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SOME ECOLOGICAL STUDIES OF AFLATOXIN-PRODUCING STRAINS OF *Aspergillus flavus* Link INFECTING CROPS IN MEXICO[1]

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ABSTRACT

Samples of nine different crops, including cereal kernels, legume seeds, and dry chili pods, were obtained from the public market and storage facilities at Toluca, Mex., a high, cold, and dry area. Samples of 12 crops including cereal kernels, legume seeds, cacao, coffee kernels, and dry chili pods were collected from the public market and storage facilities at Veracruz, Ver., a low, warm, and humid area. In general, the moisture content was found to be higher in the crops from Veracruz.

The micoflora associated with these crops was studied with emphasis on determining prevalence of species of the *A. flavus* group. *A. flavus* was most prevalent in rice, broad beans, cacao, and oat kernels.

The ability of three strains of *A. flavus* to produce aflatoxins *in vitro* was tested by inoculating 18 different crops from two areas. Aflatoxin production varied according to the substrate and fungus strain. The strain from Veracruz was more toxicogenic than the strain from Toluca, but not as toxicogenic as the strain from Texas that was used as the standard.

Aflatoxin production was found to range from no detectable amounts and trace amounts to maximum average yields on wheat kernels of 131.59 μg of aflatoxin B_1 and 336.50 μg of aflatoxin G_1/g of substrate.

Species of the *A. flavus* group were most prevalent in crops from Veracruz than in crops from Toluca.

Aflatoxin production varied among different crops and among varieties of a single crop as with corn beans, and chili pod varieties.

RESUMEN

Muestras de nueve diferentes cultivos, incluyendo granos de cereales, semillas de leguminosas y chiles secos, fueron obtenidas del mercado público y de almacenes en Toluca, Méx., un lugar de altitud elevada, frío y relativamente seco. Muestras de doce cultivos, incluyendo granos de cereales, semillas de leguminosas, cacao, café y chiles secos, fueron obtenidas del mercado público y almacenes en Veracruz, Ver., un lugar de altitud baja, caliente y húmedo. En general, el contenido de humedad de los cultivos fue más alto en aquéllos obtenidos en Veracruz.

La micoflora asociada con estos cultivos fue estudiada, poniendo especial atención en la determinación de la prevalencia de especies del grupo de *A. flavus*. *A. flavus* fue más abundante en arroz, haba, cacao y avena.

La capacidad de tres cepas de *A. flavus* para producir aflatoxinas *in vitro* fue

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probada al inocular 18 diferentes cultivos colectados en los dos lugares. La producción de aflatoxinas varió de acuerdo con el substrato y con la cepa del hongo usados. La cepa de *A. flavus* obtenida de Veracruz fue más toxigénica que la cepa obtenida de Toluca, pero no tan toxigénica como aquélla aislada de Texas que se usó como control.

La producción de aflatoxinas varió desde cantidades no detectables y trazas hasta rendimientos máximos (promedio) de 131.59 μg de aflatoxina B_1 y 336.50 μg de aflatoxina G_1/g de substrato (granos de trigo).

Las especies del grupo de *A. flavus* fueron más prevalentes en los cultivos de Veracruz que en los de Toluca. La producción de aflatoxinas varió entre los diferentes cultivos y entre variedades de un mismo cultivo como son: maíz, frijol y chiles secos.

INTRODUCTION

In recent years, scientists have focused their attention to the changes that can be produced in food and feed crops by the attack of microorganisms, particularly fungi. The common saprophytic mold *Aspergillus flavus* Link produces four similar toxins, called aflatoxins, when it grows on grains and feeds. These compounds have been responsible for outbreaks of poisoning among certain domestic animals. Aflatoxin poisoning causes loss of appetite, reduced growth rate, ataxia, convulsions, cancerous tumors, and even death. The extreme toxicity and carcinogenic properties of aflatoxins present a hazard to warmblooded animals and human health.

A surge of interest in mycotoxicoses was stimulated by the discovery in 1961 of toxins (aflatoxins) in peanuts infected by *A. flavus*. Although acceleration of research in the field of food-borne mycotoxins has taken place in the Western world during the past few years, it is of paramount importance that more research be done in this field, especially in those countries situated in tropical and subtropical regions where natural inoculum is abundant and widely dispersed and the climatic conditions highly favorable for the rapid proliferation of molds in stored foods and feedstuffs.

In Mexico, unfortunately, very little research has been carried out in this field. This country offers a unique opportunity for undertaking an ecological study because of the immense variety of environmental conditions that can be found. Although Mexico is situated in a tropical region, its irregular topography provides a wide range in elevation and consequently, in contrasting environmental conditions.

The objectives of this study were to investigate the incidence of the *A. flavus* species, as well as the incidence of other aspergilli on several crops characteristic of two geographical areas of Mexico with different ecological conditions.

Two areas of Mexico with contrasting climatic conditions were chosen for this study. Toluca, Mex., was selected to represent the high altitude, cool, dry areas. Toluca is located at about 75 Km from Mexico City at an altitude of 2675 m above sea level. Veracruz, Ver. and Cotaxtla, Ver., were selected to represent the low altitude, warm, humid areas. The former site is located on the coast of the Gulf of Mexico about 450 Km from Mexico City at an altitude of 18 m above sea level. The latter site is located in the vicinity of Veracruz, Ver., at an altitude of 130 m above sea level. Location of these sites is indicated in figure 1.

Several investigators have found that the capacity to produce aflatoxins varies over a wide range among strains of *A. flavus* (Boller and Schroeder, 1966; Die-

ner and Davis, 1966; Schroeder and Ashworth, 1966; Taber and Schroeder, 1967) and this capacity interacts with the kind of substrate and culture conditions. Therefore, representative strains of *A. flavus* isolated from various crops were evaluated for their ability to produce aflatoxins (toxicogenicity) on the test crops.

LITERATURE REVIEW

Deterioration in stored grain typical of that caused by fungi was described some decades ago (Bunting, 1930; Snow, 1945; Garren and Higgins, 1947; Bottomley, Christensen, and Geddes, 1952; Del Prado and Christensen, 1952), but the problem has been emphasized recently by the recognition of the production of toxic metabolites by certain molds such as *Aspergillus flavus* Link ex Fries (Codner, Sargeant, and Yeo, 1963) and *Penicillium puberulum* Bainer (Kulik and Holaday, 1965). These molds have been shown to produce coumarin-like compounds (Chang, Abdel Kader, Wick, and Wogan, 1963) known by the generic name of "aflatoxins", while growing on the seeds of wheat, rice, corn, oats, peanuts, soybeans, cottonseed, and other food crops or artificial media (Diener and Davis, 1966; Mayne, Ponz, Franz, and Goldblatt, 1966; Stubblefield, Shotwell, Hesseltine, Smith, and Hall, 1967).

The discovery of the aflatoxins and of their lethal and carcinogenic properties followed the outbreak of the "Turkey X" disease (Blount, 1961) in the south and east of England in 1960, which caused the death of more than 100,000 turkey poults. The cause of the disease was traced to toxic food rations containing Brazilian groundnut meal. In 1961, Sargeant *et al* showed that "Turkey X" disease was caused by toxic metabolites (aflatoxins) and that these compounds were produced by *A. flavus*.

The aflatoxins are highly toxic to rapidly growing young animals. Biological tests have been made on a variety of animals, including birds (Sargeant, Sheridan, O'Kelly, and Carnaghan, 1961; Austwick and Ayerst, 1963; Chang, Abdel Kader, Wick, and Wogan, 1963; Sargeant, Carnaghan, and Allcroft, 1963; Bampton, 1963), cows (Allcroft and Carnaghan, 1963), rodents (Lancaster, Jerkins, and Philip, 1961; Butler and Barnes, 1963), and other experimental animals such as trout (Halver, 1964). The criteria for toxicity have been, in most cases death, a reduction in growth, hepatomas and extensive bile-duct hyperplasia.

These toxins also affect plant cells. In 1965, Schoental and White reported that aflatoxins interfered with seed germination, plant growth and with the biosynthesis of chlorophyll thereby causing wilting or blanching of leaves of *Lepidium sativum*. Black and Altschul (1965) using the same plant found that aflatoxins inhibited protein synthesis and lipase activity.

The intracellular effect of aflatoxin B_1 poisoning is one of the most interesting research discoveries. Aflatoxin B_1 was reported to bind itself to DNA in the nucleus of the cell, preventing messenger RNA from leaving the nucleus and thereby inhibiting protein synthesis (Rees and Clifford, 1966).

In 1966, Burmeister and Hesseltine studied the sensitivity of microorganisms to aflatoxins. Of 329 microorganisms tested for aflatoxin sensitivity, including bacteria, fungi, algae, and one protozoan, 12 species of the genus *Bacillus*, a *Clostridium*, and a *Streptomyces* were inhibited by 30

μg/ml of crude aflatoxin (36% purity) present in the growth substrate. Some microorganisms, howere, are not only insensitive to aflatoxins, but detoxify them. Ciegler *et al* (1966) reported the degradation of aflatoxins by some microorganisms. *Flavobacterium aurantiacum* was capable of degrading aflatoxin B_1 in a modified Czapeck Dox medium at pH 5.5.

These findings exemplify the fact that metabolites of microorganisms can have deleterious effects not only on microbes, but also on cells of higher organisms, in which they can cause disease. This does not imply that man is as sensitive to the aflatoxins as the rat, the trout, or other animals, but rather indicates a need for additional investigation. Some of the food products tested and found to contain carcinogenic mycotoxins are consumed by humans (Anonymous, 1964).

The chemical characteristics of the aflatoxins have been studied and several investigations in this field and related fields have been made since Sargeant *et al.* in 1961 reported the separation of aflatoxins on thin-layer chromatograms. These investigators found that the compounds appear in a characteristic pattern of spots detectable by their property to fluoresce when irradiated with ultraviolet light. The blue fluorescent spot with the highest Rf value (0.7) was defined as aflatoxin B. Another spot, showing a greenish fluorescence and with slightly lower Rf value than aflatoxin B, was termed aflatoxin G. The latter was found to be less toxic than aflatoxin B in biological tests with day-old ducklings. In 1963, Asao *et al* elucidated the furocoumarin structure of both toxins and showed them to be related. Later, Chang *et al* (1963) isolated aflatoxin B_2 from cultures of *A. flavus* on crushed wheat. The chemical structure showed it to be dihydro-aflatoxin B_1. Further, Hartley *et al* (1963) indicated that a mixture of four closely-related compounds has now been found and characterized and their interrelationships studied (figure 2). Aflatoxin B_1 was found to be indentical with the compound previously described as aflatoxin B. The substance originally called aflatoxin G corresponded to aflatoxin G_1. These workers reported that aflatoxins B_1 and G_1 could be converted into aflatoxins B_2 and G_2 by hydrogenation.

