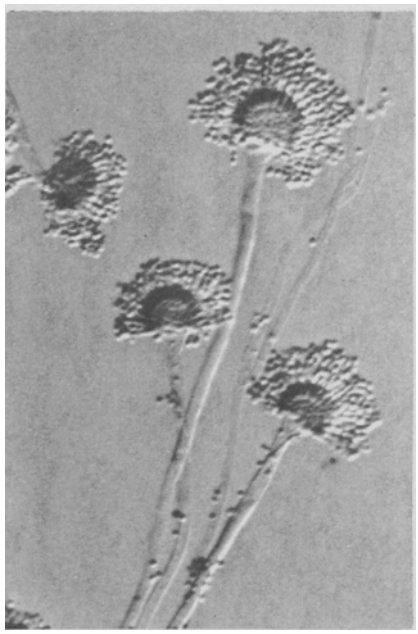
FEATURE



Aflatoxin

New approaches to research at the SRRC; a discussion of analytical instrumentation; and perspectives on the recent toughening of permissible levels in European imports

U.S. Department of Agriculture research on mycotoxins and aflatoxins centers, to a great extent, on the Southern Regional Research Center in New Orleans, Louisiana. This report by SRRC staffer Louise S. Lee describes the current thrust of research on that topic at SRRC and how the program has developed.

A front-page report in the Feb. 23, 1989, issue of the *Wall Street Journal* rekindled public concern over the aflatoxin problem. The report spoke of the potential danger to public health because "one of the most potent cancer-causing agents known to man is coursing into the nation's food supply." The implication was that because "regulatory efforts to stop the spread of tainted grain, inadequate at best, have largely failed . . . the nation's food supply is in jeopardy . . . and the food industry is left for the most part to police itself." During August 1989, the same publication reported concerns about high aflatoxin content in early harvested 1989 corn in Texas.

The problem dates back to the 1960s when a large flock of turkeys in England died after ingesting peanut meal that had been contaminated with *Aspergillus parasiticus*—a fungus that makes aflatoxin. Because peanuts were implicated in the Turkey X disease, the U.S. peanut industry instituted a policy to "police" itself through a fund set up by the Peanut Administrative Committee. The policy is still in place. The fund helps shellers with any financial loss due to aflatoxin contamination. Food industries also have internal monitoring programs. Both take advantage of the deep commitment by USDA to aflatoxin research. USDA has had research programs devoted to aflatoxin research for well over 20 years.

Soon after the problem of aflatoxin was recognized as one that could affect domestic peanuts, the USDA's laboratory in New Orleans (the Southern Regional Research Center) instituted a program on aflatoxin. Scientists in the small group at SRRC assigned to peanut research were set to work on a crash program on aflatoxin. Surveys were conducted immediately to determine the extent of the problem in domestic peanuts. Result? Aflatoxin B_1 was found in damaged peanuts from all parts of the United States where peanuts were grown. Even peanuts from the cooler parts of the country had aflatoxin. The problem was not just one of the hot, humid valleys in Brazil where the peanuts implicated in the Turkey X disease were grown. The culprit mold was everywhere. Funding for aflatoxin research increased at SRRC during the 1960s. Scientists were added to the staff and contracts were negotiated with universities in the peanut growing areas. The idea was conceived for a peanut laboratory in Dawson, Georgia, and funds were appropriated.

At SRRC, under Leo Goldblatt's leadership, Walter Pons improved analytical methods. Alva Cucullu and Louise Lee devised a microprocedure for analyzing individual peanuts or parts of peanuts. Use of this method to examine a large number of individual peanuts proved the extreme heterogeneity of the problem. A few highly contaminated peanuts in a sample could raise the toxin level of that sample above limits deemed acceptable. One peanut with 1,000,000 parts per billion (ppb) of aflatoxin would contain enough toxin to cause a 100 ppb assay in a sample taken from 10 kilograms of peanuts. Whole carloads were condemned because of a handful of bad peanuts.

