UNIVERSITÀ DI PARMA

UNIVERSITY OF PARMA

Ph.D. in Biotecnology and Life Science

XXXII cycle

DEVELOPMENT OF INNOVATIVE TECHNOLOGIES FOR THE MANAGEMENT OF AFLATOXIN CONTAMINATION

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Years 2016-2019

Alla mia famiglia…

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Introduction

1. Mycotoxins

Mycotoxins are products of the secondary metabolism of some filamentous fungi that commonly contaminate agricultural commodities and derived products. The concern of the medical-scientific community about the spread of producing microorganisms and the resulting mycotoxin contamination is due to the harmful effects that exposure to these molecules causes to humans and other animal species. Mycotoxins such as aflatoxins, ochratoxins, zeralenones and fumonisins deserves a particular attentions since all of them are known to possess a nephrotoxic, hepatotoxic, immunodepressive and immunosuppressive, mutagenic, teratogenic and carcinogenic effects *(Bennet and Klich, 2003; Gruber-Dorninger, 2019).* Among these, the most widespread contaminants on cereal matrices are species belonging to the fungal genera *Aspergillus, Fusarium* and *Penicillium (Gruber-Dorninger, 2019).*

Mycotoxigenic fungi are widely present in agricultural areas characterized by optimal temperature and humidity conditions for their development and diffusion.

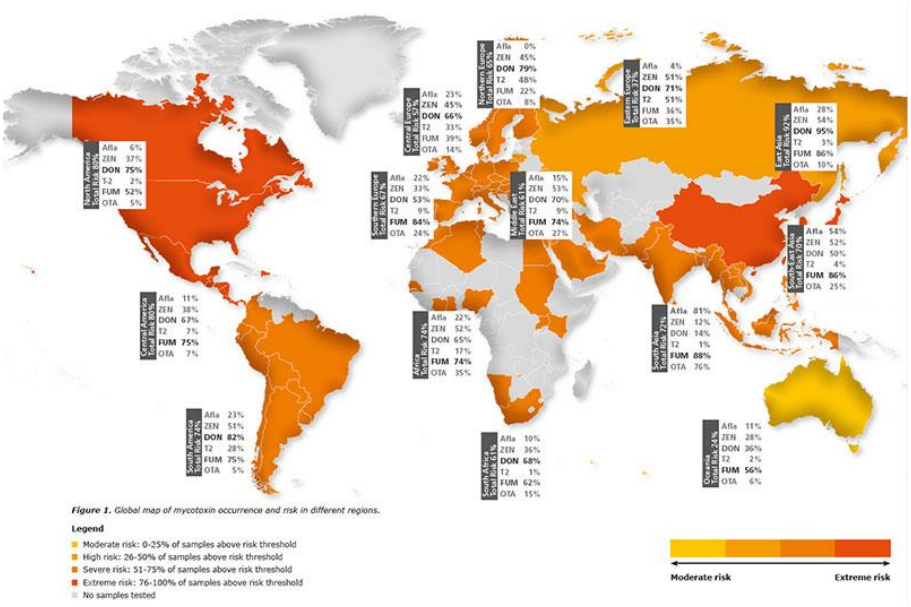


Figure 1: Global map of mycotoxins occurrence and risk in different regions. Afla = Aflatoxin; ZEN = Zearalenone; DON = deoxynivalenol; T2 = trichothecenes; FUM = Fumonisine; OTA = Ochratoxin A. (Biomin mycotoxin report 2017).

Temperature is one of the main environmental factors that influence the distribution of fungal species and, consequently, the mycotoxin contamination: as shown in *Figure 1*, in Europe trichothecenes are prevalent in samples collected from Northern Europe, while DON was detected in 74.2% of the samples and T-2 was detected in 30.3% of the samples. DON was detected for 21.5%. Also in Central Europe trichothecenes are prevalent: 69.8% and 30.7% of the samples were found

to be contaminated with DON and T-2, respectively. Fumonisins were the most prevalent mycotoxins in samples from Southern Europe (*Gruber-Dorninger, 2019)*. On the other hand, the current climate changes, and in particular the global warming, are increasing the exposure risk to mycotoxins *(Bailly et al. 2018)*. Monitoring actions have shown that fungi typically considered to be tropical can now also be found in temperate areas *(Battilani et al. 2016)*.

The risk of mycotoxins contamination in agricultural commodities is not limited to raw materials only: in fact, the intake of contaminated feed by livestock can in turn lead to the contamination of meat, milk, eggs and derivatives. Furthermore, due to their high stability to most industrial processes, such toxic compounds can arrive almost unaltered consumer's table.

Another consequence of climate change is the necessity to satisfy the food needs of a growing world population: thinking that the effects of global warming on the Mediterranean area will lead to increasing drought, it is difficult to ignore how much this will affect the availability of food as well as its quality, since there will be an increase in plant pests and pathogens occurrence *(FAO, 1996; www.fao.org)*. In this sense, it has been observed with a certain alarmism that due to these climatic changes we are witnessing a distortion of the plant-parasite-environment interaction, which inevitably triggers a remodeling of the agronomic industry, which in turn must increasingly take into account of the problem of mycotoxin contamination *(Medina et al. 2017)*. Since mycotoxins have very different chemical structures, the biological effects vary according to the type; additionally, if different fungal species can produce different mycotoxins, the same mycotoxin can be produced by several species. The toxic effects may be different depending on dose and frequency of exposure *(www.izsler.it).* The most well-known mycotoxins are **aflatoxins** (mainly produced by species belonging to the genus *Aspergillus*), **fumonisines** and **zearalenone** (produced by the genus *Fusarium*) and ochratoxin (produced by species of the genus *Penicillium*).

2. Regulation

In terms of economic impact, aflatoxins represent the main concern in the agri-food sector; it was estimated that, dating back to the early 2000s, economic losses ranged between 2 and 25 million dollars a year *(Rubens and Cardwell, 2005)*: in Asia and Africa, incalculable damages are estimated *(Yu et al. 2005)*. The definition of aflatoxin as the most potent natural carcinogen for humans (IARC, 1993) justifies the drafting of a specific legislation as unified as possible throughout the world: alarming are the costs in terms of human lives due to aflatoxicosis in developing Countries, thus numerous epidemies occurred in Kenya from 1981 to 2008 could be cited, that raised the attention

of the scientific community *(Farombi, 2006; Ngindu et al. 1982; Probst et al. 2010)*. In the United States the estimate of economic losses due to mycotoxin contamination is about a billion dollars, but aflatoxin alone is the one that determines the greatest impact. Therefore, the Food and Drugs Administration (FDA) has established as acceptable 20 ppb of aflatoxins in cereals and 0.5 ppb in milk for human food, while higher concentrations are tolerable for livestock feed (***Table 1***). On the contrary, the European directive 2003/100 / EC sets the limit of 20 ppb for raw feed materials, and the 1881/2006 directive establishes more stringent limits for cereals (2 ppb) and dairy products (0.05 ppb).

DRIED AND SHELL FRUIT	**AFB_1 (µg/Kg)**
Almonds, pistachios and apricot seeds to be sorted	12.0
Peanuts, hazelnuts, Brazil nuts, to be sorted	8.0
Almonds, pistachios and apricot seeds for direct human consumption	8.0
Other nuts and dried fruit to be sorted	5.0
Hazelnuts and Brazil nuts intended for direct human consumption	5.0
Peanuts, nuts, dried fruit and derivative products intended for direct human consumption	2.0
Other nuts intended for direct human consumption	2.0
CEREALS	**AFB_1 (µg/Kg)**
Maize to be subjected to sorting	5.0
Cereals and derived products except maize and baby food	2.0
Cereals and foods for infants and young children	0.1
OTHER FOODS	**AFB_1 (µg/Kg)**
Spices	5.0
Diet foods	0.1
FODDER	**AFB_1 (mg/Kg)**
Feed materials	0.02
Complementary and complete feed	0.01
Compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry	0.005
Compound feed for cattle, sheep, goats, pigs and poultry	0.02

Table 1: Levels of AFB_1 allowed in products intended for human consumption and in feed in Europe *(https://ec.europa.eu/food/safety/chemical_safety/contaminants/catalogue/aflatoxins_en)***.**

In developing Countries, where it is not possible to obtain a precise estimate, it is believed that the impact is even greater *(Yu et al. 2005)*. The diffusion of toxigenic species combined with the complexity of the factors that regulate their secondary metabolism, the resistance of these metabolites and management costs, make utopistic the obtaining of matrices completely free of aflatoxins.

As can be observed from these examples, regulations are not uniform in the different areas: in developing Countries the restrictions are almost absent, as is the control of storage conditions and cultivation practices that can limit the spread of contamination. As an example, consider that in Europe lots of peanuts containing more than 2 ppb of AFB_1 (or 4 ppb of total AFs) are excluded from marketing, while in India the fixed limit is 30 ppb *(Amaike and Keller, 2011)*. Given these differences, for developing Countries the problem related to mycotoxins is economically more harmful, as it limits the possibility of trading and export of raw materials to Countries with more severe limits.

3. *Aspergillus flavus*

Aspergillus genus was first described by the Florentine priest and mycologist P.A. Micheli in 1672, and its name is due to the strong observable similarity between the reproductive structure (conidiophore) and the *aspersorium*, the liturgical tool commonly used for dispersion of sacred water *(Amaike and Keller, 2011)*. Nowadays, this genus encloses more than 200 species, but the species *flavus* is considered among the most dangerous, being a potential etiological agents of systemic infections in humans and animals. It is considered an opportunistic saprophyte and a natural contaminant of certain agricultural crops, especially the oily seeds (maize, cotton, peanuts), but also of spices peanuts and derived products, particularly in areas characterized by a hot and humid climate. In fact, *A. flavus* is widely present in climatic zones between the 16th and 35th parallel while it is not very common beyond 45° latitude *(Klich, 2007)*. The *A. flavus* species was first described by Link in 1809 and was known to be a species with asexual reproduction capable of producing vegetative spores (**conidia**) and of wintering in the field in the form of asexual fruiting bodies (**sclerotia**). Recently, a sexual stage has also been discovered, and classified as *Petromyces flavus (Horn et al. 2009)*. Under favorable condition of humidity, oxygen concentration 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.

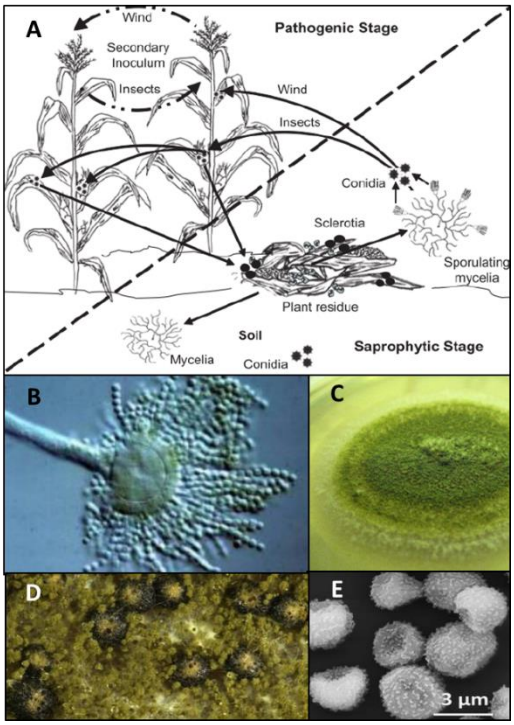


Figure 2: Life and infection cycle of A. flavus (A); Colony of A. flavus on synthetic medium (C); Characteristic structures of A. flavus: Conidiophore (B); Sclerotia (D); Conidia (E);

The fungal colony (***Figure 2-C***) develops from the conidia (***Figure 2-E***) or from the sclerotia (***Figure 2-D***) in a process known as germination: from them the hyphae depart, filamentous structures that make up the body vegetative fungus (mycelium) and that differentiate in conidiophores (***Figure 2-B***), from which new spores originate by mitotic division *(Horn et al. 2009)*. On the basis of the sclerotia diameter, strains can be grouped in L strains (group I, sclerotia with a diameter greater than 400 μm) and S strains (group II, sclerotia with a diameter smaller than 400 μm, typically around 200 μm).

