The Agribusiness Project (TAP)

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Chile peppers and mycotoxin contamination: Problems and solutions Final Report for the Agribusiness Project

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1 Contents

1 Lists of Acronyms and Definitions

DAP	Di-ammonium phosphate
EU	European Union
ELISA	Enzyme Linked Immunosorbent Assay test
N	Nitrogen
ng/g	Nanograms per gram
ppb	Parts per billion
PPD	Plant Population Density
Rs	Pakistan Rupee
TAP	The Agribusiness Project
USAID	United States Agency for International Development
USDA	Unites States Department of Agriculture
VC	Value Chain

2 Executive Summary

The dried chili industry in Pakistan has previously been one of the major success stories for smallholders throughout semi-arid areas under low-tech irrigation. Smallholders often lack the means to improve their crops with modern methods, and dried chilies have been a significant part of their annual income. However, during the past 10 years, food safety regulations have come into play in international markets that have been enforced with the result of limiting the participation of Pakistani dried chilies.

This has caused major economic disruptions due to rejection of finished product at markets in the EU and the USA where significant transaction costs have been incurred. This resulted in a strongly risk averse attitude and very important losses as Pakistan lost the bulk of the dried chili markets to other competitors more able to produce toxin free chili powder. The organoleptic qualities of Pakistani chili powder are considered superior by markets, but with unacceptable toxin levels, substitutes have had to be found.

This report is concerned with one specific problem: Aspergillus infection and subsequent poisonous fungal metabolites in chili and the remedies appropriate to different production regions. As all farms have differing limiting factors, environmental constraints and access to resources, a generalized observation of the chili industry in Pakistan is not practical. In order to address the market challenges of the chili industry, the stakeholders within the value chain need to address the toxicity levels during production and post-harvest handling.

The toxins in question are well known mycotoxins including at least 20 forms of aflatoxins (B_1, B_2, G_1, G_2), of which B_1 is most highly toxic, produced by *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus* and Ochratoxin A, a toxin produced by *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Penicillium verrucosum*, which is one of the most abundant food-contaminating mycotoxins resulting from end product metabolites of fungi.

These fungi are ubiquitous in the environment and often infect mature grains and fruits from spores and grow rapidly, producing toxic metabolites. However, due to the nature of fungi, not all members of the *Aspergillus* genus produce these extremely toxic end products of respiration and growth. Aflatoxins are present in distressed crops with pod or grain moisture higher than 7% where the spores can germinate and produce mycelial growth and subsequently, toxic metabolites.

Mycotoxins are also found in meat, eggs and milk caused by feeding contaminated feeds to animals. Fortunately, toxin producing *Aspergillus* forms are the exception rather than the rule. This makes it possible to manipulate the field environment and incur conditions that favor non-toxic strains of *Aspergillus* which pose no harm to humans, animals or the environment.

Plants under stress from under irrigation or over irrigation, virus infections, mite infestation, insect damage to fruit pods, root diseases, foliar diseases, insufficient mineral nutrition, over-fertilization with N, particularly Urea forms, weed pressure, and general poor husbandry are susceptible to becoming hosts to toxin producing strains of *Aspergillus*. The remedies for such a wide range of poor practices are complex, but manageable using local resources and changes to cultural practices.

There are a number of ways to control the presence of toxic metabolites or mycotoxins in foods and chili in particular and the following interventions are recommended to achieve the gold standard of less than 2 ng/g of any aflatoxins in any product:

1. Continuous improvement of seed using specialized methods that are simple and effective on the farm level.
2. Changes in soil preparation and plant population density to achieve optimal spacing and rectangularity.
3. Reductions in costs for unnecessary high amounts of fertilizers that promote heavy vegetation at the expense of fruit production and encourage foliar diseases. Aspergillus can feed and develop on dead leaves that accumulate when vegetation is too lush.
4. Introduction of ELISA testing at the farm gate to check toxicity levels and develop traceability protocols.
5. Changes in harvesting practices, better screening of hand labor, and use of appropriate biologically stable barrier between soil and drying chili.
6. Introduction of competitive exclusion methods whereby atoxic strains of the fungus are identified and cultivated. The private sector should be encouraged to take up the business of producing atoxic inoculant of *Aspergillus* strains to protect crops in Pakistan.

3 Background

The dried chili industry in Pakistan has previously been one of the major success stories for smallholders throughout semi-arid areas under low-tech irrigation. Smallholders often lack the means to improve their crops with modern methods, and dried chilies have been a significant part of their annual income. However, during the past 10 years, food safety regulations have come into play in international markets that have been enforced with the result of limiting the participation of Pakistani dried chilies.

This has caused major economic disruptions due to rejection of finished product at markets in the EU and the USA where significant transaction costs have been incurred. This resulted in a strongly risk averse attitude and very important losses as Pakistan lost the bulk of the dried chili markets to other competitors more able to produce toxin free chili powder. The organoleptic qualities of Pakistani chili powder are considered superior by markets, but with unacceptable toxin levels, substitutes have had to be found.

It has been said pending validation that Pakistan has been losing 100 million USD per year in lost sales for more than a decade due to contaminated chili powder and exclusion from EU markets. This does not consider the negative externalities of the problem such as idle and expensive machinery, lost jobs, idle transport infrastructure, loss of tax income for the state, and all the myriad and incredible losses that have occurred over the past ten years since the markets were lost.

A common thread in the understanding that came out of meetings with stakeholders is that aflatoxins are mainly a post-harvest and drying problem and that solutions should be concentrated on that specific area of the value chain.

However, this is a misconception. The fungus *Aspergillus* is ubiquitous in the environment and is present in all fields where it is a natural part of the microbial ecosystem. Some strains of the same species are toxigenic and some strains are atoxic, making them harmless. However, those strains that produce toxic metabolites called aflatoxins (there are many, of varying degrees of toxicity) are extremely harmful to humans and animals used for food, such as corn or peanut meal used to feed cattle and chickens.

An assessment of the toxicity levels for dried chilies was conducted during the period of assignment to develop appropriate remedies for different production regions.

4 CONSTRAINTS AND CHALLENGES

Problem 1

It is logical to begin with seed production to start the crop with the best available genetic resistance that can be found within a reasonable time. Pakistani smallholders and even large growers do not buy seed from breeders, but rather save seed from year to year. This has significant disadvantages including risks of using virus infested seed or bacterial disease infected seed, and a lack of selection for superior traits that would protect the crop on a genetic level and improve organoleptic qualities.

Problem 2

Incorrect applications and usage of pre-plant fertilizers contribute to hidden losses in uniformity and productivity of chili plantings. Many smallholders growing chili for sale to industry lack a good idea of necessary fertilization practices and few soil analyses are available. It is generally known that in many areas of Pakistan where chili is produced that the soil pH is high and microelements such as zinc are lacking. Very few producers differentiate between solid effective practices or simply following advice from vested interests such as fertilizer dealers and salesmen.

