Table I. Percentage Recovery of Nitrosamines Added to a 250-g Sample of Luncheon Meat at the 10, 2, and 1 ppb Levels

Level, ppb, of nitrosamine	10 % Recovery			2 % Recovery			1 % Recovery		
	Max	Min	Mean[a]	Max	Min	Mean[a]	Max	Min	Mean[a]
DMN	94	55	75	88	35	56	Interference could not be measured		
DEN	93	65	82	132	61	102	125	41	96
Di-BN	100	52	78	92	51	72	133	60	98
Dn-BN	73	47	58	80	51	69	106	53	77
NNPyrr	52	29	41	56	27	42	101	30	69

[a] The mean value of at least six determinations.

on the column. This corresponds to 1 ppb of the original sample after allowing for the quantities used in the method. However, these values vary on a day-to-day basis and frequent calibration of the mass spectrometer is necessary. Recalibration is essential if the mass spectrometer conditions are changed, *e.g.*, to record low-resolution spectra between nitrosamine determinations. A single measurement of the five nitrosamines used in this study takes 1 hr. Thus it is possible, in a day, to carry out several such measurements to calibrate the instrument and still analyze two or three samples. The time required to analyze a sample for each of the five nitrosamines is governed by the chromatographic conditions, which were chosen so that the nitrosamines are adequately separated from each other. The analysis of an extract for one or two nitrosamines only can be accomplished in a much shorter time by altering the chromatographic conditions.

The overall procedure was checked by adding all five nitrosamines to a typical food substrate, luncheon meat, and measuring the amount recovered. Percentage recovery values of nitrosamines added to samples at 10, 2, and 1 ppb are given in Table I. There is a wide spread in the data, especially at the lowest level. The low precision of the quantification by gas chromatography–mass spectrometry and variations in recovery in the extraction procedure are mainly responsible for the spread in the data. The recovery values which are greater than 100% arise through measurement errors and not from nitrosamines originally present in the sample, since no nitrosamines were detected in the original samples. The lack of precision of the measurements means that only semiquantitative analyses can be carried out in the 1–5 ppb range. However, the technique still offers a means of qualitative analysis at these levels.

SAFETY NOTE

Nitrosamines are highly carcinogenic compounds and all experimental work should be done in a well ventilated area. Safety gloves should be worn whenever nitrosamines are being handled.

LITERATURE CITED

Telling, G. M., Bryce, T. A., Althorpe, J., J. AGR. FOOD CHEM. **19**, 937 (1971).

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Received for review November 26, 1971. Accepted February 7, 1972.

Factor Responsible for Varietal Differences in Aflatoxin Production in Maize

Varietal differences in aflatoxin production in food grains is of considerable practical significance. The present study on seven hybrid varieties of maize indicated marked differences in aflatoxin production. Opaque-2 produced markedly low toxin, while Deccan hybrid produced maximal amounts. The possible reasons for the differences in toxin production have been investigated. Different extracts of the two varieties of maize (Opaque-2 and Deccan hybrid) have been incorporated into a standard synthetic medium and their ability to support toxin production has been examined. A 5% NaCl extract of Opaque-2 could result in almost total inhibition of the toxin production. Further fractionation of the NaCl extract revealed that the inhibitory factor is a protein of low molecular weight. The concentration of this protein is markedly higher in Opaque-2 than Deccan hybrid variety.

Aflatoxin contamination of food grains is now recognized to be a potential health hazard, in view of the known hepatotoxic and carcinogenic effects of the toxin (Goldblatt, 1969). The production of aflatoxin by toxigenic strains of *Aspergillus flavus* L. on different food grains is known to vary widely (Diener and Davis, 1969; Hesseltine *et al.*, 1966; Kriz, 1970). Maximal production of the toxin has been demonstrated on natural substrates such as rice, while meager production is obtained on soya beans (Hesseltine *et al.*, 1966). Varietal differences in aflatoxin production have been observed in peanuts (Rao and Tulpule, 1967; Doupnik, 1969) and in sorghum (Anandam, 1970). Such wide variation in toxin production in different varieties of the same crop could lead to the identification of varieties which

Table I. Influence of Different Extracts of Maize on Mat Weight and Toxin Production

