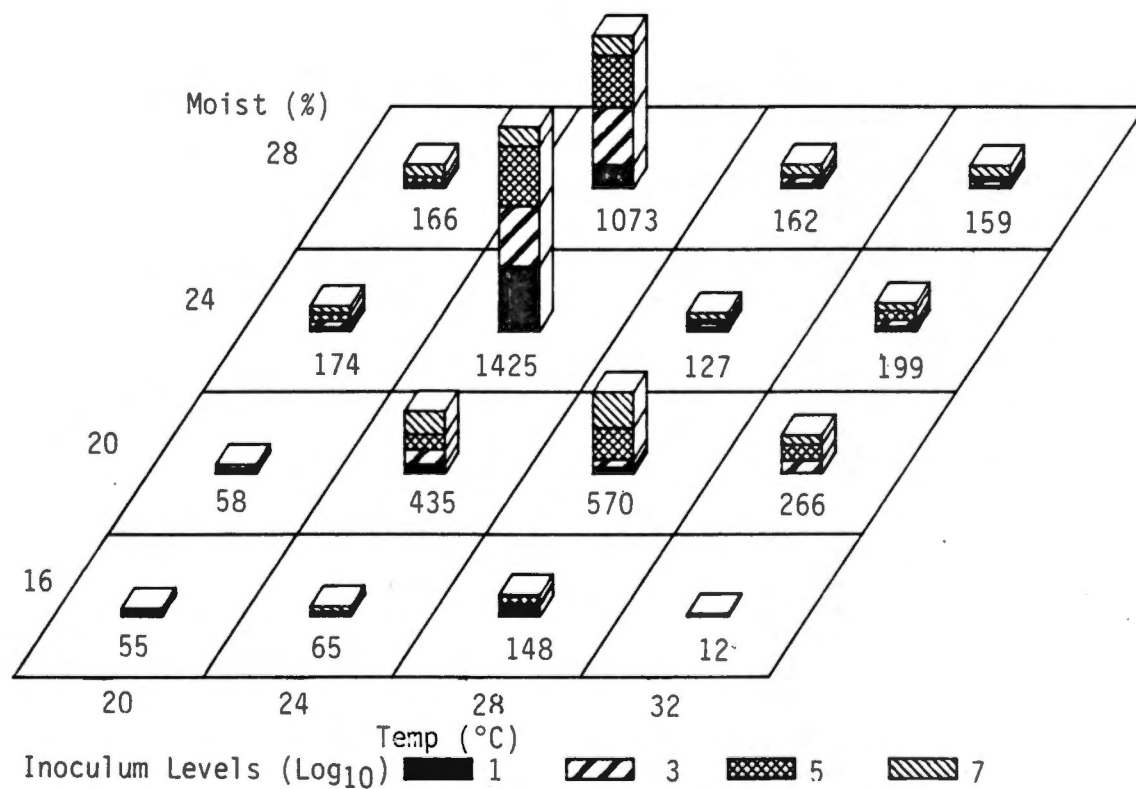


FIGURE 14. Mean level ($\mu\text{g/g}$) of total toxin (aflatoxins $B_1 + G_1 +$ ochratoxin A) produced by mixed cultures of *A. parasiticus* and *A. sulphureus* as a function of temperature and moisture content. Inoculum levels were not significantly different ($P > 0.05$).

grown as a pure culture, growth and toxin production may be more limited under natural conditions because of competition. The inoculum levels were found to be not significantly different in both competitive and non-competitive situations.

Statistical analysis showed that temperature and moisture were the main factors affecting toxin production. In all cases, temperature and moisture had significant effects ($P < 0.05$) on toxin production. Overall, inoculum was not a significant source of variation and could be considered a minor factor affecting toxin production. Significant differences in inoculum level were found for only aflatoxin G₂ and ochratoxin A in pure culture. For both aflatoxin G₂ and ochratoxin A, the 10^1 inoculum level resulted in significantly less toxin production ($P < 0.05$) than the 10^3 , 10^5 , or 10^7 levels. However, for the other toxins in both pure and mixed culture, the 10^7 , 10^5 , 10^3 , and 10^1 inoculum levels were not significantly different ($P > 0.05$).

In the field, it is important to reduce mechanical damage to the grain because broken kernels may allow more rapid invasion by fungi. It is also vital to reduce spore loads and to handle grains in as clean a manner as possible since only 10 spores/50 g corn can result in fungal growth and toxin production. However, reducing spore loads below 10 spores/50 g corn is extremely difficult to achieve because of dispersal of fungal spores by wind, rain, insects, birds, and rodents. Uncontaminated corn can quickly become contaminated with toxin-producing fungi because of the wide variety of means of spore dispersion. Proper harvesting methods, intense insect, bird,

and rodent control, and carefully selected and monitored storage conditions are necessary to reduce or prevent fungal growth and toxin production.

Interactions between temperature and moisture content were significant ($P < 0.05$) for all toxins in both pure and mixed culture except for ochratoxin A, which had no significant temperature/moisture interaction in either pure or mixed culture. Only ochratoxin A in mixed culture had a significant ($P < 0.05$) three-way interaction (temperature/inoculum/moisture).