Problem 3

The main source of fertilizer nitrogen is stated to be Urea. Urea in Solanaceous crops has specific and problematic actions in the tropics that are apparent in chili, eggplant, tomato and others. Urea management is necessary for a variety of reasons to enhance yields and quality of chili. It is stated by growers and processors that growers use excessive amounts of manures and fertilizer nitrogen (N) to the detriment of the chili crop. This has to do with the quality of the Urea, irrigation schemes and the natural photoperiod in tropical latitudes during the growing season.

Problem 4

Stakeholders are unaware of the toxicity problem within and among the value chain, or they are reluctant to ameliorate the problem with cold chains, selection methods, or sampling and rejection of contaminated lots. Aflatoxins are not an especially difficult group of toxins to detect qualitatively, but do require sophisticated instruments such as high performance liquid chromatography or gas chromatography to properly quantitate. At the farm gate, the problem is most acute as the product of many farms can comprise a single lot delivered by a consolidator for delivery to the processor.

If a heavily contaminated lot of chili is mixed with sound chili, or if samples are taken improperly, considerable costs can be incurred by the processor and others in the value chain to

rid the system of toxic materials and recoup costs for buying, transporting, testing, etc. The European Union's aflatoxins regulations for peanuts, tree nuts, dried fruit, spices, and cereals include limits as low as 4 ppb total aflatoxins and 2 ppb for aflatoxin B_1. Some EU member states have also separately established their own maximum limits on aflatoxins.

Problem 5

There is substantial need to improve chili harvest methods. At present, chili harvest is conducted with several passes over the field to gain as many red ripe chilies as possible. Workers are said to be generally female with little training or guidance other than to pick red chilies. This can be improved in several different ways, including simple eyesight tests to make sure workers can tell the difference between infected and sound chili. If a worker cannot see well due to uncorrected vision such as myopia, they cannot distinguish good product from bad in a time efficient operation.

Another method to improve quality would be to provide more training to judge the difference between obviously at risk chili and sound chili. This will require simple harvest aids that are easily obtainable in Pakistan. For example, all chilies, whether good or bad, are combined into a singular harvest container. This results in overly infected general chili crops when heavily contaminated chili spread spores and mycelium of toxigenic *Aspergillus* and as the cliché goes, "one bad apple spoils the entire barrel".

Problem 6

Aspergillus is a wide-spread microbe in the natural environment. It feeds and spreads on dead plant material, but fortunately only a few types of specific species are toxigenic. There is no practical way to avoid infection with the organism, but there are methods to reduce the potential for toxicity called competitive exclusion. The ideas and concepts behind competitive exclusion are simply that when one organism is able to out-compete another related organism in the same ecological niche, the organism that does not compete well essentially disappears.

5 RECOMMENDATIONS

5.1 Short term Interventions

Recommendation 1: Plant Selection

All farmers are generally very familiar with their crops, even on an individual plant basis. It is a good idea to select superior plants and protect them from virus vectors such as aphids and mite infestation by choosing obviously strong, well-adapted plants and covering them with a lightweight floating row cover made from edge-bonded polypropylene by many manufacturers including DuPont, called Reemay in trade terms. The insect proof grade is made of very soft, lightweight material that allows light, air and water to infiltrate, but does not allow the passage of even the smallest insects such as flower thrips.

Special care can be taken to make sure the selected plants are well watered and have adequate and complete mineral fertility and sufficient fungicide and insecticide treatments for seed crop use. This implies that the fruit will be used for future superior seed and not sold on the market, because many times the levels of pesticides used for seed crops are far more than food crops.

For example, the nematicide called oxymyl is used frequently for nematode control in Solanaceous crops, but the dosage is difficult to control without expensive and difficult to calibrate equipment. With only singular plants to treat, the dosage is greatly simplified, and it is within the means of any smallholder to protect high performance plants from the deleterious effects of nematodes and certain insects as the active ingredient is a systemic insecticide/nematicide.

During the season, farmers can take exceptionally good care of seed production plants and use the seeds from these protected pods for future crops. In this way, only a few years must pass before an equilibrium of phenotype can be observed and superior plants can continue to be selected each year. Smallholders have been doing this for the entire history of agriculture, but new methods of plant protection make the techniques even more valuable.

This continuous selection of superior plant types is a simple technique and assists the competitiveness of the Pakistani cultivars of chili for drying. Processors have very explicit guidelines for quality, ranging from color to Scoville derived heat units, and it behooves growers to select their crops to produce a quality product that can be used directly by processors without wastage or over-processing.

Each processing operation costs a certain amount of money and competitive processors know that each time they must re-process to remove foreign material, off-types or poor quality, the margin of profitability subsides. Moreover, superior chili types can often resist infection by fungal organisms giving a degree of biological protection from toxic strains of *Aspergillus*.

Recommendation 2: Appropriate Use of Fertilizers

Chili crops in particular and vegetable crops in general do not need nearly as much fertilizer nitrogen as commonly applied by smallholders. Larger growers often have application equipment and are cognizant of the high costs of over dosage of nitrogenous fertilizers on chili crops, and as a result, their problems are not as severe. However, smallholders rarely have application equipment and often simply scatter Urea to suit past experience or in a random pattern, which is the wrong approach for several reasons.

Urea as a synthetic material is derived from a very old process using natural gas from petroleum developed in Germany over 100 years ago, called the Haber-Bosch process. When Urea is applied to soil it immediately takes up water and hydrolyzes to ammonia, which is toxic to plants. Ammonia is also volatile, and losses to the environment can occur unless the material is well watered in or worked into the soil. However, the toxicity of the resulting ammonia is dealt with by the plant by forming carbon skeletons within the plant vegetation to sequester the ammonia until it can be nitrified to a form usable by the plant.

This causes very high amounts of leafy vegetation to be produced and causes a wide range of problems in crop production. Lush vegetation does not dry as fast as normal foliage and can lead to bacterial and fungal infection and disease, many times causing crop loss. Overly high leaf area does not allow for good penetration of sunlight and air, and, therefore, impedes normal photosynthesis and reduces crop yields.

Excessive fertilizer applications cause considerable crop damage in smallholder fields since much traffic is required to apply the fertilizer, which causes considerable compaction, and disproportionate amounts of water are used to incorporate the Urea before volatization losses can occur. For chili crops, a program of small frequent doses of Urea should be applied not to exceed 115 kilos of elemental N per hectare or 25 grams of Urea per square meter per crop. It is best to apply Urea in a sequence of 5 grams per square meter per 10 days. In this way, farmers can apply small doses at frequent intervals and be assured that they are not wasting fertilizer or using excessive amounts that reduce final yields and increase disease incidence.