Extracts	Opaque-2						Deccan Hybrid					
	Original extract		Dialyzed		Ultrafiltrate		Original extract		Dialyzed		Ultrafiltrate	
	Mat wt, mg	Toxin	Mat wt, mg	Toxin	Mat wt, mg	Toxin	Mat wt, mg	Toxin	Mat wt, mg	Toxin	Mat wt, mg	Toxin
Water extract	1100	+++	688	+	836	++	993	+	638	+++	730	++
5% NaCl extract	1025	Nil	735	++	906	Traces	938	+	588	+	735	+
70% alcoholic extract	719	+	619	++	768	+	749	+	522	+	676	+
0.2% alkali extract	807	+	689	++	725	++	872	+++	541	+	709	++++

Control: Basal medium only; 572 mg (mat wt); toxin produced ++ (approx 130 μg per flask). Figures relate to 50 ml medium per flask.

support low toxin production. The elucidation of the precise chemical nature of factors responsible for varietal differences in susceptibility to toxin production will help plant geneticists to breed strains with such desirable characteristics. The present communication describes varietal differences in aflatoxin production in maize and provides information regarding the actual factor responsible for inhibiting toxin production.

GENERAL METHODS

Culture. A toxigenic strain of *A. flavus* (NIN-25) isolated from rice was used throughout the investigation.

Maize Varieties. Seven authentic varieties of maize obtained from the unit of the Indian Council of Agricultural Research were used. Except for Opaque-2, the other varieties are of Indian origin. Opaque-2 and Hi-starch Makkai are white, while the other varieties are yellow.

Medium. The synthetic medium used in the series was as described by Adye and Mateles (1964). All the materials intended for inoculation were sterilized at 15 lb/in.^{2} for 15 min. Extracts to be incorporated into the medium were sterilized by passing through a sterilized Seitz filter under aseptic conditions. Samples in duplicate were inoculated with a uniform spore suspension of *A. flavus* in sterile distilled water and incubated at 28 °C for 7 days.

Extraction. After the incubation period, the spores were killed by alcoholic spray. In the case of grains, the sample was dried at 100°C and extracted with methanol. The aqueous methanolic extract was treated with basic lead acetate to remove pigments. The resulting filtrate was extracted with chloroform and evaporated to dryness. In the case of culture flasks, the mat was separated and dried at 100°C to constant weight. The culture filtrate was extracted several times with $CHCl_3$. The chloroform extracts were processed for thin-layer chromatography (tlc).

Thin-Layer Chromatography. The toxin produced (aflatoxin B_1) was assessed by tlc technique using $CHCl_3$:MeOH (95:5) as the developing solvent. The toxin produced was analyzed by visual grading of the fluorescent band corresponding to B_1 as described by the method developed by Tropical Products Institute, London (1965).

EXPERIMENTAL AND RESULTS

Varietal Differences in Toxin Production. Twenty-gram lots of seven varieties of maize were infected under laboratory conditions with the toxigenic strain of *A. flavus* and after a week's incubation were processed and the toxin produced was quantified by the tlc technique. The toxin production was markedly low in Opaque-2, Amber Composite, and Ganga-101 (0.05–0.25 ppm), while it was maximal in Deccan Hybrid and Ganga Safed (>1 ppm). Jawahar Composite and Hi-starch Makkai were intermediate in toxin production (0.25 ppm).

Biochemical Basis for Varietal Differences in Toxin Production. It was of interest to examine the possible reasons for this marked variation in the toxin-producing ability of the different varieties. A systematic examination of the different constituents in the two varieties of maize (Opaque-2 and Deccan Hybrid) was undertaken. This was achieved by preparing different extracts of the two varieties and these extracts, representing different constituents, were incorporated into a defined synthetic medium (double strength) and the toxin production was assessed. Preliminary investigations showed that ether extractives (lipid fraction) and ethyl acetate extracts (phenolic constituents) and total ash (minerals) from the two varieties appropriately processed and incorporated into the medium did not have any apparent difference in toxin production. Next, the protein fractions of the varieties were examined. Protein fractionation was carried out by successive extraction in the cold at 4°C from a 200-g lot of the ground maize variety with water, 5% NaCl solution, 70% alcohol, and finally 0.2% alkali (Osborne and Mendel, 1914). These extracts (1500 ml each) were appropriately processed and concentrated under vacuum to 150 ml. Aliquots of the extracts were dialyzed against water in the cold at 4°C for 24 hr. Five milliliters of the original extracts or its equivalent of the dialyzed extract or its ultrafiltrate was used for incorporating into a double strength medium (previously sterilized). The pH of the extracts was adjusted to 6.0 in all cases and the extracts were sterilized by passing through a sterilized Seitz filter. After the addition of the extracts, the medium in all the flasks was made up to single strength (final volume 50 ml) with the addition of appropriate amounts of sterilized distilled water. The flasks after inoculation were incubated for 7 days at 28°C, at the end of which period the mat was separated and culture filtrates were extracted with $CHCl_3$ and processed for tlc.