There were no significant differences ($P > 0.05$) between replications for aflatoxin B₁ (pure culture only) and for ochratoxin A (mixed culture only). However, significant differences ($P < 0.05$) in replication were found for aflatoxins G₁, G₂, ochratoxin A, and total toxin in pure culture, and for aflatoxins B₁, G₁, and total toxin in mixed culture. In general, replication #2 had higher amounts of toxin than replication #1. Replication #2 samples were stored at -15°C for a longer period of time than replication #1, so the length of the storage period might have influenced the amount of toxin present. The longer the samples were stored, the greater the chance for evaporation of the reconstituting solvent. This would result in a reduction in the total sample volume and concentration of the sample. The total sample volume after storage would be less than the original total sample volume of 5 ml, thus an error in toxin calculation could have been introduced with an overestimate of the amount of toxin present. This might explain why more toxin was

found in the second replication. Also, replication differences could be due to the biological variability inherent within each fungus.

Subculturing of the fungi might also have influenced the ability of the fungus to produce toxin.

CHAPTER V

CONCLUSION

The effect of temperature, initial moisture content, and level of inoculum on aflatoxin and ochratoxin production in corn was evaluated using two fungi, Aspergillus parasiticus NRRL 3145 and Aspergillus sulphureus NRRL 4077, during competition and in pure culture.

In conclusion, for aflatoxin B₁, aflatoxin G₁, ochratoxin A, and total toxin, more toxin was formed during fungal competition than when each fungus was grown alone. The presence of A. parasiticus enhanced ochratoxin A production by A. sulphureus and the presence of A. sulphureus enhanced aflatoxin production by A. parasiticus. Toxin production increased probably as a means of survival. In response to stress or unfavorable conditions, fungi produce secondary metabolites. In pure culture, the main concern of the fungus is to obtain nutrients from the substrate for growth and primary metabolism (vegetative activity), but when the nutrients become limited, large amounts of primary metabolic precursors accumulate and the fungus diverts these toxic precursors into secondary metabolites. When two fungi are competing for the same substrate, the amount of nutrients for growth and primary metabolism is much more limited, and the accumulation of toxic primary metabolites is much greater than in pure culture. To survive, one fungus must outcompete the other or they both must live together. It is likely that more toxin was produced by

A. parasiticus during competition to eliminate A. sulphureus, and that more toxin was produced by A. sulphureus to eliminate A. parasiticus. Thus, mycotoxin production may be a survival mechanism used when the fungus encounters stressful conditions. Since A. parasiticus and A. sulphureus seldom exist as pure cultures in grains, their presence as fungal competitors greatly increases the chance for mycotoxin formation in the field and in improperly stored crops. Also, the potential danger to man and animals is enhanced because fungal competition between A. parasiticus and A. sulphureus resulted in increased toxin formation.

During fungal competition, maximum production of aflatoxin B₁, ochratoxin A, and total toxin occurred at a lower temperature (24C) than in pure culture. Maximum production of aflatoxin G₁ occurred at 24C in both pure and mixed culture. For aflatoxins B₁, G₁, ochratoxin A, and total toxin in mixed culture, 24C resulted in significantly more toxin production ($P < 0.05$) than 20, 28, and 32C. In pure culture, 20C resulted in significantly less aflatoxins B₁, G₂, ochratoxin A, and total toxin production ($P < 0.05$) than 24, 28, and 32C. Significantly less aflatoxin G₁ ($P < 0.05$) was produced at 20 and 32C than in 24 and 28C in pure culture. In general for pure cultures, as the temperature increased, aflatoxin B₁ also increased, and aflatoxin G₁ decreased. This temperature dependent relationship did not hold for mixed cultures. Optimum conditions for toxin production were more limited during fungal competition (24C and 20-28% MC).

In both pure and mixed culture, 16% was the minimum moisture content for toxin production. For pure cultures, 16% MC resulted

significantly less aflatoxin B₁, aflatoxin G₁, ochratoxin A, and total toxin production ($P < 0.05$) than 20, 24, and 28% MC. Significantly more aflatoxin G₂ was produced ($P < 0.05$) at 20% MC than at 16, 24, and 28% MC. For mixed cultures, significantly less aflatoxin B₁ and total toxin were produced ($P < 0.05$) at 16% MC than at 24 and 28% MC. Significantly less aflatoxin G₁ ($P < 0.05$) was produced at 16% MC than at 24% MC, while significantly less ochratoxin A was produced ($P < 0.05$) at 16 and 24% MC than at 28% MC.

Inoculum was not a significant source of variation. Significant differences in inoculum level were found for only aflatoxin G₂ and ochratoxin A in pure culture. In these cases, the 10^1 inoculum level resulted in significantly less toxin production ($P < 0.05$) than the 10^3 , 10^5 , and 10^7 levels. No significant differences between inoculum levels were found in mixed culture.

To reduce or prevent toxin formation by A. parasiticus and A. sulphureus during storage of corn, the moisture content of the grain should be reduced to less than 16% and the storage temperature maintained below 20C. Corn should be handled in as clean a manner as possible to reduce spore loads and to minimize contamination since in many cases, 10 spores per 50 grams of corn resulted in toxin production.

