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Ph.D IN "BIOTECHNOLOGY AND LIFE SCIENCES" XXXII CYCLE

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Development of innovative technologies for the management of aflatoxin contamination

Aspergillus flavus and Aflatoxins containment: an introduction

Fungi are known to produce several secondary metabolites, which have a wide range of biological activities. Among these secondary metabolites, mycotoxins deserve a particular attention since most of them are provided with carcinogenic and mutagenic activity. *Aspergillus flavus*, a filamentous fungus that naturally contaminate cultivation such as corn, peanuts and cotton, is the major species producing aflatoxins. Aflatoxins is the most toxic and cancerogenic mycotoxin that contaminates food and feed commodities.
The exposure to this compound causes a relevant health risk to both human and animals. For this reason, the concentration of such toxsins in food for human and animal use is strictly regulated by national authorities. Under favorable condition of humidity, oxygen and temperature the spores of *A. flavus* can germinate and produce a compact mycelium, that may differentiate and produce numerous spores that are dispersed in the environment by insects and wind, and thereby can colonize the relevant crops. Aflatoxins that may be found in the food commodities are mainly aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2). Aflatoxin B1 is mainly produced by *A. flavus, A. parasiticus and A. nomius*. A not secondary sanitary and economic issue is dependent on the fact that the toxin may easily be transferred along the food chain reaching the consumer. Accordingly, the aflatoxins control is a complex problem that requires the development of a wide range of contamination control strategies along the food chain, from the field (pre-harvest and post harvest stages), to the fork.
My Ph.D project is focused on the development of integrated strategies for aflatoxin pre-harvest contamination control, to increase the sustainability of corn cultivation in Emilia Romagna Region, coping innovative technologies of plant defense with lower environmental impact, and quantifying the successful reduction of the agronomical inputs.
During this first year I was involved in two different research projects: the first is "Aflatox: a biotechnological approach for the development of new antifungal compounds to protect the environment and the human health". This project is funded by Cariplo Fundation, and its general aim is the development of a wide screening to characterize the biological activities of new bioinorganic compounds harmless to the environment and to human health but efficient as inhibitors of fungi proliferation, and particularly of aflatoxins production. Our partners from the Inorganic Chemistry unit of the Department synthesized new molecules that incorporate natural molecules, some of which known to be mould proliferation inhibitors, and endogenous metal ions: biological activity of such molecules are assessed for their effect on *A. flavus* growth and aflatoxins production. Compounds that showed an interesting antifungal and/or antimycotoxigenic potential were then subjected to cell toxicity, genotoxicity and epi-genotoxicity tests performed *in vitro* on different model systems (plant, bacterial and human cells) to assess their impact on the environment and the human health. Once identified the most promising compounds, they were modified in their structure and in their physicochemical properties (redox potential, lipophilicity, etc.) and the consequent effects on their antifungal activity will be evaluated (structure-activity relationship). Final scope of the project is the creation of a database containing all collected data in order to produce Q-SAR (Quantitative Structure-Activity Relationship) evaluation profiles for the newly synthesized compounds.

Among the obtained results, there is the identification of new metal-based antifungal substances acting directly on cells and on the aflatoxin production, the development of a protocol system for the screening on the efficacy of antifungal molecules and to improve drug design, the identification of compounds with remarkable antifungal activity without toxic and genotoxic effects on human health and the environment.
The second research project I'm involved in is "DIFESA MAIS", funded by the Emilia Romagna Agricultural Development Project. "DIFESA MAIS" proposes a green approach with low environmental impact and application costs, which main goal is the development, validation and comparative analysis of the technologies for monitoring and controlling insect/larvae infestations, and individuation of sustainable procedures to reduce aflatoxin contamination. The expected output of this project is the creation of an innovative model, displaying a high level of environmental sustainability, addressed to the development and the valorization of organic farming/ integrated production of corn. Moreover, the effects are expected be felt on all corn production chain, improving its efficacy and its control. Positive effects are expected on the maintenance of biodiversity, for example controlling the evolution of microbial and insects population, avoiding the use of phytochemicals. Beneficial effects on consumers health are also expected. Project partners from University of Bologna and other company partners are involved: their role is the implementation of good agronomic and cultural practices aimed at preventing plant stress, for example by mulching, irrigation, fertilization, crop rotation, insect control and the administration of *Bacillus turigensis* toxin (Bt). Our specific contribution to this research is the implementation of an intraspecific bio-competition strategy to reduce aflatoxin contamination of maize crops: this kind of approach, based on the application over the relevant crop fields of an atoxigenic *Aspergillus flavus* strain, is successfully used in both maize and cotton cultivations worldwide. For this purpose, a natural strain of *A. flavus* belonging to the fungal population resident on the corn of the Po Valley is used (Degola et al. 2011; Degola et al. 2012).