According to the results of several investigators, *A. flavus* is not the only producer of aflatoxins. Several species of *Aspergillus and Penicillium* may have the capacity to produce aflatoxins under certain conditions. In 1963, Codner *et al* reported that *A. parasiticus,* another species of the *A. flavus* group, produced aflatoxins. In 1964, Hodges *et al* found that these toxins were also elaborated by *Penicillium puberulum.*

In view of these studies, Kulik *et al.* screened in 1965, *P. puberulum* and other species of *Aspergillus* which they had isolated from corn kernels for production of aflatoxins. These workers reported that certain isolates of *A. flavus, A. niger, A. parasiticus, A. ruber, A. wentii, Penicillium citrinum,* and *P. variable* produced aflatoxins. *P. frequentans* and *P. puberulum* elaborated the toxins only in trace amounts.

In 1966, Parrish *et al* studied the producction of aflatoxins and kojic acid by species of *Aspergillus* and *Penicillium.* Aflatoxins were produced by strains of *A. flavus* and *A. parasiticus,* but neither aflatoxins nor kojic acid were produced by other species of *Aspergillus* and *P. puberulum.* In 1966, Schroeder also studied the relation of aflatoxin production by *A. parasiticus* as affected by different concentrations of corn steep liquor.

In spite of the variable results obtained by different investigators, these findings indicate that molds other than *A. flavus* are highly adaptable and posses the bio-

chemical capabilities to produce complex organic molecules significant in food intoxication. These molds are omnivorous, able to grow and develop on almost any substrate. They grow and may develop toxic products in food and feed tuffs and thus play a definite role in the health of man and his domestic animals. Interestingly, these toxic products are mainly produced by saprophytic fungi previously considered harmless to animal and human health.

Prior to 1952, most molds generally were considered harmless, and in some instances beneficial, as with certain molds that produce antibiotics. It was considered unlikely that fungi could be responsible for toxicoses in animals, and for the production of lethal or carcinogenic toxins (Forgacs, 1962). Soviet scientists, however, had much earlier grasped the significance of the mycotoxins and their role in animal and human pathology. Following the discovery in 1938 of a new disease in horses, stachybotryotoxicosis, caused by *Stachybotrys atra,* a saprophytic fungus, the Soviets, according to Sarkisov as cited by Forgacs (1962), established an institute at Moscow for detailed research in mycotoxins.

Since 1952, several toxicoses have been determined to be of mycotoxin origin (Forgacs, 1962). The discovery of the aflatoxins, in 1960, created an impetus to detailed study of the mycotoxins and their implications in animal and, mainly, in human health. The first review of mycotoxicoses published in English language apeared in 1962 (Forgacs, 1962). The first international symposium on mycotoxicoses was held at the Massachusetts Institute of Technology in March, 1964 (Wogan, 1964).

MATERIALS AND METHODS

Sampling. The crops investigated in this study were sampled during June of 1968. Most samples were obtained from public markets, but some were collected from storage facilities.

Samples of white corn, and "pinto" [3] corn (*Zea mays* L.), were obtained from lots stored by the Almacenes Nacionales de Depósito, S. A.; and samples of wheat *(Triticum aestivum* L.*),* and oats *(Avena sativa* L.*)* from storage facilities of the Nacional Harinera, S. A. of Toluca, Mex. Samples of "bayo" beans (*Phaseolus vulgaris* L.), and of four kinds of dry chili pods (*Capsicum annum* L.) with common names of "cascabel", "guajillo", "mulato", and "pasilla", were bought at the public market of Toluca.

From the public market of Veracruz, Ver., the following crops were obtained: cacao beans (*Theobroma cacao* L.); white corn (*Z. mays* L.); black beans (*P. vulgaris* L.); and four varieties of dry chili pods (*C. annum* L.) "pasilla", "mulato", "ancho", and "chipotle".

Samples of rice (*Oryza sativa* L.), yellow corn, and of 'terciopelo' (seeds of the legume *Canavalia* sp.), were obtained from storage through the courtesy of the Experiment Station of Cotaxtla, Ver., of the Secretaría de Agricultura y Ganadería, located in the vicinity of Veracruz.

Samples were divided into three parts: one part was used for determining moisture content; the second part for making the mycoflora isolations; and the third part as substrates for testing aflatoxins production. All subsamples were sealed in polyethylene bags to maintain their original moisture content.

Percent moisture content determination. Within a few days after the samples were obtained, ten 10 g (wet basis) replicates

[3] Spanish common names are given between quotation marks in those cases where direct translation to English is difficult.

of each different crop were dried in an oven at 70 C to constant weight. The percent moisture content was determined as follows:

$$\% \text{ moisture} = \frac{\textit{weight of moisture lost in drying x 100}}{\text{weight to oven dried sample.}}$$

Isolation and identification of the mycoflora. Depending upon size of kernels, 100 and 150 in case of the bigger kernels (cacao, broad beans, etc.), or 300 in case of the smaller kernels (rice, oats, and wheat), were randomly drawn from each sample. These were surface disinfected with a 1.0% solution of sodium hypochlorite for one minue, then rinsed twice in a sterile 7.5% solution of sodium chloride, and finally plated on petri dishes containing malt-salt (2% malt extract, 2% agar, and 7.5% NaCl) according to the technique described by Christensen (1957). This culture medium favors the isolation of a large number of fungi significant in stored grains deterioration and inhibits the growth of most of the bacteria present. Depending upon size of chili pods, 10 in the case of the bigger pods ("pasilla", "mulato", etc.) or 30 in the case of the smaller pods ("cascabel", and "chipotle"), were selected at random from each sample and plated according to the technique described above. The approximate natural size of the mentioned crops is shown in figure 3.

In all cases, 10 plates for each different crop were prepared, each plate containing, 10, 15, or 30 kernels or 1 to 3 pods. A temperature of 30 C is most favorable for the rapid apperance of the common species *A. flavus, A. glaucus,* and *A. candidus* (Christensen, 1957), therefore the plates were incubated at 30 C for 8 days and examined daily for isolation and identification of the growing fungi. The number of kernels developing colonies of *A. flavus* and/or other fungi was determined. Species of the *A. flavus* group were selected and maintained on Czapek's agar slants at 25 C for further study.

Production of aflatoxins in vitro. Production of aflatoxins by representative strains of *A. flavus* from Toluca, Mex. (strain I isolated from oats) and a strain from Veracruz, Ver. (strain VI isolated from cacao beans) was compared to that of a known toxicogenic strain (strain XVI-I from white corn in Texas).

Production of aflatoxins was compared by growing each strain on 30 g of each substrate (kernels or dry chili pods). Ten ml distilled water were added to each substrate in a 250 ml Erlenmeyer flask before sterilization in the autoclave (20 min at 120 C and 18 psi) in order to supply enough moisture for the growth of the fungus. The flasks were inoculated with 1 ml of a spore suspension obtained from the slants of *A. flavus.* The inoculated substrates were incubated for 10 days at 25 C, since this temperature was reported to favor aflatoxin production (Schroeder and Hein, 1967). After completion of the incubation period the inoculated substrates were stored at 0 C until assayed. In the cases of the chili pods, 10 ml of distilled water seemed not enough moisture for fungal growth, since up to 5 days after inoculation, no growth was apparent. Therefore, 15 ml of distilled water were added before sterilization when the experiment was repeated.

Extraction and quantification of aflatoxins. Aflatoxins were extracted by using a modified thin-layer chromatography method originally designed by Pons and Goldblatt (1965) for assaying aflatoxins in cottonseed and cottonseed products. This analytical procedure has been modified for application to many agricultural

materials and it is capable of detecting as little as 0.3 ppb of aflatoxin B.

The extraction procedure was as follows: each 30 g substrate portion was ground with 250 ml of 70% aqueous acetone (700 ml of acetone, analytical reagent grade and 300 ml of distilled water) in an electric blender for one minute in the case of the softer substrates (corn, cacao, chili pods, etc.), or two minutes for harder substrates (broad beans and coffee). Since the fungus grows into the seed, grinding is necessary for extracting the aflatoxins. Each comminuted sample was transferred to a 1000 ml Erlenmeyer flask fitted with a leakproof glass stopper and shaken on a mechanical reciprocal shaker for 30 min at such a rate that the sample material collecting in the neck of the flask was constantly washed back into the solvent (70% aqueous acetone). Each sample was then filtered through Whatman num. 4 filter paper and the clear filtrate collected in a 1000 ml Erlenmeyer flask. Furthermore, 70 ml of distilled water and 20 ml of a 10% lead acetate solution (100 g neutral lead acetate, analytical reagent grade, in one liter of distilled water and 3 ml of glacial acetic acid, analytical reagent grade) were added to each filtrate, and most of the acetone boiled off by heating each filtrate on a steam bath, evaporating it down to 175 ml. Most of the interfering pigments are removed from the crude extract by precipitation as insoluble lead derivatives. These were further eliminated by centrifugation (10 min at 2000 rpm). The supernatant was decanted into a 500 ml separatory funnel and extracted with 50 ml of chloroform (analytical reagent grade) by shaking the funnel vigorously for one min. The inorganic (water) and organic (chloroform) layers were allowed to separate and the chloroform (lower) phase was filtered through Whatman num. 4 filter paper fitted in a long stem funel and containing anhydrous sodium sulfate (analytical reagent grade) into a 250 ml beaker. Anhydrous sodium sulfate was used to remove water in the chloroform. The extraction was repeated with a second 50 ml portion of chloroform, filtering the choloroform (lower) phase through the same sodium sulfate drying funnel. The sodium sulfate was then rinsed with 20 ml of chloroform.

The chloroform extract from each sample was evaporated until the volume was reduced to almost dryness and then transferred with chloroform to small vials (7 ml capacity). The extracts in the vials were evaporated to dryness, dissolved in 200 μl chloroform, and examined immediately by thin-layer chromatography.

For the thin-layer chromatography analysis, 3 μl of each sample were spotted on preliminary thin-layer plates (20 x 20 cm Silica Gel G-HR, 0.25 mm thick) along with 5 μl of an aflatoxins standard solution, placing the spots on a line about 3 cm from the bottom of the plate. The aflatoxin standard solution used for the analysis had the following concentration in $\mu g/\mu l$: B_1-0.0119, B_2-0.0021, G_1-0.0066, and G_2-0.0004. After developing the preliminary plates in chloroform containing 15% acetone (v/v) in a chromatography tank, the chromatograms were allowed to dry for about 5 min and observed under long wave ultraviolet light.

When the thin-layer analysis indicated that the sample spots were too weakly or too intensely fluorescent for reliable measurement, adequate dilutions were made before the final analysis. Furthermore, final plates were spotted by placing 1, 2, 3, and 5 μl of each sample extract along with 1, 3, 5, and 10 μl of aflatoxins standard solution.

Concentration of aflatoxins was estimated by comparing the size and fluorescent intensity of the sample spots with those of the standard spots. The concentration of aflatoxins was calculated by following the formula used by Pons and Goldblatt for estimating aflatoxins concentration in cottonseed and cottonseed products:

Parts per billion (μg/K).

$$\text{aflatoxin } B_1 = \frac{(Vs)\ (Cs)\ (S.D.)\ (1000)}{(W)\ (X)\ (0.7)}$$

Where,

(Vs) = μl of aflatoxin standard in which B_1 spot matches the sample spot B_1

(Cs) = Concentration of aflatoxin B_1 in the standard aflatoxin solution; μg/μl

(S.D.) = Dilution of sample extract in μl

(W) = Sample weight, g

(X) = Volume of sample extract spotted in μl

the same procedure was used to calculate aflatoxins B_2, G_1, and G_2. The necessary calculations were made to report aflatoxins concentration in μg/g of substrate.