3.1. Pathogenicity

Aspergillus flavus is the etiological agent of numerous human pathologies, ranging from allergic reactions to infections associated with the entry of the fungus in the blood system. After *A. fumigatus*, it is considered the second species causing invasive and non-invasive aspergillosis *(Morgan et al. 2005)*. The first way of entry is represented by the inhalation of spores, whose larger dimensions than those of the other species favors their deposition in the upper respiratory tract *(Morrow, 1980)*. *A. flavus* is also implicated in allergic bronchopulmonary aspergillosis, and cases of native and prosthetic endocarditis *(Demaria et al. 2000; Rao and Saha 2000; Irles et al. 2004)*, craniocerebral aspergillosis in immunocompromised subjects *(Rudwan and Sheikh, 1976; Hussain et al. 1995; Panda et al. 1998)*, keratitis and skin infections in both humans and animals, have been attributed to it. As already mentioned, *A. flavus* is an opportunistic pathogen of agricultural crops; in particular those with a high content of oils, and its infection can involve different stages of production, from cultivation in the field, to storage, to derivatives. The factors that can influence its infection are many and varied, although the temperature and humidity are of particular importance. In fact, warm and humid climates are those in which fungal proliferation is favored. Another important factor in promoting infection is represented by the physical damage present on the matrices. In the case of maize, for example, the physical damage caused by parasitic insects such as *Sesamia cretica* or *Orsinia nubilalis* on the grains, represent the preferential way of entry to the substrate. Although its presence has a negative impact in economic terms, mainly due to the produced rots, it is considered to be minor. Is the synthesis and release of aflatoxins on the infected matrices to determine the greatest losses in economic terms.

3.2. Aflatoxins

.Aflatoxins are compounds derived from the secondary metabolism of several species belonging to the genus *Aspergillus*, among which the main producers are *A. flavus* and *A. parasiticus*. Among all mycotoxins, aflatoxins are of particular attention due to their acute and chronic hepatotoxicity, but above all because of their very high carcinogenicity. They were first detected in 1960, when more than 100,000 turkeys died in a UK farm due to severe aflatoxicosis. Initially it was not possible to understand the causes of the phenomenon, so that the pathology was called "Turkey X Disease", but in-depth studies revealed that the cause of those deaths was attributable to an excessive consumption of peanuts strongly contaminated by *Aspergillus flavus*. The fungal metabolites responsible for the toxic outbreak were isolated and called **aflatoxins** *(Forgacs et al. 1962)*. There are numerous compounds belonging to this group, but four are the main aflatoxins: AFB_1, AFB_2, AFG_1 and AFG_2 ***(Figure 3)***.

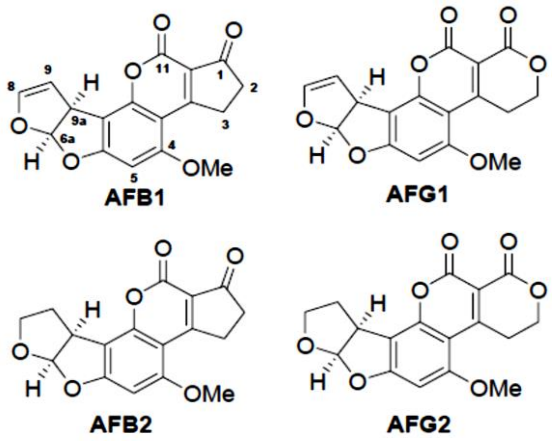


Figure 3: Chemical structure of aflatoxins B_1, B_2, G_1 and G_2.

From the structural point of view, AFs are bifuranocumarinic compounds: those belonging to group B (so called due to the blue-violet fluorescence they emit when irradiated at a wavelength of 365 nm) carry a cyclopentene ring, while group G (so called for the green fluorescence) is characterized by an δ-lactone ring (***Figure 4***). AFG_1 and AFB_1 share the presence of a dihydrofuran ring, while AFB_2 and AFG_2 possess a tetrahydrofuran ring. Aflatoxins B_2 and G_2 are the corresponding dihydroxy derivatives of B1 and G1 respectively.

AFB_1 represents the most abundant and most toxic metabolite in this group and for this reason, it is the object of the most numerous studies: in 2012, the International Agency for Research on Cancer included aflatoxin B_1 in group 1, in which are grouped all the molecules certainly carcinogenic to

humans. The development of the fungus and the subsequent contamination of the infected matrix can take place on numerous substrates that become part of the agro-food chain, and can involve different production stages: from cultivation, to storage, to processed derivatives.

The main factors involved in the accumulation of aflatoxins by the mycelium may be extrinsic, such as conditions that favor or inhibit fungal development, and intrinsic, linked instead to the capacity of the strain to synthesize aflatoxin. Among the extrinsic factors, the combination of temperature and humidity, which regulates the development and differentiation of the fungus, and the presence of damage on the matrix surface, which allows access to it, are of particular importance; in this sense, agricultural practices of crop management, grain collection and conservation are therefore decisive. In the case of maize, some biotic factors also become relevant, such as the diffusion on the cultivations of *Ostrinia nubilalis*, whose larvae, by digging tunnels inside the grain, create access routes exploitable by the fungus to complete the infection.

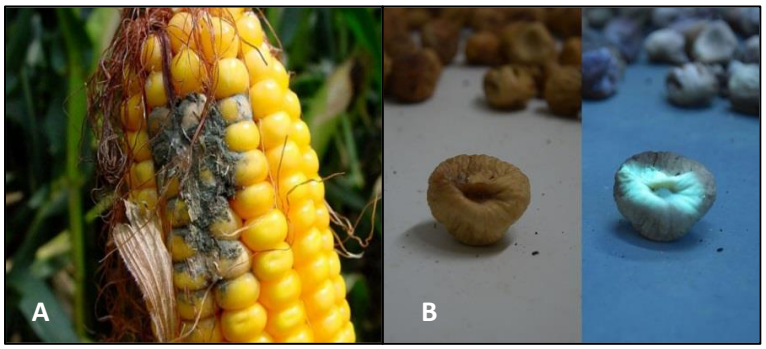


Figure 4: Ear of maize infested by A. flavus (A). Contamination by aflatoxin B_1 on dried fruit visible as fluorescence when exposure to UV source 365 nm (B).

The conditions that allow the development of the mycelium, however, may differ from those that lead to the accumulation of toxin: the requirements for the biosynthesis of aflatoxin by different toxigenic species are somewhat non-specific, they correspond to temperatures between 25 and 35 °C and to free water values between 0.82 and 0.87, but it has also been shown that the type of substrate can exert considerable influence. For example, the presence of different carbon sources (glucose, mannose, fructose and sucrose) in the substrate induces different levels of accumulation: for this reason, the quantity of AF produced by *A. flavus* in peanuts and corn is definitely higher than in other cereals, while typically not occurring in rice. The biosynthesis of AF is often conditioned by the osmotic and oxidative status of the fungal cell, both physiological and non physiological. These are not necessarily linked to environmental factors, but can derive from the plant during the attack of the fungus: in fact, it is well known that plants subjected to biotic stress often react by increasing

the production of reactive oxygen species, which can accumulate in the tissues and reach in contact with the pathogen that is developing on them. Hence, the production of AFs is quite variable depending on the growing conditions, but it is initially related to the presence of the producing microorganism. However, the high resistance of these molecules to the treatments used in the production and in the transformations to which contaminated food matrices are subjected allows their persistence even after the death of the fungus. Therefore, the apparent absence of mycelium on a given substrate is not sufficient to considered it toxin-free.

The problem of contamination of substrates such as maize kernels is very important in those Countries (such as Italy) in which corn grain represents the basic ingredient for the feeding of dairy cattle, and through the dairy chain aflatoxin can reaching up the consumer. In fact, in mammals AFB_1 is metabolized by the liver in its hydroxylated form AFM_1 ***(Figure 5)***, which is then excreted through the glandular system in milk. In this way, the toxin is transferred along the whole chain of derived products.

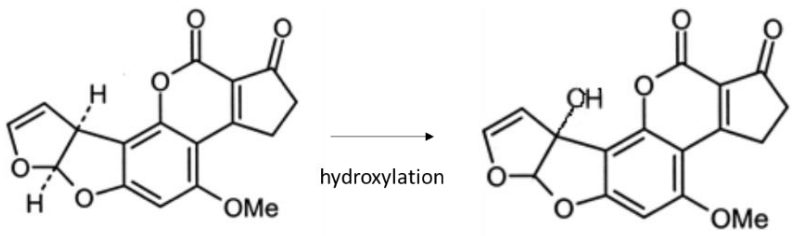


Figure 5: Conversion of aflatoxin B1 to M1

4. Mechanism of toxicity

Several metabolic pathways involved in the biotransformation of AFB_1 and other aflatoxins have been identified in humans: these can lead to the activation or detoxification of the toxin. Biotransformation plays a fundamental role in determining its toxic activity and it has been demonstrated that bioactivation is a prerequisite for many of the toxic and carcinogenic effects of aflatoxins.

In general, the metabolism of AFB_1 involves four types of reactions: reduction, hydroxylation, epoxidation and O-dealkylation ***(Figure 6)*** *(Wu et al. 2009)*.

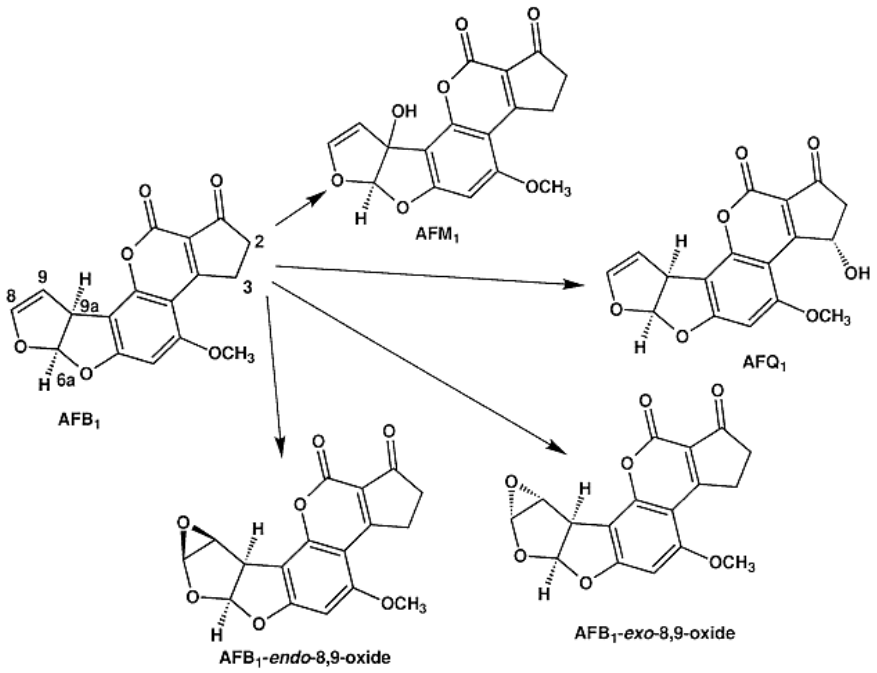


Figure 6: Oxidation products of AFB_1.

The ketonic reduction of AFB_1 in aflatoxicle is associated with the activity of NADPH-reductase, while all the other reactions are mainly carried out by the enzymes belonging to the superfamily of the cytochromes P450. The epoxidation reaction in the 8,9-exo-epoxide species is essential for the toxin to exert its mutagenicity and carcinogenicity, and the enzyme most involved in the process is cytochrome P450 3A4, which is able to metabolize aflatoxin B_1 even at very low concentrations. Another cytochrome, P450 1A2, has the potential to generate this genotoxic product but to a much lesser extent. This is due both to the fact that it is present in significantly smaller quantities compared to 3A4, and that it is involved in the formation of a non-toxic product (endo-8,9-oxide form) *(Ueng et al. 1995)*. In the small human intestine P450 3A1 is present in large quantities, so in this case the oxidation in AFQ_1 or in exo-epoxide actually represents a form of detoxification of the original toxin, and explains why the genotoxic action of the molecule is not explained in the cells of this intestinal tract. In addition to the epoxidation reactions, the P450-dependent monooxygenases can oxidize AFB1 in hydroxylated or dealkylated products such as AFM_1, Q_1, B_2 or P_1. Specifically, P450 1A2 is involved in oxidation in AFM_1 while P450 3A4 in conversion to AFQ_1, and both compounds are characterized by a much lower toxicity than AFB_1 *(Daniels et al. 1992; Daniels et al. 1990; Liu et al. 1992)*. Further data showed the possibility of activation in epoxide also through catalysis involving lipoxygenase, microsomal prostaglandin H synthetase and through a lipid hydroperoxidation mechanism ***(Figure 7)***.