1st year Research activities:

"AFLATOX" Project:

Aim of this project was the identification of new compounds to be proposed as antimycotic and antimycotoxic in the agri-food area, even if other implications could be expected for the pharmaceutical area too. The newly synthesized molecules, similar to each other for structure and belonging to thiosemicarbazones group, were tested for their effect on the phytopathogenic fungus *A.flavus*: in fact, if their use as antiviral and antimicrobial agents is well documented, only recently evidences on their antimycotic activity have been reported (Degola et al. 2015). From the beginning of the Ph.D first year, I performed analyses aimed to assess the effect on growth and production of aflatoxin: a 96-well microplates strategy was used, allowing us to contemporary test different substances in different concentration. To evaluate the effect on the toxin production, a coconut-based medium was used (Degola et al. 2011). A panel of about 52 compounds was analyzed: some showed a good antifungal activity, generally coupled with an anti-aflatoxigenic potential, and then sent to our partners for the assessment of eventual cytotoxic effects on several healthy human cell lines; finally, they were tested for the absence of genotoxic effects using plant model systems. Interestingly, few of them displayed an anti-mycotoxin effect without affecting *Aspergillus* growth: these molecules were addressed to other analyses, aimed to find their putative cellular/molecular target in *A. flavus*: with this aim, analysis of the oxidative status of fungal cells on the biogenesis of stress-responsive structures (known as *sclerotia*) and the expression of specific genes were performed.
Two molecules, the thiosemicarbazone of 2-hydroxymethylanthrachinon and the thiosemicarbazone of 2-formylanthrachinone, were found to possess a particular effect on the aflatoxin biosynthesis in strain BS07. Strain BS07, an *A. flavus* isolate that, due to a single nucleotide polimorfism, is blocked in the aflatoxin metabolism: in fact, the treatment of the atoxigenic strain with these thiosemicarbazones resulted in an accumulation of a compound that share some chemical-physical characteristics with aflatoxins, and seemed to respond to different conditions (such as nutritional balance, temperature, and the presence of inhibitors)

known to control the aflatoxin biosynthesis. Experiments inquiring the chemical structure of this compound are undergoing, while the screening of newly synthesized thiosemicarbazones goes on.

"DIFESA MAIS" Project:

In the last decade, our lab mapped and analyzed the *Aspergillus flavus* population resident on maize crops of several geographic regions of the northern Italy: a wide screening on the aflatoxin producing and non-producing strains, and on the ability of non-producing strains to be effective in lowering the toxin production by toxigenic strains was conducted. Obtained results led to the individuation of the best candidate for *in field* competition trials. In the second half of this first year of my Ph.D project, I optimized a ready-to-use procedure toobtain the conidial suspension quantity required for deliver the bio-control strain on two experimental maize fields located in Ozzano Emilia (BO) and Crespellano (BO): it is a low-cost procedure that uses recycled material such as plastic bottles sterilized by sodium hypochlorite. A total of 75 plastic bottles have been poured with solid growth medium and seeded with the atoxigenic *Aspergillus flavus* strain; after incubation, spores have been recovered and the conidial suspension was delivered on the maize plants by the use of a pesticide sprayer. Treatments were scheduled at the stage of corn silking. Fields were divided into randomized lots to test the effect of different treatments (alone or in combination), including the administration of a Bt endotoxin (Turex®) commercial product, the administration of chemical commercial product (Coragen®), mulching and fertilization. Before treatments, microbiological preliminary tests were performed in laboratory conditions to evaluate possible negative effects of Bt toxin product: growth and spore maturation of the *A. flavus* bio-competitor strain in presence of Bt was assessed.