RESULTS

Moisture content of crops. The moisture content of the crops obtained from Toluca ranged from 6.4% in wheat to 21.1% in 'mulato" chili pods (figure 4a). The crops collected from large lots in storage, such as wheat, white corn, oats, etc., contained less moisture than the crops obtained from the public market. The moisture content of the crops obtained from Veracruz ranged from 5.8% in cacao to 18.8% in "chipotle" chili pods (figure 4b). The low moisture content in cacao was probably due to the higher content of fat. In general, crops obtained from the public market had higher moisture content than crops from large storage structures. However, the former consisted mostly of "dry" chili pods which due to their fleshy mesoderm retain more water than kernels. In general, and as it was expected, crops from Veracruz contained more moisture than crops from Toluca.

Identification of the associated mycoflora. The species of *Aspergillus* isolated were identified according to the classification keys of Raper and Fennell (1965).

a) *Toluca crops.* Species of the *A. flavus* group[4] were not found on any of the crops except oats; 4% of the kernels were contaminated by these fungi (table 1). Other species of *Aspergillus* were present in higher percentages, i.e., 15.6% of oat kernels were contaminated by species of the *A. glaucus* group, and 8.3% by species of the *A. niger* group. Species of the *A. candidus* group and of *Penicillium* were very rare. On oat kernels, and particularly on wheat kernels, large numbers of colonies of *Alternaria* sp., *Curvularia* sp., and *Helminthosporium* sp. were found. In the cases of white corn and "pinto" corn, neither species of *Aspergillus* nor of *Penicillium* were found, probably due to their low moisture content, though *Fusarium* sp. was abundantly isolated from the kernels. These molds are cataloged in the table 1 as "other fungi", and no attempt was made to identify them to species. "Bayo" beans had the highest percentage of sound kernels (90%). No species of the *A. flavus* group were found on any of the four kinds of chili pods, and only a few colonies of *A. glaucus* and *A. niger* groups were isolated from "cascabel" and "pasilla" chili pods (table 3a). Species of *Rhizopus* and *Alternaria* were found among the "other fungi" on "cascabel" and "guajillo" chili pods.

b) *Veracruz crops.* In contrast with the isolates obtained from Toluca crops, those obtained from Veracruz showed a much higher incidence of species of the

[4] Raper and Fennell, in their classification system of the genus *Aspergillus*, use groups to include related species.

TABLE 1
FUNGI ISOLATED FROM TOLUCA CROPS

Crops	*A. flavus* %	*A. glacus* %	*A. niger* %	*A. candidus* %	*Penicillium spp.* %	*Other fungi* %	*Sound* %
Wheat[b]	0	0	0	1.0	0	83.0	16.0
Oats[b]	4.0	15.6	8.3	0.3	0.3	32.6	38.3
Pinto corn[a]	0	0	0	0	0	30.0	70.0
White corn[a]	0	0	0	0	0	30.0	70.0
Bayo beans[a]	0	1.3	0	0	0.6	8.0	90.0

[a]Results from 150 kernels, 15 k./plate.
[b]Results from 300 kernels, 30 k./plate.

TABLE 2
FUNGI ISOLATED FROM VERACRUZ CROPS

Crops	*A. flavus* %	*A. glaucus* %	*A. niger* %	*A. candidus* %	*A. wentii* %	*A. oriolus* %	*Penicillium spp.* %	Other *fungi* %	*Sound* %
Cacao[a]	7.0	49.0	8.0	3.0	11.0	0	3.0	19.0	0
Bread beans[a]	14.0	27.0	3.0	0	2.0	5.0	1.0	12.0	36.0
Rice[c]	14.3	19.3	0.6	0.1	4.3	0.3	0.3	13.0	47.6
Terciopelo[a] *Canavalia* sp.	1.0	22.0	2.0	0	2.0	5.0	2.0	5.0	61.0
White corn[b]	0	12.0	0	0	0	0.6	0	1.3	86.0
Black beans[b]	0.6	3.3	0	0	0	0	0	6.0	90.0
Yellow corn[b]	0	1.3	0	0	0	0	0.6	7.3	90.6
Coffee[b]	0	2.6	3.3	0	0	0	0	2.0	92.0

[a]Results from 100 kernels, 10 k./plate; [b]150 k., 15 k./plate; [c]300 k., 30 k./plate.

A. flavus group. These molds were isolated from five different substrates (table 2). The highest percentages of *A. flavus* contamination were found on rough rice kernels (14.3%) and broad beans (14.0%); even 7.0% of the cacao kernels contained these fungi although their moisture content was the lowest determined in crops from Veracruz. White corn, yellow corn, and coffee were not infected by *A. flavus,* but species of the *A. glaucus* group were found. Species of *Fusarium* (cataloged as 'other fungi") were abundant on white and yellow corn. However, these crops along with coffee had the highest percentages of sound kernels (table 2). As with Toluca crops, the ones obtained from Veracruz were more commonly infected by species of *A. glaucus* and *A. niger,* but mainly by the former. In cacao, up to 49% of the kernels were infected by *A. glaucus* spp., and 11% by species of the *A. wentii* group. Species of the *A. candidus* and *A. oriolus* groups as well as of *Penicillium* were also found, but with less frequency, on cacao, broad beans, and "terciopelo" (table 2).

In the cases of the chili pods, neither species of the *A. flavus* group nor of other aspergilli were found with the exception of *A. glaucus* which was found infecting "pasilla" and "ancho" pods. *Rhizopus* sp. was isolated from "pasilla" and "mulato" chili pods (table 3b).

As mentioned before, the culture medium used for making the mycoflora isolations favored storage fungi[5] and inhibited the growth of most field fungi[6] and bacteria present. In fact, no bacterial contamination was observed on any of the plates, and although some species of field fungi were isolated, such as *Alternaria, Rhizopus, Fusarium,* etc., the most preva-

[5] Fungi which develop on and within seeds at moisture contents often encountered in storage. They are species of *Aspergillus* and *Penicillium.*

[6] Fungi which invade the developing or mature seed while it is still on the plant, i.e., *Alternaria, Helminthosporium,* and *Fusarium.*

TABLE 3

FUNGI ISOLATED FROM DRY CHILI PODS

	A. flavus %	*A. glaucus* %	*A. niger* %	*A. candidus* %	*Penicillium* spp. %	Other fungi %
A. From Toluca						
Cascabel [a]	0	34.6	11.5	0	0	23.0
Guajillo [b]	0	0	0	0	0	11.5
Mulato [b]	0	0	0	0	0	0
Pasilla [b]	0	15.3	0	0	0	3.8
B. From Veracruz						
Pasilla [b]	0	41.1	0	0	0	5.8
Mulato [b]	0	0	0	0	0	5.8
Ancho [b]	0	47.1	0	0	0	0
Chipotle [a]	0	0	0	0	0	0

[a] Results from 30 chili pods, 3 pods/plate.
[b] Results from 10 chili pods, 1 pods/plate.

lent molds were species of *Aspergillus*. These grew without indication of antagonism to each other. However, the colonies of *A. glaucus* (the most abundant mold) always appeared before the other species of *Aspergillus*. On the third day after plating, a vigorous growth of these species was observed, whereas the *A. flavus* and *A. niger* colonies took from 5 to 8 days to appear. This was true for both Toluca and Veracruz crops.

Aflatoxin production in vitro. The production of aflatoxins by the three different strains of *A. flavus* varied greatly according to the crop used as substrate and the strain itself used for inoculation. The strain XVI-I (isolated from Texas corn) was known to be highly toxicogenic and therefore was used as a control in order to estimate the toxicogenicity of the other two strains isolated from Mexican crops (strains I and VI isolated from Toluca oats and Veracruz cacao kernels respectively).

The quantity of aflatoxins produced by the three strains of *A. flavus* while growing on the various substrates is reported in μg/g of substrate (figures 5-11). The figures at the top of each column represent the average of the production of toxins on three replicates of each kind of substrate. The results showed in figures 5-11, indicate that strain XVI-I (A) produced the most aflatoxins. Strains I (B) and VI (C) were less toxicogenic, but strain I in particular did not produce either aflatoxins B or G on white corn, wheat, rice, black beans, broad beans, coffee, and all six kinds of chili pods.

In general, wheat and oat kernels were suitable substrates for aflatoxin production, mainly by the strain XVI-I (A), although white corn, 'pinto" corn, and "bayo" beans were also suitable substrates for the production of high levels of aflatoxins (figures 5 and 6). The highest average production of aflatoxin B_1 (131.59 μg/g and B_2 (3.66 μg/g) was detected in wheat inoculated with the strains XVI-I (A) and VI (C) respectively (figure 5). The highest average production of aflatoxin G_1 (336.50μg/g) and G_2 (8.24 μg/g) was found in wheat kernels inoculated with the strain XVI-I (A) and in "bayo" beans inoculated with the same strain XVI-I (figures 9 and 10).

Cacao and coffee kernels as well as the six kinds of dry chili pods proved to be relatively poor substrates for aflatoxin production as determined in this study. Small amounts and trace amounts were detected on cacao and on some chili pods. No aflatoxins were produced by any of the isolates on coffee kernels and "ancho" chili pods (figures 7, 10, and 11).

In general, aflatoxins G were produced in larger amounts than aflatoxins B. The ratio, however, varied with the substrate and fungus strain (table 4). In all cases, the total production of aflatoxins G by the strain XVI-I (A) was several times as much as the total production of aflatoxins B. Strain I (B), as mentioned before, was the lowest producer of toxins, and even in many cases of none at all. It is important to point out that strain VI (C) did not produce aflatoxins G on most of the substrates with the exceptions of cacao kernels, "cascabel" chili pods, and "chipotle" chili pods where very small quantities of toxins (0.05 μg/g) were detected (figures 10 and 11).

No correlation between fungus growth and aflatoxin production was found. Vigorous growth was observed on coffee, where no aflatoxins were produced or detected, as well as on wheat and oat kernels, here high amounts of toxins were found. In fact, all the 18 different crops inoculated with the *A. flavus* strains supported a good growth of the fungus mycelium. A less heavier growth of the mycelium was, however, observed on "chipotle" and "mulato" chili pods and small amounts, or none, of aflatoxins were produced.

Although the aflatoxin-producing po-

TABLE 4

RATIO OF THE TOTAL AFLATOXINS B AND G PRODUCED ON 18 DIFFERENT SUBSTRATES (CROPS) BY THREE STRAINS OF *A. FLAVUS*, A (STRAIN XVI-I), B (STRAIN I), AND C (STRAIN VI) AFTER 10 DAYS OF INCUBATION AT 25°C.