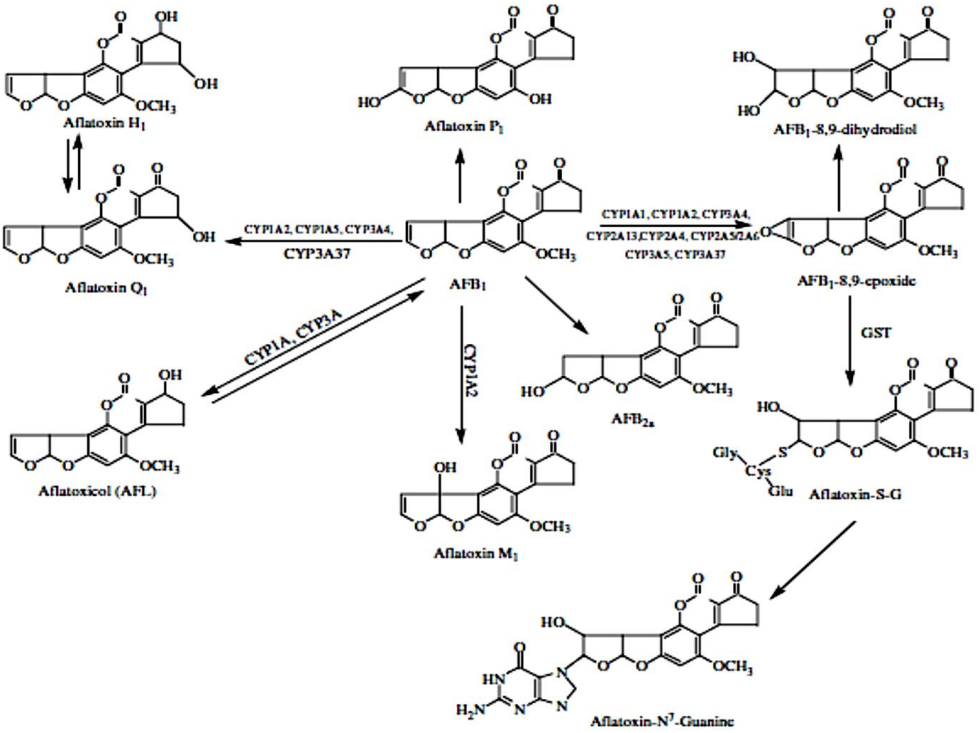


Figure 7: Representation of major pathways and key enzymes in the metabolism of aflatoxin B_1 in humans.

The enzymes involved in the activation of AFB_1 are not the only ones to determine the sensitivity to toxicity of these molecules. It is also necessary to consider the activity of detoxifying enzymes such as glutathione transferase (GST) or glucuronosyltransferase. In these processes, the toxicity is decreased and the hydrosolubility and polarity of the compounds are increased, favoring their excretion. In this regard, the conjugation of AFB1-exo-8,9-epoxide with glutathione, catalysed by GST, represents the most important detoxification phenomenon and plays a key role in protecting tissues from aflatoxin toxicity. Hydroxylated or O-dealkylated metabolites such as AFM_1, AFP_1 or AFQ_1 are readily conjugated to gluuoronic acid or sulfate and excreted in bile or urine. However, the significance of this process in tissue protection is marginal, since hydroxylated products have a very low toxicity *(Guengerich et al. 1997)*.

5. Aflatoxicosis

Toxic syndromes resulting from exposure of humans and animals to AF are called aflatoxicosis. Two forms of aflatoxicosis have been characterized: the first is a severe acute intoxication that results in direct liver damage and subsequent illness and death, while the second is a chronic asymptomatic exposure. The studies carried out on the different forms provide clear evidence that dose and

duration of exposure to AFB_1 have a direct effect on toxicity and can cause various consequences: 1) massive doses involve acute illness and death, usually due to liver cirrhosis; 2) chronic sub-lethal doses cause nutritional and immune consequences and, 3) all doses have a cumulative effect for cancer risk (*Williams, J.H. et al. 2004).* Sensitivity to AFB_1 is greater in young people and there are very significant differences between species, between individuals of the same species (in relation to their different ability to detoxify AFB_1) and the gender (in relation to testosterone concentration). The toxicity of AFB_1 also varies according to various nutritional factors, and recovery from protein deficiency is delayed by exposure to AFB_1 *(Pier. A.C. et al 1985; Adhikari, M.R. et al. 1994; Rodgers, A.P. et al. 2012)*. The main target organ of both acute and chronic AF toxicity is the liver, but injuries to kidney, stomach and lung damage have also been reported *(IARC. 1993)*. Not all the amount of ingested AF is biologically significant, as a variable proportion is detoxified. The differences in sensitivity to AFs between the different animal species, and between individuals of the same species, therefore depend, in large part, on the fraction of the internal dose that is engaged in the various possible metabolic pathways, where the greater biological risk is connected to the pathway of activation in epoxide with consequent reaction with DNA and proteins (*Williams J.H. et al. 2004).* The decreasing order of chronic and acute toxicity of the four main AF (AFB_1>AFG_1>AFB2>AFG_2) reflects the chemical characteristics of the four molecules. The lower reactivity of AFB2 and AFG2, in fact, is determined by the absence of the unsaturated double bond at the end of the furan ring which determines the possibility of forming the epoxide, while the lower reactivity of AFG_1 and AFG_2 is characterized by the lower reactivity of the lactone ring with six atoms compared to the cyclopentenone of AFB_1 and AFB_2 (*Wogan, 1996).*

5.1. Acute aflatoxicosis

Over the years, numerous cases of acute aflatoxicosis have been reported especially in populations of developing regions. The clinical manifestation of aflatoxicosis is represented by vomiting, abdominal pain, pulmonary edema and infiltration of fat and necrosis of the liver tissue. In the 1970s, the consumption of heavily contaminated maize in western India caused massive poisoning which led to 97 deaths: the histological examination of the liver revealed an extensive proliferation of bile ducts, a lesion often found in animal experiments in which it was induced toxicosis by administration of contaminated feed *(Bhat and Krishnamachari 1977; Krishnamachari et al. 1975)*. In 1995, consumption of noodles in which more than 3 mg of aflatoxin were found per serving

caused acute hepatic encephalopathy in many Malay children *(Lye et al. 1995)*. In a more recent 2004 event in rural Kenya, 317 cases were recorded with 125 victims.

5.2. Cronic aflatoxicosis

The most frequent situation, in the case of AF, is chronic exposure to low levels of contamination *(CAST 2003).* To assess chronic AF exposure in humans, two main approaches were used. The first is based on food sampling, taking samples both from prepared foods and from ingredients on the market. The simplest and most reliable source for the assessment of the exposure is represented by the analysis of prepared foods as the raw materials (ingredients) considered unsuitable can be directly discarded by the consumer. The second approach is based on the analysis of biological exposure markers. In this perspective, samples of human blood, milk or urine are collected, which are analyzed for the presence of AF derivatives, each of which is characterized by a specific body half-life. The technology is relatively recent, and the cases described refer, in particular, to restricted areas of developing countries (*Williams et al. 2004).* The prevalence and level of human exposure to AF on a global scale has been reviewed and the conclusion is that about 4.5 billion people, especially in developing Countries, but also in industrialized countries, are chronically exposed to quantities uncontrolled AF. A small number of information suggests that, at least where extensive investigations have been conducted, common exposure to AF may be reflected in alterations in immunity and nutrition, which can negatively affect health (particularly in the case of HIV infection) and account for about 40% of cases of illness in developing Countries where the average life span is short.

5.3. Growth compromission in children

Exposure to aflatoxin is linked to growth arrest in children. This phenomenon is very important because it is associated with other effects such as an increased vulnerability to infections and cognitive disorders that extend well beyond childhood. Studies conducted in Togo and West Africa have shown that the weight and height of children in relation to age are lower in a dose-dependent manner than aflatoxin exposure. In addition, two other studies carried out in the same regions have shown that the presence of high levels of albumin-conjugated aflatoxin in the maternal blood, in the umbilical cord and in the child's blood are associated with lower growth indicators as a weight greatly reduced at birth. In Iran, two other studies have shown that even high levels of AFM1

excreted in the milk of pregnant women are associated with a lower weight and height of the newborn at birth *(Khlangwiset et al. 2011) (Table 2).*

Country	% population living below national poverty line (WHO, 2010b)	GDP per capita, 2010 USD (PPP) (IMF, 2010)	Aflatoxin exposure, ng/kg bw/day (Liu and Wu, 2010)	% stunted children (WHO, 2010b)
Argentina	NA	15 030	0–4	8
China	5	7240	17–37	22
France	NA	34 250	0.3–1.3	NA
The Gambia	58	1479	4–115	28
India	29	3176	4–100	48
Kenya	52	1783	3.5–133	36
Nigeria	34	2357	139–227	43
Philippines	37	3604	44–54	34
Spain	NA	29 649	0.3–1.3	NA
Tanzania	36	1484	0.02–50	44
Thailand	13	8479	53–73	16
USA	NA	47 702	0.26	4

Note. GDP = gross domestic product per capita; NA = not available; PPP = purchasing power parity.

Table2: Relationship between exposure to aflatoxin and defects in growth in children between nations.

5.4. Immunomodulation

Many studies have examined the link between exposure to aflatoxin and some markers representative of immune system dysfunctions in humans: it has been observed in Ghana that the presence of high levels of aflatoxin-albumin in infected and non-HIV infected individuals was genus associated with low levels of regulatory CD4 + T lymphocytes, as well as of B cells. In a different study of Gambian resident children, it was found that at a high level of aflatoxin-albumin adducts corresponded low levels of IgA in saliva *(Jiang et al. 2005)*. However, it is still considered necessary to carry out other types of epidemiological studies in various regions of the world to look for other evidences that associate exposure to aflatoxin and compromise of the immune system.

5.5. Carcinogenicity

The main carcinogenic effect of aflatoxin is exerted in the liver, where it induces the development of hepatocellular carcinoma (HCC). The molecular marker of aflatoxin-induced hepatic carcinogenesis is a mutation in codon 249 in the tumor suppressor gene P53: this mutation, a nucleotide transversion on the third base of codon 249 (AGG → AGT), determines the amino acid substitution R → S. No other type of cancer has ever been shown to have such a close association

between a mutagen and a mutation so specific in TP53 as in this case: in fact, this alteration is found in 90% of hepatocarcinomas developed in individuals belonging to regions where it was high exposure to aflatoxin has been demonstrated *(**Figure 8**)*.

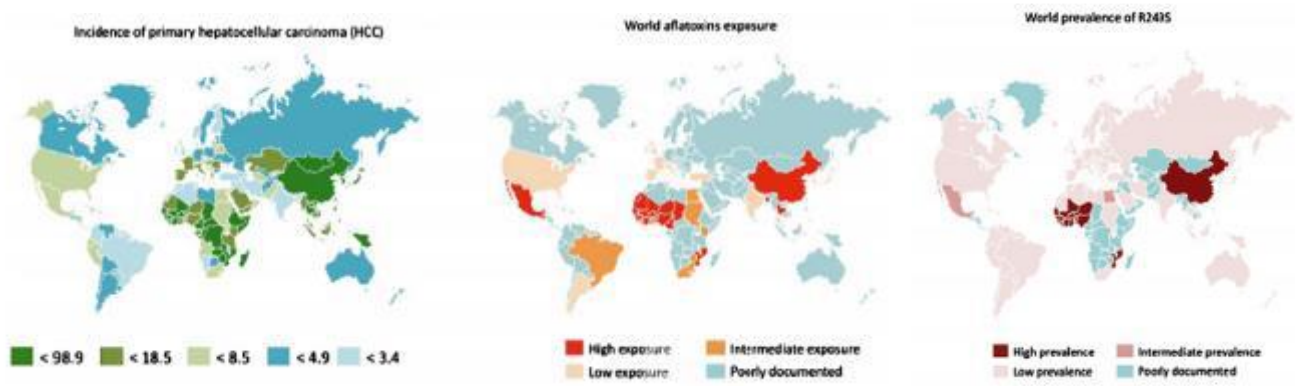


Figure 8: Geographical distribution of the incidence of HCC, exposure to aflatoxin and prevalence of the R249S mutation.

