Bundesanstalt für Landwirtschaft und Ernährung

AflaZ

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Projektträger Bundesanstalt für Landwirtschaft und Ernährung

MRI Max Rubner-Institut

JKI Julius Kühn-Institut Bundesforschungsinstitut für Kulturpflanzen

EAFF Eastern African Farmers Federation – Voice of all farmers in Eastern Africa

KALRO Kenya Agricultural & Livestock Research Organization

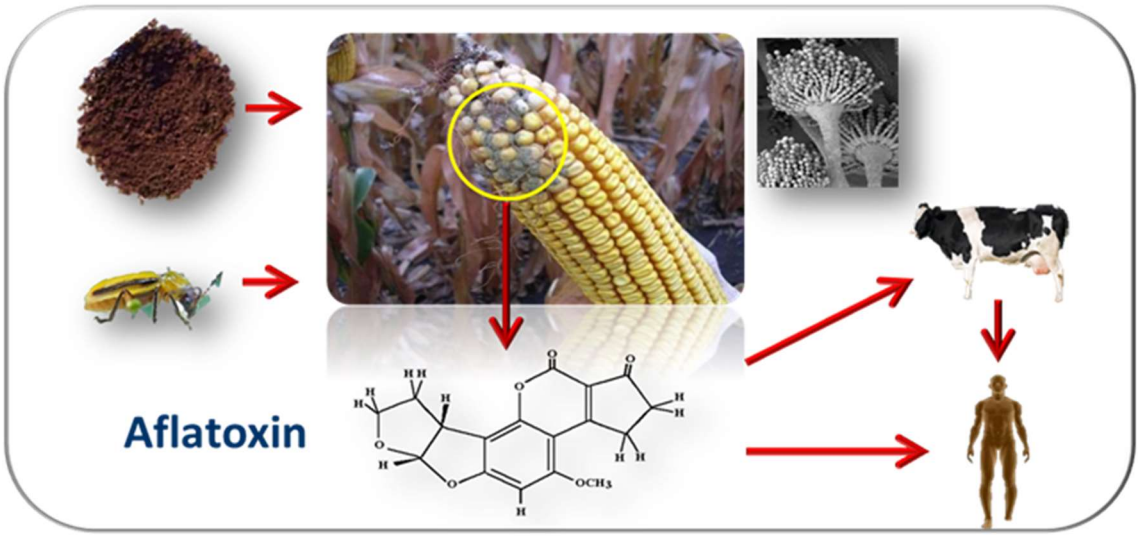
UNIVERSITÄT KOBLENZ · LANDAU

FRIEDRICH-LOEFFLER-INSTITUT FLI Bundesforschungsinstitut für Tiergesundheit Federal Research Institute for Animal Health

Project-Update

Project title:	Development and implementation of sustainable strategies to improve food-safety and retain nutritional values by reducing fungal infestation and aflatoxin contamination in the food-chain in Kenya as model region for Sub-Saharan Africa (AflaZ)
Geographical focus:	Kenya
Call reference:	Reference: 323-06.01-03-2816PROC11 Funding code: 2816PROC11
Cooperating partners:	Max Rubner-Institut; Julius Kühn-Institut; Friedrich-Loeffler-Institut; Universität Koblenz-Landau; Kenya Agricultural & Livestock Research Organisation; East African Farmers Federation
Duration:	1.10.2018 - 31.12.2022
Budget:	1.505.039,13 €

Diagram of the core topics that AflaZ focuses on:



Aim of the project:

The population of Kenya and other African countries are regularly exposed to aflatoxin levels far above recommended limits through the consumption of foods sometimes heavily contaminated with aflatoxins (especially the staple foods maize and milk). Nevertheless, the consumption of these products is steadily increasing. Children and sick people, in particular, are especially at risk from the health effects associated with mycotoxin ingestion.

The BLE-funded project, AflaZ, focuses on improving food safety and quality standards of milk, maize and derived products. Kenya was chosen as a model region because it is a high-risk area for aflatoxin contamination due to fungal infestation in the food sector. The AflaZ project will develop effective and sustainable methods to analyze and monitor fungal infestations and aflatoxin contamination, both in the field and in storage, in order to reduce them in a sustainable manner. In addition, the AflaZ research program includes extensive capacity building strategies. These include cooperation with local institutions, farmers, students and other stakeholders, thus enabling sustainable knowledge transfer (dissemination) that ensures cultural acceptance of the recommendations and sustainable integration of the new methods by the local population.