The effect of these extracts was examined in terms of dry mat weight and toxin production. The results of these investigations, summarized in Table I, showed the following features. The mat weight and, therefore, the growth was not adversely affected by the extracts. In fact, there was stimulation of growth in most cases. The toxin production was not correlated to the mat weight. The toxin production pattern showed that each variety of maize contained both stimulatory as well as inhibitory factors. The 5% NaCl extract of Opaque-2 variety resulted in almost total inhibition of toxin production while, in the case of the Deccan variety, the degree of inhibition was markedly lower than Opaque-2.

Fractionation of the NaCl Extract. Since the 5% NaCl extract of Opaque-2 resulted in almost total inhibition of

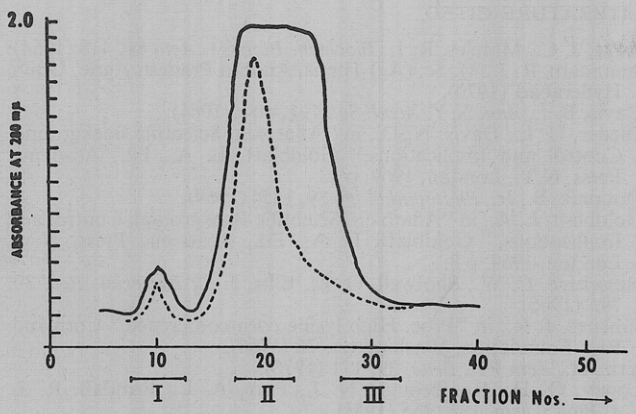

Figure 1. Elution pattern from Sephadex-G-75. ———, absorbance at 280 mμ; – – – – –, protein level by Biuret test

Table II. Influence of Different Fractions of Opaque-2, NaCl Extracts

	Mat wt, mg	Toxin
Control (basal medium only)	600	++
Opaque-2, NaCl extract diffusate	656	Traces
Protein fraction I (from Sephadex)	560	++
Protein fraction II (from Sephadex)	550	Traces
Fraction III	536	++

(Final volume 50 ml medium per flask).

toxin production, it was of interest to fractionate this extract further to locate the actual principle responsible for such inhibition. Subsequent experiments by dialysis showed that the inhibitory effect was almost fully recoverable in the ultrafiltrate or the diffusate. Degradation of the organic moiety in the diffusate by ashing resulted in loss of the inhibitory capacity. This indicated that the inhibitory principle resided in the organic moiety of the diffusate. The diffusate on routine analysis showed characteristics for the presence of some protein material as indicated by positive reaction for Biuret test, heat coagulation, weak ninhydrin positive reaction, and characteristic staining for protein by bromophenol blue after electrophoresis on paper at 250 V for 4 hr using standard buffers (pH 3.6 and 8.0), showing two bands positive for protein.

Fractionation on Sephadex. Since the diffusate showed two protein bands on paper electrophoresis, the diffusate was further fractionated by passing an aliquot (15 ml containing protein at 7 mg/ml) through a column of Sephadex G-75 (50 × 1.5 cm) and eluted with phosphate buffer pH 7.2 of increasing molarity (from 0.01 *M* to 0.2 *M*) at a flow rate of 30 ml/hr. The elution pattern is shown in Figure 1. The fractions from the column were monitored by measuring the absorbance at 280 mμ and pooled into three lots and concentrated under vacuum to original volume. These fractions were sterilized by Seitz filter and incorporated into the medium, and toxin production was assessed. This series showed that the protein in fraction II had the inhibitory activity. The results of these series are shown in Table II. Electrophoretic studies using Whatman No. 1 paper (Smith, 1960) and acrylamide gel, as described by Davis (1964), revealed that the protein in fraction II was a single homogenous component (Figure 2). Further work is in progress to examine the other characteristics of this protein.