Peanuts were the first crop to be investigated, but evidence soon accumulated that implicated cottonseed. Trout in Oregon

developed liver cancers after having consumed feed that contained cottonseed meal. Since cotton was also a crop studied at the SRRC, work intensified on this commodity. An entirely new method for analysis of aflatoxin in cottonseed or cottonseed products was devised by Pons. The method won him the USDA's Superior Service award and is now official for both AOAC and AOCS. Rather than the time-consuming (four-day) procedure used for the initial survey on peanuts, analysts were able to complete assays in less than three hours. Minicolumns were developed for quick qualitative assays and instrumentation was introduced to improve quantitation. The densitometer is now the workhorse of aflatoxin analysis. High pressure liquid chromatography was introduced at SRRC for aflatoxin separation and quantitation. Derivatization of the already highly fluorescent aflatoxins enhanced that fluorescence and increased sensitivity. With better methods for analysis, toxins were found much more often. The problem still has no easy solution. The mold has been ubiquitous. Field conditions in the irrigated deserts of the Southwest were conducive to toxin formation in cottonseed and the drought stressed fields of the Southeast made fungal invasion and toxin formation in peanuts and corn a common occurrence. What was to be done?

If you can't prevent its formation, why not treat the toxin? SRRC initiated a major research program on solvent extraction and chemical detoxification of peanut or cottonseed meals. Henry Vix, Homer Gardner and Stan Koltun were the engineers involved. When peanuts or cottonseed were de-oiled, the oil was virtually toxin free while toxin levels nearly doubled in the oil-free meal. Meals had to be detoxified. The most fruitful approach to meal detoxification was ammoniation. Peanut or cottonseed meals were treated with gaseous ammonia under pressure with heat—a procedure developed at SRRC. The procedure required less than an hour contact time of meal with the ammonia vapors. Toxin levels were reduced from 300-1000 ppb to around 1-3 ppb. Moreover, treated meals had a nutritive value almost equal to that of non-treated, nonaflatoxin controls. Almost 20 years of research time and money went into extensive feeding trials conducted by the cottonseed crushing industry and USDA. Test results obtained proved the efficacy of the ammoniation procedure for meals. Model studies on ammonia treatment of aflatoxin without the meal matrix showed that the chemical structure was irreversibly changed by the treatment. Tests for mutagenicity, closely associated with carcinogenicity, indicated a 450-fold reduction for the ammonia-altered compound from that of aflatoxin B_1. This giant research effort by industry and government—both USDA and FDA—is an example of real research cooperation over many, many years. Treatment by ammoniation is approved domestically in several states for in-state use and is used extensively abroad. A petition for final approval for interstate use in the U.S., however, is still under review by the FDA.

Practical research in the 1970s was complemented by basic studies on how the fungus makes aflatoxin—aflatoxin biosynthesis. Joan Bennett, currently on the faculty at Tulane University and the president-elect of the American Society for Microbiology, served for two years as a postdoctoral fellow at SRRC. Her training was as a fungal geneticist. She isolated pigmented mutants of *Aspergillus parasiticus*; some of these pigments were later identified as aflatoxin precursors by Lee. It is these well-researched mutants that are the backbone of some of the current sophisticated biological studies now under way at SRRC.