Substrate	*A. flavus* strains	*B* : *G ratio*
1. White	A	1 : 3.35
	B	0 : 0
	C	1 : 0
2. Yellow corn	A	1 : 2.19
	B	1 : 1.90
	C	1 : 0
3. "Pinto" corn	A	1 : 1.41
	B	1 : 0.84
	C	1 : 0
4. Wheat	A	1 : 2.52
	B	0 : 0
	C	1 : 0
5. Oats	A	1 : 1.75
	B	1 : 1
	C	1 : 0
6. Rice	A	1 : 1.37
	B	0 : 0
	C	1 : 1
7. "Bayo" beans	A	1 : 2.27
	B	1 : 2
	C	1 : 0
8. Black beans	A	1 : 6.05
	B	0 : 0
	C	1 : 0
9. Broad beans	A	1 : 3.03
	B	0 : 0
	C	1 : 0
10. "Terciopelo"	A	1 : 2.70
	B	1 : 2
	C	1 : 0
11. Cacao	A	1 : 8.14
	B	0 : 0
	C	1 : 2.5
12. Coffee	A	0 : 0
	B	0 : 0
	C	0 : 0
13. "Cascabel" chili pods	A	1 : 5.36
	B	0 : 0
	C	1 : 0.55
14. "Guajillo" chili pods	A	1 : 2.35
	B	0 : 0
	C	0 : 0
15. "Mulato" chili pods	A	1 : 2.28
	B	0 : 0
	C	0 : 0
16. "Pasilla" chili pods	A	1 : 2.05
	B	0 : 0
	C	1 : 0
17. "Chipotle" chili pods	A	0 : 0
	B	0 : 0
	C	1 : 0.55
18. "Ancho" chili pods	A	0 : 0
	B	0 : 0
	C	0 : 0

tential of the *A. flavus* strains varied greatly, macroscopic and microscopic examination of their reproductive structures did not show differences in morphology or taxonomy, and all of them were identified as *Aspergillus flavus* Link ex Fries (Raper and Fennell, 1965). The criteria followed in this determination were the following: a) *colonies* in yellow-green shades when young and not shifting to brown on Czapek's agar in aging; b) *conidial heads* typically radiate, splitting into several poorly defined columns; c) *conidiophores* heavy walled, uncolored, coarsely roughened and of about one mm in length; d) *vesicles* subglobose to globose; and e) *conidia* typically globose and conspicously equinulate (figures 12a,b).

DISCUSSION

Environmental factors, such as temperature and relative humidity, as well as moisture content of the substrate influenced the incidence of the *Aspergillus* species

on the various crops. Since these environmental factors were affected by the climate of the localities where collections were made, it will be discussed first.

The climate of these localities was classified according to the Climatic Classification System of García (García, 1964), which is a modification to the Köppen System adapted to the conditions of Mexico.

The climate of Toluca was classified as a $Cw_2(w)$ big type, characterized as a temperate climate. The temperature of the coldest month is below 18 C, and the temperature of the hotest month is above 18 but below 22 C. As the difference between the lowest and the highest temperatures is less than 5 C, it is classified as isothermal. The highest temperature is registered before the summer solstice (June, 23). This locality has a cool and long summer, which includes most of the rainy period (May-October). This type is considered to be less than humid because the total anual precipitation does not exceed 1000 mm. Some meteorological data of Toluca are shown in table 5.

The climate of Veracruz was classified as a $A(W_2")(w)(i')$ type, characterized as a hot climate. The temperature of the hotest month is over 22 C, with the highest temperature registered after the summer solstice. It has slight oscillation of temperatures during the year (the difference is from 5-7 C). "Canicula" (a dry period between the rainy period) is present. The total annual precipitation is over 1000 mm, therefore it is considered a humid climate. There are few rains during the winter (less than 5% of the total annual precipitation).

The exact climatic characterization for Cotaxtla, Ver., is not given because of the lack of a meteorological station at that locality. However, the existence of two meteorological stations near Cotaxtla, namely "Los Capulines" and "Tinajas", provides an estimate of the type of climate in this locality. Thus Cotaxtla also has a hot climate, but with slightly greater oscillation of the temperature than that of the climate of Veracruz, Ver. It is consired humid and with few rains during the winter. "Canicula" is also present. Therefore, it is very similar to the climate of Veracruz. Some meteorological data of Veracruz are shown in the table 6.

The influence of the climate on the incidence of *A. flavus* and other species of *Aspergillus* in the various crops was clearly seen. It seems that a complex of conditions influences the invasion of stored crops by fungi. Physical factors of the environment, such as temperature and relative humidity, prevalent in the areas where collections were made, seem to determine fungal development in stored agricultural products.

Aspergilli have been reported to develop under limited conditions of atmospheric moisture. According to Austwick and Ayerst (1963), *A. niger, A. flavus,* and other species such as *A. tamarii,* will not grow at below 80-85% relative humidity.

The relative humidity prevalent in Toluca during June, when collections were made, is about 72%, even though the rainy period is present (table 5). The lower humidity and temperature found in this area might account for the low incidence of *A. flavus* (4.0% on oat kernels) and other aspergilli. Species of the *A. glaucus* group, which seem to require lower humidity, appeared in larger numbers (table 1). On the other hand, the higher humidity and temperature found in Veracruz (about 80% and 28 C) during June seemed to favor fungal development. Higher incidence of species of the *A. flavus* group was found in several crops from Veracruz (table 2).

The fact that samples were collected during June, when warmer temperatures and higher humidities are registered, could have influenced the mycoflora. Perhaps the incidence of mycoflora species would be lower if collection of the crop samples

TABLE 5

CLIMATIC CHARACTERISTICS OF TOLUCA, MEX.[a]

	Mean temperature [b]	*Maximum extreme temperature* [b]	*Minimum extreme temperature* [b]	*Relative humidity* [c]	*Total precipitation* [c]
	C	C	C	%	mm
June [d]	14.8	22.2	7.4	72.0	137.0
Annual	13.0	25.3	—3.4	63.8	716.0

[a] Data obtained from the S.M.M. (Mexican Meteorogical Service).
[b] The figures for the mean, maximum extreme, and minimum extreme temperatures represent the average of the readings obtained in 27 years (1941-1967).
[c] The figures for relative humidity and precipitation represent the average of the readings taken in 25 years (1941-1965).
[d] Month during which the sampling was done.

TABLE 6

CLIMATIC CHARACTERISTICS OF VERACRUZ, VER.[a]

	Mean temperature [b]	*Maximum extreme temperature* [b]	*Minimum extreme temperature* [b]	*Relative humidity* [b]	*Total precipitation* [b]
	C	C	C	%	mm
June [c]	28.0	32.9	21.9	80.2	284.2
Annual	25.9	34.7	12.5	79.6	1558.8

[a] Data obtained from the S.M.M. (Mexican Meteorological Service).
[b] The figures represent the average of the readings obtained in 27 years (1941-1967).
[c] Month during which the sampling was done.

was made during the winter months when lower temperatures are prevalent. In Toluca, during the winter, below zero C temperatures are registered, and also in Veracruz the temperatures drop below 10 C. Under these conditions even high humidities do not permit extensive growth of fungi (Dickens and Pattee, 1965).

A complex of conditions influences the invasion of stored crops by fungi. Although the most important ones are the moisture content of the crops in equilibrium with the relative humidity of the air, and the temperature, other factors may also affect invasion, i.e., amount of previous infection of the crops by the fungi concerned, storage time, and the activity of various grain inhabiting insects. The prevalence of different species of molds with the kind of crop and environmental conditions illustrates the complex ecologic relationships encountered where a variety of organisms are competing in a dynamic, fluctuating, biological environment.

It has been reported that seeds containing a moisture content of about 15-17% permit the heavy invasion of *A. candidus* and *A. flavus* (Christensen, 1957). The

precise safe upper limit of moisture to which kernels need be dried has to be determined, but evidence puts it somwhere within the range of 9.3% (Austwick and Ayerst, 1963) to 15% (Ashworth, Schroeder, and Langley, 1965). However in this study, crops with low moisture content were found to be contaminated by *A. flavus*. Some examples are cacao kernels (5.8%), oat kernels (8.6%), broad beans (10.1%), and rice kernels (11.0%). In the case of cacao kernels the low moisture content, as determined in this work, might be not so low if we had excluded the fat portion and determining the moisture content in relation to the starchy portion. In the other crops with lower moisture content and *A. flavus* contamination, the explanation could be that a higher moisture content could have been present in such crops before they were collected. On the other hand, crops with higher moisture content, i.e., "chipotle" chili pods .... (18.8%), "mulato" chili pods (21.1%), etc., were found to be free from *A. flavus* contamination. This indicates that the moisture content is a very important factor, but that its influence is limited by other components of the substrate.

The production of aflatoxins under controlled laboratory conditions varied according to the substrate and fungus strain used. Wheat and oat kernels were among the most suitable substrates for aflatoxins production (figure 5 and 6). Similar results were reported by Stubblefield *et al* (1967). These workers obtained optimal yields of 900 μg of aflatoxin B_1 and 900 μg of G_1/g of substrate (wheat and oat kernels) by using a highly toxicogenic strain of *A. flavus*.

The production of aflatoxins by the *A. flavus* strains was studied in still cultures, and only a daily shaking of the cultures was done during the incubation period. This might have affected yields of aflatoxins, since areation in shake cultures has been found to increase aflatoxin production (Schroeder, 1965; Hayes, Davis, and Diener, 1966). The daily manual shaking may have been insufficient to promote aeration. It is of importance to point out that in the cases of coffee and cacao, large quantities of unknown materials were extracted by the procedure utilized. These unknown materials (alkaloids?) might have masked any aflatoxin present. The same thing might have happened in the cases of some chili pods. Perhaps a different extraction procedure should be applied to those cases.

A different potential for aflatoxin production was found in the three *A. flavus* strains used for the inoculation of the various crops. This does not necessarily indicate, however, that these strains behave under natural conditions in the same manner. The nature of the substrate, fluctuating environmental conditions, and microbial competition may influence their physiology and hence their metabolic capabilities for aflatoxin production. The fact that the strain of *A. flavus* isolated from Veracruz proved to be more toxicogenic than the strain isolated from Toluca, suggests the possible influence of different environment upon toxicogenicity.

The production of aflatoxins by strain of *A. flavus* seems to be the result of the interaction of the genotype of the strain and its environment. Therefore, in order to evaluate the capacity of a strain to produce aflatoxins a broad range of environmental conditions must be studied. The ecological studies related to the contamination of stored crops by *A. flavus* in the two areas of Mexico studied, are by no means ended. Detailed studies on storage conditions, such as temperature, relative humidity, insect injuries of grains, etc., must be done in the future. Furthermore, this type of studies must be extended to other areas of Mexico which offer contrasting environmental conditions and storage problems.