LITERATURE CITED

LITERATURE CITED

1. Agnihotri, V. P. 1964. Studies on aspergilli XVI. Effect of pH, temperature, and carbon and nitrogen interaction. *Mycopathologia* 24:20.
2. Allcroft, R. 1969. Aflatoxicosis in farm animals. In "Aflatoxin. Scientific Background, Control, and Implications," (Ed.) Goldblatt, L. A., Academic Press, New York, NY.
3. Applebaum, R. S., Brackett, R. E., Wiseman, D. W., and Marth, E. H. 1982. Aflatoxin: toxicity to dairy cattle and occurrence in milk and milk products--a review. *J. Food Prot.* 45:752.
4. Applegate, K. L. and Chipley, J. R. 1974. Daily variations in the production of aflatoxins by Aspergillus flavus NRRL 3145 following exposure to ⁶⁰Co irradiation. *J. Appl. Bacteriol.* 37:359.
5. Armolik, N. and Dickson, J. G. 1956. Minimum humidity requirement for germination of conidia of fungi associated with storage of grain. *Phytopathology* 46:462.
6. Arseculeratne, S. N. and Bandunatha, C. H. S. R. 1972. Variation of aflatoxin content of cultures of Aspergillus flavus with duration of incubation and its relation to studies on aflatoxin production. *J. Appl. Bacteriol.* 35:43.
7. Ashworth, L. J., Jr., Schroeder, H. W., and Langley, B. C. 1965. Aflatoxins: environmental factors governing occurrence in Spanish peanuts. *Science* 148:1228.
8. Austwick, P. K. C. and Ayerst, G. 1963. Groundnut microflora and toxicity. *Chem. Ind. (London)* 2:55.
9. Ayerst, G. 1969. The effects of moisture and temperature on growth and spore germination in some fungi. *J. Stored Prod. Res.* 5:127.
10. Bacon, C. W., Sweeney, J. G., Robbins, J. D., and Burdick, D. 1973. Production of penicillic acid and ochratoxin A on poultry feed by Aspergillus ochraceus: temperature and moisture experiments. *Appl. Microbiol.* 26:155.
11. Bennett, J. W., Fernholz, F. A., and Lee, L. S. 1978. Effect of light on aflatoxins, anthraquinones, and sclerotia in Aspergillus flavus and A. parasiticus. *Mycologia* 70:104.

12. Bennett, J. W. and Lee, L. S. 1979. Mycotoxins--their biosynthesis in fungi: aflatoxins and other bisfuranoids. J. Food Prot. 42:805.
13. Bennett, J. W., Lee, L. S., Shoss, S. M., and Boudreaux, G. H. 1980. Identification of averantin as an aflatoxin B₁ precursor: placement in the biosynthetic pathway. Appl. Environ. Microbiol. 39:835.
14. Bennett, J. W., Dunn, J. J., and Goldsman, C. I. 1981. Influence of white light on production of aflatoxins and anthraquinones in Aspergillus parasiticus. Appl. Environ. Microbiol. 41:488.
15. Biollaz, M., Buchi, G., and Milne, G. 1970. The biosynthesis of the aflatoxins. J. Am. Chem. Soc. 92:1035.
16. Block, S. S. 1953. Humidity requirements for mold growth. Appl. Microbiol. 1:287.
- * 17. Boller, R. A. and Schroeder, H. W. 1973. Influence of Aspergillus chevalieri on production of aflatoxin in rice by Aspergillus parasiticus. Phytopathology 63:1507.
- * 18. Boller, R. A. and Schroeder, H. W. 1974. Influence of Aspergillus candidus on production of aflatoxin in rice by Aspergillus parasiticus. Phytopathology 64:121.
19. Bottomley, R. A., Christensen, C. M., and Geddes, W. F. 1950. Grain storage studies IX. The influence of various temperatures, humidities, and oxygen concentrations on mold growth and biochemical changes in stored yellow corn. Cereal Chem. 27:271.
20. Bottomley, R. A., Christensen, C. M., and Geddes, W. F. 1952. Grain storage studies X. The influence of aeration, time, and moisture content on fat acidity, nonreducing sugars, and mold flora of stored yellow corn. Cereal Chem. 29:53.
21. Buchanan, R. L., Jr. and Ayres, J. C. 1975. Effect of initial pH on aflatoxin production. Appl. Microbiol. 30:1050.
22. Bullerman, L. B. and Buchanan, R. L. 1981. Mycotoxins other than aflatoxins--their relationships to food safety. J. Food Prot. 44:701.
23. Butler, W. H. and Clifford, J. I. 1965. The extraction of aflatoxin from rat liver. Nature 206:1045.
24. Butler, W. H. 1970. The toxicology of aflatoxin. In "Symposium on Mycotoxins in Human Health," (Ed.) Purchase, I. F. H., MacMillan Press, London.