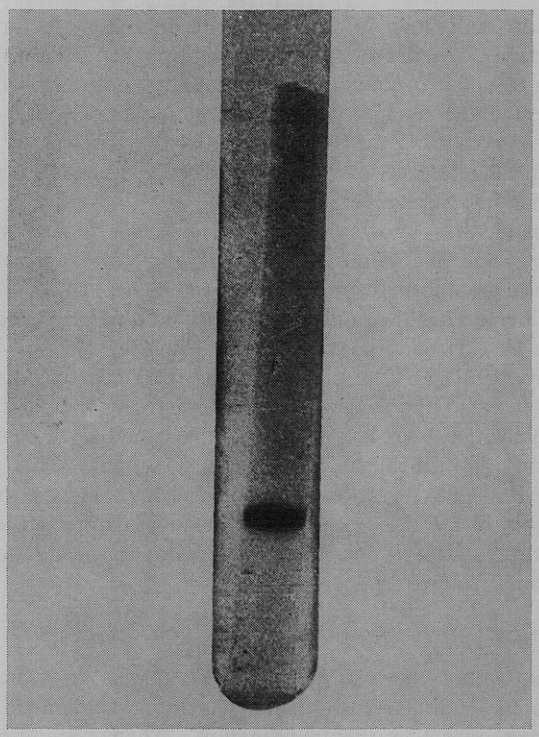

Figure 2. Polyacrylamide gel electrophoresis of fraction II, stained with Amido Black

Table III. Protein Levels mg/ml in NaCl Extract Diffusates

	Opaque-2	Deccan
NaCl extract diffusate	7.0	2.97
Fraction I from Sephadex	0.59	0.197
Fraction II from Sephadex	3.40	1.97
Fraction III from Sephadex	2.50	...

(Protein by the method of Lowry *et al.*, 1951).

Similar experiments on the diffusate of the NaCl extract of Deccan Hybrid variety of maize have shown that this variety also has similar protein but its concentration is markedly low (Table III).

DISCUSSION

Earlier work had shown that there are varietal differences in aflatoxin production in ground nuts (Rao and Tulpule, 1967). Similar differences have now been observed in different varieties of maize. Among the seven varieties of maize screened, Opaque-2 produced markedly low toxin, while Deccan Hybrid produced maximum. Two other Indian varieties, Amber Composite and Ganga-101, also produced relatively low toxin. Such variations are also noticed in varieties of sorghum (Anandam, 1970). Obviously, there could be some biochemical basis for such wide variations in the toxin-producing potential. A systematic examination of the two varieties of maize (Opaque-2 and Deccan Hybrid) has been made to locate the actual fraction responsible for inhibition or stimulation of the toxin production. These investigations have clearly shown that each variety of maize contains both the inhibitory and stimulatory factors. The present study shows that differences in toxin production potential are not related to lipid or phenolic constituents or the total mineral pattern of the two varieties of maize. Studies with crude protein fractions revealed that a potent inhibitor is present in the 5% NaCl extract (globulin fraction). Further fractiona-

tion of this extract indicated that the inhibitory factor is a dialyzable material of low molecular weight. Elution pattern on Sephadex column and electrophoretic studies strongly suggest that it is a protein of low molecular weight. This protein possessing the inhibitory capacity is present in both Opaque-2 and Deccan variety. However, its concentration in Opaque-2 variety is markedly high.

Opaque-2 is known to contain markedly higher water-soluble and NaCl-soluble nitrogen and lower prolamine fraction than normal variety of maize (Jimenez, 1966). Striking differences in the protein fractions have also been observed in developing endosperm of the two varieties (Murphy and Dalby, 1971). According to these workers, the striking differences in the NaCl-soluble nitrogen in Opaque-2 and normal variety were largely accountable by a dialyzable nitrogeneous constituent of low molecular weight. This is in line with the observation of the present investigation on the presence of a protein probably of identical nature but in addition possessing a powerful inhibitory action in relation to aflatoxin production.

ACKNOWLEDGMENT

The authors express their sincere thanks to Coluchur Gopalan, National Institute of Nutrition, Hyderabad, for his keen interest in this investigation. Thanks are also due to P. G. Tulpule and D. N. Roy for their helpful suggestions.