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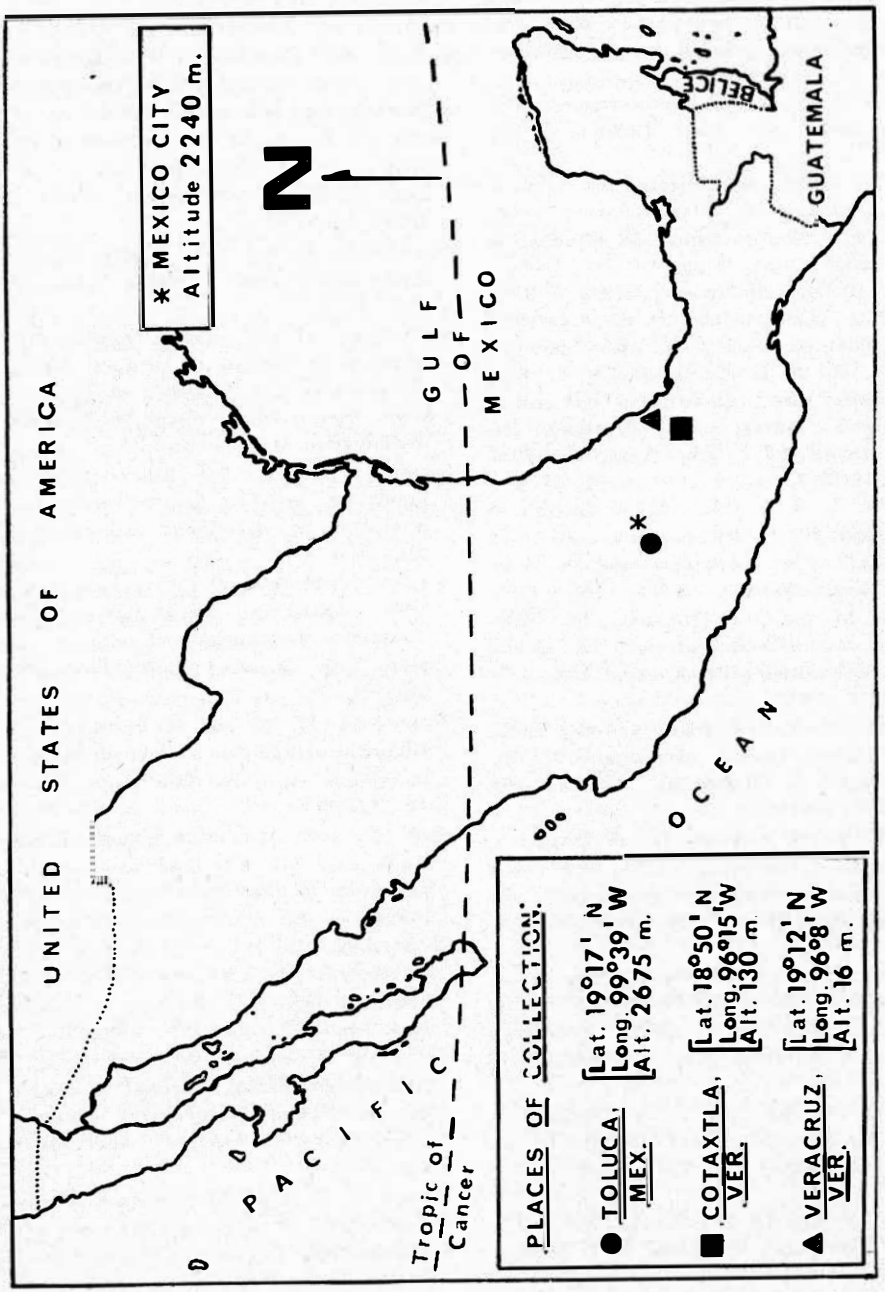
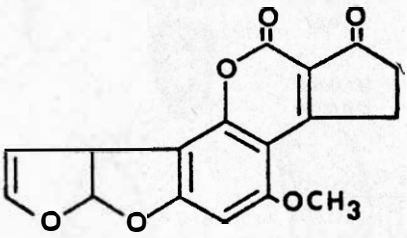
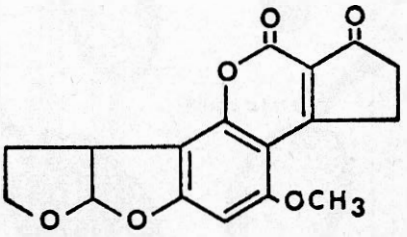


Figure 1. Map of Mexico showing locations where collections of food products were made.



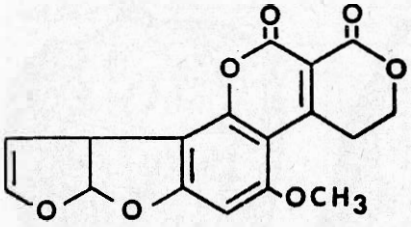
Aflatoxin B_1

$C_{17}H_{12}O_6$



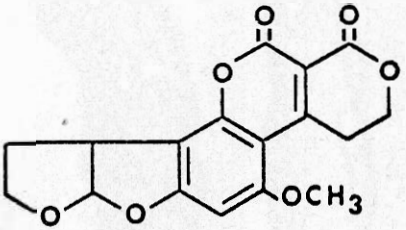
Aflatoxin B_2

$C_{17}H_{14}O_6$



Aflatoxin G_1

$C_{17}H_{12}O_7$



Aflatoxin G_2

$C_{17}H_{14}O_7$

Figure 2. Structures of the four primary aflatoxins.

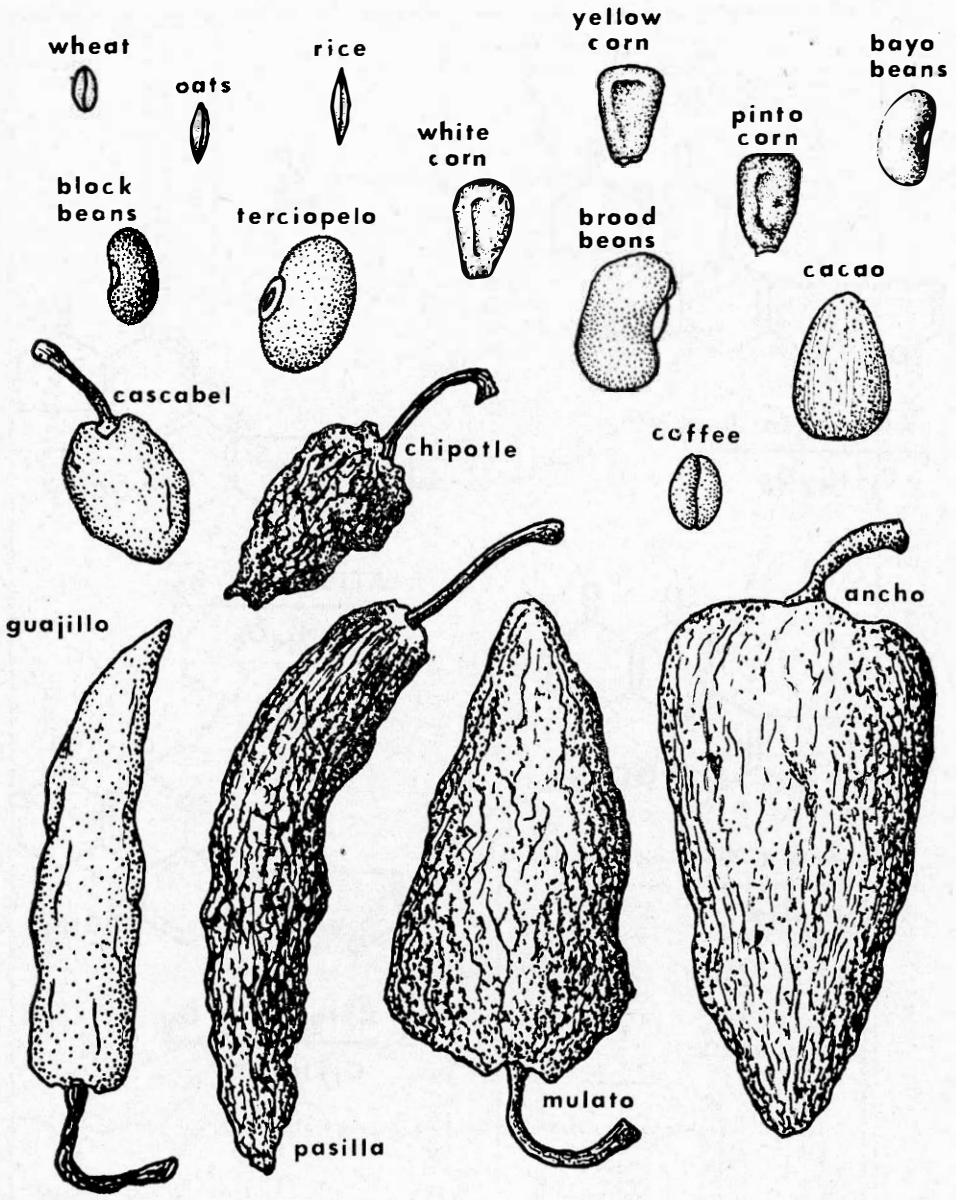


Figure 3. Approximate natural size of the stored crops used for studying their associated mycoflora and as substrates for aflatoxins production.

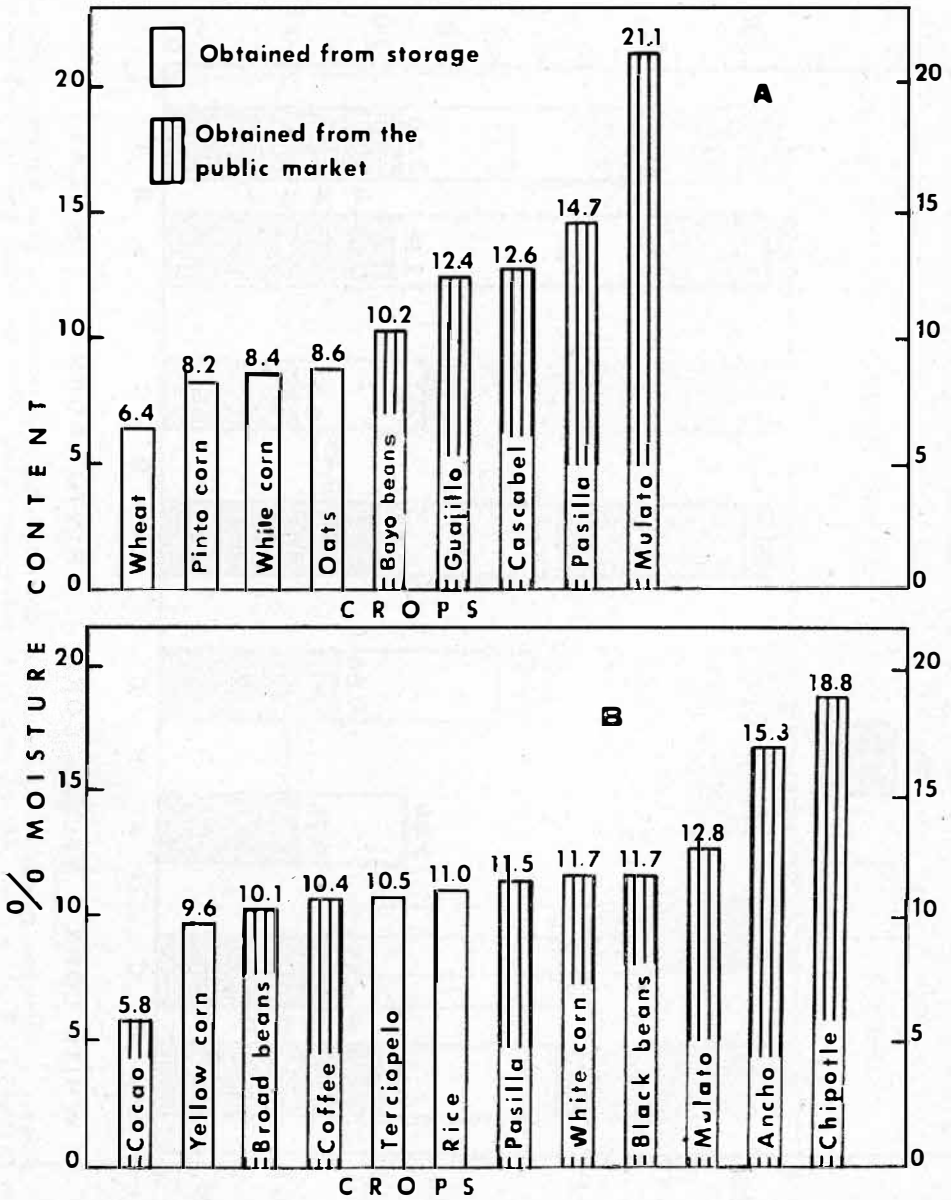


Figure 4. Percent moisture content in different crops from A) Toluca, Mex., and B) Veracruz, Ver., Mexico.