Moreover, high vegetative production and subsequent dying and dropping of leaves to the ground, coupled with frequent irrigation encourages fungi such as *Aspergillus* to grow and proliferate, making infection via spores of flowers and young fruit pods almost inevitable. If these *Aspergillus* species are of the toxic strains, much harm can be done and can even render the crop poisonous and unsalable.

This presents a public health safety issue as farmers may be unaware of the nature of the toxicity and sell the crop, thereby contaminating entire lots of otherwise clean chili pods. Since aflatoxins are measured in nanograms per gram (ng/g) or parts per billion (ppb), it only takes a small amount to make an entire lot toxic and a human health hazard.

Recommendation 3: Soil Preparation and Plant Density

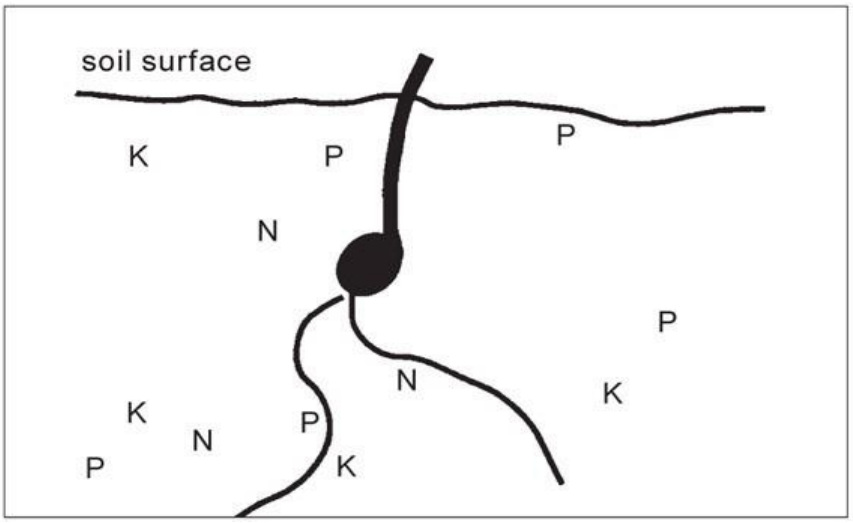
Planting systems for chili in Pakistan generally have very good to optimal plant population density (PPD). It is important that the transplants be planted when they are of uniform size and weight to diminish the chances for sub-standard populations, weed intrusion, and dis-uniformities in growth. One way to improve this early uniform growth is to change the application of di-ammonium phosphate (DAP) typically used by most smallholders and large growers alike in Sindh.

According to local sources, DAP is generally spread over the soil surface and plowed in to mix it thoroughly with the soil. However, this is a poor use of the material. Phosphate fertilizers form two species of phosphoric acid, H_3PO_4 and H_2PO_4, which move only minutely within the soil, and may only move by diffusion 1-2 mm per year from the source of placement irrespective of soil type or rainfall.

This means that there is a poor probability that young roots from newly transplanted chili plants will encounter sufficient labile phosphorus (P) to increase root growth and get the plants off to a strong and steady start. It is important in all vegetable crops that there be no checks in growth, or yield will suffer. Since the chili plants are planted in a dual row configuration on basic raised beds, it is a better strategy to apply the DAP in a band sufficiently close to the transplant roots to enable immediate supplies of labile P to encourage root growth and subsequent shoot and leaf coverage. The method is simple and works well when planned correctly. Otherwise, consider the current situation; 1 hectare of land contains approximately 1 million kilos of soil in the topmost 25 cm where plant growth takes place. If 300 kilos are perfectly and evenly distributed throughout this 1 million kilos of soil, it would be a very lucky root indeed that encounters the available P and gets off to a good start. The probability of all the plants encountering sufficient P for uniform and superior growth is essentially nil when such a small amount of DAP is mixed with such a large amount of soil.

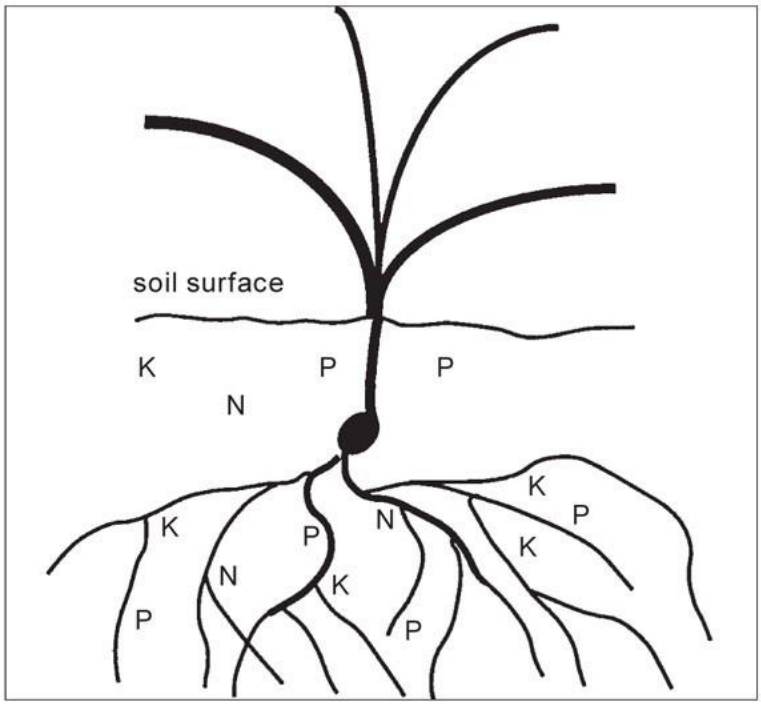
For example, if the soil analysis calls for 60 kilograms of elemental P per hectare, it translates to 300 kilos of DAP. The DAP material can be spread in a band no closer than 5cm from the plant roots and 5cm below the plant roots or transplant depth. After the transplants are planted, and before they are watered in, a shallow trench can be dug using ordinary hand tools 5cm to the inside of the ridge and 5cm below the transplant depth. See Figures 1 and 2 below which illustrate the concept of a concentrated band of mineral fertility for early use by the plant after seeding or transplanting.

5.1.1 Figure 1: DAP Application Near Plant Roots



Unless DAP is banded in close proximity to young chili transplant roots, the available or native mineral fertility is diffuse and difficult to absorb by primary roots.

5.1.2 Figure 2: Banded DAP



Banded DAP (5 cm below roots and 5 cm to one side) provides concentrated mineral fertility for early use by young chili transplants.