The factors that could explain this unique situation are two: an extreme specificity of the induced mutation or the definition of p.R249S as a protein that contributes to hepatocarcinoma through a preferential way. Usually, regions with a high risk of exposure to aflatoxins, such as the Quidong region in China or some areas of Sub-Saharan Africa, show a dramatic incidence of HCC *(Hsu et al. 1991)*.

As already mentioned, the product with the highest genotoxic power is represented by the metabolite AFB_1-exo-8,9-epoxide, capable of binding DNA to form adducts with an efficiency of 98% *(Johnson et al. 1997)*, and its reactivity is about 1000 times greater than its isomer *(Lyer et al. 1994)*. The epoxide can intercalate between the bases of the DNA forming a covalent bond with the nucleic acid through an electrophilic attack on the N7 of the guanine, which leads to the formation of the AFB_1-N7-guanine adduct. This adduct is very unstable due to the positive charge that is generated on the imidazole ring: as a result, it undergoes several processes that include both the purification (which leads to the formation of an apurinic site) and the opening of the imidazole ring (generating a much more stable adduct, AFB_1-FAPY) *(Smela et al., 2001)*. It has also been proposed that AFB_1 may cause DNA damage even through an indirect mechanism, ie by increasing the production of reactive oxygen species by mitochondria *(Bedard et al. 2006)*. Both the above mentioned adducts can be repaired by the Nucleotide Excision Repair (NER) cell repair system, but not all with the same efficiency while the apurinic site can undergo repair under the Base Excision Repair (BER) system.

However, if the lesion is not repaired, it is fixed and results in a mutation: both the two adducts and the apurinic site lead to a G-T transversion. *(Bedard et al. 2006).*The crystallized structure of the wild-type p53 protein complexed with DNA shows that arginine at position 249 forms four interactions with the residues present in the L2 and L3 loops of the same protein, but does not form any direct link with DNA ***(Figure 9)***.

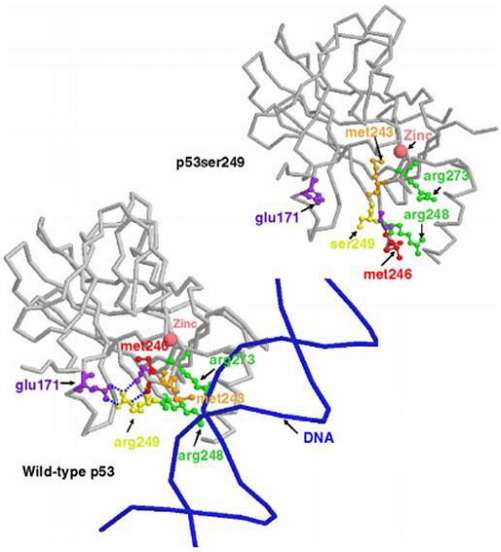


Figure 9: Crystallized structure of the wild-type p53 and of the p.R249S

Arginine replacement with serine thermodynamically destabilizes the protein and compromises the ability to bind DNA *(Cho et al. 1994)*. The NMR analysis shows that the structure of the p.R249S protein core is very similar to the wild type, but there is a distortion around the position changed in the L3 loop which increases the flexibility of the structure. The major effect of serine is to induce a reorientation of the residue M243: in the wild-type protein, this residue is exposed to the solvent when the protein is in dissociated form, but is enclosed within the interface of between the p53 oligomers when complexed with target DNA. On the contrary, in the mutated M243 protein it displaces the M246 residue from its hydrophobic pocket of the zinc-binding region, leading to the formation of a short alpha helix and a conformational change that displaces the R248 residue responsible for DNA binding. This effect may well explain the loss of the ability to bind DNA in the protein p.R249S *(Friedler et al. 2004)*.

6. Aflatoxin's genetics and metabolism

The biological role of aflatoxins has not yet been clarified, but what is certain is that they are not indispensable to the growth of the fungus. Rather, they seem to be a response to certain environmental stimuli, not necessarily of stress. Since their discovery to date, countless studies have allowed us to shed light on biosynthesis, starting from the identification of a 72 Kb gene cluster containing 25 ORFs *(**Figure 10**)*, 17 of which were identified as genes directly involved in the synthesis of toxin. In the Aspergillus genus, DNA information is organized in eight chromosomes *(Robinow et al. 1969).* Genes responsible for aflatoxin production are situated in the 54th gene cluster, located at 80 kb from the telomere of the chromosome 3 *(Georgianna et al, 2009).*

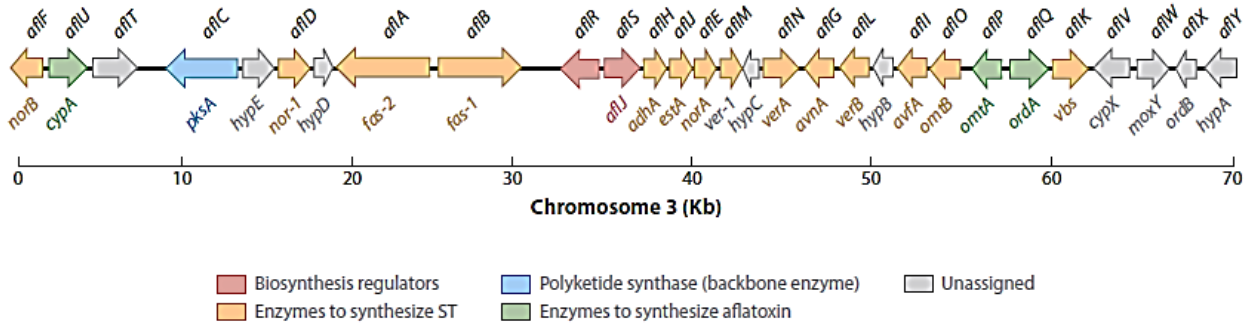


Figure10: Aflatoxin cluster in Aspergillus parasiticus. *The vertical line represents the 82-kb aflatoxin biosynthetic pathway gene cluster and sugar utilization gene cluster in A. parasiticus and A. flavus. The new gene names are given on the left of the vertical line and the old gene names are given on the right.Arrows along the vertical line indicate the directionof gene transcription. The ruler at far left indicates the relative sizes of these genes in kilobases.* ***(Yu Li et al. 2004).***

These can be divided into three categories:

• **Structural**, whose product are enzymes or enzymatic subunits implicated in the catalysis of the various biosynthetic intermediates;

• **Regulators**, with the only gene currently discovered ***aflR***, which encodes a zinc-protein responsible for binding a specific DNA sequence and acts as a transcriptional activator;

• **Co-regulators**, with the only gene currently discovered ***aflS***. The inactivation of this gene leads to a drastic transcriptional decline of numerous functional genes, hence the hypothesis that it performs co-regulatory functions in the transcription of cluster genes.

The aflatoxin biosynthesis genes are found grouped in a cluster, which many studies have shown to be regulated by the specific transcription factor encoded by the *aflR* gene. The AFLR protein, defined as zinc cluster DNA-binding protein, binds the *aflR* gene promoter, self-regulating, and some TCGN5CGA motifs within the promoter of other pathway genes; the C-terminal portion of the protein probably makes contact with RNA polymerase II by activating its transcription *(Chang et al. 1999).*

The ***omtB*** gene is one of the enzymes of the aflatoxin pathway and encodes an O-methyltransferase involved in the conversion of demethylsterigmatocystine (DMST) and of dihydrodemethylsterigmatocystine (DHDMST) in sterigmatocystina (ST), aflatoxin precursor.

The ***dmtA*** is a gene encoding a methyltransferase that acts through the methylation of the cysteine position 5. A significant decrease in aflatoxin conidiation and biosynthesis was observed in *A. flavus ΔdmtA* strains, a consequence of a lower expression of genes such as *omtB* and *aflR*, thus indicating a possible positive role of *dmtA* in the expression of aflatoxin pathway genes; it is similarly involved in the negative regulation of sclerotia production *(Kunlong Yang et al. 2016)*.

The ***mtfA*** is a gene that codes for a histone methyltransferase involved in morphogenesis, mycotoxin biosynthesis and pathogenicity of *A. flavus*; specifically it catalyzes an arginine methylation reaction in post-translational modification processes. Also in this case its activity results in a decrease in the conidiation and in an increase in the biogenesis of sclerotia, increasing the expression of genes such as *NsdC* and *NsdD.* In the aflatoxin biosynthesis process, on the other hand, its transcription appears to be up regulated, resulting in an increase in the expression of genes such as aflR. He is also involved in mechanisms of response to stress, such as that caused by membrane lesions, osmotic and redox *(Yu Li et al. 2017)*.

The ***NsdD*** and ***NsdC*** coding respectively for a transcription factor with zinc-finger domain GATA-type and a DNA binding-protein with zinc-finger domain C2H2, are two genes characterized for the first time in *A. nidulans*, but also present in *A. flavus* and *A. parasiticus*, are necessary for the transcriptional regulation of genes involved in sclerotia biogenesis and conidiation. In addition, since a relationship between morphogenesis and secondary metabolism is known, it is believed that the proteins encoded by the *Nsd* genes may, by extension, also influence the biosynthesis of aflatoxins.

Studies conducted on *ΔNsdCΔNsdD* knockout mutants have shown that the deletion of these genes leads to severe morphological alterations of colonies and conidiophores, a decrease in spore

production and an altered pigmentation, tending to ocher or black, the loss of sclerotia, and a significant reduction in the amount of aflatoxins produced *(Cary et al. 2012)*.

The ***VeA*** gene is considered the global regulator of secondary metabolism in fungi such as *A. flavus* and codes for a protein of 574 amino acids belonging to the family defined as "velvet family proteins"; forms a heterotrimer with *VelB* and *LaeA,* required for the coordination of fungal development *(Sarikaya-Bayram et al. 2015)*.

The mere presence of the cluster does not in itself determine the ability to accumulate aflatoxin: in fact, there are frequent strains belonging to aflatoxigenic species that are incapable of producing it. There are several events that can determine the loss of this capacity: for example, single nucleotide polymorphisms (SNPs) are known which introduce stop codons in the sequences of some genes, causing the loss of the function of the relative gene product. 0ne of these events has been identified in the non-toxic species *A. sojae* at the level of *aflR*, whose AFLR protein is truncated and is incapable of promoting the transcription of most genes of the biosynthetic pathway *(Matsushima et al. 2001)*. Similarly, at the base of the non-oxygenic character of the *A. flavus* BS07 strain there is a SNP at the level of the *aflC* gene (which codes for one of the very first enzymes of the aflatoxin biosynthetic pathway) which determines a pre-termination of the amino acid sequence, preventing the accumulation of the necessary precursor *(**Figure 13**)*. The inability of all members of the *A. flavus* species to produce AFG_1 and AFG_2 defines a further critical point in the progression of the biosynthetic pathway. Unlike what happens in the "cousin" species *A. parasiticus*, in this species the cluster has a gap in the *aflF*/*aflU* region that covers both the promoter regions of the two genes (to which the transcription factor AFLR is normally bound), and the initial portions of the genes themselves. This means that *aflF* and *aflU* gene products prevent the conversion of O-methylsterigmatocysteine and dihydro-O-methylsterigmatocysteine to AFG_1 and AFG_2 respectively *(**Figure 11**)*. The two enzymes do not seem indispensable for the biosynthesis of AFBs *(Ehrlich et al. 2004).*

