MYCOTOXIN RESEARCH IN INDIA

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Judging from personal contacts and from a literature survey on mycotoxins in India, the five major centers working on mycotoxins are the Central Food Technological Research Institute at Mysore; the Department of Biochemistry, Vallabhbhai Patel Chest Institute, University of Delhi; Biochemical Laboratories, A. C. College, Madras; Nutrition Research Laboratories, Hyderabad; and Department of Botany and Microbiology, S.V. University, Tirupati. Practically all the work is being devoted to studies on various aspects of the aflatoxin problem. Although the Department of Plant Pathology and Botany at the University of Allahabad, Allahabad, has conducted related studies on the mold flora of various grains of India, it has never been directly involved in mycotoxins.

Each center has worked in a different area. The University of Delhi has concentrated primarily on the biochemistry of the aflatoxins under the direction of T. A. Venkitasubramanian. Work at Mysore has been somewhat biochemical, although major efforts have been in surveying such food products as peanuts, peanut oil and meal and in developing analytical methods. The work at Madras under E. R. B. Shanmugasundaram has been on mycotoxins other than aflatoxin. At Hyderabad, research is directed toward animal studies and resistance to aflatoxin formation in varieties of peanuts. At Tirupati, work has been devoted more to mycology, in addition to investigation of the aflatoxin problem in peanuts.

All centers have published both in Indian and in foreign journals. Major discoveries reported by Indian authors include: (1) Unrefined Indian peanut oil contains aflatoxins B1 and B2 but not G1 and G2; (2) hydrogen peroxide, coupled with a heat treatment at 80°C, destroys aflatoxin in peanut meal; (3) aflatoxin affects monkeys; in fact, some of the first studies (1964) were made in India; (4) both nontoxic and toxigenic strains of Aspergillus flavus occur on peanuts, surveyed in 1965; (5) peanut, corn, and soybean varieties have different degrees of resistance to the formation of aflatoxin by A. flavus; this demonstration led to considerable research in finding genetic resistance in other countries in peanuts and corn; (6) a blue fluorescent compound, often occurring in tapioca, was not aflatoxin; (7) aflatoxin forms in sunflower seeds, representing the first study conducted on sunflower seed; (8) studies on the mycotoxins produced by Aspergillus candidus, Penicillium piceum, and Penicillium oxalicum; (9) resistance to aflatoxin formation in soybeans depends upon phytic acid, which, reportedly, binds zinc in the soybean and so inhibits aflatoxin formation; and (10) when rats are fed aflatoxin, a low-protein diet leads to long-term resistance to liver cancer.

The funding for considerable amount of this work has come from PL 480 funds. The U.S. Cooperator and his counterpart Principal Investigator agree on the broad outline of a mutually agreeable problem. This work is funded by U.S. owned Indian rupees which cannot be converted into dollars but may be spent in India for agricultural research.

To cover all the Indian work would be impossible in this paper. Therefore, I will omit much good work on biochemistry, analytical methods, toxicity studies in animals, and other related animal work. I have assembled a list of these publications which will be made available to interested persons.

Occurrence of Aflatoxins in India

India is an ideal country for problems of aflatoxin to develop since it has high temperatures, high moisture levels during the monsoon season, and often inadequate