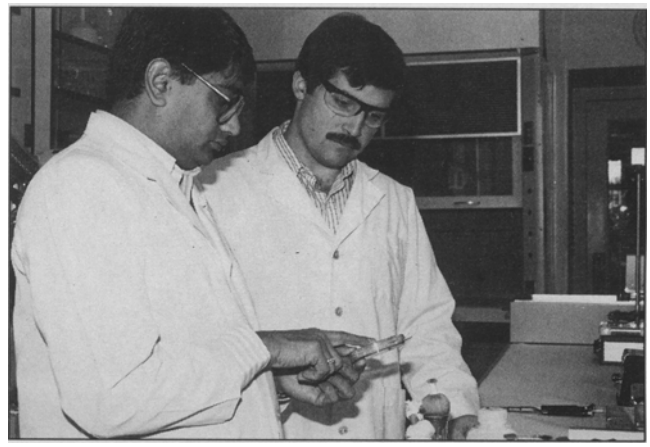
Yes, molecular biology and genetic engineering have become important tools at SRRC. Biology has made deep inroads where traditional chemistry once prevailed. Young "super scientists" have taken over research on the problem of aflatoxin. They bring their newly developed skills and expertise for a fresh approach to problem solution. Phrases such as "Southern or Northern blot" or "gene library" are now common at SRRC.

Maren Kilch (left) and Peter Cotty inspect plants that are part of experiments to determine environmental factors affecting aflatoxin production in cottonseed.

The new researchers ask, "Why not alter the fungus?" They reason that *A. parasiticus* can be genetically engineered to no longer produce aflatoxin. How? Deepak Bhatnagar and Ed Cleveland are taking the approach that removal of the genes, or altering the genes, that make the enzymes that make the toxin would remove the source of the problem. Bhatnagar has succeeded in the difficult isolation of the enzyme responsible for the conversion of one of the aflatoxin precursors found in the late stages of the pathway to aflatoxin B_1. The enzyme is a methyltransferase. His report was the first to describe the purification to homogeneity of an

enzyme involved in toxin synthesis. This methyltransferase was used to prepare an antibody to be used as a probe for gene identification. The enzyme was isolated from one of the mutants produced by Bennett. Bhatnagar also identified O-methyl-sterigmatocystin as the last known precursor in the biosynthetic pathway. Yet another enzyme, an oxidoreductase that catalyzes the conversion of O-methyl sterigmatocystin to aflatoxin B_1 also has been identified by these scientists. Cleveland, research leader for Food and Feed Safety at SRRC, is midway toward "getting out" the gene that makes this enzyme. One gene/one enzyme is the rule. Thus a single gene could control the release of this key enzyme. Once the gene is located, it could be removed or the mechanism by which the gene is responsible for the production of the enzyme could be altered. Cleveland stated in a recent interview that this process could take five years of research. One goal of this research is to produce large amounts of a genetically engineered fungus—one that will not make aflatoxin. This innocuous fungus could then compete with the potent toxin-producing strains for a niche in the environment. That environment could be a cornfield in Illinois, a cotton field in Arizona, or even a peanut field in Georgia.

Another young SRRC scientist, plant pathologist Peter Cotty, is taking a slightly different approach to biological control of aflatoxin. Cotty reasons that natural nontoxin producers could be used in competition with fungi that do produce aflatoxins. On trips to Arizona, Cotty has collected a large number of cotton bolls contaminated with *A. flavus*. Some strains are toxin producers and some are not. He has selected the nontoxin producers that are extremely virulent—that is, strains that grow faster than the toxinproducing strains. His greenhouse experiments with these virulent nonaflatoxin-producers have been highly successful. When cotton bolls were inoculated with these nontoxigenic strains prior to or at the same time as inoculation with toxigenic strains, aflatoxin in cottonseed was reduced to very low levels. He feels that application of these aggressive, nontoxigenic, biocompetitive strains in the field could lead to a drastic reduction in aflatoxin contamination. Experiments are in progress in Arizona.



Deepak Bhatnagar (left) and Ed Cleveland investigate the biochemical mechanisms of aflatoxin production.

Other young SRRC scientists propose alteration of the cotton plant. Again, genetic engineering is proposed—this time to introduce genes that code for a resistant trait. Jay Mellon is following up on results reported by Susan McCormick during her recent postdoctoral tour at SRRC. She determined the presence of a large molecular weight material, probably a glycoprotein, in the seedcoats of cottonseed at a particular stage in the seed's development. The material does not stop fungal growth but does inhibit synthesis of aflatoxin. Mellon is following through on the difficult task of purifying the material to homogeneity so that an antibody to it can be developed and used as a probe. Then begins the tedious task of finding that one gene in cotton that codes for this compound. Once that goal is reached, it is entirely possible that the cotton plant could be "engineered"—that is, made resistant with its own built-in defense mechanism.