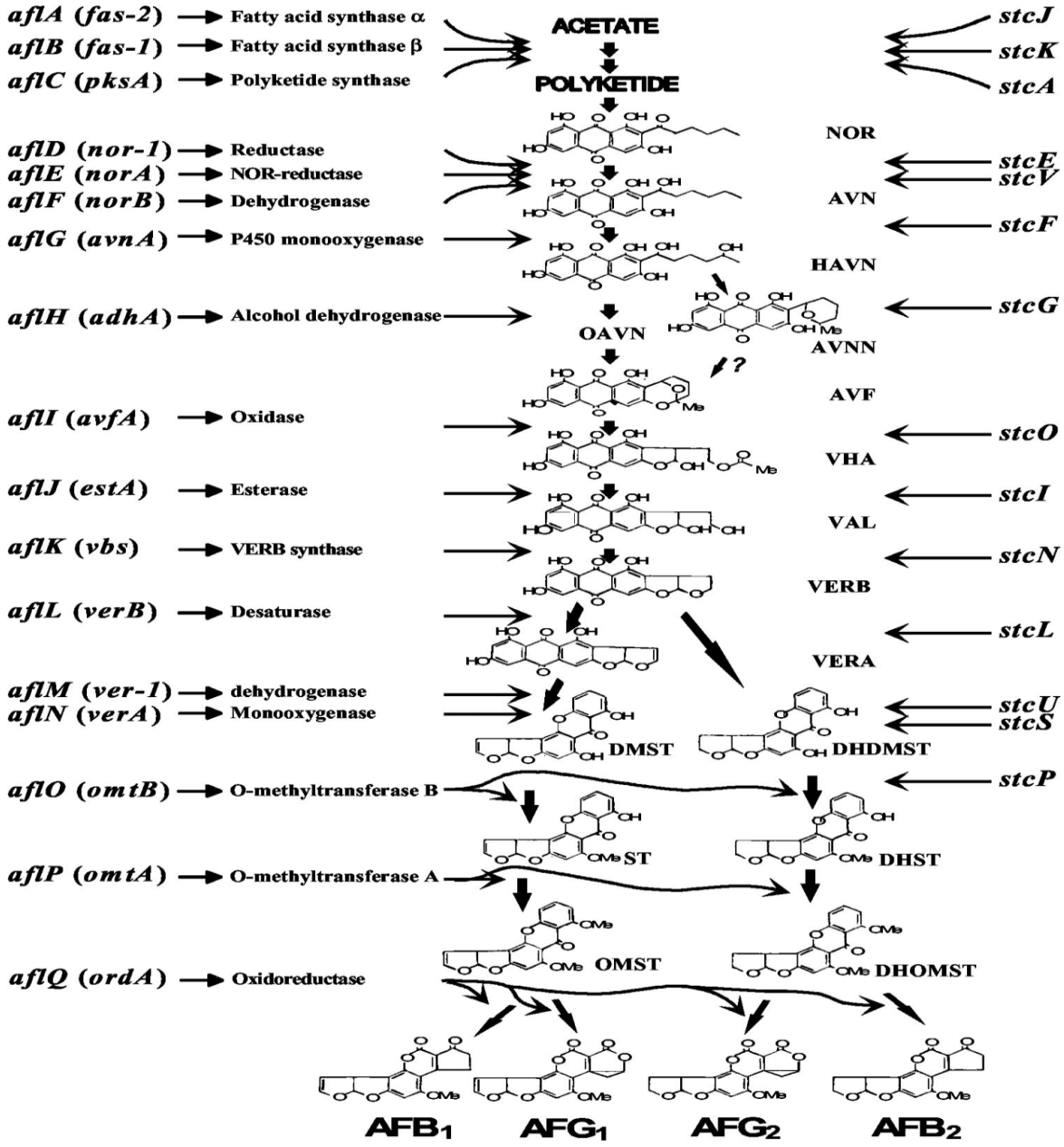


Figure 11. Gene cluster and aflatoxin pathway. *Arrows indicate the connections from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and from the intermediates to the products in the aflatoxin bioconversion steps.* ***(Yu Li et al. 2004).***

7. Contamination management and containment strategies

To date, analytical techniques for the quantification of mycotoxins in the matrices include thin layer chromatography (TLC), high-pressure liquid chromatography (HPLC) and gas chromatography (GC-mass). Quantifications in the order of tenth of ng/mL are possible through Rapid immuno-assay (RIA) and immunological analysis (ELISA). However, if the optimization of the diagnostic aspect remains fundamental, the importance of the prevention of contamination and of the spread of the producing fungi directly on the crops and on the products in storage is of increasing importance. There are many strategies that can be used to limit the exposure of both animals and consumers to aflatoxins: for example the de-contamination of commodities using chemical (treatments with ammonia, sodium bisulfite or oxidizing agents), physical (solvent extraction, adsorption, heat inactivation or irradiation) and biological methods (microorganisms capable of metabolizing / inactivating AFs). Unfortunately, these strategies present some drawbacks such as: high costs, limited effectiveness, safety problems related to reaction products, organoleptic changes of the matrices, difficulties of application on a large scale. Different and varied strategies are available to limit the spread on cereals of both infection and toxin contamination. For example, pre-harvest, using good agronomical practices (crops rotations, optimal seeding time, seeding density, correct use of chemical fertilizers). We can also to reduce sources of infection using corn hybrids resistant to fungal attack and treatments with insecticides. Also, the stress reduction using suitable irrigation interventions, draining excess water and balanced fertilization. For example, one of the most important agronomic tools for the control of infections in the field is irrigation, since a high-risk condition is the presence of water stress following the waxy ripening of the grain. Also the factors that cause lesions of the grain surface (hail, insect attacks, mechanized harvesting, etc.) increase the risk of infection. For example, the larvae of the European borer (*Ostrinia nubilalis*) or other lepidoptera can transport the conidia from the leaf surface to the tunnels dug in the ear. For this reason, anti-phytophagous treatments become essential for the prevention of aflatoxin contamination. A significant risk reduction can be obtained at the time of harvest, avoiding the threshing of crops in which the grain has a humidity of not less than 22%.

There are also, strategies that we can adopt post-harvest such as the control of temperature and humidity in storage and also during transformation process of the raw material to the fork. The target can be variable: ranging from the competitive exclusion of toxigenic species directly in the field, for example by applying specific antagonists (antibiosis) or intraspecific biocompetitors (AflaGuard®) to the affected crops, containing physical stress and mechanical damage caused by

phytophagous, through insecticidal treatments, to the effective elimination of the fungus obtained with fungicides and/or antifungals. These are currently divided into two classes: non-specific and site-specific. However, both are increasingly affected by the phenomenon of resistance. To obviate the consequent problems, the interest in the identification and selection of new compounds is increasingly growing, with the most specific action possible and capable of interfering at several levels in the metabolism of the fungus. In general the fungicides of agronomic interest show a higher diversity in the chemical structure and in the mode of action than those of medical use, and are classified by the International Fungicides Resistance Action Committee (FRAC) on the basis of their target *(Jamplilek, 2015)*. For example, a very popular target is the chitin, one of the main components of the fungal cell wall (of which it constitutes about 3%). It consists of a homopolymer of N-acetylglucosamine covalently linked to β(1,3)-D-glucans through a β(1,4) bond *(Lorand et al. 2007)*. Alterations in the abundance and distribution of this polymer cause the cell to lose its resistance, becoming more fragile and susceptible to the action of external agents, and it is on this principle that the antifungal activity of some UDP-N analogues is based -acetylglucosamine which when administered compete for the active site of the enzyme chitin-synthetase *(Castelli et al. 2014)*. Interferents of pyrimidine metabolism are also used, such as flucytosine (5-fluorocytosine), a fluorinated pyrimidine analogue that interferes with the synthesis of both RNA/DNA and proteins *(Onishi et al. 2000)*. Other compounds are known to have a highly specific mode of action, for example at the level of signal transduction (quinazolinones), production of ATP (thiophene-3-carboxamides) or assembly of tubulin during mitosis (arylcarboxyamides). Another field of specialization is that which operates in the research/definition of compounds with a non-purely fungicidal but antitoxigenic action, aiming at specific targets of secondary metabolism rather than growth; currently, the prevalence of these compounds has a natural origin. Another recent alternative is instead the use of natural compounds (such as essential oils and other metabolites) produced by plants and microorganisms: for example, resveratrol and cinnamonic oil were particularly effective in containing the production of mycotoxins from species belonging to to the genera *Fusarium*, *Penicillium* and *Aspergillus*. However, an obstacle to the use of these substances is that the cost is too high *(Magan, 2006)*. Precisely because addressed to targets that are not essential for the development and growth of the fungus, the use of specific inhibitors of the aflatoxin biosynthetic pathway could represent a better choice for the control and prevention of contamination, obviating the problem of resistance development *(Tian and Chun, 2017)*. On the other hand, the use of fungicides alone is not able to guarantee an adequate protection against mycotoxins, since in addition to the fact that the metabolites persist in the contaminated product

even after the disappearance of the producer, in some cases this practice can be reveal counterproductive. It has been observed that some fungicides can sometimes even increase the biosynthesis of mycotoxins *(Schmidt-Heydt et al. 2013)*.The ecological role of mycotoxins still remains unclear, but a strong link between their biosynthesis and the oxidative state of the fungus has been demonstrated. In *A. parasiticus* the excess of some reactive oxygen species seem to trigger the biosynthesis of AFs *(Reverberi et al. 2008)*. On the other hand, various substances with antioxidant properties (such as ascorbate, BHT, eugenol, ethylene and methyl-jasmonate) are able to exert an inhibiting action on it *(Jayashree et Subramanyam, 1999; Passone et al. 2005; Huang et al. 2008; Huang et al 2009; Meimaroglou et al. 2009; Caceres et al. 2016)*. The inhibitory effect of antioxidants seems to be exerted through the stress of the enzymatic activity of SOD, CAT and GPX, reducing the accumulation of aflatoxin through the down-regulation of the expression of genes of the biosynthetic pathway *(Reverberi et al. 2005)*.

Three possible ways of inhibiting aflatoxin biosynthesis can be roughly identified:

1) Interference with the physiological environment of the fungus. An example is eugenol, the most abundant phenolic compound present in the essential oils of many spices. The evidence suggests that it acts as a modulator of the cellular oxidative-reduction state, producing a decrease in the activity of the enzymes involved in the response to oxidative stresses (glutathione peroxidase, superoxide dismutase) which is known to act in the regulation of differentiation and secondary metabolism *(Jayashree et al. 1999)*.

2) Interferences in the transduction of signals such as the regulation of upstream gene expression of aflatoxin biosynthesis. There is much evidence to support the idea that a calcium-dependent signaling system is important in the modulation of the aflatoxin *pathway (Praveen et al. 2000)*. For example, at the C-terminal of the AFLR cluster regulator and of the secondary metabolism regulator A putative calmodulin binding domain has been identified as VEA, which reflects the possibility that this may influence the transcription of cluster structural genes *(Juvvadi et al. 2006)*. In fact, the use of molecules such as trifluoperazine, a drug used in the treatment of schizophrenia capable of sequestering Ca2 + ions, determines an inhibition of aflatoxin biosynthesis *(Praveen et al. 1999)*.

3) The third way is represented by the possibility of blocking the activity of pathway enzymes: numerous reports show how different terpenoids, natural products of vegetable origin, possess the ability to inhibit the accumulation of aflatoxin. Among these, alpha-carotene is effective in blocking the synthesis of norsolorinic acid, the first stable aflatoxin precursor, by blocking the upstream

enzymatic activity, thus preventing the accumulation of subsequent intermediates *(Greene-McDowelle et al. 1999)*.

Recently, many strategies based on the control of toxin production were developed. The most promising regard the use of synthesized or natural compounds, that can inhibit both fungus growth and/or aflatoxin biosynthesis, and the application of intraspecific bio-competitor of atoxigenic strains. In this sense, my Ph. D project is focused on two main projects: the first, Aflatox® Project, was funded by Cariplo Foundation, and was aimed at characterizing the biological activities of new bioinorganic compounds, harmless to the environment and the human health but efficient as inhibitors of fungi proliferation and aflatoxins biosynthesis. The second, 'DIFESAMAIS Project, was funded by Emilia Romagna Agricultural Development Plan and was aimed at the evaluation of the efficacy of an atoxigenic *A. flavus* strain as a bio-control agent for aflatoxin containment in maize fields.