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1 Presented at the Japanese Association of Mycotoxicology meeting, Tokyo, Japan, September 27, 1975.
storage facilities. Another important factor is that most of the cereals are used in human food. The harvesting and storage of peanuts is described by Sreenivasamurthy et al. (1965). Study of strains of *A. flavus* for their ability to produce aflatoxin and methods of analysis are given but no data is presented on the natural occurrence of aflatoxin in peanuts. In 1969 the same laboratory (Dwarkanath et al.) reported a study of aflatoxin in peanut oil. Peanut oil production amounts to 1.32 million tons annually in India and 98% is used for edible purposes. Thirty-two percent is used in hydrogenated fat, the other 68% is consumed as unrefined oil. Examination of oils from freshly harvested peanuts showed an aflatoxin content of from 0.02–0.2 ppm, with an average of 0.10 ppm. For oils made from peanuts stored for 6 months the range was 0.06–0.26 ppm, with an average of 0.14 ppm. The highest level encountered was 0.4 ppm. The number of samples examined was not specified, but assay of unrefined peanut oil over two seasons showed no significant difference in aflatoxin levels. Both B1 and B2 were detected in the oil but no G1 or G2. No aflatoxin was detected in refined oil. When peanut oil was heated to 120°C for 10 min, 50% destruction of aflatoxin occurred. Native foods prepared from wheat and pulses and fried in aflatoxin contaminated oil took up some of the toxin.

Nagarajan et al. (1973b) at Hyderabad investigated aflatoxin in 20 samples of tapioca obtained in Kerala State. Most of the samples had visible mold growth, with *A. flavus, A. glaucus,* and *A. niger* present along with *Penicillium* and *Rhizopus*. Chloroform extracts of almost all the samples showed a blue fluorescent spot behaving like pure B1. However, the material in biological tests was not toxic.

Aflatoxins in sorghum grains infected with head molds were reported by Tripathi (1975). After a heavy rain in October 1969 in Pantnagar, about 13% of the sorghum heads were infected with *A. flavus*. No aflatoxin could be detected 3 days after the rain but on the seventh day aflatoxin was detected in the sorghum heads in the field as well as in heads stored for the same period. The levels of aflatoxin detected were not given. According to Mehan & Chohan (1973), Wagle (1970) surveyed 500 samples of groundnuts of India from the West Coast and found that nearly 50% contained aflatoxin. He found levels of aflatoxin to be 0.10–0.25 ppm, with almost 75% in the 0.10–0.50 ppm range. Narasimhan (1968) reported the occurrence of aflatoxin in copra, but his paper did not give the levels of toxin. Gopal et al. (1968) described an outbreak of aflatoxicosis in a dairy herd; of 126 animals, 58 died. Aflatoxin was found in the groundnut cake as well as the mold. A second outbreak occurred in cattle fed pellets containing cottonseed cake in which high levels of aflatoxin were demonstrated.

Aflatoxin in Humans

Amla et al. (1971) reported on children who were suffering from protein caloric malnutrition, who had accidentally consumed aflatoxin in low-fat peanut flour. The children had eaten this material for periods of 5 days to 4 weeks. They developed hepatic lesions which showed a gradual transition from an increase in central and peripheral fat to fibrosis and cirrhosis. These observations are identical to the lesions seen in Indian childhood cirrhosis. The role of aflatoxin in Indian cirrhosis was investigated by Yadgiri et al. (1970). They made chloroform extracts of urine and liver and found a blue-violet fluorescence spot on TLC on silica gel having an Rf almost identical to that of aflatoxin B1. Further tests, including the day-old duckling test, showed this spot not to be aflatoxin B1.

In 1975, Krishnamachari et al. described an outbreak of aflatoxicosis in man in Western India. This epidemic affected as many as 200 villages in the states of Gujarat and Rajasthan in late October 1974 and lasted about 2 months. Involved in the outbreak were 396 patients, of which 106 died. The aflatoxin source was maize grown in chronically drought-stricken regions; in October unseasonal rains drenched the standing crop and caused it to mold.

The clinical picture showed a brief febrile episode, vomiting, and anorexia. In some, the disease was mild and recovery complete. In more severe cases, ascites appeared in 2 to 3 weeks followed by oedema of the legs. Both the liver and spleen became enlarged and jaundice was present. Death was usually sudden and, in most instances, preceded by massive gastrointestinal bleeding. Males were more affected than females. Dogs sharing the food with the affected families likewise developed ascites and icterus and died in 2 or 3 weeks. The highest number of deaths occurred in December with the death rate distributed through the age group from 5–30; there were no infant cases.

