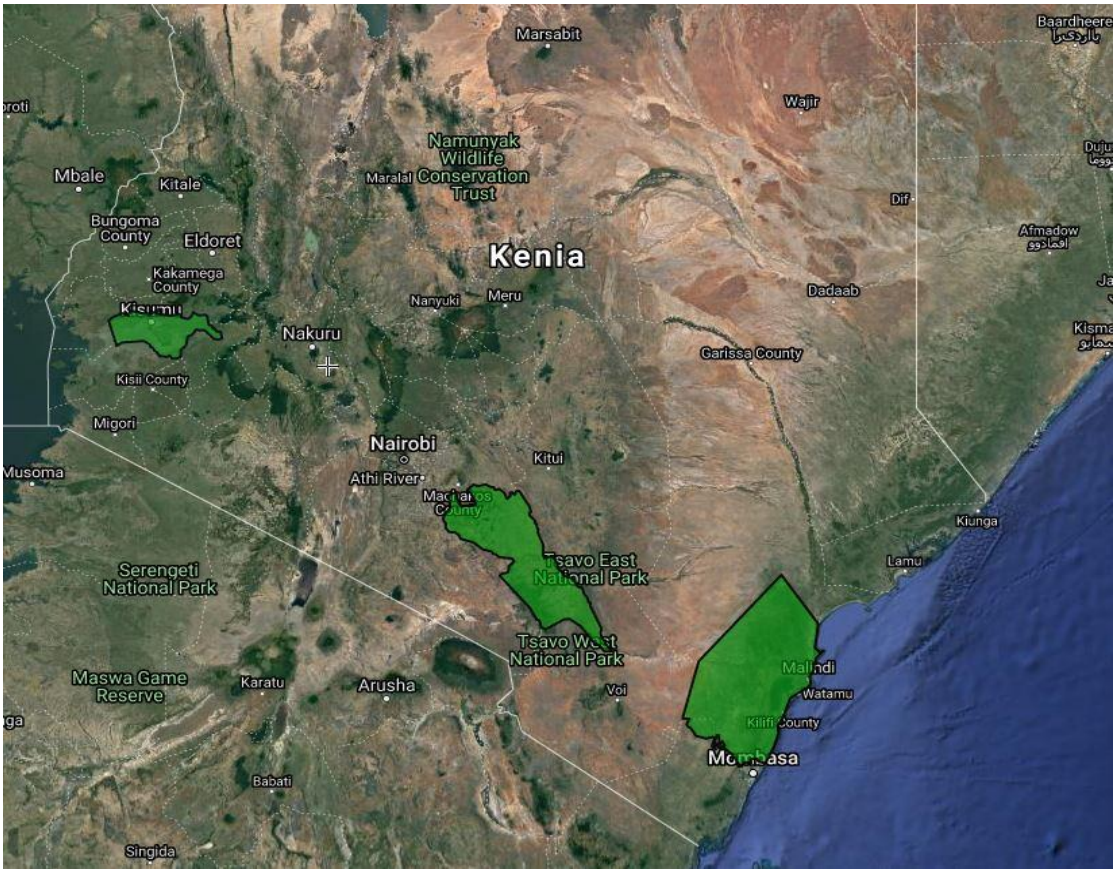
Projektupdate

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:	Announcement No.18/16/32
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:	1.324.664,27 €

Map of target region (Makueni, Kisumu, Kilifi):



Aim of the project:

Due to the consumption of foods heavily contaminated with aflatoxins (especially the staple food maize and milk), the population of Kenya is regularly exposed to toxin levels that are well above the recommended limit values. Nevertheless, the consumption of these products is constantly increasing. Especially children, old and sick people are particularly at risk due to the health effects of ingesting mycotoxins.

The BLE-funded project AflaZ focuses on improving food safety and the quality standard of milk, maize and products made of them. Kenya was selected as a model region, since it is a high-risk area for aflatoxin contamination and food infestation by mycotoxigenic fungi. As part of the AflaZ project, effective and sustainable methods are developed to analyze and monitor aflatoxin contamination both in the field and in the storage in order to effectively reduce fungal contamination. In addition, AflaZ implements extensive strategies for capacity building, which include cooperation with local institutions, farmers, students and other participants, and thus enables sustainable knowledge transfer, cultural acceptance of the recommendations and the effective integration of new methods through the local population.