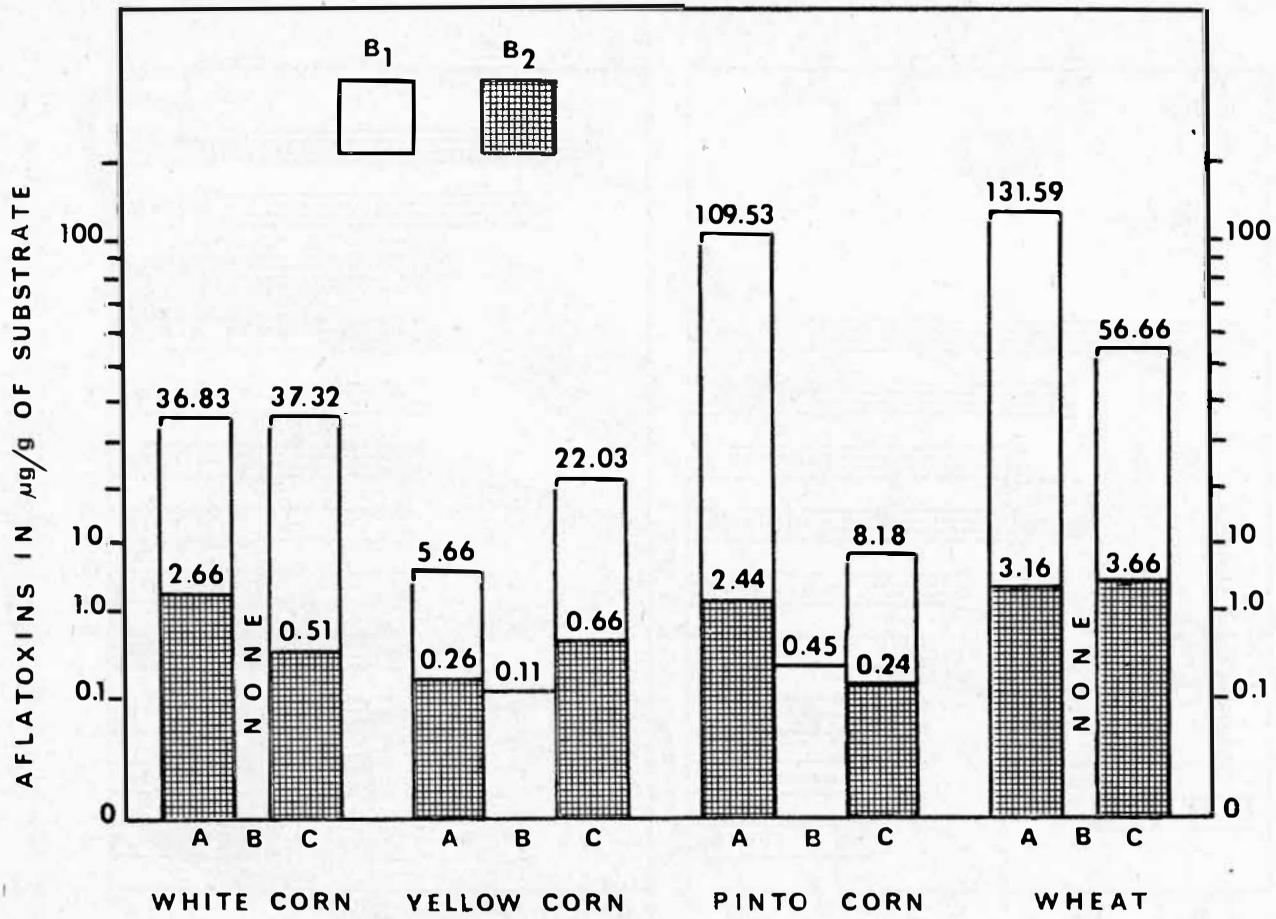


Figure 5. Average (3 replicates) production of aflatoxins B1 and B2, in/µg/g of substrate, by three different strains of *A. flavus*. A (XVI-I), B (I) and C (VI) on white corn, yellow corn, "pinto" corn, and wheat after 10 days of incubation at 25 C.

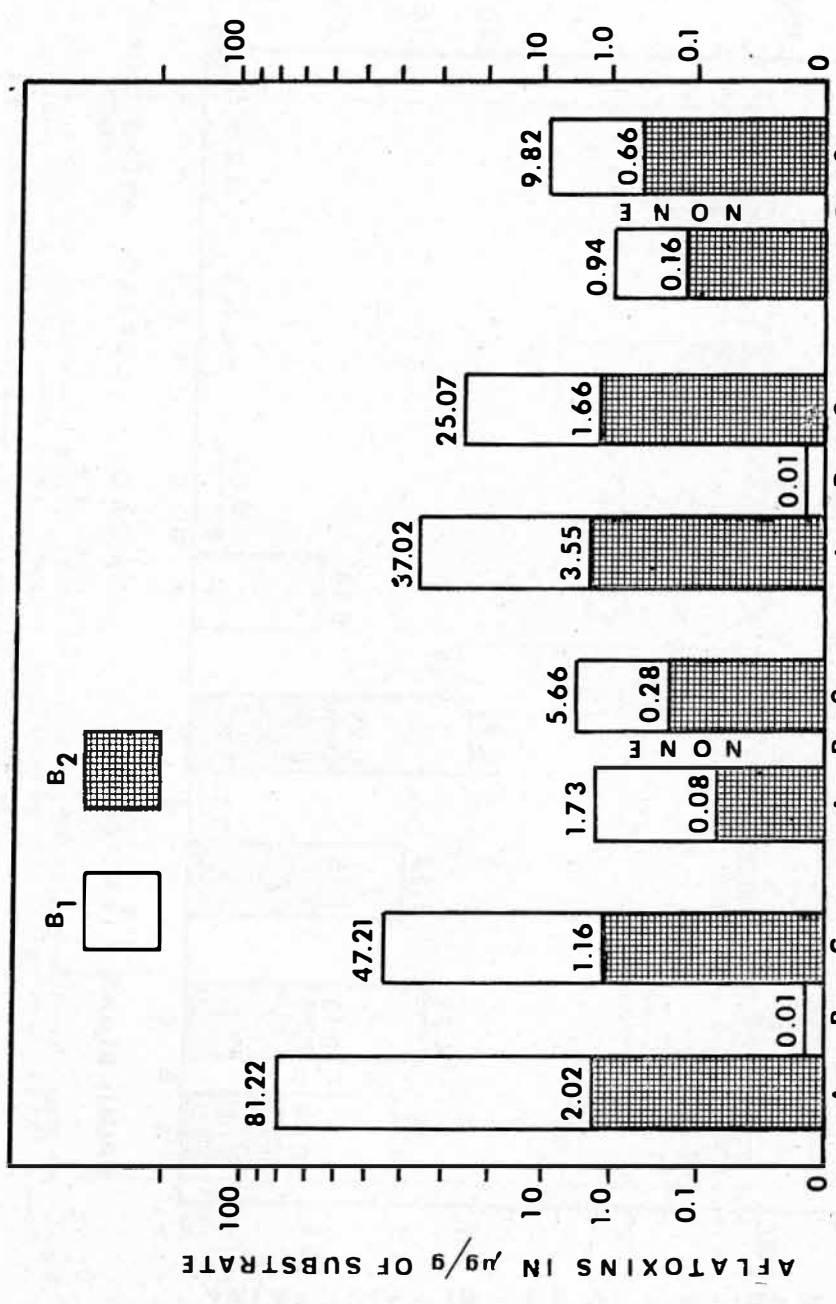


Figure 6. Average (3 replicates) production of aflatoxins B1 and B2, in μg/g of substrate, by three different strains of *A. flavus* (XVI-I), B (1), and C (VI) on oats, rice, "bayo" beans, and black beans after 10 days of incubation at 25 C.