The food was collected from the affected households, including maize, sorghum, wheat, and Kodo millet. The presence of *A. flavus* and aflatoxin was determined, and the latter was confirmed by the day-old duckling test.
The affected maize was pale and shriveled and all samples showed the presence of *A. flavus*, but it was not found in wheat or sorghum. In the five maize samples, aflatoxin ranged between 6.25 and 15.6 ppm. An adult in the region consumes about 350 g of maize daily; therefore, adults were ingesting from 2–6 mg of aflatoxin. The disease disappeared from the villages when the supply of maize was exhausted. The histopathology revealed extensive bile duct proliferation; however, no aflatoxin could be found in the liver examined, although two of seven serum samples showed detectable amounts of aflatoxin.

**Role of Insects on Transmission of *A. flavus* Spores**

Since we know in the U.S. that *A. flavus* infects the kernels of corn in the field before harvest, people are interested in how the ear corn becomes infected. Part of the answer may be found in some recent Indian work. In three papers from the Central Food Technological Research Institute at Mysore, Majumder and coworkers, have done some pioneering work showing that certain insects do transmit spores of *A. flavus* to stored grain. The first, an abstract by Srinath et al. (1971), described the isolation of internal fungi from 10 species of insects occurring in stored grain. All 10, except for their eggs, carry fungi internally: among the fungi are *A. flavus*, *A. ochraceus*, and *Penicillium islandicum*, all mycotoxin-producing fungi. The numbers of fungi present in the grain has no correlation with the number of adult insects having internal fungi. They conclude with the statement 'The results of the study revealed that these stored-product insects can act as a potential vector for transmitting several toxigenic species of fungi to the grain bulk.'

The second paper by the same authors (1973) examined the food values of several fungi to the rice weevil (*Sitophilus oryzae* L.). Sterilized grain sorghum was inoculated with pure cultures of five species of *Aspergillus*. After the grain was covered with the mold, 30 adult rice weevils were released in each flask. The results showed *A. flavus* completely retarded breeding, whereas *A. versicolor* and *A. ruber* gave significantly higher populations when compared with the control (uninfected sorghum). *A. nidulans* and *A. ochraceus* had no significant effect on insect reproduction. However, further study found that an isolate of *A. flavus* from maize did not inhibit breeding of the rice weevil.

The third paper (Ragunathan et al., 1974) noted that the rice weevil is the most common pest in sorghum, wheat, and rice under tropical conditions. Ten samples of each of these cereals naturally infected with this insect were secured from commercial channels. The grain was soaked in acid fuchsin to identify the egg plugs. From these grains, eggs, grubs, and pupae were removed, surface sterilized, and cultured on malt agar. In wheat, 32–100% of the adults showed the presence of fungi, and 25–100% in sorghum. For the most, the molds were *Aspergillus* with *A. flavus* found in all three cereals. All stages of the insect contained fungi except the eggs. *A. flavus* was the predominant fungi in weevils from rice and wheat.

**Natural Resistance in Plants to Aflatoxin Formation**

The first paper published on resistance in peanuts to aflatoxin formation was one by Rao et al. (1967). They obtained 60 different varieties originating from 15 different countries. Each peanut variety was inoculated aseptically in flasks with a toxigenic strain of *A. flavus*, incubated for 10 days, and then analyzed for aflatoxin. All peanut varieties supported good growth of *A. flavus* and only one strain showed no toxin, U.S. No. 26. This work was never confirmed, but it resulted in other workers investigating the problem of varietal resistance. It now is known that some peanut varieties do have resistance to aflatoxin formation. Nagarajan & Bhat (1972) using U.S. 26 and TMV-2 (the most widely used variety in India) investigated the nature of resistance in peanuts. Each variety was inoculated with three strains of *A. flavus* and two of *A. parasiticus*. Twenty-gram lots of each peanut variety were moistened, autoclaved, and inoculated and each flask was incubated at 28 C for 7 days. The five mold cultures produced aflatoxin on all varieties including U.S. 26, but there were differences in yield between the two peanut varieties with amounts much less for all cultures growing on U.S. 26. For instance NRRL 2999 gave 51,600 on TMV 2 as compared to 25,000 on U.S. 26. However, the authors consider *A. parasiticus* to be no problem in India, since they had examined 100 isolates from peanuts and 2500 from cottonseed but had not found any strains of this species. The same authors studied varietal resistance of maize in India (Nagarajan & Bhat, 1972), examining seven varieties and using one strain of *A. flavus* to establish levels of aflatoxin. The same general methods were used as for peanuts. Toxin levels were low for Opaque 2 and high for hybrid Deccan. A systematic examination was
made of these two maize varieties to determine how they differed. An inhibitory factor was found but was not identified. It is a protein of low molecular weight and occurs at markedly higher levels in Opaque-2 than in Deccan.