TOPIC 1:

The Aflatox® Project

Inhibition of aflatoxin production by natural compounds and their derivatives

“Aflatox®: a biotechnological approach for the development of new antifungal compounds to protect the environment and the human health”

In 2015, the CARIPLO Foundation selected to be funded a project proposal from group : its general aim was the development of a battery of newly synthesized compounds, followed by a wide screening to characterize their biological activities in order to evaluate a possible employment as inhibitors for fungal proliferation and mycotoxins containment agents The Alfatox® Project was characterized by a multidisciplinary approach: in fact, from the synthesis of compounds to the final assessment of non-harmfulness against human health, every step of the flowchart needed different expertise in several fields: synthetic chemistry, medicinal and bio-inorganic chemistry, biology, micro-biology, toxicology and genetics. Thus, a group of 20 people (professors, researchers, post-doc fellows and Ph.D students) from both Parma and Brescia Universities were involved in the project.

Amongst the project goals, the main were:

1. The identification of new natural scaffold-based antifungal substances acting directly on the fungal cells and/or on the aflatoxin production;
2. The development of a protocol for the screening of their biological activities and for the improvement through a drug-design approach;
3. The identification of compounds with remarkable antifungal and/or antiaflatoxigenic activity but safe for the human health and the environment *(Zani et al. 2015)*.

The use of synthetic fungicides is still the most effective and common way to intervene, but this generates well-known concerns about long-term residues in food and in the environment (EFSA 2009). For this reason, the development of new substances specifically aimed at preventing the production of aflatoxin with little impact on the environment is a topic of primary importance. The Project was based on a "funnel approach" in which the number of compounds subjected to the next step decreases gradually during the stages. The Inorganic Chemistry unit of the Department synthesized new compouds, based on the scaffold of various natural molecules, some of which already known to be inhibitors for the proliferation of moulds; these molecules were tested for their effect on the mycotoxigenic and phytopathogenic fungus *Aspergillus flavus*, and in particular on its growth and the biosynthesis of aflatoxins. 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). Only the compounds that showed an interesting activity on *A. flavus* were then addressed to the toxicology units (from both our Department and the University of Brescia Department of Medical-surgical specialties, radiological sciences and public health) to be subjected to *in vitro* tests performed on different model systems (plant, bacterial and human cells), to evaluate cell toxicity, genotoxicity and epi-genotoxicity and to assess their impact on the environment and the human health. Final scope of the Project was the creation of a database containing all collected data used for the creation of a Q-SAR model (Quantitative Structure-Activity Relationship) for the newly synthesized compounds. The infographic of Aflatox® Project flowchart is reported in ***Figure 12***.

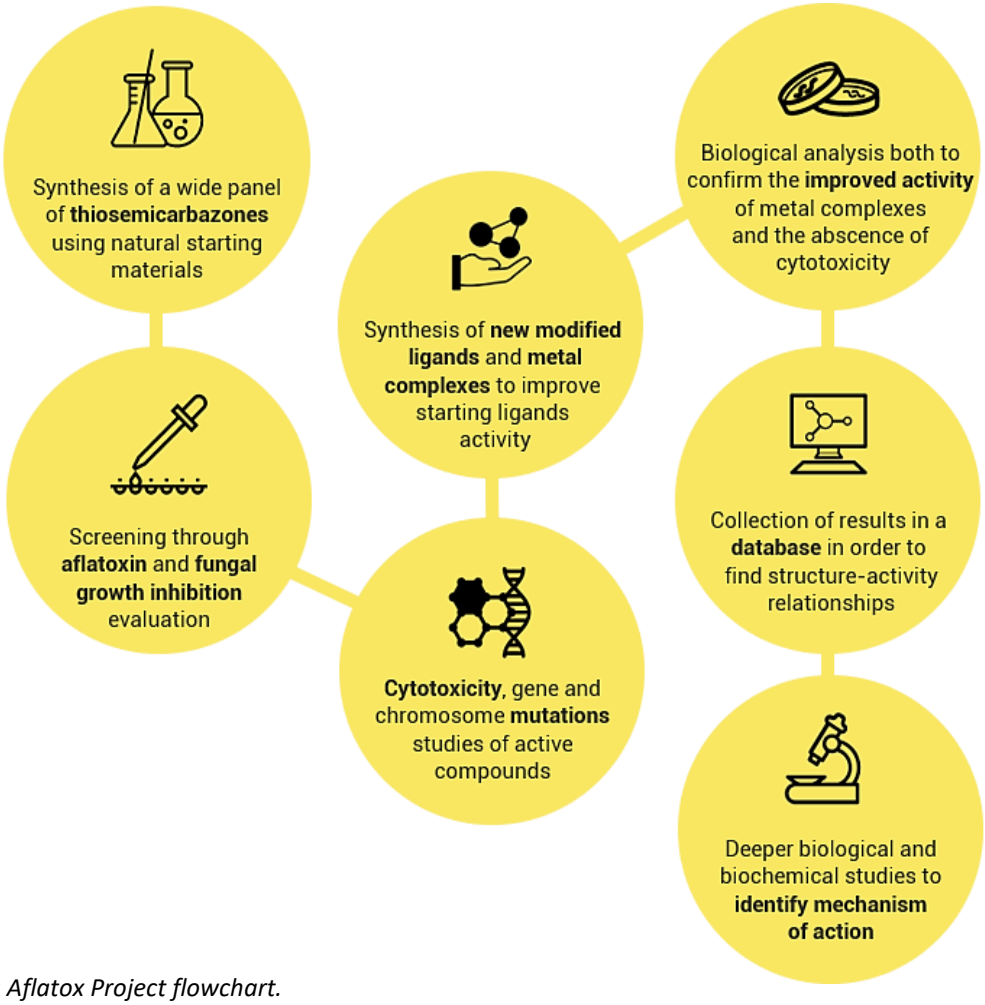


Figure 12: *Aflatox Project flowchart.*

1. Design, synthesis and characterization of new parent compounds. To obtain active and not toxic candidates we used thiosemicarbazones derived from natural aldehydes and ketones.

2. Evaluation of fungistatic and/or antimycotoxigenic activity in *A. flavus.* Each compound was tested to evaluate its ability to inhibit fungal growth and aflatoxin production. *A. flavus* cultures were exposed to different concentration of substances and then analyzed. The fungal growth inhibition was evaluated measuring the optical density of samples and, only for very interesting compounds, also the radial growth. The aflatoxin production inhibition was estimated through microplate fluorescence emission measurements *(Degola et al. 2011; 2012*), allowing us to test at the same time different substances in different concentration (from 10 to 100 µM). Compounds were also tested for the effectiveness in inhibiting sclerotia biogenesis. The data obtained from the evaluation of each of these three physiological parameters were compared with the control, and results were then expressed as percentage of inhibition.

3. Evaluation of toxicity, genotoxicity and epi-genotoxicity. The compounds resulted interesting from the previous steps were tested on three different human cell lines: HFL1 (lung tissue), Crl1790 (colon tissue) and Hs27 (skin tissue). These lines were chosen to simulate the three typical level of exposure of the stakeholder and the consumer to the analyzed compounds.

The cytotoxicity was tested through MTS assay, and effects were measured as GI_{50} (50% cell growth inhibition) values, whereas the Ames test performed on *Salmonella typhimurium* S9 cells was used to assess the compound's ability to induce mutations at the gDNA level. Genotoxicity of compounds were evaluated through the Comet Test Assay, a method to measure deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. The analysis of the average DNA damage after the exposure to a compound is a measure of its genotoxic potential.

4. Assessment of mutagenic potential evaluation and chromosome aberrations induction in *A. cepa* model. Molecules effective in inhibiting *A. flavus* growth and/or aflatoxin accumulation but unaccompanied by cytotoxic and mutagenic effects were then tested on plant and bacterial cells models. The *A. cepa* Assay on root apices of *A. cepa* is used to evaluate the chromosomal aberrations eventually caused by the tested molecules; the effect is expressed as EC_{50}, that is the compound concentration which induces the 50% of *A. cepa* root growth inhibition. The MI (mitotic index) result, represents the number of cells undergoing mitosis in a population: if this value is different from the control, problems in the cell cycle due to the presence of the compound might be occurred.

5. Compound derivatization. Non-toxic candidates were used as parent compounds to create families of derivatives. Different thiosemicarbazones (TSs) were obtained by modifying the original aldehyde or the ketone, changing the functional groups in N^2 and N^4, or replacing the sulphur atom with oxygen. Additionally, some molecules were coordinated with metal ions to obtain metal complexes.

6. Screening of the biological activity of derivatives. Each family of derivatives was tested on both *A. flavus* and human cells to obtain sets of data suitable for Structure-Activity Relationships studies (SAR).

7. Creation of a database. All the data obtained for each compound, including the physicochemical proprieties, were collected and organized in a database. This tool was useful to record and manage the large volumes of data obtained, allowing to analyze and compare results coming from different research fields.

8. Study of the mode of action of active molecules. Promising compounds were analyzed more deeply to understand the underlying mechanism for their anti-aflatoxigenic effect. We used specific experimental designs to clarify possible mechanisms of action and potential cellular/molecular target of compounds. As a side tool, computational chemistry to describe interactions between pesticides and targets and to create SAR models was used.

1. BACKGROUND

1.1. Thiosemicarbazones

Thiosemicarbazones (TSs) are an extremely versatile class of compounds. They possess a variety of interesting physico-chemical properties: peculiar of most of these sulphur-containing organic molecules, are the extensive electronic delocalization and the presence of a thione-thiol tautomerism *(**Figure 13**)*.

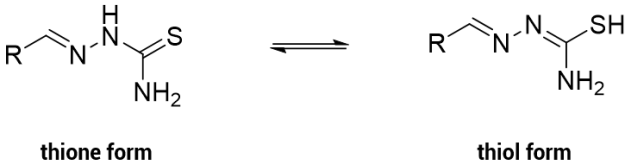


Figure 13: Thione-thiol tautomerism.

The idea was to conjugate the already known antimicrobial properties of some natural aldehydes and ketones (the active component of spices, used for centuries to preserve food) and those of metal ions (for example some metal salts, in particular copper, have been extensively used in agriculture to protect plants from molds). Therefore, TSs are usually obtained from the condensation between the hydrazine group of a thiosemicarbazide with the carbonyl group of an aldehyde or ketone in protic solvents, like ethanol or methanol. The thiosemicarbazide easily reacts with the carbonyl group of the natural aldehydes, possessing the right donor atoms to chelate metal ions and thus allowing the incorporation in a single molecule of three potentially active components. One of the major assets of these compounds is the ease with which the TS structure can be modified, simply by using differentially substituted aldehydes (or ketones) or different aldehydes. The synthesis is usually fast, and the product is obtained usually in 12-24 hours. The most common nomenclature adopted to describe a TS structure follows a scheme in which every nitrogen is numbered as reported in ***Figure 14***.

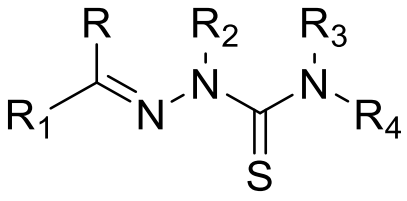


Figure 14: Thiosemicarbazone general structure.

Another crucial characteristic of TSs is the fact that they effectively act as bidentate ligands, using the sulphur and the iminic nitrogen, hence forming a 5-term coordination 2 ring. When the N^2 is not alkylated, TSs can also act as an anionic ligand through the deprotonation of the hydrazinic nitrogen (N^2). The TSs show high affinity to many different metals thanks to the presence of mixed hard-soft N-S donor atoms. Moreover, thanks to the N^2 deprotonation, Thiosemicarbazone's can also balance the positive charges of the metal. This electrostatic effect makes TSs very effective in chelating not only transition elements, but also alkaline and alkaline earth metals. Sulphur creates effectively metal-to- ligand charge-transfer (MLCT), an additional electrostatic stabilizing force for the final complex. The formation of a metal complex between a TS and a transition metal is usually indicated by a color transition which can be explained by the well-known crystal field theory.

1.2. Applications of TSs

TSs (and their metal complexes) have attracted scientists for many years, as they have been successfully adapted for different applications in many fields. For example in ***Figure 15***, the acetonethiosemicarbazone is commonly used in the plastic industry as "stopper" in the polyvinyl chloride polymerization, while the Triapine® (3-aminopyridine-2-carboxaldehyde thiosemicarbazone) is a patented drug used in the treatment of cancer which has successfully reached the phase II of clinical trials *(Ocean et al.2011)* .

