Bundesanstalt für Landwirtschaft und Ernährung

AflaZ

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Projektträger Bundesanstalt für Landwirtschaft und Ernährung

MRI Max Rubner-Institut

JKI Julius Kühn-Institut

EAFF

KALRO

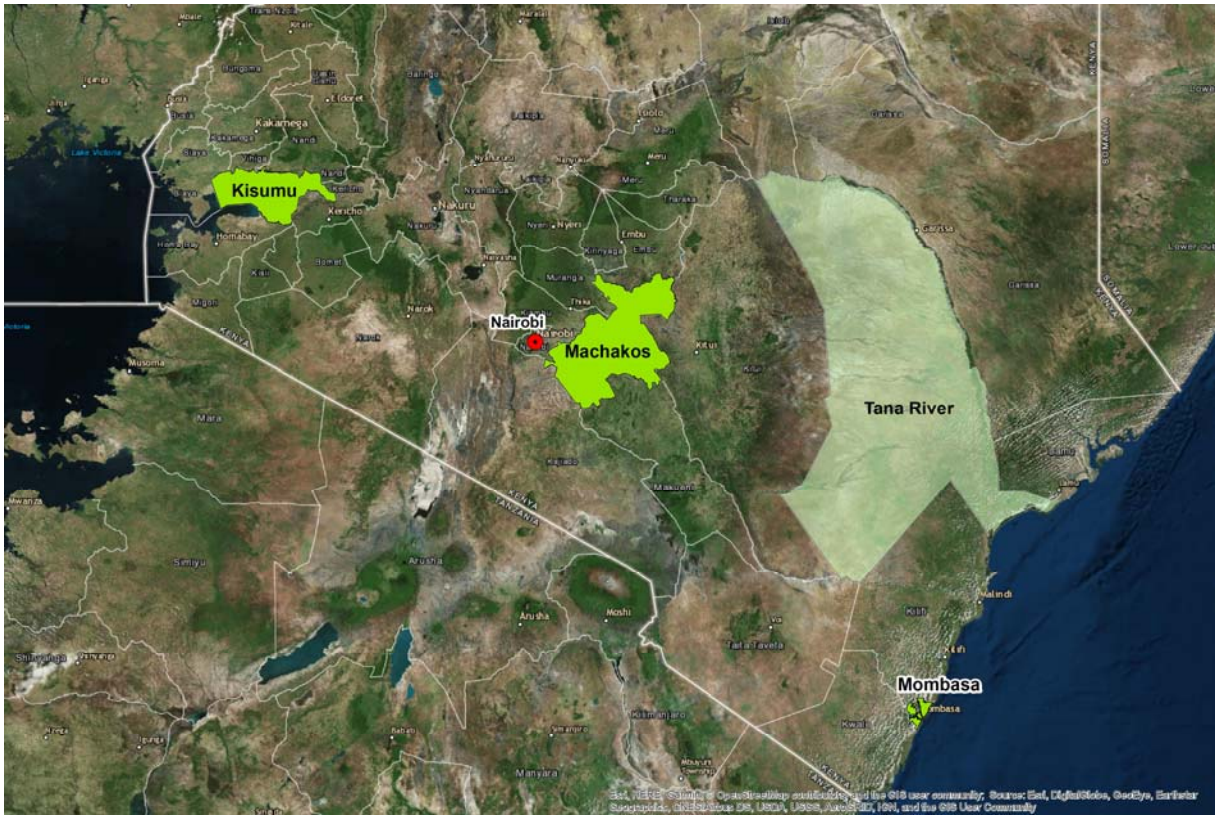
UNIVERSITÄT KOBLENZ · LANDAU

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:	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:



(bitte hier die Logos der kooperierenden Partner einfügen)

Stand Januar 2020

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 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, corn and products made from it. Kenya is a model region, since it is a high-risk area for aflatoxin contamination and mold in the food sector. As part of the AflaZ project, effective and sustainable methods will be developed to detect, analyze and effectively reduce fungal and aflatoxin contamination both in the field and in the warehouse. 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 from Kenya, as well as their microbiological and molecular biological characterization for the development of effective and sustainable detection and avoidance 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 in the aflatoxin content in the subsequent processing of the milk into cheese and yoghurt. Identification of an aflatoxin biomarker in the blood of dairy cows and development of a suitable analysis method.

• Analysis of aflatoxin derivatives, which can contribute significantly to "masked" contamination; Development and implementation of fast screen tests and an APP for mobile analysis.

• Determination of soil parameters as vitality parameters for soil organisms and for maize plants and of insects as vectors for spore distribution.

• Capacity building together with African PhD students, quarterly dissemination of the project results via the PAEPARD blog.