A measure containing the adequate amount is best used in field circumstances when DAP applications are made by hand. Scales are unlikely to be widely available, and only a few grams are necessary per linear meter. With a few minutes of practice by workers, with perhaps a string to use as guidance at first, the operation goes quickly and smoothly. In this example, a dual row configuration with rows 1.5 meters center to center requires 15 grams DAP per linear meter for each row of plants on the ridge or a single band of 30 grams or less between the ridges. Such a measure is often found with a small plastic scoop often sold with pesticides or in local markets.

If the transplants are large and have a large root mass, which is unlikely, a single application of 30 grams per linear meter can be made down the center of the ridge using the same techniques and tools. It is important to acquire a simple measure that holds the required amount (in this case 15 grams of DAP) to avoid over-dosage or ineffective dosages.

Care must be taken to place the DAP at least 5 cm from and below the root system of the transplant as the ammonium portion of DAP is toxic and the fertilizer has a high osmotic potential which can dry out young roots quickly and kill them. Also, the DAP application will raise the soil pH until some of the ammonia is nitrified; this may cause localized but temporary microelement deficiencies or leaf discoloration. After application of DAP, a heavy initial watering will ensure transplant survival and fast growth in the younger stages. This insures fast uniform growth, good fruit set, and lowered probability of infection by toxic strains of *Aspergillus* spp. The risk of damaging young transplants is minimal compared to the fast, uniform start that all vegetable crops need to reach their maximum productive potential.

5.2 Medium term Interventions

Recommendation 4: ELISA Test Kits

At present, there are simple Enzyme Linked Immunosorbent Assay (ELISA) test kits that indicate the presence of mycotoxins with simple methods and materials. Some are sufficiently sophisticated for quantitative measurement of the most common aflatoxins, while others only indicate the presence of dangerous mycotoxins. For example, one firm sells a simple qualitative test kit that can be used under rustic conditions and detect mycotoxins at levels as low as 10 ppb. The main advantages of such a method used at or near the collection center or farm gate are:

- Speed – Results in as little as 3 minutes
- Simplicity — No special training or equipment required
- Durability — Long shelf life; requires no refrigeration
- Economics — Inexpensive first step in your testing protocol
- Versatility — Choice of two cutoff procedures; 10 ppb and 20 ppb

This specific test kit is sold by Vicam, a US firm located at:

Address : 34 Maple Street
Milford, MA 01757
USA
T el: +1 800.338.4381, +1 508.482.4935
Fax: +1 508.482.4972
Email: vicam@vicam.com

This firm specializes in ELISA test kits. The specific model referred to here is: **AflaCheck**.

AflaCheck

AflaCheck™ is a qualitative one-step test kit for the detection of aflatoxin.

AflaCheck uses highly specific reactions between antibodies and aflatoxin to detect aflatoxin in a variety of samples. The test strips can be used to detect the presence of aflatoxin at two different cutoff levels: 10 ppb or 20 ppb, depending on the protocol followed. The costs of testing at the farm gate can be managed using a dedicated vehicle designed with a covered area to conduct sensitive lab tests quickly and efficiently.

It is estimated that 4 hours are required to properly conduct a random sample test to concur with modern statistical methods, crush the chili samples and go through the simple ELISA analysis. The lot can then be positively identified with simple technology such as bar-coding to denote the GPS coordinates of the crop, the grower by name, and the variety and level of contamination by aflatoxin and other contaminant such as mud or foreign materials.

In this way, a traceability record is generated and kept for future use and analysis. A simple computer utilizing a GPS program for mapping farms and chili lots and an SQL database will more than suffice for these needs. It is also a simple matter to generate unique barcodes and affix labels to the bags or bales of dried chili for continuous traceability.

Very little equipment is required to make field personnel responsible for primary quality control, and even the ELISA testing strips do not need refrigeration lower than 20^{o} Celsius. However, the back of the van or vehicle serving as a field laboratory must be somewhat climate controlled for efficiency and a low degree of contamination by technicians. The work is not delicate, but it is not especially robust either and some poisonous material must be used and stored in the lab-vehicle such as reagent grade methanol and triple distilled water. Any refrigeration necessary can be easily accommodated using CNG vehicles and appropriate equipment.

Recommendation 5: Harvesting

The most important idea that can be implemented is to provide dedicated workers with cotton harvest bags to remove infected chilies from the field immediately after harvesting from the plant. These workers would be given vision tests to ascertain visual acuity of at least 20/20 to spot small areas of sporulation, unsound fruit, or infections and work damage, and would take care to place them in the cotton harvest bags. The workers' vision must be good enough to catch infected pods in the field at harvest and remove them in order to not contaminate the good chili. This obviously requires more cost, but the cost is a small expense when returns are sufficiently high. If these obviously bad chilies are mixed with the sound chili crop, the total quality is seriously diminished. These workers should only harvest bad fruit and concentrate on keeping the sound fruit separate from fruit that will deteriorate its quality.

In addition, there is a practice of spreading harvested chilies on bare soil, cow dung, and any hard surface available. This is totally unacceptable in terms of food safety and international standards for sound food products that meet purity and toxicity minimums. A better idea is to treat a single ridge with ethephon (Ethrel; Bayer Crop Science) to ripen the entire fruiting plant at once. Ethrel is a plant growth regulator used to ripen fruit and causes flowering in many different crops. The material breaks down on exposure to the plant into ethylene, a natural plant hormone that accelerates ripening and causes the entire plant to ripen at once. It is commonly used in processing tomatoes and other crops. It is harmless to workers and the environment.

However, ethephon will cause the entire row of chilies to ripen at once, allowing a single once-over harvest along one ridge or bed with a dual row of chili plants. After the plants are completely harvested in this row, it is possible to remove the plants, level and smooth the ridge or bed and use this area for effective drying. A material is required to provide a barrier to spores and mycelium of *Aspergillus* already present in the soil. Harvested chilies should not be allowed to touch the bare soil. This presents a conundrum; materials sufficiently robust to protect the drying chilies are expensive. However, there is an alternative with decades of use all around the world for drying fruit; continuous paper used for drying raisins. A picture is included in the Annex.

There is 50 pound test Kraft paper available in various widths that holds up to the environment and does not require collection after the chilies are dried because the paper is biodegradable and can be simply plowed into the soil. It is at first impermeable, but cannot stand up to heavy rain, so it is urgent to get the chilies harvested and dried as efficiently as possible.

In fact, it is completely logical to spray the ripening agent Ethrel to ripen all the chilies to the deep red favored by processors and perform a single one time destructive harvest to gain warmer days with bright sun to dry the chilies quickly to ±7% moisture, which is out of the *Aspergillus* infection danger zone.