Chip Zeringue's interest is also in improving the cotton plant so that it will be more resistant to fungal invasion. He found that a number of phytoalexins are formed in response to injury and/or fungal challenge. Phytoalexins are substances of plant origin that defend against foreign invaders. Locating the genes responsible for production of these substances involved in cotton defense mechanisms is a formidable task; but, again, the new techniques of molecular biology (familiar to the new group) may accomplish the task. In other highly creative studies, Zeringue has demonstrated fungal and toxin reduction from a new source. He has found volatile elicitors and gaseous phytoalexins involved in fungal/plant interactions. Volatile compounds have been identified that are released from damaged cotton tissue. These compounds elicit resistance chemicals in cotton tissues remote from the site of injury. Results could lead to the development of procedures to enhance natural evolution of volatile signals, or to the development of methods to apply volatile chemicals directly to plants in order to induce natural resistance chemicals in other plants "under attack" by the fungal pest. Other natural volatiles from cotton tissues have been identified that are directly inhibitory to fungal growth and/or toxin production. These volatiles could have tremendous value in crop protection during storage or in the field. As one of his research goals, Zeringue has chosen the development of plant protection by increased phytoalexins or beneficial volatiles.

Drought stress is conducive to toxin formation in the field. In recent field studies in Arizona, Maren Klich found that a narrow range

of water potential in cotton at the time of flowering predisposes the plant to increased susceptibility to *A. flavus* in seeds at harvest. Water potential is a measure of plant water stress. Her field studies on mode of entry of the fungus into cotton have verified that the fungus can enter through natural openings such as nectaries of either flowers or young bolls. Her research in the cotton fields of Arizona has interested growers and made them cognizant of the possibility of early season control of the fungus. Breeders may now try to develop nectariless varieties of cotton. At SRRC, Klich is using DNA technologies to help classify toxigenic fungi.

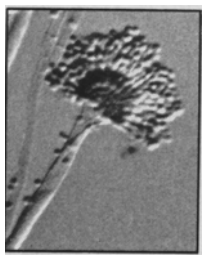
Lee has joined the young, tougher scientists in travelling to Arizona. (Travel and research assistance are supported by funds from the National Cottonseed Products Association (NCPA) with Lynn Jones as director of research.) Lee related current field studies on the position of toxin-containing seed inside a cotton lock back to the earlier observations on the heterogeneity of toxin contamination in cottonseed and peanut samples where the position of the seeds relative to each other was not known. Not only are toxin-containing seeds distributed unevenly in a sample of cottonseed, the distribution of toxin-containing seed to nontoxin seed in a lock is also heterogeneous. Seeds with toxin contents well over 100,000 ppb can exist in a lock where adjacent seeds have no toxin. The observation that relatively few seeds are the problem-causers points to the need for development of novel means of control. Growers can afford only a limited number of insecticide or fungicide applications to prevent fungal invasion; they cannot treat a whole field again and again to get rid of just a few bad seeds. Therefore, sophisticated and precise, innovative biotechnological solutions are needed that will alter the plants so that they are more fungal resistant, or alter the fungus so that it is not so dangerous an adversary.

In another research group at SRRC, sophisticated and innovative technologies now aid SRRC engineers Bob Hron and Sam Kuk in their development of a novel two-step alcohol extraction of cottonseed flakes. Alcohol can be produced from a renewable resource whereas solvents such as hexane are petroleum based. Kuk has added a reverse osmosis membrane unit, an important part of their two-step process. Reverse osmosis is a high pressure process in which a membrane retains low molecular weight materials such as aflatoxin. The goal is to produce a meal that

is free of oil, gossypol, and aflatoxin and high in nutritive value. The oil should be light. Hron and Kuk are making exciting strides toward this goal. Again, NCPA provides financial support and intense interest.