Results of first year of the project (2019):

At the **Max Rubner Institute** (MRI) in Karlsruhe, work began on isolating *Aspergillus* strains from maize samples from Kenya. The different strains were examined for their ability to form aflatoxin and compared with each other. In order to better understand the gene expression of aflatoxin and cyclopiazonic acid specific genes as well as the production of aflatoxin and cyclopiazonic acid under standard conditions (at 25 ° C) before testing different temperatures and water activities, in **WP1** *A. flavus* and two other selected strains were cultivated on laboratory medium and on corn

kernels at various temperatures and water activities and the aflatoxin formation and gene expression has been analyzed using a newly developed ddPCR system. This ddPCR system will be used in the later phase of the project to investigate the interaction between toxin forming and non-forming *A. flavus* strains. The background of this approach is the method of using AflaSafe in Kenya, which uses non-forming strains of *A. flavus* to competitively suppress forming strains on maize. For the development of the ddPCR system, specific probes with two different fluorescent dyes, one for the non-forming strain (*A. flavus* ATCC96045 / AF36 from WP2) and one for the forming strain (*A. flavus* MRI19 / M19 from WP2) were developed from a DNA area of the polyketide synthase, a key gene for aflatoxin biosynthesis. In the search for new prevention methods for the aflatoxin biosynthesis of *A. flavus*, tests with arginine were carried out, since previous studies implemented by the work group showed that the amino acid arginine can inhibit the mycotoxin biosynthesis of *Penicillium* species. It could effectively be shown, that *A. flavus* growth decreased on PDA containing at least 80mM arginine and was completely inhibited on maize medium containing 60mM arginine. Aflatoxin biosynthesis was reduced while cyclopiazonic acid biosynthesis was increased.

As part of **WP2** and **WP3**, various aflatoxin-forming Aspergilli from Kenyan corn kernels were isolated and identified. A non-aflatoxin-producing *A. flavus* was also acquired from the ATCC stock collection. These fungi were made available to WP1 and WP7 for their work and were further characterized in WP2 on a physiological level. The genomes of these fungi were sequenced and characterized using paired-end sequencing technology. In addition, conditions were investigated under which aflatoxin-forming Aspergilli form aflatoxin and under which conditions the formation of aflatoxin or the growth of these fungi is reduced. The results could, for example, be used to reduce the use of chemical fungicides in agriculture, which is costly and can lead to undesirable residues. With the most important aflatoxin-producing fungal species *Aspergillus flavus* and *A. parasiticus*, it could be shown that by reducing the water availability in the nutrient medium (water activity) and the influence of light of a certain wavelength, both growth, mycotoxin biosynthesis and the formation of spores can be drastically reduced. Analysis of a homologue of the HOG1 protein (high osmolarity glycerol) in *Aspergillus*, called SAKA, which regulates besides other stresses, the reaction to osmotic stress in the fungus, shows a negative-proportional correlation between aflatoxin or cyclopiazonic-acid (CPA) formation and SAKA activity (phosphorylation) in *Aspergillus flavus* and *A. parasiticus*. This illustrates, that there is a regulatory link between the SAKA stress signalling pathway and aflatoxin respectively CPA formation. Aspergilli can infect young maize plants via injured roots and thus penetrate into the plant's vascular bundle. In these and further investigations into the pathways of infection of Aspergilli and maize plants in **WP2**, it was shown *in vivo* by using Scanning Electron Microscopy (SEM), that the fungus grows in the stomata openings of the plant in a directional way and must therefore have appropriate sensor systems in order to find the stomata openings. In a laboratory test it was also shown that aflatoxin can be absorbed into the plant via the roots (leaching) and was detectable in the sprout of the maize plants after only a few days. Further investigations will follow.

As a further option of intervention, the mycoparasitic fungus *Trichoderma afroharzianum* is used against aflatoxin-forming fungi as part of the **WP3** part of the MRI Karlsruhe. This approach has been shown to be very successful in the course of the work of WP3. The fungus *Trichoderma afroharzianum* strongly inhibits and reduces not only aflatoxin-forming fungi, but also other mycotoxin-forming and partly phytopathogenic fungi of the genera *Fusarium*, *Alternaria* and *Penicillium*. With regard to the use of *Trichoderma* as a harmless biocide, initial analyzes of the genome

have shown that there are no toxic secondary metabolite clusters in this *Trichoderma* species, as described for example for the trichothecenes in other *Trichoderma* species.

At the **Julius Kühn** Institute (JKI) the work on **WP3** started in 2019 by method development. Bioassays (petri dish assays and microdilution assays) have been adapted to test the antifungal activity of aqueous and methanolic extracts and essential oils from leaves of Kenyan plants. The growth and mycotoxin production of various types of fungi (*Fusarium culmorum, Fusarium graminearum, Aspergillus sidowii*) was tested in combination with the extracts of various plants (*Callistemon rigidus, Annona senagelensis, Lippia adoensis, Parthenium hysterophus*). Furthermore, various sampling methods for measuring fungal infestation on plants were examined.

At the **MRI** in Detmold as part of **WP4**, a maize sampling plan for Kenya was developed. In addition, after some preliminary work, the optimization of eluent and flow parameters for the aflatoxin determination methods on two chromatographic systems (LC-MS / MS -HRQToF) was established. Furthermore, the rapid test systems were obtained, tested and prepared for use in Kenya. This system will be made available for permanent use in Kenya, also after the AflaZ project has been finished.