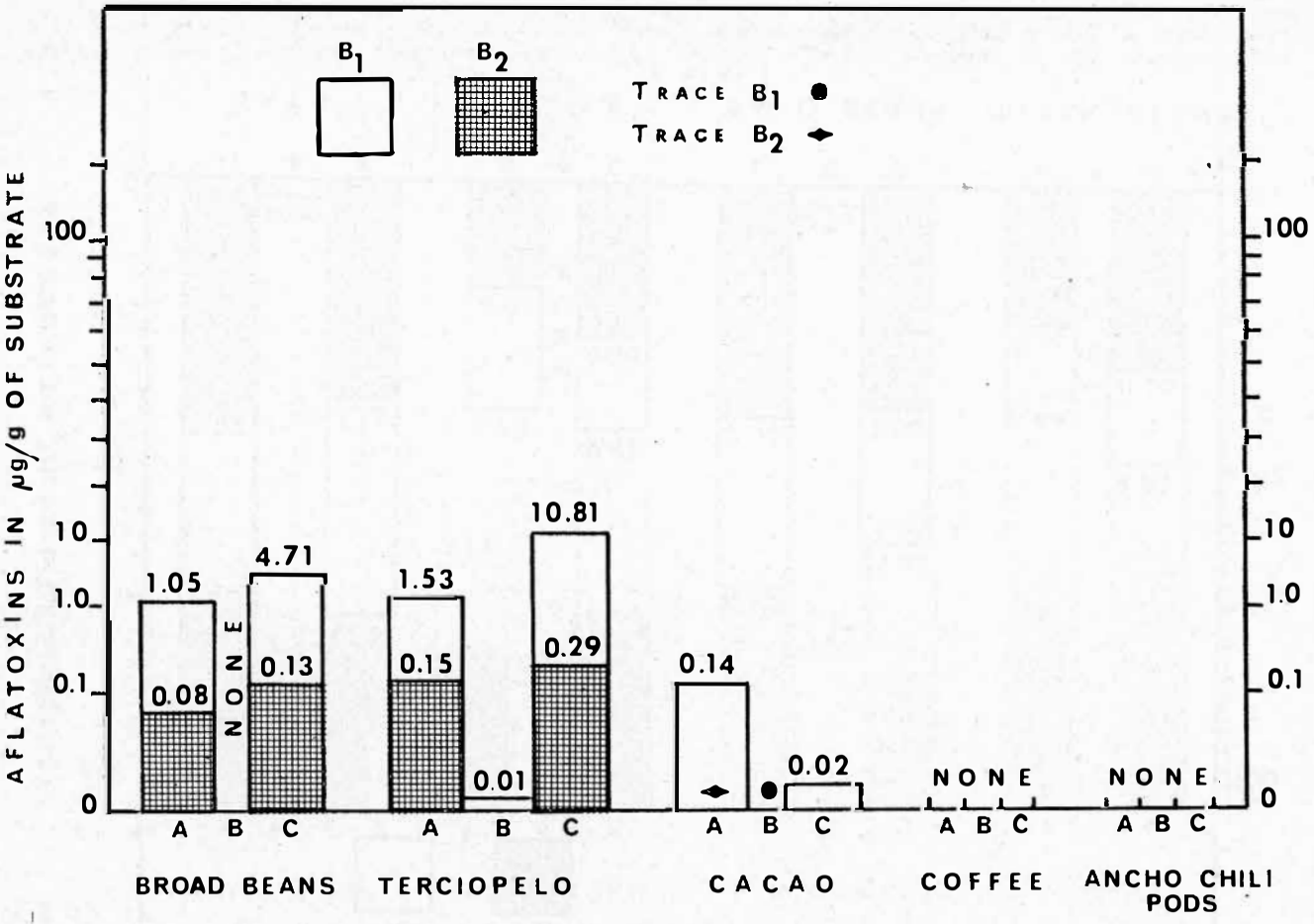


Figure 7. Average (3 replicates) production of aflatoxins B1 and B2, in µg/g of substrate, by three different strains of *A. flavus*, A (XVI-I), B (I), and C (VI) on broad beans, "terciopelo", cacao, coffee, and "ancho" chili pods after 10 days of incubation at 25 C.

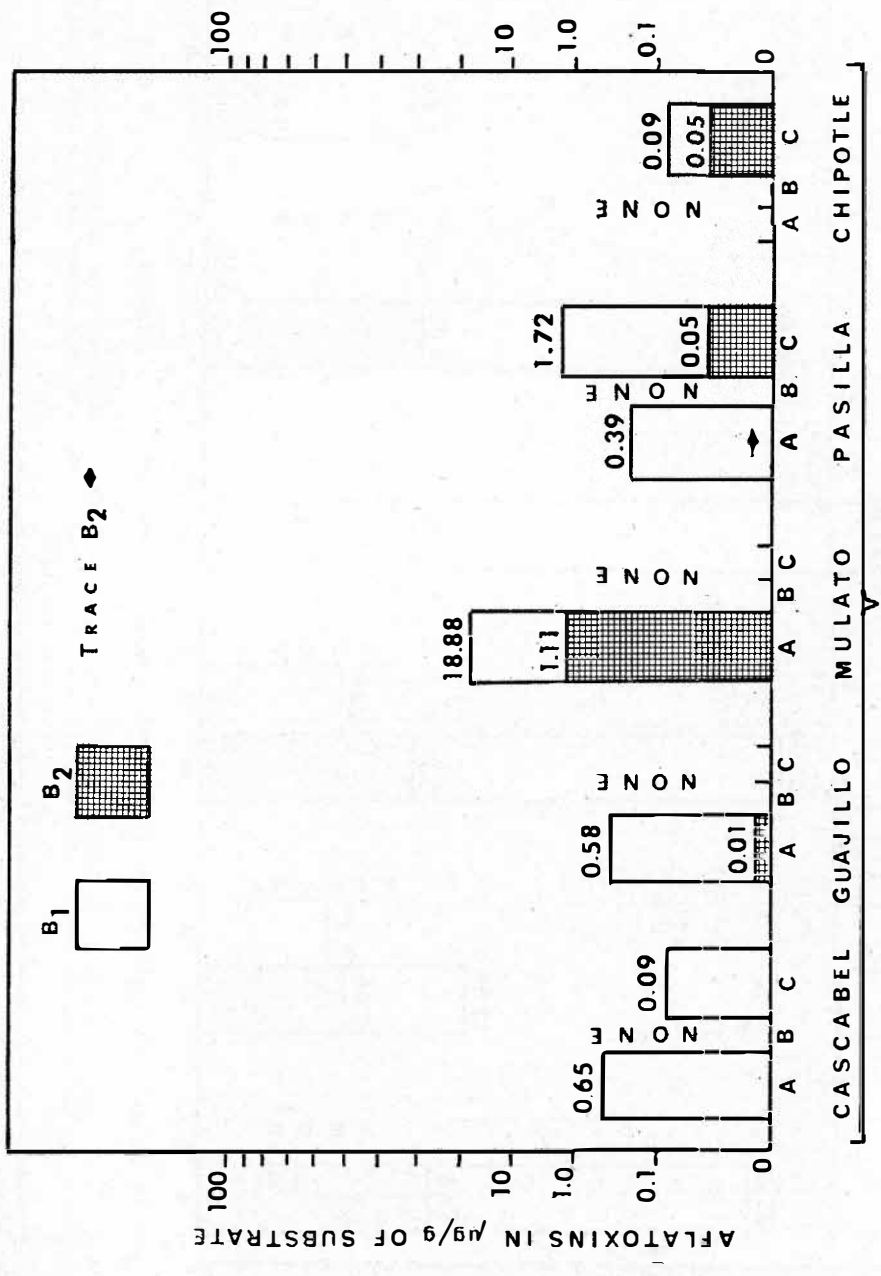


Figure 8. Average (3 replicates) production of aflatoxins B1 and B2, in $\mu g/g$ of substrate, by three different strains of *A. flavus*, A (XVI-I), B (I) and C (VI) on five kinds of chili pods: "cascabel", "guajillo", "mulato", "pasilla", and "chipotle" after 10 days of incubation at 25 C.

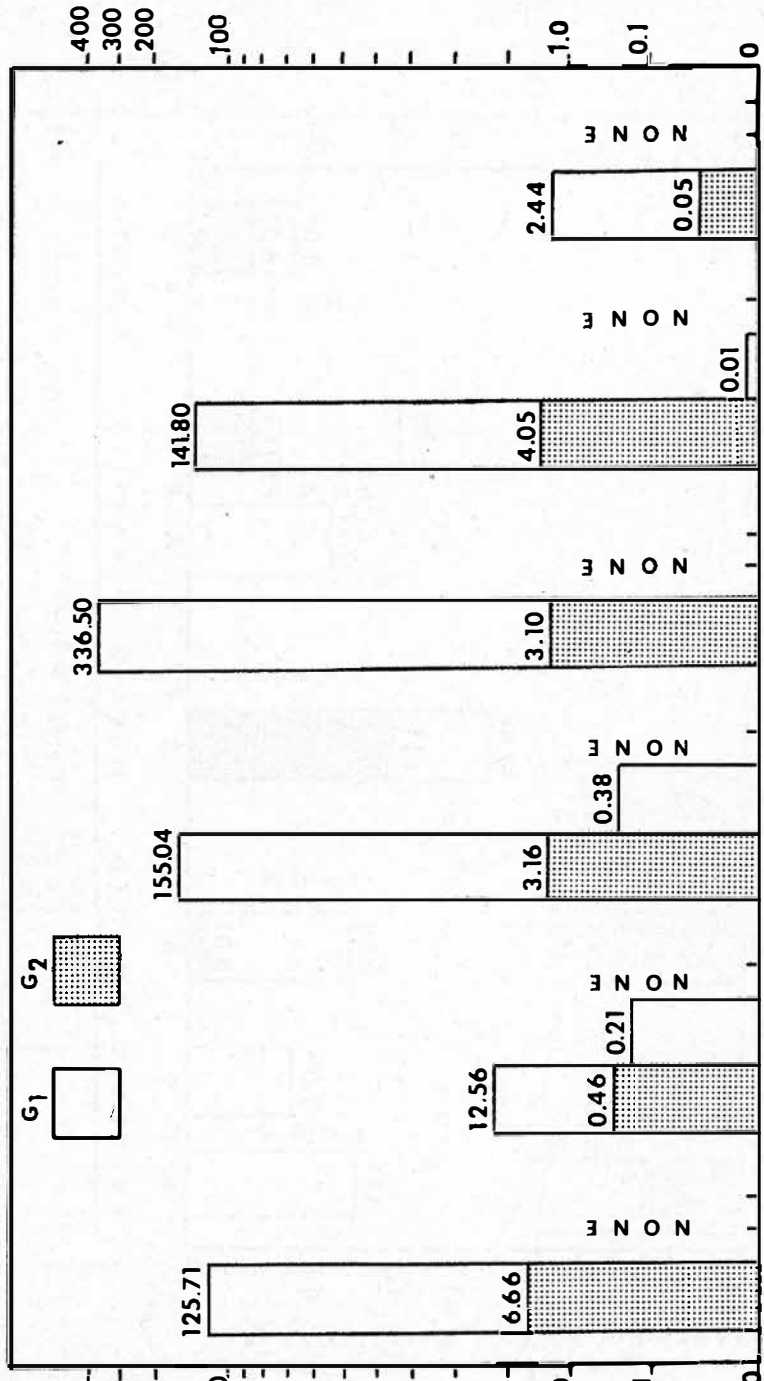


Figure 9. Average (3 replicates) production of aflatoxins G1 and G2, in µg/g of substrate, by three different strains of *A. flavus*, A (XVI-I), B (I), and C (VI) on white corn, yellow corn, "pinto" corn, wheat, oats, and rice after 10 days of incubation at 25 C.