Increased funding at SRRC in the 1960s was generously supplied by Congress for aflatoxin research. Internal funds were shifted in the late 1970s to give a boost to biological research at SRRC. That shift in funds, with Alex Ciegler in charge of Food & Feed Safety, allowed for the hiring of two young scientists (Mellon and Klich). New funds came later in the mid-1980s to add to SRRC's brain power (Bhatnagar, Cleveland and Cotty). They were brought on board by Eivind Lillehoj. It is these plant biochemists, mycologists and plant pathologists who are doing the different, innovative aflatoxin research at SRRC. All research in Food & Feed Safety at SRRC has the solution of the aflatoxin problem as its ultimate goal. Even though the engineers have a more diverse program, aflatoxin removal is an important segment of that program.

Partly because of last year's drought and increased toxin levels detected, the NCPA, the corn industry and the peanut industry are asking for new funds for USDA research to eliminate the aflatoxin problem. They are confident that one of the many new approaches will be successful. The young scientists at SRRC are giving these new molecular biology techniques a real chance to solve a problem that has plagued us for nearly 30 years.



Contamination issues, technology

Aflatoxin made major news in U.S. agricultural circles last year as growing conditions led to outbreaks in various crops. In this report, Douglas L. Park and Henry Njapau of the Department of Nutrition and Food Science at the University of Arizona, Tucson, describe recent history on aflatoxin and talk about methods and instruments used to detect its presence in raw materials.

The scientific literature is replete with reports concerning the actual or probable occurrence of mycotoxin in foods and acute and subacute poisonings of man and animals after the ingestion of such foods. Aflatoxins, potent carcinogenic and toxic metabolites produced by the fungal species *Aspergillus flavus* and *A. parasiticus*, can contaminate animal feeds as a result of the currently unavoidable invasion by the molds before and during harvest, or because of improper storage of feeds. The need to limit aflatoxin in feeds is based on two major concerns: (a) the adverse effects of aflatoxin-contaminated feeds on animal health and productivity, and (b) the presence of aflatoxin residues or toxic metabolites in animal tissues used as human foods. U.S. crops most susceptible to unavoidable aflatoxin contamination are corn, peanuts and cottonseed. Although the overall incidence and levels are low, numerous surveys of animal feeds in the U.S. have shown that specific regions consistently have high preharvest aflatoxin contamination. The Southwest with cottonseed and the Southeast with corn are the areas in the United States most adversely affected by aflatoxin contamination. The 1988 corn crop from the Midwest and South, however, has shown unusually high levels of aflatoxin. This contamination has highlighted the importance of having a good program to monitor aflatoxin levels in agricultural products and of having adequate analytical tools. Also, the high aflatoxin contamination levels prompted the Food and Drug Administration (FDA) to re-evaluate current action levels for aflatoxins for corn. The primary goal of an effective food safety monitoring program is the protection of human health and the enhancement of food resources.

TABLE 1

Current Aflatoxin Action Levels Established by the Food and Drug Administration (μg/kg, ppb)

Human foods (except milk)	20.0
Milk	0.5
Animal feeds (except cottonseed meal)	20.0
Cottonseed meal (used for mature beef, swine and poultry rations)	300.0

Regulatory programs

The manner in which the FDA managed risks from aflatoxin is well documented. Current aflatoxin action levels for human foods and animal feeds are presented in Table 1. Through the years, however, when unusually high levels of aflatoxin occurred, the agency was required to revise the action levels previously established to minimize aflatoxin risks associated with interstate commerce or shipment of aflatoxin-contaminated products of the new crop and preserve an adequate food/feed supply for that particular year. As a result of the high levels of aflatoxin in the 1988 corn crop, FDA revised the action levels for that crop and has also in-