25. Butler, W. H. 1974. Aflatoxin. In "Mycotoxins," (Ed.) Purchase, I. F. H., Elsevier Science Publishing Co., New York, NY.
26. Calvert, O. H., Lillehoj, E. B., Kwolek, W. F., and Zuber, M. S. 1978. Aflatoxin B₁ and G₁ production in developing Zea mays kernels from mixed inocula of Aspergillus flavus and A. parasiticus. Phytopathology 68:501.
17. Campbell, T. C. and Stoloff, L. 1974. Implication of mycotoxins for human health. J. Agric. Food Chem. 22:1006.
28. Carlton, W. W. 1976. Mycotoxicoses in animals and humans. Proc. Am. Phytopath. Soc. 3:140.
29. Chang, H. G. and Markakis, P. 1981. Effect of moisture content on aflatoxin production in barley. Cereal Chem. 58:89.
30. Christensen, C. M. 1962. Invasion of stored wheat by Aspergillus ochraceus. Cereal Chem. 39:100.
31. Christensen, C. M. and Lopez, L. C. 1965. Relation of moisture content and length of storage to changes in the microflora and germination percentage of rough rice. Phytopathology 55:953.
32. Christensen, C. M. 1969. Influence of moisture content, temperature, and time of storage upon invasion of rough rice by storage fungi. Phytopathology 59:145.
33. Christensen, C. M. 1971. Mycotoxins. Crit. Rev. Environ. Control 2:57.
34. Christensen, C. M. and Mirocha, C. J. 1976. Relation of relative humidity to the invasion of rough rice by Aspergillus parasiticus. Phytopathology 66:204.
35. Chu, F. S. 1971. Interaction of ochratoxin A with bovine serum albumin. Arch. Biochem. Biophys. 147:359.
36. Ciegler, A., Peterson, R. E., Lagoda, A. A., and Hall, H. H. 1966. Aflatoxin production and degradation by Aspergillus flavus in 20 liter fermentors. Appl. Microbiol. 14:826.
37. Ciegler, A. 1972. Bioproduction of ochratoxin A and penicillic acid by members of the Aspergillus ochraceus group. Can. J. Microbiol. 18:631.
38. Ciegler, A. 1975. Mycotoxins: occurrence, chemistry, biological activity. Lloydia 38:21.

39. Coker, R. D. 1979. Aflatoxin: past, present and future. *Trop. Sci.* 21:143.
40. Cole, R. J. 1976. Aspergillus toxins other than aflatoxin. In "Mycotoxins and Other Fungal Related Food Problems," (Ed.) Rodricks, J. V., Am. Chem. Soc., Washington, D.C.
41. Cox, R. H., Churchill, F., Cole, R. J., and Dorner, J. W. 1977. Carbon-13 nuclear magnetic resonance studies of the structure and biosynthesis of versiconal acetate. *J. Am. Chem. Soc.* 99:3159.
42. Davis, N. D., Diener, U. L., and Eldridge, D. W. 1966. Production of aflatoxins B₁ and G₁ by Aspergillus flavus in a semisynthetic medium. *Appl. Microbiol.* 14:378.
43. Davis, N. D., Diener, U. L., and Agnihotri, V. P. 1967. Production of aflatoxins B₁ and G₁ in chemically defined medium. *Mycopathologia* 31:251.
44. Davis, N. D. and Diener, U. L. 1968. Environmental factors affecting the production of aflatoxin. In "Proceedings of the First U.S.-Japan Conference on Toxic Microorganisms," (Ed.) Herzberg, M., U. S. Government Printing Office, Washington, D. C.
45. Davis, N. D., Searcy, J. W., and Diener, U. L. 1969. Production of ochratoxin A by Aspergillus ochraceus in a semisynthetic medium. *Appl. Microbiol.* 17:742.
46. Davis, N. D. 1981. Sterigmatocystin and other mycotoxins produced by Aspergillus species. *J. Food Prot.* 44:711.
47. Detroy, R. W. and Hesseltine, C. W. 1970. Secondary biosynthesis of aflatoxin B₁ in Aspergillus parasiticus. *Can. J. Microbiol.* 16:959.
48. Diener, U. L. and Davis, N. D. 1966. Aflatoxin production by isolates of Aspergillus flavus. *Phytopathology* 56:1390.
49. Diener, U. L. and Davis, N. D. 1967. Limiting temperature and relative humidity for growth and production of aflatoxin and free fatty acids by Aspergillus flavus in sterile peanuts. *J. Am. Oil Chem. Soc.* 44:259.
50. Diener, U. L. and Davis, N. D. 1969. Aflatoxin formation by Aspergillus flavus. In "Aflatoxin. Scientific Background, Control, and Implications," (Ed.) Goldblatt, L. A., Academic Press, New York, NY.