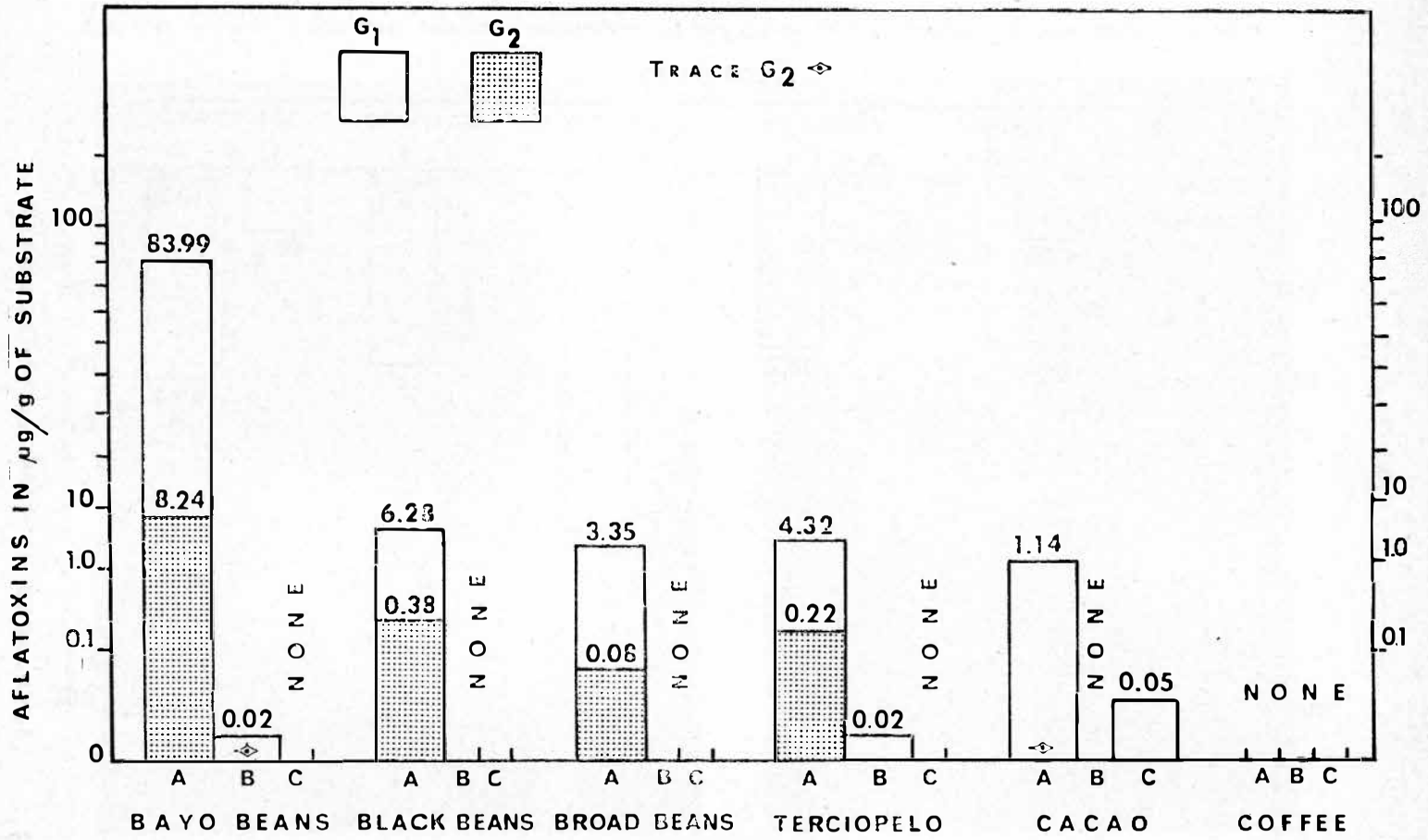


Figure 10. Average (3 replicates) production of aflatoxins G1 and G2, in µg/g of substrate, by three different strains of *A. flavus*, A (XVI-I), B (I), and C (VI) on "bayo" beans, broad beans, "terciopelo", cacao, and coffee after 10 days of incubation at 25 C.

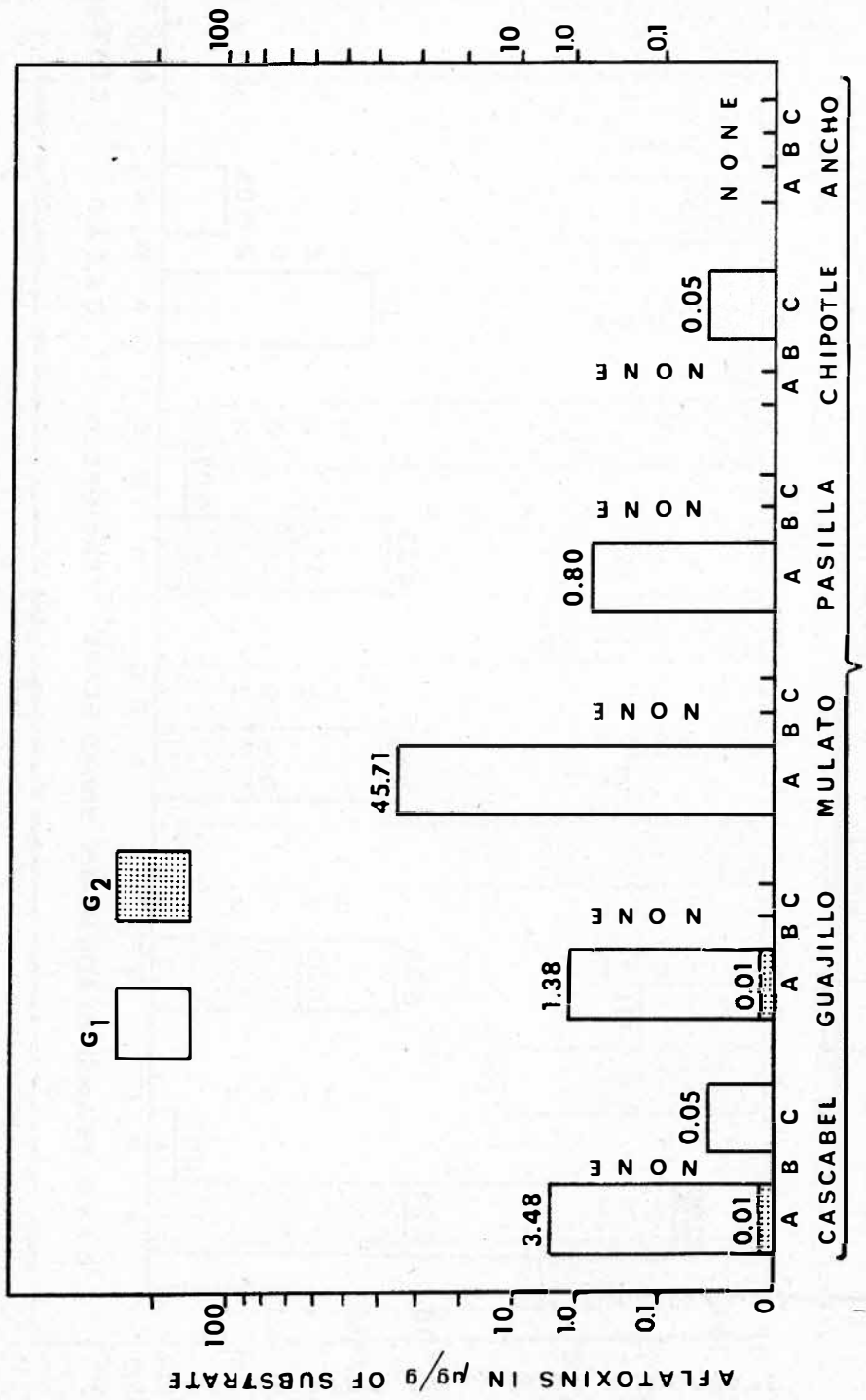


Figure 11. Average (3 replicates) production of aflatoxins G1 and G2, in $\mu g/g$ of substrate, by three different strains of *A. flavus*, A (XVI-I), B (I), and C (VI) on six kinds of chili pods: "cascabel", "guajillo", "mulato", "pasilla", "chipotle", and "ancho" after 10 days of incubation at 25 C.

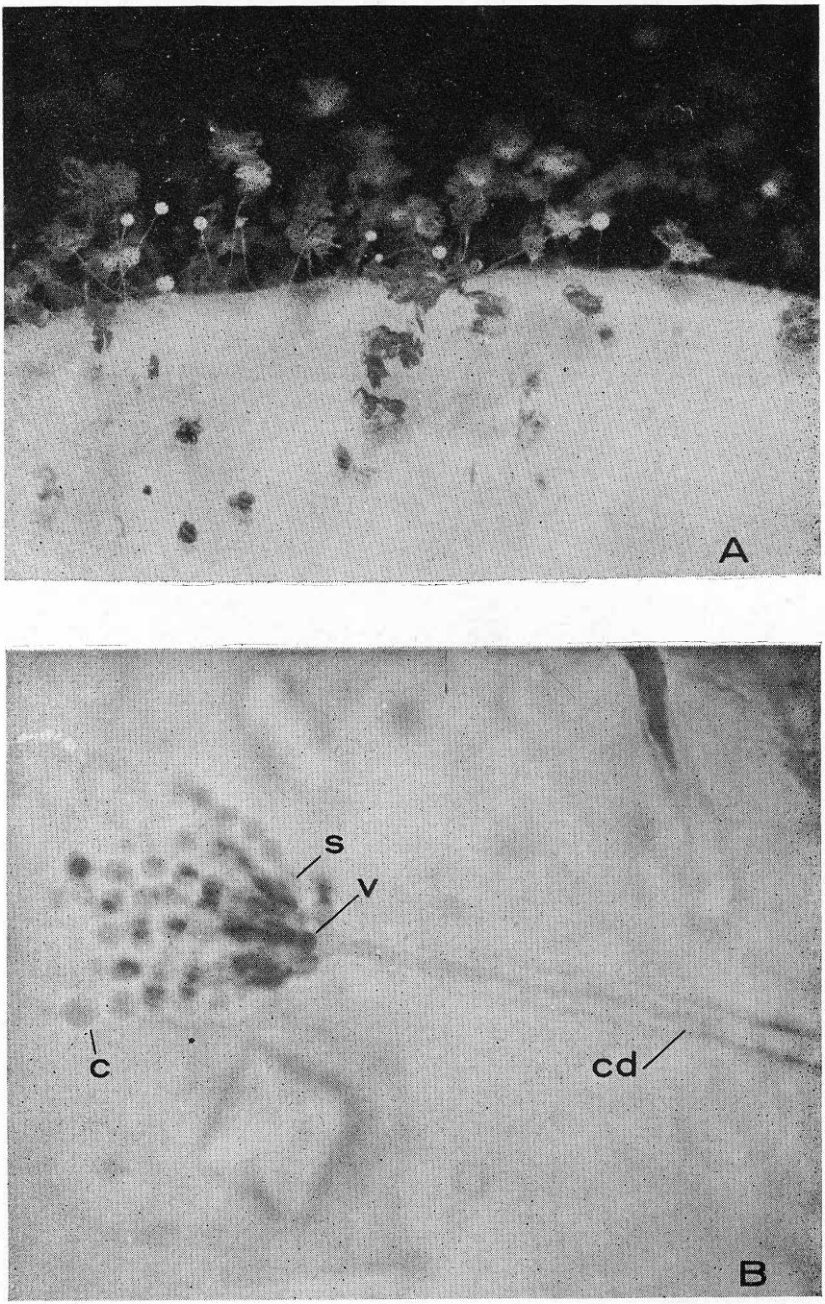


Figure 12. *A. flavus*. A: conidial heads growing on the surface of a corn kernel, 18 ×; B: young conidial head showing, *v*: vesicle, *s*: sterigmata, *c*: conidia, *cd*: conidiophore, 500 ×.