The following core topics are in focus of the AflaZ project:

• Isolation and identification of aflatoxin-producing fungi from maize and associated soil samples from Kenya, as well as their microbiological and molecular biological characterization for the development of effective and sustainable monitoring and prevention strategies.

• Analysis of the transfer of aflatoxin from the feed to cow's milk, so-called carry over, as well as analysis of a possible reduction/degradation in the aflatoxin content in the subsequent processing of the milk into cheese and yoghurt. Identification of an aflatoxin-specific biomarker in the blood of dairy cows and development of a suitable analysis method.

• Analysis of aflatoxin derivatives formed by the fungus or plant through metabolization that may contribute significantly to "masked" aflatoxin contamination; implementation of fast-screen tests for aflatoxin, and connection of the test with an APP for mobile aflatoxin analysis in the field.

• Determination of soil aflatoxin content and physico-chemical parameters as vitality factors for soil organisms and for maize plants, as well as field insects as pests on the maize plant and vectors of the spread of spores of aflatoxin-producing fungi.

• Training and guidance (capacity building) of Kenyan PhD students of the AflaZ project and farmers as multipliers of AflaZ research results. Strengthening of communication and cooperation between Kenyan partner institutions and other local research institutions. Dissemination of the project results via the eGRANARY platform and the PAEPARD blog spot, as well as recommendations to governmental and non-governmental organizations in Kenya. A TV documentary about the AflaZ project and topic-specific radio programs is developed and will be broadcast to reach broader sections of the population.

Results of third year of the project (2021):

The Corona pandemic has been causing restrictions in many living and working situations since the spring of 2020, including the work taking place as part of the AflaZ project in 2021. In addition, there were unusually heavy rains over an extended period in Kenya, which further complicated the work in some cases. Nevertheless, the project objectives did not change during the course of project implementation due to reorganization and compensatory measures. However, the work had to be adapted to the Covid19-related norms.

At the **Max Rubner-Institut** (MRI) in Karlsruhe, a comprehensive monitoring of the aflatoxin biosynthesis of *Aspergillus flavus* was carried out within the framework of **WP1** and the aflatoxins formed as well as precursors of aflatoxins were investigated. It was found that in addition to B aflatoxins, the fungus also forms aflatoxin M_1 and M_2, which are primarily known to be contaminants in milk and dairy products as degradation products of B aflatoxins by metabolism in cows. However, formation of M-aflatoxins by the fungus is poorly described. The results show that M-aflatoxins can also be formed in cereals such as maize and that it is therefore advisable to expand the aflatoxin monitoring of such foods to include M-aflatoxins. In addition, a relatively high concentration of certain precursors of aflatoxins was measured, especially aspertoxin and O-methylsterigmatocystin, compounds that are regularly not in research focus but already have shown toxic effects. Both compounds have the double bonds required for the carcinogenic effect of aflatoxin B_1 and could thus also have carcinogenic activity.

The work under **WP2** compared the genomes of different aspergilli to draw conclusions about the differences in aflatoxin biosynthesis and thus their impact on food safety of the affected products. A deletion was discovered in the aflatoxin gene clusters of *A. minisclerotigenes* strains, which includes the genes necessary for aflatoxin G biosynthesis. This may explain why *A. minisclerotigenes* strains isolated from Kenyan samples do not produce aflatoxin G, which has been described for the majority of these species. Furthermore, a stop codon was discovered in the aflatoxin gene cluster of an *A. parasiticus* strain, which explains the inability to form aflatoxin B_1 and G_1 but the simultaneous formation of aflatoxin B_2 and G_2 of this strain. These new research results have a high relevance for the evaluation of the safety of food contaminated by the mentioned fungi.

Competition trials with a mycoparasitic fungus, *Trichoderma afroharzianum*, and *Aspergillus flavus* in **WP3** were extended. The effectiveness in particular of an MRI isolate of *T. afroharzianum* as a biocontrol species was determined. Further analyses were conducted to investigate the role of mycotoxins in the mechanism of competition. In addition, initial competition experiments were also conducted with *Fusarium verticilloides*, another filamentous fungus that is pathogenic to humans and plants and often serves as a pioneer-species for other species that are not highly plant pathogenic, such as *A. flavus*. *F. verticilloides* forms the very toxic mycotoxin fumonisin and very commonly infects maize and other crops. During studies at MRI, corresponding *Fusarium* species were found in both maize and soil samples from Kenyan experimental fields.