The following core topics are dealt with within the framework of AflaZ:

• 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; development and application of fast-screen tests for aflatoxin, and an APP for mobile aflatoxin analysis in the field.

• Determination of soil 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.

Results of second year of the project (2020):

The Corona pandemic has been causing restrictions in many life and work situations since spring 2020, including the work taking place as part of the AflaZ project. The project objectives have not changed during the course of the project implementation. However, the work had to be adapted to the new Covid19-related norms.

At the **Max Rubner Institut (MRI), Karlsruhe**, both the effects of temperature and water activity on aflatoxin biosynthesis of *Aspergillus minisclerotigenes* on maize kernels were determined as part of **WP1**, and the assertiveness between aflatoxin-producing and non-aflatoxin-producing *Aspergillus flavus* strains was investigated using a droplet digital PCR system (ddPCR). Maize kernels were inoculated with a non-aflatoxin-producing *A. flavus* strain approved as a biocontrol strain (AflaSafe) and an aflatoxigenic *A. flavus* strain at different spore ratios, and then the changing ratio was molecularly monitored using ddPCR. Subsequent analysis clearly showed that the aflatoxin-producing *Aspergillus* prevailed over the non-aflatoxin-producing *Aspergillus* at almost all spore ratios. The aflatoxin formation itself, however, could be clearly inhibited already at a ratio of 20 % of spores of the non-aflatoxigenic fungus. An increase in the number of spores of the biocontrol strain used did not lead to a further reduction in aflatoxin formation.

An essential factor for molecular monitoring of aflatoxin biosynthesis in aflatoxin-producing fungi is the time of onset of aflatoxin biosynthesis to determine when inhibition of toxin formation would be required. To this end, gene expression of aflatoxin-specific genes and aflatoxin biosynthesis were measured virtually in real time in samples of *A. flavus* and *A. minisclerotigenes*. Aflatoxin formation was shown to be preceded by a peak in the expression of several genes of the aflatoxin gene cluster. After only 36 hours, aflatoxin could already be measured in *A. flavus*. At this time, the fungus was still very small and was not yet clearly recognizable as *A. flavus*, which shows that even if no clear fungal formation is yet recognizable, aflatoxins can already be formed on cereals or nuts.

In the course of the analyses within the AflaZ project, as well as in current studies, it became apparent that *A. minisclerotigenes* plays an essential role in aflatoxin contamination in Kenya. Therefore, the analyses in **WP1** focused primarily on this fungus and it was sequenced in **WP2** in addition to *A. flavus* and *A. parasiticus* using a MiSeq sequencer (short reads) and PacBio technology (long reads). Detailed and extensive comparative genome analyses **(WP2)** of different isolates of *A. flavus* and *A. minisclerotigenes* will be performed now to elucidate variations in the architecture of the aflatoxin gene clusters and to identify differences between these two aflatoxin-producing fungal species with respect to their prevalence of aflatoxin production. Finally, on the basis of the knowledge gained from these analyses, effective and targeted prevention strategies against these particularly abundant fungal species can be developed.

Within the framework of **WP3**, extensive competition experiments with the mycoparasitic *Trichoderma afroharzianum* and the aflatoxin-producing *Aspergillus flavus* were carried out on culture media at the **MRI-Karlsruhe** and the growth rates of the fungal strains in competition with each other were determined. It was shown that *T. afroharzianum* is capable of effectively and permanently inhibiting the growth of *A. flavus* and, after a sufficient incubation period, also overgrowing it.

Together with those genome data on *T. afroharzianum* obtained in **WP2** (no secondary metabolites known to be harmful to humans, such as mycotoxins, are formed), *T. afroharzianum* is considered to be less of a concern and more effective in eliminating aflatoxin-producing *Aspergillus* in the field than the comparative use of non-aflatoxin-producing *Aspergillus* species (which often have the ability to produce toxic cyclopiazonic acid, another mycotoxin, as does the fungal strain used in biocontrol formulations). The system has also been shown to work on maize and in soil.