It is possible that this Kraft paper for raisin drying is already available in Pakistan as there are large quantities of raisins exported each year, and some are exported to Japan. Japan is the gold standard for food safety, and if the Japanese accept Pakistani raisins, there must be a mechanism whereby the raisins are protected from soil contact during drying or are dried on the vine and machine harvested, although this is a very new technique. In any event, the odds are good that the materials and methods already exist in Pakistan, and it is important to focus on finding them and using them for the project's purposes.

5.3 Long term Interventions

Recommendation 6: Developing non-toxic strains of *Aspergillus*

Recent advances have been made in Africa to eliminate *Aspergillus* toxicity in maize and other crops due to several disastrous episodes that killed hundreds of people from eating infected grain in large amounts. Those not affected acutely have chronic health problems such as liver dysfunction, cirrhosis and liver cancer. Recently, a group of scientists from different institutions and agencies got together and developed the solution of growing atoxic *Aspergillus* on corn or sorghum and spreading or inoculating the non-toxic material throughout the fields prior to flowering. The cultivated atoxic *Aspergillus* was grown on energy rich grain and produced mycelium and fruiting bodies and sporulated rapidly in the field, completely overwhelming the toxic strains of *Aspergillus*. This is now a commercial enterprise, selling 10 kg packets to farmers to improve the food safety of the community and to allow a sustainable effort to come into being and solve the serious problem.

The atoxic strains of *Aspergillus* were isolated from the wild in Nigeria and have been used with great success in Kenya, Uganda, and much of Francophone Africa. There is similar scope to develop this technology in Pakistan. Recently in Karachi, I presented this idea to the scientists gathered for our focus group meeting. The idea was solidly affirmed as technologically viable. There were molecular biologists present able to discuss the necessary means to distinguish atoxic strains of *Aspergillus* using polymerase chain reaction technology to screen for toxic and atoxic metabolites. Alternatively, it is possible to simply import via air prepared non-toxic *Aspergillus* grown on grain sorghum for field testing until Pakistani production can be started. The chemical engineering is not a problem for Pakistan, and the mycology is not difficult. However, a great deal of funding will be required in order to produce very large quantities of sterilized grain with atoxic *Aspergillus* for field inoculation to solve the proximate cause of the problem and regain lucrative markets in Europe, Japan, and the USA.

Photo 1: African maize farmer with commercial AflaSafe™ atoxic *Aspergillus*

6 ANNEXURES

Source: All annexures are downloaded from
http://www.icrisat.org/aflatoxin/anamika_Effects_Aflatoxins.asp#top.

6.1 Annex 1: Estimation of Aflatoxins in Food Samples

Effects of Aflatoxins on Human and Animal Health

Aspergillus flavus and *Aspergillus parasiticus* are the molds that produce Aflatoxin. These fungi can produce their toxic compounds on almost any food that will support growth. The metabolites produced by these fungi are named AFB_1, AFB_2, AFG_1, and AFG_2, all which occur naturally. Of the four, AFB_1 is found in highest concentrations followed by AFG_1, AFB_2 and AFG_2. *Aspergillus flavus* only produces AFB_1 and AFB_2 and *Aspergillus parasiticus* produces these same metabolites along with G_1 and G_2. Aflatoxins are secondary metabolites that are highly mutagenic and toxic for human and also animal health.

Effect on human health:

Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Such exposure is difficult to avoid because fungal growth in foods is not easy to prevent. Even though heavily contaminated food supplies are not permitted in the market place in developed countries, concern still remains for the possible adverse effects resulting from long-term exposure to low levels of aflatoxins in the food supply . Evidence of acute aflatoxicosis in humans has been reported from many parts of the world , namely countries, like Taiwan, Uganda, India, and many others. The syndrome is characterized by vomiting, abdominal pain, pulmonary edema, convulsions, coma, and death with cerebral edema and fatty involvement of the liver, kidney, and heart. Conditions increasing the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that favor fungal development in crops and commodities, and lack of regulatory systems for aflatoxin monitoring and control.

The expression of aflatoxin related diseases in humans may be influenced by factors such as age, sex, nutritional status, and/or concurrent exposure to other causative agents such as viral hepatitis (HBV) or parasite infestation. Ingestion of aflatoxin, viral diseases, and hereditary factors have been suggested as possible aetiological agents of childhood cirrhosis. There are evidences to indicate that children exposed to aflatoxin breast milk and dietary items such as unrefined groundnut oil, may develop cirrhosis. Malnourished children are also prone to childhood cirrhosis on consumption of contaminated food. Several investigators have suggested aflatoxin as an aetiological agent of Reye's syndrome in children in Thailand, New Zealand etc. Though

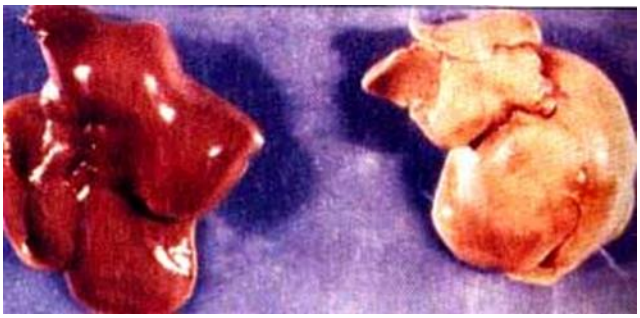
there is no conclusive evidence as yet. Epidemiological studies have shown the involvement of aflatoxins in Kwashiorkor mainly in malnourished children. The diagnostic features of Kwashiorkor are edema, damage to liver etc. These out breaks of aflatoxicosis in man have been attributed to ingestion of contaminated food such as maize, groundnut etc. Hence it is very important to reduce the dietary intake of aflatoxins by following the procedures for monitoring levels of aflatoxins in foodstuffs.

Effects on animals:

There are differences in species with respect to their susceptibility to aflatoxins, but in general, most animals, including humans, are affected in the same manner.

Acute toxicity:

Acute toxicity is less likely than chronic toxicity. Studies have shown that ducklings are the species most susceptible to acute poisoning by aflatoxins. The LD50 of a day old duckling is 0.3mg/kg bodyweight.



The principal target organ for aflatoxins is the liver. After the invasion of aflatoxins into the liver, lipids infiltrate hepatocytes and leads to necrosis or liver cell death. The main reason for this is that aflatoxin metabolites react negatively with different cell proteins, which leads to inhibition of carbohydrate and lipid metabolism and protein synthesis. In correlation with the decrease in liver function, there is a derangement of the blood clotting mechanism, icterus (jaundice), and a decrease in essential serum proteins synthesized by the liver. Other general signs of aflatoxicosis are edema of the lower extremities, abdominal pain, and vomiting.