Nagarajan et al. (1974) likewise investigated aflatoxin production in five seed varieties of sunflower. The varieties were tested for the ability to support aflatoxin synthesis by two strains of \textit{A. flavus} and three of \textit{A. parasiticus}. Sunflower varieties exhibited variation in toxin-producing capacity. Furthermore, sunflower varieties appear to support less aflatoxin synthesis than other oilseeds such as peanuts and soybeans. However, if broken seeds are used, yields are comparable to that found on peanuts and soybeans. They conclude 'These data appear to suggest strongly that the ‘armoured seedcoat’ perhaps interferes with the penetration of the invading fungus and subsequent ability to produce toxin.'

The final paper in this series by Nagarajan et al. (1973a) examined aflatoxin production in five varieties of soybeans using two isolates each of \textit{A. flavus} and \textit{A. parasiticus}. The yields ranged from 19.5 to 31.5 ppm when NRRL 2999 was used. Variety Lee produced the lowest aflatoxin levels. Apparently the levels of aflatoxin in soybeans depend on the variety and the toxigenic potential of the fungal isolate.

The factors determining resistance to aflatoxin synthesis in soybeans were the subject of an investigation by Gupta \\& Venkitasubramaniam (1975). Lee soybeans were ground to a fine powder and 20 g placed in 500-ml flasks, and moistened with 20 ml of water. One group of flasks was autoclaved and the second was not autoclaved. Flasks were inoculated with NRRL 3240, \textit{A. parasiticus}, and incubated at 26\textdegree{}C for 8 days. Total aflatoxin production on unautoclaved soybeans was 0.125 as compared to 1.9 mg/100 g on autoclaved beans. Soybeans are known to be high in phytic acid (690 mg/100 g) and phytic acid is known to bind zinc, an element highly stimulatory to aflatoxin formation. The high levels of aflatoxin obtained are probably due to the destruction of phytic acid by heat. In a further experiment, unautoclaved soybeans were inoculated as before with \textit{A. flavus} but three levels of ZnSO\textsubscript{4} were added. The results were as follows:

<table>
<thead>
<tr>
<th>ZnSO\textsubscript{4}</th>
<th>Aflatoxin (mg/100 g)</th>
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<tbody>
<tr>
<td></td>
<td>B\textsubscript{1} + B\textsubscript{2}</td>
</tr>
<tr>
<td>0.0</td>
<td>0.13</td>
</tr>
<tr>
<td>1.0</td>
<td>0.70</td>
</tr>
<tr>
<td>2.0</td>
<td>1.60</td>
</tr>
<tr>
<td>5.0</td>
<td>2.30</td>
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</table>