At the **University of Koblenz-Landau** as part of **WP5**, a reliable, simple and inexpensive extraction and analysis method for aflatoxins in the soil was developed. For this purpose, various quick and simple generic extraction methods (solid-liquid extraction, ultrasonic extraction, QuE-ChERS) in combination with different aflatoxins (AFB1, AFB2, AFG1, AFG2) were compared. A “worst-case” soil was used as the soil to be tested, i.e. a soil with a high proportion of clay and organic material, which therefore offers particularly unfavorable conditions for the extraction of aflatoxins. The reasoning behind this is that if the method works under the worst possible conditions, it is also suitable for all other types of soil. The extracts were analyzed by LC-MS (Liquid chromatography mass spectrometry) using a matrix-adapted calibration and the detection limits and quantification were determined. In a next step, the optimized method will be validated on four agricultural reference soils and a Kenyan trial soil. It can be assumed that many Kenyan / African institutes do not have access to MS systems and therefore quantitative determination of aflatoxins in the soil is only possible using conventional methods (HPLC fluorescence). Therefore, an HPLC-FLD method which reaches determination limits which make the method applicable for real environmental samples is being developed. In addition, a study is being carried out to investigate the toxic effects of aerobic biotic degradation of aflatoxins on soil microorganisms.

In October 2019, a researcher from the **JKI** travelled to Kenya to prepare the work for **WP6.** He met with his kenyan doctoral student on site to methodically structure and discuss the entomological examinations and the requirements for the test areas in the field. During his stay, with the support of KALRO, he visited the test areas in the regions of Kisumu, Machakos and Mombasa and was able to adapt the test setup based on the newly gained impressions. In addition, he was able to collect valuable information on the real conditions, cultivation methods and possible problems on site for all project partners.

The goal for 2019 at the **Friedrich-Loeffler-Institut** (FLI) as part of **WP7** was to support the feeding experiments of the Institute for Safety and Quality in Milk and Fish at the MRI. An ELISA test kit ("QUANTITATIVE ASSAY FOR - AFLATOXIN M1 IN URINE" from Helica Biosystems) was evaluated and subjected to a validation test. The validation was carried out using urine samples

from 12 dairy cows and an Aflatoxin M1 standard. The method was checked for the validation parameters of accuracy, precision, detection- and quantification-limits and dilution linearity. The next step was to develop an improved method using UHPLC with an FLD detector. The first batches of samples were prepared analogously to the ELISA method using urine samples from 12 dairy cows and an Aflatoxin M1 standard and were analyzed using the developed method. The evaluation of the qualitative assessment of the results and the quantitative assessment of the recovery shows, that the method is generally suitable. Researchers of the FLI within AflaZ are in close contact with Kenyan researchers of ILRI (Livestock Research) to share and compare results of kenyan cow varieties.

In 2019, the **Kenya Agricultural & Livestock Research Organization** in **WP8**, in collaboration with **EAFF, WP9**, carried out a gender-specific survey in 112 households using a questionnaire developed by EAFF to learn about the farming and processing practices of farmers and their knowledge of aflatoxin. Based on the results of the surveys (e.g. topography of the farmland, size of the farmland, willingness of the farmer to participate in the experiments), the final selection of the three regions in which the test fields are located was made in cooperation with EAFF. In each of the 72 farms in which field tests are carried out, soil samples and maize samples from the previous harvest from the involved farms were taken and analyzed. In the course of the year, the creation of the trial fields, their cultivation and the harvest has been monitored and supported by the EAFF. EAFF uses its own information and communication platform, e-Granary, to extensively communicate the project results. In 2019, EAFF reached 13,000 farmers from different counties to train them on knowledge about the effects of aflatoxin contamination and application of aflatoxin prevention methods in maize and soybean.

In April 2019, the first **AflaZ partner meeting** took place at the MRI in Karlsruhe with the participation of almost all project participants.

Key statements and policy advice:

1) It is possible to inhibit mycotoxin-forming fungi with a combination of inhibiting influences in storage and to some extend also already in the field by the use of natural competitor fungi.
2) Antifungal phytochemicals and the amino acid arginine, which can be easily sprayed on, are a promising approach to inhibit aflatoxin-producing fungi.
3) Infestation and effectiveness of prevention methods can be checked and monitored in the laboratory using molecular ddPCR technology.
4) Rapid aflatoxin detection methods for the field are being developed and the transmission rate of aflatoxin in cow's milk is being investigated, as well as a possible aflatoxin reduction in the milk products yoghurt and cheese. A biomarker for measuring the aflatoxin load in dairy cows is under development for the use of an effective analysis method for aflatoxin load in cow blood.
5) Corn fields are cultivated using conventional methods and the methods developed in the AflaZ, the infestation with aflatoxin is compared and the farmers are trained in the new methods. In this way, a sustainable reduction in aflatoxin is possible and achievable. By training African doctoral students in the AflaZ project, the multiplication of the findings from AflaZ is guaranteed on site.