The **Julius-Kühn-Institut** (JKI) has continued to chemically extract and analyse field associated and medical plants in **WP3.2**. In the new established BSL2 laboratory of the JKI in Berlin, which was completed and registered in March 2021, it was now also possible to work with the human pathogenic *Aspergillus flavus*. Results on the antifungal effect of the associated plants *Lippia adoensis* and *Ocimum gratissimum* could now also be confirmed for *A. flavus*. The influence of the origin and cultivation conditions on the ingredients and the antifungal effect was shown.

At **MRI Detmold**, methods for the determination of aflatoxins and their plant metabolites, which could possibly have an influence on the rapid test, were further established. The Kenyan PhD student Beatrice Tenge performed comparative measurements on maize samples using two rapid test systems (**WP4**). However, due to the travel restrictions, only limited contact with the farmers was possible, so that only limited samples could be analyzed on site in Kenya.

At the **University of Koblenz-Landau**, the occurrence of aflatoxins and *Fusarium*-toxins in soils from fields with maize cultivation in Germany was investigated in 2021 as part of **WP5**. In addition, the persistence of aflatoxins in soil was investigated in three scenarios: Photolytic degradation, microbial degradation, and degradation under sterile conditions and absence of light (control). In addition, soil samples from Kenya were analyzed by HPLC-fluorescence (HPLC-FL) and LC-mass spectroscopy (LC-HRMS). The mycotoxins analyzed were: Aflatoxins, ochratoxin A, deoxynivalenol, nivalenol, T-2 toxin, and zearalenone. A total of 319 samples were analyzed, corresponding to the four agricultural treatments, two depths and two positions. The survey conducted among Kenyan farmers (n=116) is currently being analyzed. Based on the statistically evaluated data of the survey, a model will be developed in the current year that relates the knowledge about mycotoxins to the occurrence of grain infections.

The Kenyan PhD student Ginson Riungu (**WP6**) sampled more than 100 insect species in Kenya and so far, the 3 species *Sitophilus zeamis, Carpophilus sp*. and *Camponotus nearcticus sp*. have been detected as spore carriers. A large part of the spores (60 %) is carried by the appendages of the insects (legs and antennae). The sampled spores are viable in cultures and 30 % of them are aflatoxigenic.

At the **MRI in Kiel**, the feeding trial with fungi-contaminated maize (MRI-KA-isolates), which began in November 2020, was completed in January 2021 as part of **WP7**. The production of yogurt from the milk obtained in the trial was started and the preparations for the subsequent analytical work required were largely completed. Yogurts were produced from the milk samples obtained and analyzed, demonstrating the stability of the toxin AFM_1 during yogurt production. In addition, Edam-style cheese was produced from the milk to map the fate of the *Aspergillus* toxin cyclopiazonic acid in this important food technology process.

At the **Friedrich-Loeffler-Institut** (FLI), 208 samples of serum were obtained (4 groups of 4 animals each, 13 points in time) during the animal experiment as part of **WP7**, which were analyzed for the biomarker aflatoxin-lysine by LC-MS/MS. Aflatoxin-lysine could not be detected in any of these samples, with a detection limit of 0.05 ng/mL. Furthermore, clinical chemistry parameters and selected electrolytes were determined in these samples. The analysis of these data is still pending.
The development of the HPLC method for the determination of aflatoxin M_1 (AFM_1) in urine was completed and the method validated. The method has a working range of 0.05 to 20 ng/mL with a detection limit of 0.2 ng AFM_1/mL urine. The recovery rate is 93 %. In the experimental samples (n=48), AFM_1 was detected only in the toxin-producing *Aspergillus flavus* group after 14 days of exposure (n=4), with concentrations ranging from 0.33 to 0.95 ng/mL.
Furthermore, these urine samples were analyzed for AFM_1 by ELISA. Also, by this method, AFM_1 was detected only in the above-mentioned samples, with concentrations ranging from 0.8 to 2.0 ng/mL.
The collected liver biopsies from the animal experiment were examined with respect to their lipid content and histologically. The determination of the liver transcriptome is currently being prepared. The final sequencing and analysis of the data are still pending.

The **Kenya Agricultural & Livestock Research Organization (KALRO), WP8**, managed AflaZ trial fields in 2021 in the three counties of Kisumu, Makueni and Kilifi, which are considered hotspots for aflatoxin contamination in maize. During the 2021 project period, the sample fields in Kilifi and Makueni were harvested by February and new maize fields were established from March to demonstrate the differences in the different farming practices (conservative

tillage, push and pull, use of *Trichoderma harzianum* and conventional tillage). In addition, the results from the trials conducted in previous years were further analyzed and evaluated in 2021: The effect of the four treatment methods on soil and corn plant contamination by *Aspergillus* species and other filamentous fungi, as well as on maize aflatoxin levels, was studied over a total of three cropping seasons. In addition, soil fertility was determined and analysis of nutrient-content was performed in the soil chemistry laboratories of KALRO Kabete. The pH of the soils collected during the baseline survey was determined.