Acetonethiosemicarbazone **Triapine ®**

Figure 15: Example of TSs

Thiosemicarbazones find also vast applications in analytical chemistry as sensing agents to detect metals in solution, acting as spectrophotometric or electrochemical indicators *(Raman et al. 2009)*. In particular, the most recent application explored in this field is the use of TS's as sensing agents to detect metals in food *(Janardhan Reddy et al. 2007)*.

Triapine®, that as stated before showed promising anticancer ability, it is not the only TS with interesting biological properties: since their discovery in the 1950s, TSs have proven to be a very

interesting bioactive agents, possessing antibacterial and antiviral activity *(Vahma et al. 1967, Padmanabhan et al. 2017)*, antifungal and anti-aflatoxigenic properties *(Gingras et al. 1965, Rogolino et al. 2017;Degola et al. 2015)* and antitumoral capacity *(Pelosi et al. 2010)*.

1.3. Thiosemicarbazones as antifungal agents

To our knowledge, *in 1960 Benns et al.* published the first example of TS designed and tested as antifungal agents:they evaluated the antifungal activity of a panel of 40 TSs and copper complexes on *A. niger* and *Chaetomium globosum* cultures; the compounds were compared with two commercial fungicides: the 2,2'-dihydroxy-5,5'-dichlorodiphenylmethane and the copper/8-hydroxyquinolinolate mixture. The results showed that several TSs resulted significantly more effective than their corresponding copper complexes, indicating that the antifungal potential was not only due to the metal ion but also to the TS structure. Starting from this discovery, the exploration of TSs capacities for antifungal purposes has grown rapidly, and many other interesting TSs were characterized. Their mechanism of action is still debated: some results ascribed the antifungal activity to the ability of TSs to modify the redox equilibrium in target cells, acting both as anti-oxidant or ROS stimulating agents. Other studies revealed that TSs seem to induce changes in the biosynthesis of cell membrane sterols: in particular, it was reported that specific TSs can influence the regulation and biosynthesis of ergosterol, an essential vitamin for fungal cells strictly connected with the metabolism of lipids and membrane structure *(De Araújo Neto et al. 2017)*.

As previously indicated, TSs are also interesting because of their effectiveness in chelating metal ions; the synthesis of TS metal complexes can thus be used to improve the TS biological effects through the modification of the original scaffold chemical and physical properties, such as water solubility, membrane permeability, bioavailability and cell uptake. In this context, and with regard to our research interest, the addition of a metallic nucleus to the molecule has been explored for the improvement of TS antifungal and anti-aflatoxigenic effect.

1.4. Metal ions in agriculture

In agriculture, metals are usually applied as fertilizers but also as fungicides and antibacterial agents. Since some metals are essential micro-nutrients, their biological effects are strictly connected with their amount, and the level of each metal must be balanced to grant its homeostasis.

Copper compounds are the most employed, especially copper(II)sulphate. It has been known since the XVIII century that the use of a mixture of copper(II)sulphate and lime in water – called "Bordeaux mixture" - has a strong fungistatic effect. If sprayed on crops, it inhibits mould growth and makes seeds unattractive for birds. The fungicidal action of copper is often explained in terms of capacity to interfere with the redox processes which regulate respiration in cells. In fact, due to the easy redox interconversion between Cu(I)/Cu(II), it raises the number of reactive oxygen species (ROS) in cells inducing high levels of oxidative stress.

Zinc is widely used as fertilizer combined with other macronutrients (like potash, phosphate and nitrogen) and supports root growth increasing leaf size and resilience during stressful growing conditions. However, zinc is not only applied as fertilizer. Zinc dimethyldithiocarbamate is a broad-spectrum fungicide applied on the plant surface where it forms a barrier which inhibits the fungal growth on plant.

From the fungus perspective, the role of copper and zinc in the aflatoxin biosynthesis is still poorly investigated, but there are evidences that they play a significant role *(Cuero et al. 2005).* In addition, the production of mycotoxins is well known to be strictly connected with the redox equilibrium in fungal cells. Even though the molecular details of this correlation are still unclear, evidence is coming out that the production of ROS (from both fungus and host) during the mould/plant interaction are able to modulate the biosynthesis of aflatoxins *(Rogolino et al. 2017)*.

2. Newly synthesized compounds

With regard to the above mentioned evidences, the Inorganic Chemistry unit synthesized newly TSs derived from specific natural aldehydes and ketones, chosen for their previously assessed biological properties. In the ***Table 3*** were reported the complete list of the natural starting materials.

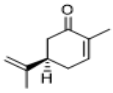
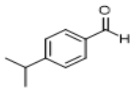
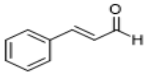
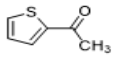
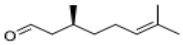
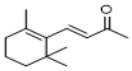
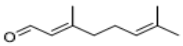
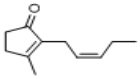
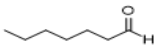
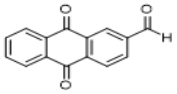
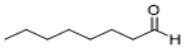
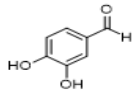
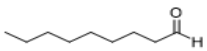
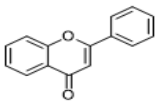
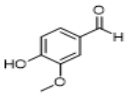
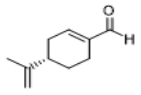
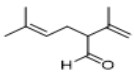
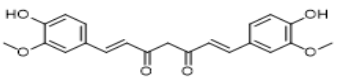
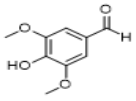
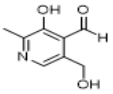
raw material	natural source	raw material	natural source
 benzaldehyde	bitter almonds, apricot and peach seeds	 carvone	dill and spearmint leaves
 cuminaldehyde	cumin seeds	 cinnamaldehyde	cinnamon essential oil
 2-acetylthiophene	tobacco leaves	 citral	lemon, lemongrass and orange essential oil
 ionone	rose essential oil	 citronellal	kaffir lime essential oil
 *cis*-jasmone	jasmine essential oil	 heptanal	clary sage, lemon, bitter orange and hyacinth essential oils
 2-acethylanthraquinone	dyes in rhubarb root, alder tree bark, and aloe leaves	 octanal	citrus essential oil
 protocatechualdehyde	cork	 nonanal	citrus essential oil
 flavone	red berries	 vanillin	vanilla beans
 perillaldehyde	perilla leaves	 fenchone	fennel essential oil
 lavandulol	lavender essential oil	 curcumin	curcuma essential oil
 syringaldehyde	oak wood	 pyridoxal	vitamin B_6 derivative

Table 3: Natural ketones and aldehydes used in the Aflatox Project.

In order to better describe the results obtained, all compounds have been grouped in families on the basis of their structure, including natural starting compounds, ligands, and structural modifications.

The families are listed below:

1. **Cuminaldehyde derivatives**
2. **Cinnamaldehyde derivatives**
3. **Benzaldehyde derivatives**
4. **Jasmonic acid derivatives**
5. **Anthraquinone derivatives**
6. **2-acetylthiophene and 2-acetylfuran derivatives**
7. **Phenone derivatives**
8. **Fenchone derivatives**
9. **Camphorquinone derivatives**
10. **Long hydrophobic chain thiosemicarbazones derivatives**
11. **Non effective derivatives**

My personal contribution…

My contribution to the Aflatox® Project was the evaluation of antifungal and antitoxigenic activity on *Aspergillus flavus* strains. A panel of over 180 molecules was analyzed. Biological activities of compounds on *A. flavus* were assessed by the evaluation of different physiological parameters, such as: 1) the fungal growth, differentiation, and sclerotia biogenesis, and 2) the secondary metabolism, in particular aflatoxin accumulation. The most promising compounds were then analyzed for their capacity to inhibit fungal colonization of maize kernels. To elucidate the cellular/molecular target of some compounds, I 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 technology. Molecular analyses were completed by some functional analyses aimed to the evaluation of morphogenic effects and by the use of other microorganism as model system such as *S. cerevisiae*.

3. Detailed studies on the promising compounds

3.1. Cuminaldehyde derivatives

Cuminaldehyde, a molecule present in the true cinnamon tree's bark at high concentration, is also found in the shoot of *Artemisia salsoloides*, in the leaf of *Aegle marmelos,* and in some essential oils from cumin *(Tsai et al. 2016)*. The chemical compound is stable, soluble in ethanol, and commercially available. *Cuminum cyminum*, commonly known as cumin, has been traditionally used in Thai traditional medicine and traditional food flavoring *(Wongkattiya et al. 2019)*. Until now, very little research on cuminaldehyde has been published on its beneficial effect. Therefore, this molecule was used as lead compound for the design of several derivatives ***(Figure 16)***.

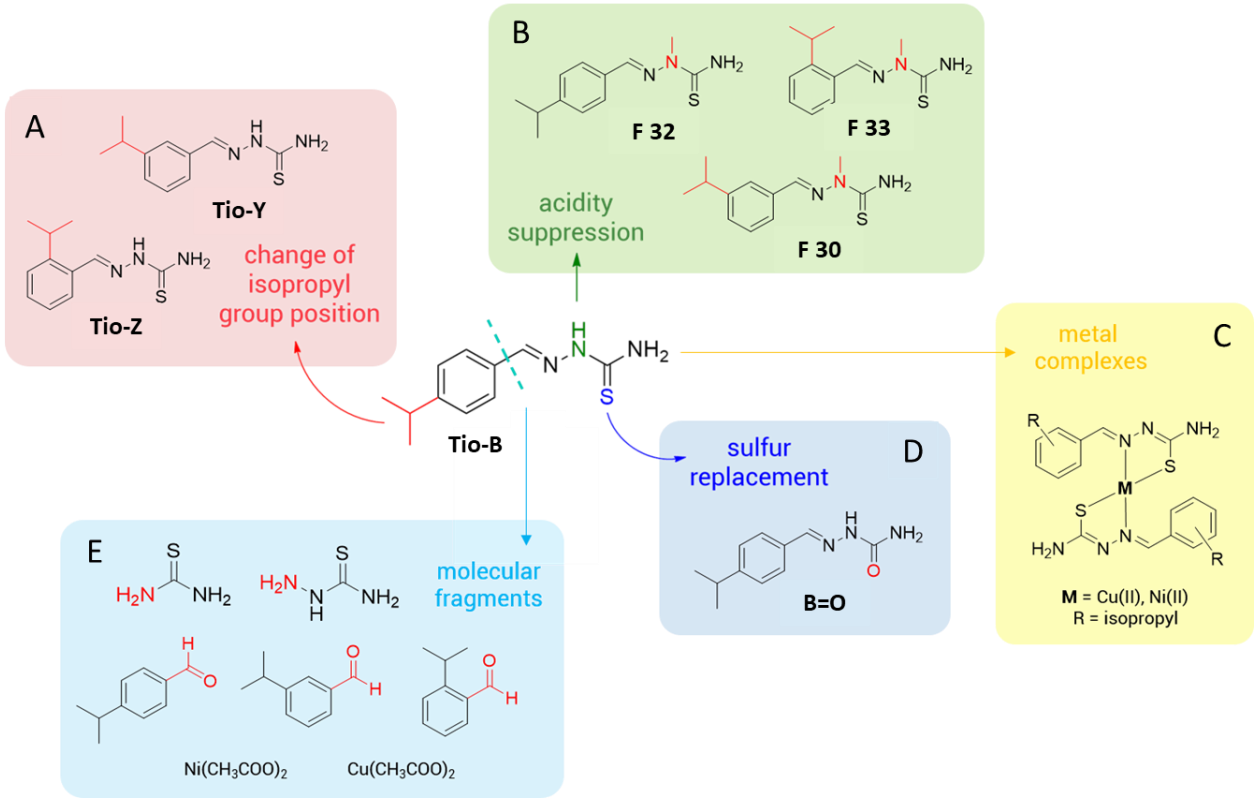


Figure 16: Components of the cuminaldehyde family.