Post-harvest analyses were conducted in order to map the population of *A. flavus* resident on the different parcels, mainly in response to bio-competitor administration. *Aspergillus s*trains, were isolated and molecularly and physiologically characterized in order to individuate the relevant bio-competitor. Results are being processed.

Molecular characterization of African *Aspergillus* isolates:

I was accorded of a scholarship for a stage abroad from 15 september to 22 october 2017: during this short stay I was hosted in the "*Toxalim laboratory"*, at the *Research Center for Food Toxicology* of the *Institut National de la Recherche Agronomique (INRA),* in Toulouse (France), where I l performed genetic and phylogenetic analyses on several strains isolated from maize grains collected in Madagascar, and not univocally classified as belonging to *A. flavus* species yet.

2nd year Research activities:

"AFLATOX" Project:

During my second year of Ph.D. school, the number of tested compounds reached the number of 110 compounds. To evaluate the effect on the toxin biosynthesis a 96-well microplates strategy was used, allowing us to contemporary test different substances in different concentration in coconut-based medium. Our ability to quantify accumulation of aflatoxin in culture medium depends on a detection system based on fluorescence emission at a wavelength of 365nm after six days from inoculum. While providing a high throughput fluorimetric dosage this method brings the disadvantage that the use of any compounds emitting or absorbing fluorescence at the wavelength characteristic of aflatoxins makes impossible to estimate the real toxin's level in the medium. For this reason, the preliminary phase of the screening involved the determination of emission/shielding properties of tested compounds: those that emit or absorb fluorescence in the test conditions were discarded. However, for some compounds a semiquantitative dosage of aflatoxin was conducted by TLC. All data were expressed as percentage of inhibition. To evaluate the effect on fungal growth a synthetic medium (Yeast extract containing 5% sucrose) was used. After 40 hours from inoculum,

the OD (Optical Density) increase of treated cultures was compared to the untreated cultures (control). Also in this case the data were expressed as percentage of inhibition. All compounds were also tested for their effect on sclerotia differentiation. Sclerotia are vegetative structures formed by undifferentiated hyphae and produced by some strains under specific environmental conditions. Biogenesis of this structures is strategic for the diffusion of the fungus in the environment. The differentiation process is linked to secondary metabolism and even if the details are still not clear, several regulator checkpoints are shared with the aflatoxin biosynthesis patway.
To elucidate the cellular/molecular target of some compounds, we analyzed the transcriptomic and proteomic changes in treated cultures: gene expression was analyzed via Real Time PCR, while proteome study was performed using 2DE-PAGE.
On the basis of their structure, compounds have been grouped in families including natural starting compounds, binders, modifications and complexes with metals.
An important group of compounds includes Benzaldehyde and Cinnamaldehyde derivatives. Benzaldehyde and cinnamaldehyde differ for the presence of a carbon atom and a double bond on the chain. This two thiosemicarbazones have been further modified, for a total of 6 molecules. The analysis of their biological activity showed a large variability as far as the antifungal and antimicotoxigenic effects.
Another family of molecules includes phenone derivatives. These molecules slightly inhibit germination but only at the highest concentration. However, they are effective on aflatoxin biosynthesis. We also found that the tested compounds impaired sclerotia biogenesis. Two thiosemicarbazones of this family were further modified encapsulating them in the nanoparticles (NPs). The nanoparticles encapsulated do not differ in their effect from the compounds alone, both on growth and aflatoxin production, while an important inhibitory effect was observed on sclerotia production. For this reason, these compounds only can be proposed for an "in-field" strategy to propagation of fungus on crops.
The isopropylbenzaldehyde derivatives represent another family of molecules that was considered. These compounds differ for the position of the isopropyl group on the aromatic ring. The antimycotoxigenic activity seems to rely on the position of the isopropyl group. One of these molecules was considered the most interesting due to its high antitoxigenic capacity coupled with a low inhibition of the fungal growth. An excellent candidate for functional studies on the regulation of aflatoxin metabolism.
Seven hydrophobic long chain thiosemicarbazones and the relevant aldehydes are actually under investigation.