Chronic toxicity:

Animals which consume sub-lethal quantities of aflatoxin for several days or weeks develop a sub acute toxicity syndrome which commonly includes moderate to severe liver damage. Even with low levels of aflatoxins in the diet, there will be a decrease in growth rate, lowered milk or egg production, and immunosuppression. There is some observed carcinogenicity, mainly related to aflatoxin B_1. Liver damage is apparent due to the yellow color that is characteristic of

jaundice, and the gall bladder will become swollen. Immunosuppression is due to the reactivity of aflatoxins with T-cells, decrease in Vitamin K activities, and a decrease in phagocytic activity in macrophages.

Cellular affects:

Aflatoxins are inhibitors of nucleic acid synthesis because they have a high affinity for nucleic acids and polynucleotides. They attach to guanine residues and for nucleic acid adducts. Aflatoxins also have been shown to decrease protein synthesis, lipid metabolism, and mitochondrial respiration. They also cause an accumulation of lipids in the liver, causing a fatty liver. This is due to impaired transport of lipids out of the liver after they are synthesized. This leads to high fecal fat content. Carcinogenisis has been observed in rats, ducks, mice, trout, and subhuman primates, and it is rarely seen in poultry or ruminants. Trout are the most susceptible. In fact, 1ppb of aflatoxin B_1 will cause liver cancer in trout. Carcinogenisis occurs due to the formation of –8,9-epoxide, which binds to DNA and alters gene expression. There is a correlation with the presence of aflatoxins and increased liver cancer in individuals that are hepatitis B carriers.

Specific species affects:

PIG: Aflatoxicosis in swine is mainly due to the fact that corn is a large part of their diet Piglets are more susceptible than adults are and it has been shown that feeding sows AFM1, during lactation, can cause stunted growth in litter. The groundnut cake implicated in the suspected aflatoxicosis of pigs had aflatoxin levels estimated at over 20000 mg /kg.

Large doses of aflatoxins have been shown to produce hepatic necrosis. The effects of aflatoxicosis can be compounded with the addition of stress. This can lead to ataxia and induced hemorrhaging. The hemorrhaging is due to the prolonged blood clotting time caused by lack of Vitamin K utilization.

Poultry: Aflatoxicosis has the same toxic effects in poultry as it does in mammals. The aflatoxicosis problem was mainly noticed in 1960 in turkey poults in England with the outbreak of a disease known as Turkey X disease. The affected birds lost appetite, became lethargic, and died within 7 days after onset of symptoms. Livers of diseased turkeys were severely damaged. A similar disease of ducklings and also chickens was reported in Kenya. Later it was discovered that it was the contaminated groundnut meal which was included in their diet was the main cause of the disease. The groundnut meal was contaminated with a mold called as Aspergillus flavus and the disease was caused by the toxin produced by the fungus while growing on the meal, the toxin is named as aflatoxin. A dose of 0.25ppm in turkey poults and ducklings impairs growth, and a dose of 1.5ppm in broilers and 4ppm in Japanese quail also has a negative effect on growth. An increase in blood clotting time increases the susceptibility of the carcass to bruising even at doses below that to have an effect on growth. In poultry, aflatoxins impair the availability

of bile salts, which decreases Vitamin D_3 production. This causes a decrease in the absorption of fat-soluble vitamins. Aflatoxins also decrease the production of Vitamin A in the liver, and it has secondary effects such as decreased blood calcium levels, bone strength, tissue and serum tocopherol levels. This decrease in tocopherol levels can lead to Vitamin A and E deficiencies.

Cattle: The effects of aflatoxicosis in ruminants are similar to those of non-ruminants. Calves are more sensitive than yearlings and adults. The first symptomatic effect of continuous ingestion of toxic groundnut meal in calves was a reduction in growth rate followed by unthriftiness, and loss of appetite, and the terminal symptoms were characterized by severe tenesmus. A dose of 0.2mg/kg body weight can cause a decrease in weight gains. This can be attributed to poor feed utilization and a dramatic increase in alkaline phosphate activity in the rumen. Chronic aflatoxicosis in adult ruminants can cause anorexia, drying and peeling of the skin on the muzzle, rectal prolapse, and abdominal edema. Aflatoxicosis has also been shown to cause decreased fertility, abortion, and lowered birth weights in sheep.

6.2 Annex 2: Properties of Aflatoxin and It Producing Fungi

By S.V. Reddy and Farid Waliyar

Many agricultural commodities are vulnerable to attack by a group of fungi that are able to produce toxic metabolites called mycotoxins. Among various mycotoxins, aflatoxins have assumed significance due to their deleterious effects on human beings, poultry and livestock. The aflatoxin problem was first recognized in 1960, when there was severe outbreak of a disease referred as "Turkey 'X' Disease" in UK, in which over 100,000 turkey poults were died. The cause of the disease was shown due to toxins in peanut meal infected with Aspergillus flavus and the toxins were named as aflatoxins.

Natural occurrence:

Food products contaminated with aflatoxins include cereal (maize, sorghum, pearl millet, rice, wheat), oilseeds (groundnut, soybean, sunflower, cotton), spices (chili, black pepper, coriander, turmeric, ginger), tree nuts (almonds, pistachio, walnuts, coconut) and milk.

Physical and chemical properties:

Aflatoxins are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites by the fungus *Aspergillus flavus* and *A. parasiticus* on variety of food products. Among 18 different types of aflatoxins identified, major members are aflatoxin B_1, B_2, G_1 and G_2. Aflatoxin B_1 (AFB_1) is normally predominant in amount in cultures as well as in food products. Pure AFB_1 is pale-white to yellow crystalline, odorless solid. Aflatoxins are soluble in methanol, chloroform, actone, acetonitrile. *A. flavus* typically produces AFB_1 and AFB_2, whereas *A. parasiticus* produce AFG_1 and AFG_2 as well as AFB_1 and AFB_2. Four other aflatoxins M_1, M_2, B_2A, and G_2A which may be produced in minor amounts were subsequently isolated from cultures of *A. flavus* and *A. parasiticus*. A number of closely related compounds namely aflatoxin GM_1, parasiticol and aflatoxicol are also produced by *A. flavus*. Aflatoxin $M_1$and M_2 are major metabolites of aflatoxin B_1 and B_2 respectively, found in milk of animals that have consumed feed contaminated with aflatoxins.