The farmers involved in the project are supervised by the **East African Farmers Federation (EAFF), WP9**, for the entire duration of the project. The entire cultivation of maize, from sowing to harvesting and storage, was monitored in 2021. During the growth of the maize, the germination percentage of the crop was recorded, insects and pests were identified for early control, and weed control and rainfall patterns were monitored, as all these factors can affect the harvest. Storage and harvesting technologies developed under the project that can lead to reduced aflatoxin contamination of food and feed were demonstrated to farmers in on-site training sessions when possible, or otherwise disseminated in digital form. Results from the AflaZ project are also being incorporated into a currently produced AflaZ TV-documentary. The TV-documentary captures the activities of all partners. All project partners provided their own information including film footage to ensure comprehensive coverage of the entire project. As physical meetings in 2021 were limited due to Covid-19 restrictions, the e-GRANARY platform was used to disseminate information and reach farmers with relevant issues regarding AflaZ and contamination of the maize crop. The stakeholders involved are more and more aware of the problem of aflatoxin contamination in food and feed thanks to the educational work. Those farmers who already use the new technologies pass on their newly acquired knowledge to other farmers as multipliers and thus make a sustainable contribution to the broader application of this knowledge and technology.

The **AflaZ annual meeting** in 2021 was held online with active participation of all project partners.

Key statements and policy advice:

1) The AflaZ project focuses on the problem of infestation of maize by aflatoxin-producing fungi such as *A. flavus* and *A. parasiticus*. In the course of the investigations, as well as in recent studies, it has become apparent that *A. minisclerotigenes* also plays a significant role in the aflatoxin contamination of maize in Kenya. In addition, in studies of both maize and soil samples from Kenyan trial fields, various *Fusarium* species were also found. Therefore, it was decided to include selected *Fusarium* strains in the investigations, since *Fusarium* strains are often highly pathogenic to plants, infecting maize, cereals and other crops such as fruits and vegetables, producing toxic mycotoxins and often paving the way for less pathogenic species such as *Aspergillus*.
2) It is possible to effectively inhibit mycotoxin-producing fungi with a combination of synergistic acting inhibitory influences in storage and partly already in the field by using natural antagonistic fungi. In this context, the use of biocontrol strains (*A. flavus*) already used commercially, has to be assessed critically for various reasons. The alternative use of a commercial product with *Trichoderma harzianum*, which was investigated in AflaZ, shows good, but partly ambivalent results regarding the infestation with *A. flavu*s in the field. The use of an MRI isolate of *T. afroharzianum* as a biocontrol strain seems promising.
3) Antifungal plant ingredients from local Kenyan associated plants, as well as the amino acid arginine, which could be easily sprayed on, showed promise as another approach to inhibit aflatoxin-producing fungi.
4) Infestation and effectiveness of prevention methods (e.g., assertiveness of biocontrol species against toxin-producing species) can be tested and monitored in the laboratory using molecular ddPCR technology.
5) Rapid aflatoxin detection methods continue to be used and implemented in field trials in Kenya. A survey of farmers and demonstration of the rapid tests showed that a test with smartphone app evaluation is preferred. However, it also showed that due to the high cost of the required test strips, an app development does not seem to be sufficiently feasible and promising at this point in time. Discussions with individual company representatives regarding a cooperation to reduce the costs have not been promising so far. Further considerations should therefore be carried out in addition to the project, e.g. an organization and realization of maize tests for aflatoxins by EAFF.
6) A method for the determination of aflatoxin concentration in soil/plant was developed and successfully validated. The method was further optimized for the analysis of plant matrices (food) to be used in the study of the soil/plant system. The transmission rate of aflatoxin in cow milk is investigated in a feeding study, as well as a possible aflatoxin reduction in the downstream dairy products yogurt and cheese. In addition, a specific biomarker to measure aflatoxin exposure in dairy cows is also under development.
7) Maize fields are cultivated under conventional methods as well as with the methods developed in the AflaZ, the infestation with aflatoxin is compared and the farmers are trained in the new methods as well as knowledge about the health problems of aflatoxin intoxication. In this way, a sustainable aflatoxin reduction is possible and feasible. The training of Kenyan PhD students within the AflaZ project, partly also additionally of Erasmus PhD students from Kenya and partly in tandem with German PhD students, but also of farmers, ensures the multiplication of the AflaZ findings on site.