TOOL BOX

Developing aflasafe™

Joseph Atehnkeng, j.atehnkeng@cgiar.org, Joao Augusto, Peter J. Cotty, and Ranajit Bandyopadhyay

Farmers discuss the aflatoxin menace. Photo by J. Atehnkeng, IITA.

Aflatoxins are secondary metabolites mainly produced by fungi known as *Aspergillus flavus, A. parasiticus*, and *A. nomius*. They are particularly important because of their effects on human health and agricultural trade. Aflatoxins cause liver cancer, suppress the immune system, and retard growth and development of children. Aflatoxin-contaminated feed and food causes a decrease in productivity in humans and animals and sometimes death. Maize and groundnut are particularly susceptible to aflatoxin accumulation, but other crops such as oilseeds, cassava, yam, rice, among others, can be affected as well. Aflatoxin accumulation in crops can lower income of farmers as they may not sell or negotiate better prices for their produce. Because of the high occurrence of aflatoxin in crops, many countries have set standards for acceptable aflatoxin limits in products that are meant for human and animal consumption.

Natural populations of *A. flavus* consist of toxigenic strains that produce variable amounts of aflatoxin and atoxigenic strains that lack the capability

Joseph Atehnkeng, aflasafe™ Country Manager; Joao Augusto and Ranajit Bandyopadhyay, Plant Pathologists, IITA, Ibadan, Nigeria; Peter J. Cotty, Research Plant Pathologist, USDA-ARS.

to produce aflatoxin. Carefully selected and widely distributed atoxigenic strains are applied on soil during crop growth to outcompete and exclude toxigenic strains from colonizing the crop. The biocontrol technology has been used extensively in the USA with two products AF36 and afla guard® available commercially. In Africa, aflasafe™ was first developed by IITA in partnership with the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) and the African Agriculture Technology Foundation (AATF). It is currently at different stages of development, adoption, and commercialization in at least nine African countries. Multiyear efficacy trials in farmers' fields in Nigeria have showed reduced aflatoxin concentration by more than 80%.

Survey to collect and dispatch samples

Product development begins with the collection of crop samples in farmers' stores across different agroecological zones in each country. Samples collected are mainly maize and groundnut because they are the most susceptible to aflatoxin accumulation at crop maturity, during processing, and storage. Soil samples are collected from fields where these crops were grown to determine the relationship between the *Aspergillus* composition in the soil and the relative aflatoxin concentration in the crop at maturity.

Import and export permits are required if crop and soil samples are shipped outside a country. The crop samples are analyzed for aflatoxin to obtain baseline information on aflatoxin levels in the region/country and the relative exposure of the population to unacceptable limits of aflatoxin.

Isolation and characterization of *Aspergillus* species

Aspergillus species are isolated from the crop samples to identify the non-aflatoxin-producing species of *A. flavus* for further characterization as biocontrol agents. The isolates are identified and grouped into L-strains of *A. flavus,* S_{BG}, *A. parasiticus*, and further characterized for their ability to produce aflatoxin by growing them on aflatoxin-free maize grain. Aflatoxin is extracted from the colonized grain using standard protocols to determine isolates that produce aflatoxin (toxigenic) and those that do not produce aflatoxin (atoxigenic). The amount of aflatoxin produced by toxigenic

Sorting isolates into Aspergillus species. Photo by R. Bandyopadhyay, IITA.

Aspergillus flavus, Unknown taxon S_{BG}, and Aspergillus parasiticus. Photos by J. Atehnkeng, IITA.

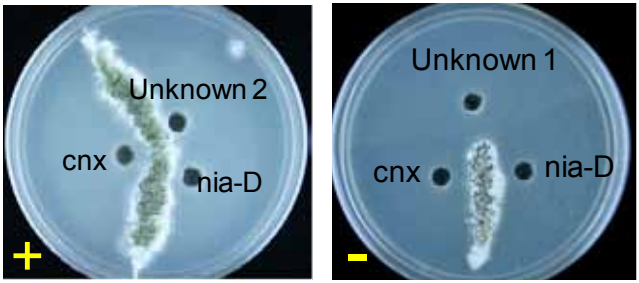
strains is usually quantified to determine the most toxigenic strains that will be useful for competition with atoxigenic strains.

Understanding genetic and molecular diversity

The genetic diversity of the atoxigenic strains is also determined molecularly by examining the presence or absence of the genes responsible for aflatoxin production in each strain. The absence of these genes explains why potential biocontrol isolates would not produce aflatoxin after release into the environment. Amplification of any given marker is taken to mean that the area around that marker is relatively intact, although substitutions and small indels outside the primer binding site may not be detected. Non-amplification could result from deletion of that area, an insertion between the primers that would result in a product too long to amplify by polymerase chain reaction (PCR), or mutations in the priming sites. Non-amplification of adjacent markers is probably best explained by very large deletions.