- * 51. Diener, U. L. 1976. Environmental factors influencing mycotoxin formation in the contamination of foods. *Proc. Am. Phytopath. Soc.* 3:126.
- 52. Draughon, F. A. 1983. Personal communication.
- 53. Dvorak, R., Jagos, P., Bouda, J., Piskac, A., and Zapletal, O. 1977. Changes in the clinico-biochemical indices in the rumen liquor and urine in cases of experimental aflatoxicosis in dairy cows. *Vet. Med. (Prague)* 22:161.
- * 54. El-Gendy, S. M. and Marth, E. H. 1981. Growth and aflatoxin production by Aspergillus parasiticus in the presence of Lactobacillus casei. *J. Food Prot.* 44:211.
- 55. Engstrom, G. W., Richard, J. L., and Cysewski, S. J. 1977. High-pressure liquid chromatographic method for detection and resolution of rubratoxin, aflatoxin, and other mycotoxins. *J. Agric. Food Chem.* 25:833.
- 56. Fanse, H. A. and Christensen, C. M. 1966. Invasion by fungi of rice stored at moisture contents of 13.5 to 15.5%. *Phytopathology* 56:1162.
- 57. Fehr, P. M. and Delage, J. 1970. Effect of aflatoxin on fermentation in rumen. *C. R. Acad. Sci. Ser. D* 270:550.
- 58. Ferreira, N. P. and Pitout, M. J. 1969. The biogenesis of ochratoxin. *J. Suid-Afrikaanse Chem. Inst.* 22:51.
- 59. Fitzell, D. L., Singh, R., Hsieh, D. P. H., and Motell, E. L. 1977. Nuclear magnetic resonance identification of versiconal hemiacetal acetate as an intermediate in aflatoxin biosynthesis. *J. Agric. Food Chem.* 25:1193.
- 60. Galloway, L. D. 1935. The moisture requirements of mold fungi with special reference to mildew in textiles. *J. Textile Inst.* 26:123.
- 61. Gilman, J. C. and Barron, D. H. 1930. Effect of molds on temperature of stored grain. *Plant Physiol.* 5:565.
- 62. Goldblatt, L. A. 1969. "Aflatoxin. Scientific Background, Control, and Implications," (Ed.) Goldblatt, L. A., Academic Press, New York, NY.
- * 63. Haggblom, P. 1982. Production of ochratoxin A in barley by Aspergillus ochraceus and Penicillium viridicatum: effect of fungal growth, time, temperature, and inoculum size. *Appl. Environ. Microbiol.* 43:1205.

64. Harwig, J. 1974. Ochratoxin A and related metabolites. In "Mycotoxins," (Ed.) Purchase, I. F. H., Elsevier Science Publishing Co., New York, NY.
65. Harwig, J. and Munro, I. C. 1975. Mycotoxins of possible importance in diseases of Canadian farm animals. *Can. Vet. J.* 16:125.
66. Hayes, A. W., Davis, N. D., and Diener, U. L. 1966. Effect of aeration on growth and aflatoxin production by Aspergillus flavus in submerged culture. *Appl. Microbiol.* 14:1019.
67. Heathcote, J. G., Dutton, M. F., and Hibbert, J. R. 1976. Bio-synthesis of aflatoxins. Part II. *Chem. Ind. (London)* 1976: 270.
68. Hesseltine, C. W., Shotwell, O. L., Ellis, J. J., and Stubblefield, R. D. 1966. Aflatoxin formation by Aspergillus flavus. *Bacteriol. Rev.* 30:795.
69. Hesseltine, C. W., Vandergraff, E. E., Fennell, D. I., Smith, M. L., and Shotwell, O. L. 1972. Aspergilli as ochratoxin producers. *Mycologia* 64:539.
70. Hesseltine, C. W. 1974. Natural occurrence of mycotoxins in cereals. *Mycopathol. Mycol. Appl.* 53:141.
71. Hesseltine, C. W. 1979. Effect of environmental conditions upon mycotoxin production. In "Proceedings of the Fifth International Conference on Global Impacts of Applied Microbiology," (Ed.) Matangkasombut, P., Bangkok, Thailand.
72. Hesseltine, C. W., Rogers, R. F., and Shotwell, O. L. 1981. Aflatoxin and mold flora in North Carolina in 1977 corn crop. *Mycologia* 73:216.
73. Holzaphel, C. W., Steyn, P. S., and Purchase, I. F. H. 1966. Isolation and structure of aflatoxins M₁ and M₂. *Tetrahedron Letters* 25:2799.
74. Hsieh, D. P. H., Seiber, J. N., Reece, C. A., Fitzell, D. L., Yang, S. L., Dalezios, J. I., LaMar, G. N., Budd, D. L., and Motell, E. 1975. ¹³C nuclear magnetic resonance spectra of aflatoxin B₁ derived from acetate. *Tetrahedron* 31:661.
75. Hsieh, D. P. H., Lin, M. T., Yao, R. C., and Singh, R. 1976. Biogenesis of aflatoxins: conversion of norsolorinic acid and other hypothetical intermediates into aflatoxin B₁ by Aspergillus parasiticus. *J. Agric Food Chem.* 24:1171.