"DIFESA MAIS" Project:

During my second year of Ph.D. school, I analized the population of *A. flavus* on maize fields that were treated as described in the 1st report. *Aspergillus* strains were isolated and molecularly characterized in order to evaluate the persistence of the relevant bio-competitor. For each experimental treatment 30 isolation plates were screened, for a total of 120 plates for each lot. Isolated strains were classified as atoxigenic (Afla-) or toxigenic strains (Afla+): In all treatments a prevalence Afla- strains (~80%) was observed. Only Coragen® treatment resulted in lower ratio of Afla-/Afla+ strain (60%). Atoxigenic strains were genotipized by RAPD-PCR to identify and quantify the presence of the bio-competitor in the atoxigenig population. The chemical dosage of aflatoxin in harvested grains confirmed that the relevant treatments resulted in a reduction of aflatoxins accumulation.

Molecular characterization of African *Aspergillus* isolates:

During my short stage I was hosted at Toxalim (the Research Center for Food Toxicology) of the Institut National de la Recherche Agronomique (INRA), in Toulouse (France). My stage involved on the genetic and phylogenetic analyses of some *Aspergillus* section Flavi strains isolated from maize grains from the Lokobe Natural Reserve, Madagascar. These strains, were compared with strains isolated from Italian maize kernels. The main goal of the stage was to characterize five Madagascar strains at the species level, and in particular, to determine whether they belong to the *flavus* or *A. oryzae* species. *A. oryzae* is almost genetically identical

to *A. flavus*, and at present, they are classified as separate species because of their morphological differences and economic and food safety concerns. *A. oryzae* has been used as the koji starter culture for the production of fermented foods and alcoholic beverages, and for the production of many industrial enzymes used in food processing (Machida et al., 2008). *A. oryzae* is considered "as safe" by the U.S. Food and Drug Administration. No *A. oryzae* isolates have been found to produce aflatoxin. However, *A. oryzae* is phylogenetically very close to *A. flavus*, from which it can be distinguished by some SNPs and a unique deletion in the norB-cypA region in the aflatoxin gene cluster (Chang et al., 2006; Ehrlich et al., 2004; Geiser et al., 2000). Specific sequences of genes tubA and cmdA from strains were amplified and sequenced, then analyzed with a sequence alignment tool (BioEdit Sequence Alignment Editor). Evolutionary models for both genes were tested using JModelTest, and phylogenetic relationships were inferred using MrBayes (Bayesian stat). Topologies for both genes were obtained. In addition to the phylogenetic inferred analyses, we analyzed: 1) the deletion in the CypA-NorB region, which presents different lenght in *A. oryzae* as compared to *A. flavus* (Chang et al. 2015). 2) the genomic region samA-rosA (Chang et al 2015). This region includes genes encoding for a cellular morphogenesis protein with a sterile alpha motif (samA) and the repressor of sexual development (rosA).
The characterization of African strains included the determination of aflatoxin production and of other parameters such as: 1) phenotypic characteristics (pigmentation of conidia); 2) Production of sclerotia and their size; 3) Mating type; 4) Presence of discriminating SNPs and of deletions along the aflatoxin gene cluster 5) aflatoxin cluster genes expression.

3rd year Research activities:

"DIFESA MAIS" Project:

During my third year of Ph.D. school, I have analyzed the population of *A. flavus* colonizing maize fields that were treated according to an experimental design that has been described, in detail, in the first and second year reports. Here, results of the second year (2018) of experimentation were reported. *Aspergillus* strains were isolated and molecularly characterized in order to evaluate the persistence of the relevant bio-competitor strain: for each experimental treatment, 30 isolation plates were screened, for a total of 120 plates for each lot. Isolated strains were classified as atoxigenic (afla-) or toxigenic strains (afla+). In all treatments, a prevalence afla- strains (80%) was observed, in agreement with what already reported: compare the results of the first treatment (year 2017). DNA extracted from atoxigenic strains were analyzed by RAPD-PCR to identify and quantify the presence of the bio-competitor strain among the afla- population. The chemical dosage (HPLC-based procedure) of aflatoxin contamination in harvested grains confirmed that the relevant treatments resulted in a reduction of aflatoxins accumulation. More than 2000 strains of *A. flavus*, both afla+ and afla-, have been isolated as a result of the two years experimentation here reported. A total of 30 afla+ strains and 30 afla- strains were selected from this large sample to perform a new set of intra-specific competition assays. The afla+ strains were in vitro co-inoculated with the bio-competitor strain to test its ability to reduce aflatoxin production by the "real time" population of afla+ strains colonizing the relevant field. Conversely, the isolated afla- strains were assayed, in co-inoculation experiments with afla+ strains, to test their efficacy as bio-competitors. The results of the above reported experimentation confirmed that 1) the anti-aflatoxigenic activity of our bio-competitor strain and 2) many new isolated afla- strains, were provided with a bio-competition activity comparable to that displayed by our bio-competitor strain. It would be worth to test if a pool of afla- strain, recovered from the field of a previous season, could be able to effectively reduce the aflatoxin production by the population of Afla+ strains colonizing the field during the following season(s). *In vitro* experiments to challenge this hypothesis are now in progress.