When 5.0 g of ZnSO\textsubscript{4} was added to autoclaved soybeans, the aflatoxin levels increased from 6.5 mg/100 g of soybeans to 9.6. Zinc is known to be low in soybeans (0.01 \(\mu\)g/g) while cereals such as rice have 18–19 \(\mu\)g/g. The authors next did an experiment to show that phytic acid, in fact, blocks aflatoxin synthesis by binding zinc.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Aflatoxin (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean</td>
<td>0.12</td>
</tr>
<tr>
<td>Soybean + 1 g ZnSO\textsubscript{4}</td>
<td>0.65</td>
</tr>
<tr>
<td>Soybean + 1 g ZnSO\textsubscript{4} + 200 mg phytic acid</td>
<td>0.28</td>
</tr>
<tr>
<td>Soybean + 1 g ZnSO\textsubscript{4} + 400 mg phytic acid</td>
<td>0.16</td>
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The same results were obtained in a purely synthetic medium in which zinc was again bound by phytic acid so that the yield was drastically reduced.

**Detoxification**

The research group at Mysore has proposed that peanut meal containing aflatoxin may be detoxified with the use of hydrogen peroxide. Sreenivasamurthy et al. (1967) reasoned that the lactone ring in the aflatoxin molecule opens in an alkaline medium and is susceptible to oxidation. The treatment consists of placing defatted peanut meal in water to give 10\% solids, and adjusting the pH to 9.5 with a strong alkali. The suspension of solids is treated with H\textsubscript{2}O\textsubscript{2} and then heated for one-half hr at 80\textdegree{}C. Data indicated that for every 10 \(\mu\)g of pure toxin, 0.5 ml of 6.0\% H\textsubscript{2}O\textsubscript{2} was required to reduce the extractability to zero. Treated material with H\textsubscript{2}O\textsubscript{2} resulted in the loss of toxicity as determined by the duckling test. Detoxification studies of toxic peanut meal revealed that 5 ml of
6% H$_2$O$_2$ was necessary to detoxify 5 g of toxic meal containing 90 ppm. At this level of aflatoxin 97% was destroyed. The treated product can be spray-dried with no residual smell or taste. Such treatments open the lactone ring of both aflatoxins B and G and each is destroyed. There was no significant difference in the PERs between the H$_2$O$_2$ treated and untreated meal.

**Mycology of Aflatoxin Strains**

A survey of freshly harvested groundnuts in 1964 from the coastal districts of Andhra Pradesh showed that 36 of 288 samples contained aflatoxin (Rao et al., 1965). The peanuts were raised under irrigation and the conditions of temperature and humidity were favorable for quick and proper maturity of the nuts. Examination of the shells indicated that 6 to 14% of the pods were visibly damaged. From these peanuts a number of strains of *A. flavus* were isolated. Each was grown aseptically on 300 g of peanuts for 10 days and analyzed for levels of aflatoxin and, if positive, the toxin was confirmed by use of the duckling assay. Aflatoxin-producing strains failed to form G$_1$ and G$_2$. Of the 29 strains of *A. flavus* isolated, only 6 were toxigenic with yields from 1 to 5 ppm.

Maggon et al. (1969) conducted one of the few, if not perhaps the only, studies of soil isolates for their ability to produce aflatoxin. Nine soil isolates came from near Delhi. The isolates were grown for 7 days at 25° on both liquid and agar media and the aflatoxin levels determined. Of the 9 strains, 5 formed large amounts of toxin (13–19.5 mg/50 ml medium), two gave small amounts (0.6–1.4 mg/50 ml), and two were negative. Each producing strain formed only B$_1$ and B$_2$ and not G$_1$ or G$_2$. The study also indicated the need for trace elements in synthetic media to produce toxin. Only sclerotia-forming strains were aflatoxin producers.