76. Hsieh, D. P. H. 1979. Basic metabolic effects of mycotoxins. In "Interactions of Mycotoxins in Animal Production," Am. Soc. Anim. Sci., Am. Dairy Sci. Assoc., Committee on Anim. Nutr., Natl. Res. Council, Natl. Acad. Sci., Washington, D. C.
77. Huff, W. E. and Hamilton, P. B. 1979. Mycotoxins--their biosynthesis in fungi: ochratoxins--metabolites of combined pathways. J. Food Prot. 42:815.
78. Hunter, J. H. 1969. Growth and aflatoxin production in shelled corn by the Aspergillus flavus group as related to relative humidity and temperature. Ph.D. Thesis, Purdue University, W. Lafayette, IN.
79. Hutchison, R. D., Steyn, P. S., and Thompson, D. L. 1971. The isolation and structure of 4-hydroxyochratoxin A and 7-carboxy-3,4-dihydroxy-8-hydroxy-3 methylisocoumarin from Penicillium viridicatum. Tetrahedron Letters 43:4033.
80. Irving, G. W., J. 1968. Perspectives on the mycotoxin problem in the United States. In "Proceedings of the First U.S.-Japan Conference on Toxic Microorganisms," (Ed.) Herzberg, M., U. S. Government Printing Office, Washington, D.C.
- * 81. Jarvis, B. 1971. Factors affecting the production of mycotoxins. J. Appl. Bacteriol. 34:199.
82. Joffe, A. Z. and Lisker, N. 1969. Effects of light, temperature, and pH value on aflatoxin production in vitro. Appl. Microbiol. 18:517.
83. Koehler, B. 1938. Fungus growth in shelled corn as affected by moisture. J. Agric. Res. 56:291.
84. Krogh, P., Axelsen, N. H., Elling, F., Gyrd-Hansen, N., Hald, B., Hyltdgaard-Jensen, J., Larsen, A. E., Madsen, A., Mortensen, H. P., Moller, T., Petersen, O. K., Ravnshov, U., Rostgaard, M., and Aalund, O. 1974. Experimental porcine nephropathy. Changes of renal function and structure induced by ochratoxin A-contaminated feed. Acta Pathol. Microbiol. Scand. Sect. A., Suppl. No. 246:1.
85. Krogh, P. 1977. Ochratoxins. In "Mycotoxins in Human and Animal Health," (Ed.) Rodricks, J. V., Hesselstine, C. W., and Mehlman, M. A., Pathotox Publishers, Inc., Park Forest South, IL.
86. Lai, M., Semeniuk, G., and Hesselstine, C. W. 1970. Conditions for production of ochratoxin A by Aspergillus species in a synthetic medium. Appl. Microbiol. 19:542.

87. Landers, K. E., Davis, N. D., and Diener, U. L. 1967. Influence of atmospheric gases on aflatoxin production by Aspergillus flavus in peanuts. *Phytopathology* 57:1086.
88. Lehninger, O. L. 1975. "Biochemistry," Worth Publishers, Inc., New York, NY.
89. Lin, J. K., Miller, J. A., and Miller, E. C. 1977. 2,3-dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B₁, a major acid hydrolysis product of aflatoxin B₁-DNA or-ribosomal RNA adducts formed in hepatic microsome-mediated reactions and in rat liver in vivo. *Cancer Res.* 37:4430.
90. Lin, Y. C., Ayres, J. C., and Koehler, P. E. 1980. Influence of temperature cycling on the production of aflatoxins B₁ and G₁ by Aspergillus parasiticus. *Appl. Environ. Microbiol.* 40:333.
91. Lopez, L. C. and Christensen, C. M. 1967. Effect of moisture content and temperature on invasion of stored corn by Aspergillus flavus. *Phytopathology* 57:588.
92. Lutey, R. W. and Christensen, C. M. 1963. Influence of moisture content, temperature, and length of storage upon survival of fungi in barley kernels. *Phytopathology* 53:713.
93. Maggon, K. K. and Venkitasubramanian, T. A. 1973. Metabolism of aflatoxins B₁ and G₁ by Aspergillus parasiticus. *Experientia* 29:1210.
94. Maggon, K. K., Gupta, S. K., and Venkitasubramanian, T. A. 1977. Biosynthesis of aflatoxins. *Bacteriol. Rev.* 41:822.
- * 95. Mayberry, D. 1980. Molds compete to control aflatoxin. *Agric. Res.* 28:10.
96. Mertens, D. R. 1979. Biological effects of mycotoxins upon rumen function and lactating dairy cows. In "Interactions of Mycotoxins in Animal Production," Am. Soc. Anim. Sci., Am. Dairy Sci. Assoc., Committee on Anim. Nutr., Natl. Res. Council, Natl. Acad. Sci., Washington, D.C.
97. Miller, D. D. and Golding, N. S. 1949. The gas requirements of molds. V. The minimum oxygen requirements for normal growth and for germination of six mold cultures. *J. Dairy Sci.* 32:101.
98. Mirocha, C. J. and Christensen, C. M. 1976. Mycotoxins and the fungi that produce them. *Proc. Am. Phytopath. Soc.* 3:110.