Development of a LAMP assay for the differentiation of afla- and afla+ strains of *Aspergillus flavus*:

During my stage, I was hosted at Technical University of Munich (TUM) in Freising (Germany). My stage consisted of the development of a LAMP (loop-mediated isothermal amplification) assay for the differentiation of afla- and afla+ strains of *Aspergillus flavus* isolated from maize kernels collected in the fields of the Po valley in Italy and from the Lokobe Natural Reserve (Madagascar).

LAMP is a molecular technology that, relying on the use of a thermophilic *Bst DNA polymerase*, amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions; this method employs a DNA polymerase and a set of four primers that hybridize to six different parts of the target DNA sequence (*Notomi, 2000*); additional primers (loop-primers) can be used to increase the efficiency of amplification of the target. Nowadays, LAMP assay is frequently applied as an alternative to classical PCR-based analyses (*Notomi, 2000*), and recently, LAMP signal detection was achieved by using pH-sensitive dyes in a weakly buffered LAMP master mix that change color upon acidification of positive LAMP reactions during DNA biosynthesis (*Tanner et al 2015*): in this study, LAMP signals were detected using calcein as a fluorescent indicator under UV light, as well as neutral red as a visual indicator under daylight conditions.

During my experience at the TUM laboratories, the main objective of stage was to design six primers for the LAMP assay, and to test them for their efficacy in differentiating *A. flavus* aflatoxin producers (Afla+) from non-producers (Afla-) strains. A total of 26 Italian strains and 15 African strains were analyzed. The primer-mix was also tested on other species belonging to *Aspergillus* and on species belonging to other fungal genera (namely *Penicillium* and *Fusarium*).

The *HexB* gene (also known as *aflB* and *fas-2*) is annotated in the genome of *A. flavus* as a fatty acid synthase beta subunit (hexanoate synthase beta subunit). The comparison between the *HexB* sequence in both producers and non-producers *A. flavus* strains revealed several single nucleotide polymorphisms (SNPs): on the basis of this observation, we postulated the hypothesis athat afla- and afla+ strains could be discriminated by the presence of two stable SNPs in *HexB* gene; thus, we decided to include both SNPs characteristic of *HexB* sequence of afla- strains in one of the primers used for the LAMP assay, in order to demonstrate the effectiveness of a selective detection, in culture, of aflatoxins non-producing strains.

Six primers were designed on the *HexB* gene sequence of *A. flavus* AF36 (a non- aflatoxigenic strain isolated and patented in USA) using the PrimerExplorer software. The LAMP master mix for neutral red-based and also for calcein-based detection was set up containing the components per 25 µl of reaction volume. LAMP assay was optimized identifying the correct temperature and the exact amount of formamide. Genomic DNA of all afla- and afla+ strains from Italy and from the Lokobe natural reserve were tested in the LAMP reaction using both calcein and neutral red as indicators. Positive signals were indicated by a color change of reactions from yellow to pink as a result of acidification during DNA biosynthesis.