LITERATURE CITED

Adye, J. C., Mateles, R. I., *Biochem. Biophys. Acta* **86,** 418 (1964).
Anandam, R. J., M. Sc. (Ag) Thesis, Andhra Pradesh Agric. Univ., Hyderabad (1970).
Davis, B. J., *Ann. N.Y. Acad. Sci.* **121,** 404, (1964).
Diener, U. L., Davis, N. D., in "Aflatoxin, Scientific Background, Control and Implications," Goldblatt, L. A., Ed., Academic Press, N.Y., London, 1969, p 13.
Doupnik, B., Jr., *Phytopathology* **59,** 1554 (1969).
Goldblatt, L. A., in "Aflatoxin, Scientific Background, Control and Implications," Goldblatt, L. A., Ed., Academic Press, N.Y., London, 1969, p 8.
Hesseltine, C. W., Shotwell, O. L., Ellis, J. J., *Bacteriol. Rev.* **30,** 795 (1966).
Jimenez, J. R., in "Proc. High lysine corn conference," Corn Ind. Res. Foundation, Washington, 74 (1966).
Kriz, H., *Acta Vet. Brno.* **39,** 171 (1970).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
Murphy, J. J., Dalby, A., *Cereal. Chem.* **48,** 336 (1971).
Osborne, T. B., Mendel, L. B., *J. Biol. Chem.* **18,** 1 (1914).
Rao, K. S., Tulpule, P. G., *Nature (London)* **214,** 738 (1967).
Smith, I., *Chromatogr. Electrophor. Tech. II* 170 (1960).
Tropical Products Institute, Recommended Procedures for Detection and Estimation of Aflatoxin B_1 in Groundnuts, T.P.I. Rep. No. G 13, (1965).

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Received for review December 8, 1971. Accepted March 7, 1972.

Identification of Carotenoid Constituents in *Hibiscus syriacus* L.

The ten carotenoid pigments found in the bud, leaf, and flower of *Hibiscus syriacus* L. were each identified and quantitated. Those most abundant in the buds and leaves were β-carotene and lutein; lutein 5,6-epoxide predominated in the flower. Carotene hydrocarbons comprised 19% of the total carotenoids in the flowers, with 37% in the buds and 33% in the leaves. *Hibiscus syriacus* contained a much lower percentage of the colorless phytoene precursors to the carotenoids than cotton (*Gossypium hirsutum* L.). However, three carotenoid pigments, cryptoxanthin, chrysanthemaxanthin, and antheraxanthin, were present in *H. syriacus* but not in cotton, and two, flavoxanthin and violaxanthin, were found in cotton but not in *H. syriacus*.

Until the discovery by Coad (1914) of the malvaceous plant *Hibiscus syriacus* L. (Rose of Sharon) on which the boll weevil (*Anthonomus grandis* Boheman) could feed, oviposit, and develop, this insect was considered monophagous on cotton (*Gossypium hirsutum* L.)

Until recently there has been general disagreement regarding the classification of the *Hibiscus* and *Gossypium* genera within the Malvaceae family. Fryxell (1968) redefined the tribe Gossypieae (Malvaceae), which includes the genus *Gossypium* (cotton), and specifically separated it from the malvaceous tribe Hibisceae, to which the genus *Hibiscus* belongs. His separation is based on the presence of pigment glands which are associated with the triterpene pigment gossypol (the Gossypieae appear to be unique in possessing these glands).

Recent developments in research on the boll weevil indicate that eradication of this insect may become feasible, but before a wide scale control or eradication program can be initiated, it is essential to determine whether alternate host plants would serve as an endemic source for reinfestation of cotton. Rose of Sharon, because it is used as an ornamental on many farms and is abundant throughout the South and Southeast, is the most important of the alternate hosts. Methods have since been developed for a chemical evaluation of the plant components affecting insect response to the two species and the taxonomic relationship between the species. Thompson *et al.* (1968) identified the carotenoids in buds, leaves, flowers, and other tissues of the cotton plant, and we now report a similar study of the carotenoids of *H. syriacus*. Also, Rose of Sharon is grown as a significant commercial ornamental in the southeastern United States. Since the desirability of varieties is influenced by bloom color, an assessment by plant breeders of the extent to which the carotenoids modify the anthocyanin color could provide background information for the development of new varieties.

MATERIALS AND METHODS

Plant Material. Flowers, leaves, and bracted buds of *H. syriacus* were harvested from mature plants grown in field plots at Mississippi State University.

Pigment Extraction. The plant material was macerated in an electric blender with acetone, the acetone was removed