At the **Julius Kühn-Institut (JKI)**, work on **WP3** began in 2020 with the establishment of an L2 laboratory in order to be able to test plant extracts and their inhibitory effect on the relevant *Aspergillus* species.
For this work, a larger number of associated and medical plants were identified through literature studies and interviews with Kenyan sources. In addition, some plants could be selected through travel reports by AflaZ scientists (**WP6**). Nine associated plant species found in Kenya were grown. Extracts were then prepared and tested for antifungal and insecticidal activity. An evaluation of the maize fields and surrounding vegetation is to be carried out using AflaZ's own drone.

Mycotoxins can be present in a "masked" form (**WP4**). Through metabolization of aflatoxin by the plant or the fungus itself, the structure of the toxin is altered in such a way that it cannot be detected by conventional analytical methods. As a result, a total load of aflatoxin in the food being tested may be undetectably elevated. A kenyan AflaZ doctoral student has been trained on aflatoxin rapid test systems during her stay at **MRI Detmold** as part of **WP4**. The rapid test equipment acquired through AflaZ is now being tested on maize samples by the PhD student in Kenya. The results of the aflatoxin quantification with the two kits, AccuScan Gold-Reader and RIDA Smart APP, showed the same result trend: Kilifi with the highest aflatoxin levels, followed by Makueni and Kisumu with the lowest levels. Furthermore, analytical methods for aflatoxins and their derivatives were optimized and, for example, maize samples from **WP7** for feeding to dairy cows were analyzed for their aflatoxin content using the previously established quantification method.

At the **University of Koblenz-Landau**, within the framework of **WP5**, an analytical method for aflatoxins in soil was developed and successfully validated according to official criteria. The method was optimized for the analysis of plant matrices (food) to be used in the investigation of the soil/plant system. In addition, the method was extended to fluorescence analysis, since mass spectrometric detectors are rarely available in Kenya. In a first model experiment, the occurrence of aflatoxins and *Fusarium* toxins in maize cultivation soils in Germany was investigated. Soil samples were first taken at the plant growth stage to test whether mycotoxin production was due to a stress factor, as there is competition between the plant and microorganisms for soil substrates during this period. The second sampling was done at the time of harvest to check if the occurrence of mycotoxins in soil was due to mobilization from the plant. Due to the climatic conditions in Germany, the work was extended to the occurrence of *Fusarium* toxins. The second model aims to analyze the persistence of aflatoxins in soils and to estimate the effects of aflatoxin on soil microorganisms and the associated biogeochemical processes.

A kenyan AflaZ PhD student from **WP6** established the field trials in 2020 and carried out insect sampling in the AflaZ trial fields in 2 seasons. A part of the captured insects were sent to the **JKI**; a determination of the insects takes place in parallel in Germany and Kenya. At the **JKI-ÖPV** (Institute for Ecological Chemistry, Plant Analysis and Stock Protection), additional methods for the transmission/spreading of fungi on/in maize cobs and their effect on mycotoxin formation were tested.

At the **MRI in Kiel**, the planning, preparation and implementation of a feeding study was the focus of **WP7**. As a result, an aflatoxin model animal-feed could be produced and analytically characterized for this purpose. Further results are the collection of milk and other biological samples from the trial, the analysis of which will provide the actual results. In preparation for the feeding trial, batches of maize of about 10 kg each were inoculated with three different fungal cultures. The spore suspensions of the corresponding fungal species were prepared by partner **MRI Karlsruhe (WP2)** and then sent to MRI partner Kiel. These were an AFB_1-producing strain (*Aspergillus flavus*), a non-AFB_1- producing so-called "biocontrol" *Aspergillus* strain and the strain *Trichoderma afroharzianum*, which is to be established as an alternative biocontrol strain. During the feeding trials, in addition to the usual feeding, four animals each, divided into three groups, received boli over a period of 14 days consisting of maize meal pre-incubated with one of the three fungal species mentioned above. A fourth experimental group acted as a control and received no boli.