At first, genomic DNA was extracted using the method described by *Cenis et al. (1992*), to obtain a specific portion of *HexB* gene that was then sent to sequencing. Once aligned, the *HexB* sequences showed that all afla+ strains of *A. flavus* shared a thymine in the nucleotide positions (4176 and 4182), while two afla- strains possessed a thymine instead of guanine in the same positions. It was speculated that these two afla- strains were originally aflatoxin producers, but they lost their ability to synthesize aflatoxin due to deletion/other mutations in other genes located in the aflatoxin genic cluster. All tested afla- strains gave a positive result in the LAMP assay; only 4 afla- strains resulted negative for the LAMP assay. Among the 22 afla+ strains analyzed with the LAMP assay, only 9 were negative in the assay, as expected, but 13 of them gave a positive LAMP result. This result shows that the reaction conditions for the LAMP assay are still not fully optimized in order to discriminate all afla+ strains from the afla- strains.

The LAMP reaction was also tested using conidia as template. Results showed that conidia must be washed three times before addition to the assay in order to remove compounds from the growth medium that interfere with the assay. Analysis of serially diluted conidia showed that the limit of detection for conidia was $5*10^3$ spores per reaction; however, while LAMP assay based on neutral red signal detection proved to be discriminant for the direct individuation of afla- strains in afla+/afla- mixture of conidia, the assay based on calcein signal detection did not provide reliable results.

To determine further the specificity of the afla- LAMP assay, reactions were performed under the assay conditions previously described using purified DNA of other Aspergillus spp.; in total forty-eight species of *Aspergillus* were tested. Only *A. arachidicola, A. flavus, A. oryzae, A.parasiticus, A. sojae and A. toxicarius* resulted in a positive LAMP reaction; all other *Aspergillus* species were negative in the afla- LAMP assay. Also DNA isolated from 16 *Penicillium spp*. and 13 *Fusarium spp*. was tested for cross-reactions in the afla- LAMP assay, giving no signal in the afla- LAMP assay. Results obtained during the current study have shown that the developed LAMP assay can profitably discriminate afla- strains from afla+ strains of *A. flavus*.

***Aspergillus flavus* mycovirome:**

The presence of viruses in mycotoxigenic fungi has been recently documented (Kotta-Loizou el al.2017; Nerva et al. 2019). Such presence of viruses is suggested to regulate the fungal metabolism, even interfering with some metabolic pathways; hence, in an ecological perspective aimed at comprehend all the possible biological variables linked to the aflatoxin biosynthesis and the intraspecific biocompetition between toxigenic and atoxigenic *A. flavus* strains, we decided to analyze the mycovirome characterizing our *A. flavus* population. For example, some authors hypothesized that a viral infection in an afla+ strain could results in an increase of the capability to accumulate the toxin.

A battery of 106 afla+ and afla- *A. flavus* strains (both from the Po Valley and the Lokobe Reserve) was subjected to RNAseq analysis; as results of sequence alignment in specific databases, all viral RNAs have been identified in the whole battery. The presence of viruses in single strains was verified identified by Real Time PCR with specific primers. The viral population identified resulted quite heterogeneous, but completely different between the two geographical areas: In African strains we identified two viruses: Aspergillus flavus negative single-stranded RNA 1 and Aspergillus flavus negative single-stranded RNA 2. In African Aspergillus tamarii we identified only Aspergillus flavus RNA virus 1. In Italian strains, instead, we identified several different viruses such as: Aspergillus flavus polymycovirus 1, Aspergillus flavus ourmia-like virus 1, Aspergillus flavus ourmia-like virus 2, Aspergillus flavus ourmia-like virus 3, Aspergillus flavus flexivirus 1, Aspergillus flavus narnavirus 1, Aspergillus flavus narnavirus 2, Aspergillus flavus clostero-like virus 1, Aspergillus flavus partitivirus 2, Aspergillus flavus RNA virus 1, Aspergillus flavus negative single-stranded RNA 1 and Aspergillus flavus negative single-stranded RNA 2.

Once characterized the virome of these two *A. flavus* populations, we investigated the persistence of specific viruses along successive generations of the strain, trying to shed light on the mechanisms that govern transmission of virus particles from mycelium to conidia. Thus, parameters such as growth, aflatoxin production and sclerotia biogenesis in naturally infected strains were evaluated. In addition, two afla+ strains were artificially transfected with two different viral strains. To validate the persistence viruses in the fungus, we cultivated the transfected strains up to the fifth generation in a nutrient culture medium, in two different conditions (shaking and static growth). Through the various generations, the presence of specific viral sequences was checked by Real Time PCR.