99. Munro, I. C., Scott, P. M., Moodie, C. A., and Willes, R. F. 1973. Ochratoxin A--occurrence and toxicity. J. Am. Vet. Med. Assoc. 163:1269.
100. Nelson, G. H. 1979. Biological effects of mycotoxins (other than aflatoxin) on swine. In "Interactions of Mycotoxins in Animal Production," Am. Soc. Anim. Sci., Am. Dairy Sci. Assoc., Committee on Anim. Nutr., Natl. Res. Council, Natl. Acad. Sci., Washington, D.C..
101. Northolt, M. D., Verhulsdonk, C. A. H., Soentoro, P. S. S., and Paulsch, W. E. 1976. Effect of water activity and temperature on aflatoxin production by Aspergillus parasiticus. J. Milk Food Technol. 39:170.
102. Northolt, M. D., van Egmond, H. P., and Paulsch, W. E. 1977. Differences between Aspergillus flavus strains in growth and aflatoxin B₁ production in relation to water activity and temperature. J. Food Prot. 40:778.
103. Northolt, M. D., van Egmond, H. P., and Paulsch, W. E. 1979. Ochratoxin A production by some fungal species in relation to water activity and temperature. J. Food Prot. 42:485.
104. Northolt, M. D. and Bullerman, L. B. 1982. Prevention of mold growth and toxin production through control of environmental conditions. J. Food Prot. 45:519.
- *105. Olutiola, P. O. 1976. Some environmental and nutritional factors affecting growth and sporulation of Aspergillus flavus. Trans. Br. Mycol. Soc. 66:131.
106. Osborne, B. G. 1982. Mycotoxins and the cereals industry--a review. J. Food Technol. 17:1.
107. Panassenko, V. T. 1941. Mold fungi of confectionary goods and their control. Microbiology (USSR) 10:470.
108. Park, K. Y. and Bullerman, L. B. 1981. Increased aflatoxin production by Aspergillus parasiticus under conditions of cycling temperatures. J. Food Sci. 46:1147.
109. Park, K. Y. and Bullerman, L. B. 1983. Effects of substrate and temperature on aflatoxin production by Aspergillus parasiticus and Aspergillus flavus. J. Food Prot. 46:178.
- *110. Paster, N. and Chet, I. 1980. Effects of environmental factors on growth and sclerotium formation in Aspergillus ochraceus. Can. J. Bot. 58:1844.

111. Pettit, R. E. and Taber, R. A. 1976. Introduction and historical perspectives in mycotoxicology research. *Proc. Am. Phytopath. Soc.* 3:99.
112. Pitout, M. J. 1970. Biochemical studies on ochratoxin A. In "Symposium on Mycotoxins in Human Health," (Ed.) Purchase, I. F. H., MacMillan Press, London.
113. Prakash, C. M. and Siradhana, B. S. 1978. Effect of temperature hydrogen-ion concentration and some media on four Aspergillus species isolated from maize grains. *Indian Phytopathol.* 31:545.
114. Purchase, I. F. H. 1972. Aflatoxin residues in food of animal origin. *Food Cosmet. Toxicol.* 10:531.
115. Qasem, S. A. and Christensen, C. M. 1958. Influence of moisture content, temperature, and time on the deterioration of stored corn by fungi. *Phytopathology* 48:544.
116. Qasem, S. A. and Christensen, C. M. 1960. Influence of various factors on the deterioration of stored corn by fungi. *Phytopathology* 50:703.
117. Rabie, C. J. and Smalley, E. B. 1965. Influence of temperature on the production of aflatoxin. In "Symposium on Mycotoxins in Foodstuffs, Pretoria," Agric. Aspects. Pretoria. Dept. Agric. Technol. Serv. Session 2.
118. Reddy, T. V., Viswanathan, L., and VenkitaSubramanian, T. A. 1971. High aflatoxin production on a chemically defined medium. *Appl. Microbiol.* 22:393.
119. Ribelin, W. E., Fukushima, K., and Still, P. E. 1978. The toxicity of ochratoxin to ruminants. *Can J. Comp. Med.* 42:172.
120. Sanders, T. H., Davis, N. D., and Diener, U. L. 1968. Effect of carbon dioxide, temperature, and relative humidity on production of aflatoxin in peanuts. *J. Am. Oil Chem. Soc.* 45:683.
121. Sansing, G. A., Davis, N. D., and Diener, U. L. 1973. Effect of time and temperature on ochratoxin A production by Aspergillus ochraceus. *Can. J. Microbiol.* 19:1259.
122. Sauer, D. B. and Burroughs, R. 1980. Fungal growth, aflatoxin production, and moisture equilibration in mixtures of wet and dry corn. *Phytopathology* 70:516.