Identification of vegetative compatible groups

Vegetative compatible group (VCG) is a technique used to determine whether the highly competitive atoxigenic isolates are genetically related to each other. In nature *A. flavus* species that are genetically related belong to the same VCG or family; those that do not exchange genetic material belong to different VCGs. This is an important criterion for selecting a good biocontrol agent to ensure that the selected biocontrol strains do not "intermate" with aflatoxin-producing strains after field application. With this technique, the distribution of a particular VCG within a country or region is also determined.



Sorting isolates into VCGs: Positive (left) and negative (right) complementation. Photos by J. Atehnkeng, IITA.

A VCG that is widely distributed is likely to be a good biocontrol agent because it has the innate ability to survive over years and across different agroecologies. On the contrary, atoxigenic VCGs that have aflatoxin-producing members within the VCG are rejected; atoxigenic VCGs that are restricted to a few locations may also not be selected.

Initial selection

The in vitro test determines the competitive ability of the atoxigenic isolate to exclude the toxigenic isolate on the same substrate. The competition test is conducted in the laboratory by co-inoculating the most toxigenic isolate with atoxigenic strains on aflatoxin-free maize grains or groundnut kernels. Grains/kernels inoculated with the toxigenic strain or not inoculated at all serve as controls. After incubation and aflatoxin analysis, atoxigenic isolates that reduce aflatoxin by more than 80% in the co-inoculated treatments are selected for unique vegetative compatible grouping.

Selection of candidate atoxigenic strains and multiplication of inocula

aflasafe™ is composed of a mixture of four atoxigenic strains of *A. flavus* previously selected from crop samples. To select the four aflasafe strains, initially 8-12 elite strains belonging to atoxigenic VCGs are evaluated in large farmers' fields. Two or three strain mixtures, each with 4-5 elite strains, are released in separate fields by broadcasting at the rate of 10 kg/ha in maize and groundnut at about 30-40 days after planting. The atoxigenic strains colonize organic matter and other plant residues in the soil in place of the aflatoxin-producing strains. Spores of the atoxigenic strains are carried by air and insects from the soil surface to the crop thereby displacing the aflatoxin-producing strains. The four best strains to constitute aflasafe™ are selected based on their ability to exclude and outcompete the toxin-producing isolates in

Colonized maize (left) and groundnut during competition experiment. Photos by J. Atehnkeng, IITA.

Strain selection criteria followed in developing competitive aflatoxin biocontrol agents

Create a strain library

- Collect ~500 maize/groundnut samples from farmers' fields/stores from diverse agroecologies in the field
- Isolate *Aspergillus* species (about 10 per sample) from the crop samples in the lab

In the laboratory evaluate ~5,000 strains to select those that:

- Do not produce aflatoxin
- Are in VCG/SSR group with
  - Wide geographic distribution
  - No toxigenic member
- Have defects in one or more aflatoxin & CPA biosynthesis genes
- Outcompetes toxigenic strains

8-12 native strains selected for field tests

4 native strains formulated into the final product

After field application, select strains that have:

- Superior capacity to colonize, multiply and survive in soil
- Superior frequency of isolation from grains
- Superior capacity to reduce aflatoxin

Strain selection criteria followed in developing competitive aflatoxin biocontrol agents.

the soil and grain, move from the soil to colonize the maize grains or groundnut kernels in the field, and occur widely and survive longer in the soil across many agroecological zones. The use of strain mixture in aflasafe™ is likely to enhance the stability of the product as more effective atoxigenic strains replace the less effective ones in specific environments. The long-term effect is the replacement of the toxigenic strains with

Left: Broadcasting aflasafe™ in maize fields. Right: Broadcasting aflasafe™ in groundnut fields. Photos by J. Atehnkeng, IITA.

the atoxigenic VCGs over years.

Assessing relative efficacy of aflasafe™

Field deployment to test efficacy of aflasafe™ is carried out in collaboration with national partners and most often with the extension services of the Ministry of Agriculture. Awareness is created by organizing seminars with extension agents and farmers. During the meetings presentations are made on the implication of aflatoxin on health and trade thereby increasing their knowledge on the impact of aflatoxins. aflasafe™ is then introduced as a product that prevents contamination and protects the grains before they are harvested and during storage. Efficacy trials are carried out in fields of farmers who voluntarily agree to test the product. Field demonstrations on the use of aflasafe™ are supervised and managed by the extension agents and farmers. Farmers are trained not only on the biocontrol technology but also on other management practices that enhance better crop quality.

Farmers learn about aflatoxin management practices. Photo by R. Bandyopadhyay, IITA.

aflasafe™ in 5-kg containers at IITA. Photo by R. Bandyopadhyay, IITA.

Farmers are also educated on the need to group themselves into cooperatives, aggregate the aflasafe™-treated grains to find a premium market with companies that value good quality products. Market linkage seminars and workshops are organized between aflasafe™ farmers, poultry farmers, and the industries to ensure that the farmers get a premium for producing good quality grains and the industries get value for using good quality raw materials for their products.