The feeding trial was conducted in two staggered runs of four experimental groups of two animals each (three treated maize feeds and one control). The boli were given in the morning and in the evening after milking time. On the analytical side, the existing HPLC-FLD method was extended to check for AFB_1 levels in feed. Likewise, the ELISA method for the analysis of AFM_1 in milk was partially revalidated and its applicability for feed analysis was tested.

At the **Friedrich-Loeffler-Institute (FLI)**, after approval of the animal experiment, the experiment and its sampling were planned and prepared in detail together with the **MRI Kiel** within the framework of **WP7**. During the "control sampling" (individual samples not influenced by the treatment), a briefing on the collection of liver biopsies was given at the **MRI** Schädtbek site. All sampling (blood, urine, liver tissue) was performed by the staff at **MRI Kiel**. Two blood samples were sent by express to Braunschweig on each sampling day. From these, measurement of red and white blood-cell count, preparation for the comet assay and phenotyping of B and T cells by flow cytometry were performed. Furthermore, the AFM_1 method for urine was further optimized. Validation of the HPLC method and the associated sample processing method will take place in February/March 2021. For a better characterization of the urine samples, the pH value and the creatinine content will also be determined.

The **Kenya Agricultural & Livestock Research Organization (KALRO), WP8**, managed the trial fields in 2020 in the three counties of Kisumu, Makueni, and Kilifi, which are hotspots for aflatoxin contamination of maize and were selected as sites for project activities in the previous year. Plantings of the planned 2020 trials were successfully completed. Germination counts, grain moisture content at harvest and after farmers dried the maize kernels were determined and differences between counties were statistically evaluated. The effect of four treatments on soil, different soil depths and maize plant contamination by *Aspergillus* species and other filamentous fungi, as well as aflatoxin levels in maize, was tested over three growing seasons. Samples from the experimental sites were provided to the PhD students in the AflaZ project and/or the students were also involved in the sampling themselves.

The farmers involved in the project are supervised by the **East African Farmer's Federation (EAFF), WP9**, for the entire duration of the project. In 2020, the entire cultivation of maize, from sowing to harvesting and storage, was monitored by **EAFF**. 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. Regarding harvesting and storage, technologies that have emerged from the project so far and can lead to significantly lower aflatoxin contamination of food and feed were presented and explained to farmers. After harvest, samples of maize stalks and maize kernels are taken in collaboration with **KALRO** to test for contamination with Aspergilli. During the second season, soil samples were taken to measure soil contamination with *Aspergillus flavus*. By examining the crop, the crop harvest and the soil, it is now possible to validate whether a new method actually reduces contamination or not. The necessary analyses are carried out locally in Kenya and at the partner institutes in Germany. During the on-site training, the farmers were given extensive knowledge of the aflatoxin reduction technologies used in agriculture and developed in AflaZ. The farmers' understanding of the possible health consequences of aflatoxin contamination for humans and livestock was deepened. The educational efforts are already leading to an increased engagement of farmers to apply the new methods and technologies on their farms. Those farmers who are already using the new technologies are passing on their newly acquired knowledge to other farmers as multipliers, thus making a sustainable contribution to the broader application of this knowledge and technology.

At the beginning of 2020, a physical **AflaZ annual meeting** was to be held, which unfortunately did not take place due to the covid19-pandemic. However, the meeting was then held online with lively participation.

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.
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 use of *T. afroharzianum* as a biocontrol strain, which was investigated within the framework of AflaZ, shows promise.
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 can be verified and monitored by molecular ddPCR technology in the laboratory.
5) Rapid aflatoxin detection methods are currently being applied and further developed in field trials in Kenya. A method for the determination of aflatoxin concentration in soil/plant has been developed and successfully validated. The method has also been optimized for the analysis of plant matrices (food) to be used in the investigation of the soil/plant system. The transmission rate of aflatoxin in cow's milk is investigated in a feeding study, as well as a possible aflatoxin reduction in the downstream milk products yoghurt and cheese. In addition, a specific biomarker to measure aflatoxin exposure in dairy cows is under development.
6) 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, partly in tandem with German PhD students, within the AflaZ project, but also of farmers, ensures the multiplication of the AflaZ findings on site.