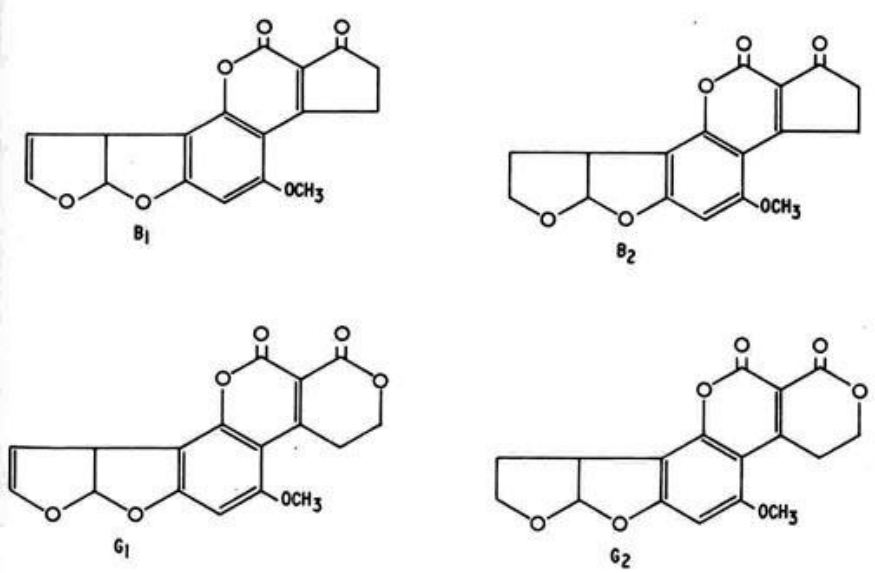


Fig. 1 Structures of aflatoxins B_1, B_2, G_1, and G_2.

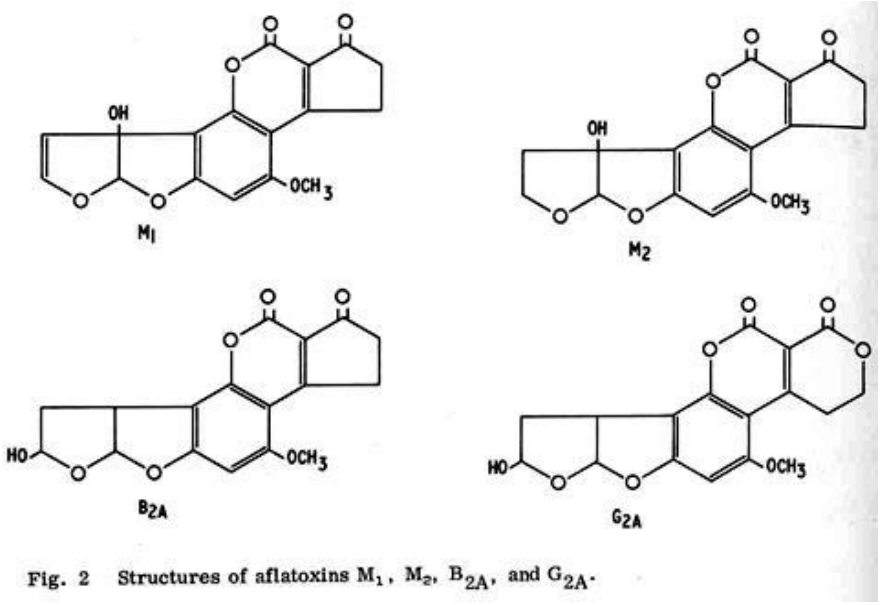


Fig. 2 Structures of aflatoxins M_1, M_2, B_{2A}, and G_{2A}.

Aflatoxins are normally refers to the group of difuranocoumarins and classified in two broad groups according to their chemical structure; the difurocoumarocyclopentenone series (AFB1, AFB2, AFB2A, AFM1, AFM2, AFM2A and aflatoxicol) and the difurocoumarolactone series (AFG1, AFG2, AFG2A, AFGM1, AFGM2, AFGM2A and AFB3). The aflatoxins display potency of toxicity, carcinogenicity, mutagenicity in the order of AFB1 > AFG1 > AFB2 > AFG2 as illustrated by their LD50 values for day-old ducklings. Structurally the dihydrofuran moiety, containing double bond, and the constituents liked to the coumarin moiety are of importance in producing biological effects. The aflatoxins fluoresce strongly in ultraviolet light (ca. 365 nm); B1 and B2 produce a blue fluorescence where as G1 and G2 produce green

fluorescence.

Chemical and physical properties of aflatoxins

Aflatoxin	Molecular formula	Molecular weight	Melting point
B_1	$C_{17}H_{12}O_6$	312	268-269
B_2	$C_{17}H_{14}O_6$	314	286-289
G_1	$C_{17}H_{12}O_7$	328	244-246
G_2	$C_{17}H_{14}O_7$	330	237-240
M_1	$C_{17}H_{12}O_7$	328	299
M_2	$C_{17}H_{14}O_7$	330	293
B_{2A}	$C_{17}H_{14}O_7$	330	240
G_{2A}	$C_{17}H_{14}O_8$	346	190

Chemical reactions of aflatoxins

The reaction of aflatoxins to various physical conditions and reagents have been studied extensively because of the possible application of such reactions to the detoxification of aflatoxins contaminated material.

Heat:

Aflatoxins in dry state are very stable to heat up to the melting point. However, in the presence of moisture and at elevated temperatures there is destruction of aflatoxin over a period of time. Such destruction can occur either with aflatoxin in oilseed meals, aflatoxin in roasted peanuts or aflatoxin in aqueous solution at pH 7. Although the reaction products have not been examined in detail it seems likely that such treatment leads to opening of the lactone ring with the possibility of decarboxylation at elevated temperatures.

Alkalis:

In alkali solution hydrolysis of the lactone moiety occurs. This hydrolysis appears to be reversible, since it has been shown that recyclization occurs following acidification of a basic solution containing aflatoxin. At higher temperatures (ca. 100^oC) ring opening followed by

decarboxylation occurs and reaction may proceed further, leading to the loss of the methoxy group from the aromatic ring. Similar series of reactions also seems to occur with ammonia and various amines.

Acids:
In the presence of mineral acids, aflatoxin B1 and G1 are converted in to aflatoxin B2A and G2A due to acid-catalyzed addition of water across the double bond in the furan ring. In the presence of acetic anhydride and hydrochloric acid the reaction proceeds further to give the acetoxy derivative. Similar adducts of aflatoxin B1 and G1 are formed with formic acid-thionyl chloride, acetic acid-thionyl chloride and trifluoroacetic acid.

Oxidizing agents:

Many oxidizing agents, such as sodium hypochlorite, potassium permanganate, chlorine, hydrogen peroxide, ozone and sodium perborate react with aflatoxin and change the aflatoxin molecule in some way as indicated by the loss of fluorescence. The mechanisms of these reactions are uncertain and the reaction products remain unidentified in most cases.