Twenty-one strains of *A. flavus* were isolated from surface-sterilized seeds of cotton, maize, and wheat (Mehan and Chohan, 1973). Each strain was grown on liquid media for aflatoxin production. The incident of *A. flavus* in seeds of cotton, maize, and wheat were respectively 16, 22, and 3%. Sixteen isolates formed aflatoxin B$_1$. Levels of aflatoxin ranged from 0.85 to 1.72 mg/50 ml of medium. When five isolates were tested individually on peanuts, maize, rice, soybeans, and wheat, the lowest yields of aflatoxin were present in soybeans and the highest on maize. All strains formed B$_1$, but apparently no G$_1$ was detected in any isolate. Their paper gives a good review of several other reports and abstracts on strains in India and their ability to produce only aflatoxin B$_1$. Sreenivasamurthy et al. (1965) found that only 4 out of 150 isolates of *A. flavus* from peanuts formed aflatoxin B$_1$. According to Mehan & Chohan (1973), Indulkar et al. (1971) isolated 1800 cultures of *A. flavus* from cottonseed. Although 90% were toxigenic, only one isolate produced G$_1$ and G$_2$. Subrahmanyam and Rao (1974b) isolated *A. flavus* from 63 groundnut samples from seven Indian States. Of the 240 strains made from these samples, only 72 were toxigenic; of these, 16 produced B$_1$ only while 48 produced B$_1$ and G$_1$, a condition not reported previously from India. Their paper also reports that more aflatoxin was produced on groundnuts, copra, and castor than on sunflower, safflower, niger seed, and gingelly when these oilseeds were sterilized and inoculated.

A *Streptomyces* sp. isolated from a human respiratory tract was claimed to produce aflatoxin (Mishra and Murthy, 1968). The evidence that this Actinomycete produced aflatoxin is based on three facts: (1) When material was injected into mice, they died with a very high degree of cirrhosis of the liver; (2) when the culture was grown in a synthetic medium and the medium extracted with chloroform and spotted in TLC plates with aflatoxin standards, the material was identical with aflatoxin B$_1$ and G$_1$; and (3) the ultraviolet absorption spectra was similar to aflatoxin. Lalithakumari et al. (1970) reported that a culture of *A. tamari* produced aflatoxin.

**Other Mycotoxins**

The occurrence of citrinin in peanuts was reported by Subrahmanyam & Rao (1974b). While studying fungal infection of groundnuts before harvest, they noted a yellow pigment in rotted pods. Such kernels emit a golden yellow fluorescence when exposed to UV light. Four peanut samples were examined, all of which contained aflatoxin. Three of the samples had citrinin at levels of 10, 950, and 86 μg/kg. All isolates of *P. citrinum* (26) and *P. jenseni* (20) from peanuts when cultured produced citrinin; 38 isolates out of 52 of *A. flavus* produced aflatoxin.

*Aspergillus candidus*, a common inhabitant of cereal flours, when grown at room temperature on Richards solution and the filtrate extracted with chloroform, gave a concentrate that was lethal to chicks via the oral route (Ramadoss & Shanmugasundaram, 1971). It was likewise toxic to mice via the oral route. The authors ruled out the presence of citrinin.
Parthasarathy & Shanmugasundaram (1973a) investigated the toxins produced by *Penicillium piceum* by growing the mold on Czapek's Dox medium for 20 days. The culture filtrate either extracted with chloroform or the original concentrated filtrate produced 100% mortality in chicks with only two or three feedings. Histological examination revealed mild hepatic and severe kidney damage in mice. In isolating the toxic principle, a yellow material was isolated which possessed a UV absorption maxima at 255 and 420 nm.

In a further paper by the same authors (1973b) they stated that although chicks are readily killed by the toxin, mice are not, although they showed both pathological cell changes in the kidneys and liver. The presence of citrinin was eliminated. Glomerulonephrites occurred tending toward glomerulosclerosis. This pathological expression is usually considered to be a secondary result of streptococcal infection. The authors note that this condition has not been reported before from animals consuming contaminated toxic food.

Parthasarathy et al. (1972) reported that the rice moth, *Corcyra cephalonica* St., is an excellent assay organism for testing for mycotoxins. For example, citrinin produced an inhibitory effect on the metamorphosis of the insect *Corcyra cephalonica* St. (rice moth larva) as a convenient test organism in mycotoxicological studies. A rapid procedure for the isolation, identification and estimation of citrinin. Anal. Biochem. 52: 482–488.