123. Schindler, A. F., Palmer, J. G., and Eisenberg, W. V. 1967. Aflatoxin production by Aspergillus flavus as related to various temperatures. Appl. Microbiol. 15:1006.
124. Schindler, A. F. and Nesheim, S. 1970. Effect of moisture and incubation time on ochratoxin A production by an isolate of Aspergillus ochraceus. J. Assoc. Off. Anal. Chem. 53:89.
125. Schindler, A. F. 1977. Temperature limits for production of aflatoxin by twenty-five isolates of Aspergillus flavus and Aspergillus parasiticus. J. Food Prot. 40:39.
126. Schroeder, H. W. and Hein, H., Jr. 1967. Aflatoxins: production of the toxins in vitro in relation to temperature. Appl. Microbiol. 15:441.
127. Scott, P. M. 1978. Mycotoxins in feeds and ingredients and their origin. J. Food Prot. 41:385.
128. Searcy, J. W., Davis, N. D., and Diener, U. L. 1969. Biosynthesis of ochratoxin A. Appl. Microbiol. 18:622.
129. Seitz, L. M., Sauer, D. B., Mohr, H. E., and Aldis, D. F. 1982. Fungal growth and dry matter loss during bin storage of high-moisture corn. Cereal Chem. 59:9.
130. Shank, R. C. 1970. Dietary aflatoxin loads and the incidence of human hepatocellular carcinoma in Thailand. In "Symposium on Mycotoxins in Human Health," (Ed.) Purchase, I. F. H., MacMillan Press, London.
131. Sharma, A., Behere, A. G., Padwal-Desai, S. R., and Nadkarni, G. B. 1980. Influence of inoculum size of Aspergillus parasiticus spores on aflatoxin production. Appl. Environ. Microbiol. 40:989.
132. Shih, C. N. and Marth, E. H. 1974. Some cultural conditions that control biosynthesis of lipid and aflatoxin by Aspergillus parasiticus. Appl. Microbiol. 27:452.
133. Sinda, J. F. and DeBernardis, J. F. 1973. Naturally occurring halogenated organic compounds. Lloydia 36:107.
134. Singh, R. and Hsieh, D. P. H. 1977. Aflatoxin biosynthetic pathway: elucidation by using blocked mutants of Aspergillus parasiticus. Arch. Biochem. Biophys. 178-285.
135. Sorenson, W. G., Hesseltine, C. W., and Shotwell, O. L. 1967. Effect of temperature on production of aflatoxin on rice by Aspergillus flavus. Mycopathologia 33:49.

136. Spensley, P. G. 1963. Aflatoxins, the active principle in turkey "X" disease. *Endeavor* 22:75.
137. Steyn, P. S., Holzapfel, C. W., and Ferreira, N. P. 1970. The biosynthesis of ochratoxins, metabolites of Aspergillus ochraceus. *Phytochemistry* 9:1977.
138. Steyn, P. S., Vleggaar, R., Wessels, P. L., and Scott, D. B. 1975. Biosynthesis of aflatoxin B₁ from 2-¹³C and 1-¹³C acetate. *J. Chem. Soc. Chem. Commun.* 1975:193.
139. Steyn, P. S. 1977. Mycotoxins, excluding aflatoxin, zearalenone, and the trichothecenes. In "Mycotoxins in Human and Animal Health," (Ed.) Rodricks, J. V., Hesseltnine, C. W., and Mehلمان, M. A., Pathotox Publishers, Inc., Park Forest South, IL.
140. Stoloff, L. 1977. Aflatoxins--an overview. In "Mycotoxins in Human and Animal Health," (Ed.) Rodricks, J. V., Hesseltnine, C. W., and Mehلمان, M. A., Pathotox Publishers, Inc., Park Forest South, IL.
141. Stoloff, L. 1979. The three eras of fungal toxin research. *J. Am. Oil Chem. Soc.* 56:784.
142. Stutz, H. K. and Krumperman, P. H. 1976. Effect of temperature cycling on the production of aflatoxin by Aspergillus parasiticus. *Appl. Environ. Microbiol.* 32:327.
143. Thomas, R. 1965. Biosynthetic pathways involving ring cleavage. In "Biogenesis of Antibiotic Substances," (Ed.) Vanek, Z. and Hostalek, Z., Academic Press, New York, NY.
144. Tong, C. H. 1982. Effects of antimicrobial food additives on growth and ochratoxin A production by Aspergillus sulphureus and Penicillium viridicatum. M. S. Thesis, The University of Tennessee, Knoxville, TN.
145. Trenk, H. L. and Hartman, P. A. 1970. Effects of moisture content and temperature on aflatoxin production in corn. *Appl. Microbiol.* 19:781.
146. Trenk, H. L., Butz, M. E., and Chu, F. S. 1971. Production of ochratoxins in different cereal products by Aspergillus ochraceus. *Appl. Microbiol.* 21:1032.
147. Turner, W. B. 1971. "Fungal metabolites," (Ed.) Turner, W. B., Academic Press, New York, NY.

148. van Walbeek, W. 1973. Fungal toxins in foods. Can. Inst. Food Technol. J. 6:96.
- * 149. Weckbach, L. S. and Marth, E. H. 1977. Aflatoxin production by Aspergillus parasiticus in a competitive environment. Mycopathologia 62:39.
- * 150. Wicklow, D. T. and Hesseltine, C. W. 1979. Fluorescence produced by Aspergillus flavus in association with other fungi in autoclaved corn kernels. Phytopathology 69:589.
- * 151. Wicklow, D. T., Hesseltine, C. W., Shotwell, O. L., and Adams, G. L. 1980. Interference competition and aflatoxin levels in corn. Phytopathology 70:761.
152. Wilson, B. J. 1978. Hazards of mycotoxins to public health. J. Food Prot. 41:375.
- * 153. Wiseman, D. W. and Marth, E. H. 1981. Growth and aflatoxin production by Aspergillus parasiticus when in the presence of Streptococcus lactis. Mycopathologia 73:49.

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