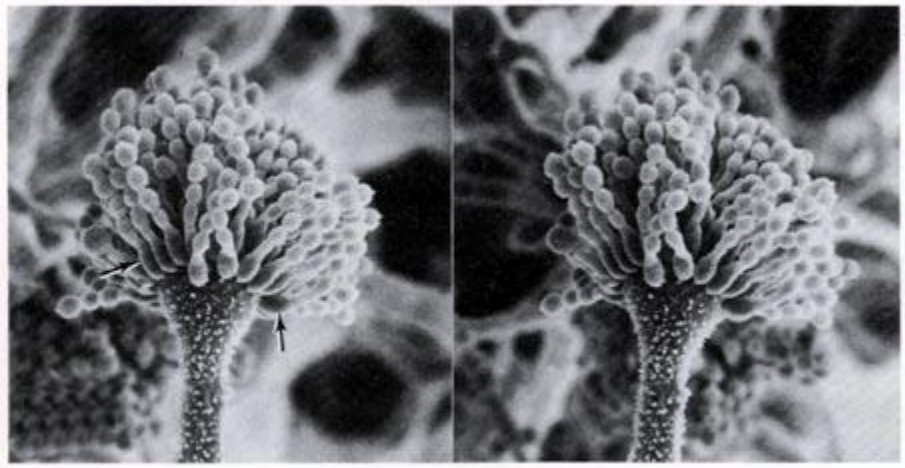
Reduction:

Hydrogenation of aflatoxin B1 and G1 yields aflatoxin B2 and G2 respectively. Further reduction of aflatoxin B1 by 3 moles of hydrogen yields tetrahydroxyaflatoxin. Reduction of aflatoxin B1 and B2 with sodium borohydride yielded aflatoxin RB1 and RB2 respectively. These arise as a result of opening of the lactone ring followed by reduction of the acid group and reduction of the keto group in the cyclopentene ring.

Biology of A. flavus Link ex Fr. and A. parasiticus Spear:

The two fungi A. flavus and A. parasiticus are closely related and grow as a saprophyte on plant debris of many crop plants left on and in the soil. They are distributed worldwide, with a tendency to be more common in countries with tropical climates that have extreme ranges of rainfall, temperature and humidity. Members of the genus Aspergillus are characterized by the production of non-septate conidiophores, which are quite distinct from hyphae and which are swollen at the top to form a vesicle on which numerous specialized spore-producing cells, known as phialides or sterigmata are borne either directly (uniseriate) or on short outgrowths known as metulae (biseriate). Sometime difficulty may arise especially to determine because the primary sterigmata are tiny and are easily obscured by spores or other sterigmata. Colonies of A. flavus are green-yellow to yellow-green or green on Czapek's agar. They usually have biseriate sterigmata; reddish-brown sclerotia are often present, conidia are finely roughened, variable in size and oval to spherical in shape. Colonies of A. parasiticus dark green on Czepak's agar,

remain green with age. Sterigmata are uniseriate, sclerotia are usually absent; conidia are coarsely echinulate, uniform in shape, size and echinulation.

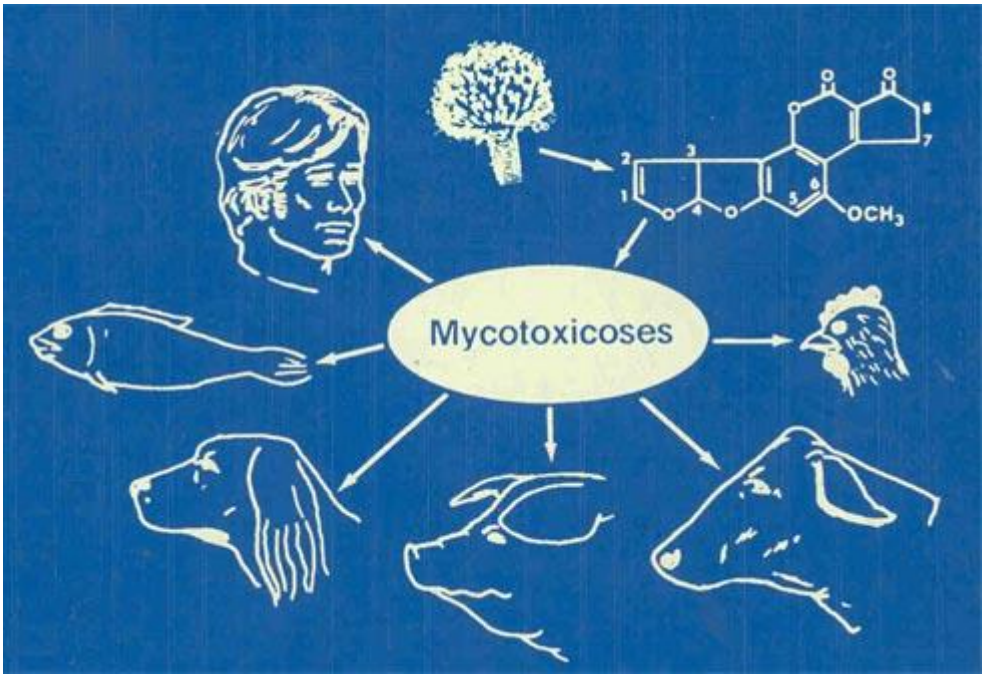


Terminal portion of a conidiophore of A. flavus showing the basal portion of the vesicle and distribution of radiation phialides (arrows). X 1000.

Phialides and chains of conidia of A. parasiticus illustrating basipetal development of conidia. Those at the base of the chains (arrows) are least mature. X 3000.

Effect of A. flavus and aflatoxins contamination:

Deteriorate in grain quality due to A. flavus growth and become unfit for marketing and consumption. In groundnut, seed and non-emerged seedling decay and aflaroot disease was observed due to fungus attack. Aflatoxins contamination in grain poses a great threat to human and livestock health as well as international trade. According to FAO estimates, 25% of the world food crops are affected by mycotoxins each year. And also crop loss due to aflatoxins contamination costs US producers more than $100 million per year on average including $ 26 million to peanuts ($69.34/ha).


Mycotoxicoses
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6.3 Annex 3: ISM-Mycored International Conference Europe 2013

The ISM-MycoRed International Conference Europe 2013 will be held from **27** to **31 May 2013** in Apulia, **Martina Franca, Italy.**

The conference is organized by **Antonio F. Logrieco and Angelo Visconti**, chairmen, **CNR ISPA** – National Research Council, Institute of Science of Food Producsion, in cooperation with **ISM** - International Society for Mycotoxicology.
This appointment will close a cycle of conferences successful performed in Austria, Malaysia, South Africa, Argentina and Canada from 2009 up to now. It will represent an opportunity to strengthen cooperation and presenting the final results and outcomes arising from MycoRed project.

Oral and poster presentations, satellite meetings and exhibitions will be organized to favor fruitful meetings among scientists, industrial representatives coming from several countries worldwide.
You all are warmly invited to attend the conference presenting the results of your research progress, and enjoy the flavors and colors of our **Mediterranean Apulia**!

The Conference website is on line!

Antonio F. Logrieco & Angelo Visconti