We first modified the cuminaldehyde-TS **(Tio-B)** by change of isopropyl group position. The choice of the *ortho*- **(Tio-Z)** and *meta*- **(Tio-Y)** isopropyl derivatives of Tio-B was based on the fact that these analogs possess a hydrophobicity very similar to cuminaldehyde, but show different structural

geometry. This comparison was aimed at understanding if the position of the isopropyl group plays a generic role, as an hydrophobic fragment that simply favors the molecule to enter the cell, or if its position on the benzene ring is involved in a specific molecular recognition mechanism (***Figure 16, box A***). As a second step, we explored the effect of the substitution in N^2 of the acidic proton (involved in the thione–thiol tautomerism) with a methyl group (***Figure 16, box B***): this modification of the thiosemicarbazide-fragment structure significantly changes the molecule hydrophilicity, and therefore its ability to chelate metals **(F30 F32 F33).** Then, we used all the modified TSs as ligands for the synthesis of their Zn(II) and Cu(II) complexes (***Figure 16, box C***). In general, sulfur forms weaker hydrogen bonds than oxygen due to a lower electronegativity and is more subject to oxidation, through the formation of disulfide bridges. In fact thiosemicarbazones, thanks to their thione-thiol tautomerism, can behave in their thiolic form as reducers, and therefore also as reactive oxygen species (ROS) generators or radical scavengers *(Degola et al. 2017)*. Hence, in order to speculate on the role played by sulfur and oxygen, their ability to form weak/strong hydrogen bondings and also by their redox potentials, we analyzed the biological activity of the **(B=O)** compound, an analog of cuminaldehyde thiosemicarbazone in which the sulfur is replaced with oxygen (***Figure 16, box D***).

As control, the biological effects of the molecular fragments (cuminaldehyde, urea, thiourea, thiosemicarbazide, copper(II) and nickel(II) acetate were also tested (***Figure 16, E***).

3.1.1. Effect on *A. flavus* growth and aflatoxins accumulation

The analysis of the molecular fragments (***Figure 17***) did not provide any significant activity, confirming that the TS complete structure is essential to could observe the biological effect. The absence of any effect was obtained when the sulphur atom was replaced with oxygen. The cuminaldehyde-TS (**Tio-B**) was firstly identified as the most interesting derivative of the cuminaldehyde, showing the over the 95% of mycelium growth and aflatoxin production inhibition at the concentration of 100 µM. To understand which factors provides to the molecule its extreme efficiency, we started by modifying the moieties of the two part constituting the thiosemicarbazone: the aldehyde and the thiosemicarbazide (***Figure 16, box A, B and D***). The fungistatic and anti-aflatoxigenic activity of derived compounds were determined and compared with Tio-B.

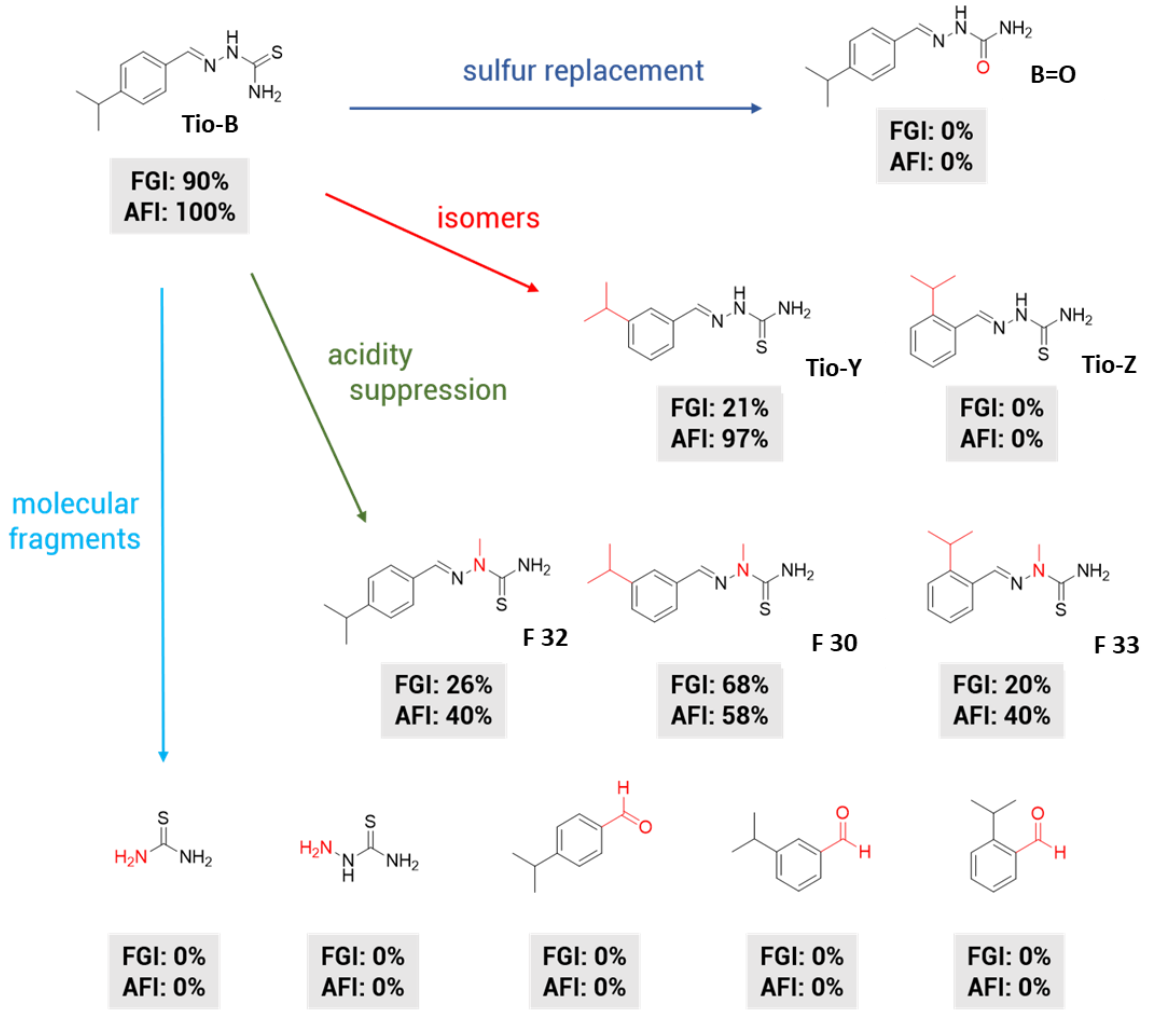


Figure 17: Effects of the cuminaldehyde structural modification on A. flavus. *FGI: fungal growth inhibition percentage at 100 µM. AFI: aflatoxin production inhibition percentage at 100 µM.*

The most surprising result came from the isomers of Tio-B, namely Tio-Y and Tio-Z: in these derivatives, the shift of the isopropyl group along the benzene ring determined significant changes of biological effects. The reference compound *para*-isomer (Tio-B) was both fungistatic and anti-aflatoxigenic, whereas the *meta*-isomer **(Tio-Y)** showed a complete aflatoxin inhibition uncoupled with any fungistatic effect; the *ortho*-isomer **(Tio-Z)** resulted less active in respect with the other two isomers. The more specific antiaflatoxigenic effect of **Tio-Y** suggests that its activity is related to the isopropyl group position on the aromatic ring, and, additionally, that the compound presumably has a cellular target more specific than Tio-B.

The combined effect of the isopropyl shift along the aromatic ring and the acidic suppression of the thiosemicarbazide fragment was compared in three derivatives, obtained by the substitution in Tio-B, Tio-Y and Tio-Z of the H atom in N^2 with a methyl group (**F32, F30** and **F33** respectively). Our screening showed that all of these isomers are less active than the corresponding N^2 free analogues

(***Figure 17***), demonstrating that in these derivatives, the isopropyl position loose its relevance in determining the biological properties. Therefore, the observation that these TSs share the same mild and not-specific effect suggests the crucial role of the free NH group in the activity against *A. flavus*.

The metal complexes gave different results depending on their metallic nucleus: copper complexes showed a lower antiaflatoxigenic power than the corresponding free ligands, whereas the zinc derivative was able to decrease the fungistatic effect of Tio-B (***Figure 18***).

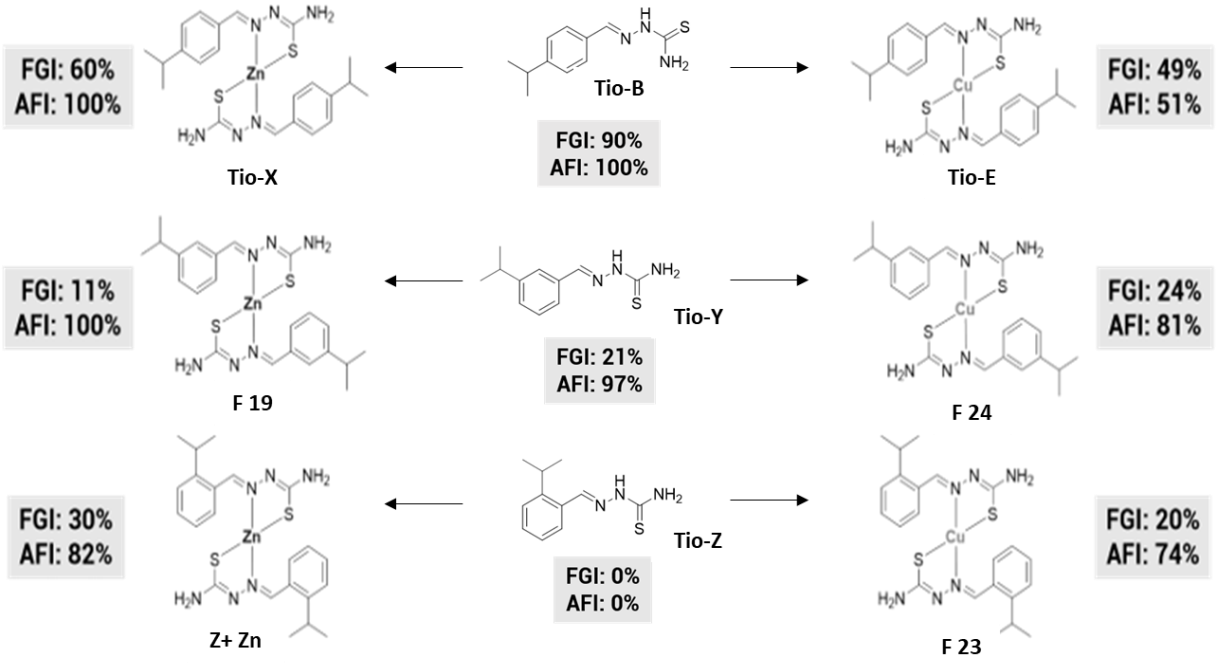


Figure 18: Effects of the cuminaldehyde family metal complexes on A. flavus. *FGI indicates fungal growth inhibition percentage at 100 µM and AFI indicates the percentage of aflatoxin inhibition at 100 µM.*

The analysis of above discussed 16 different cuminaldehyde-TS derivatives demonstrated that the presence of the sulfur is responsible for the functionality of thiosemicarbazone. We hypothesized that the key role of the S atom is due to its propensity in acting as a reducer and/or as a ROS generator. On the other hand, the characteristic that mostly affect the biological activity of parental compound **Tio-B**, revealing to be critical for the antifungal and antiaflatoxigenic effect on *A. flavus*, is the position of isopropyl group, whereas the N^2 methylation did not increase (or make more specific) the action of the molecule. With regard to this, the specificity of activity provided by the

para to *meta*-position shift of isopropyl group, which uncouples the antiaflatoxigenic and the antifungal effect, was suggested to be attributed to a different molecular target affected by **Tio-Y**. In turn, being such a highly selective antimycotoxigenic compound with no significant interference on fungal growth, this molecule is a perfect candidate to analyze in deep those cellular and molecular mechanisms that govern and regulate the aflatoxins biosynthesis metabolism.

3.1.2. Unraveling the cellular/molecular target of Tio-Y

For these reasons, we focused our attention on **Tio-Y,** that in the subsequent analyses carried out by the toxicology units was confirmed to be not cytotoxic for the three healthy cell lines HFL1, Hs27, Crl1790 nor genotoxic. We thus moved to investigate, also throught the use of *Saccharomyces cerevisiae* yeast as model system, the cellular and the molecular target of the compound.

Since several studies reported that many synthetic and natural compounds effective in contrasting the AFs biosynthesis possess an high anti-oxidant power, and that the accumulation of the toxin seems to be related to the redox balance into the fungal cell (and, as a consequence, a significant scavenging activity against ROS could prevent AFs production). We evaluated and compared the **anti-oxidant potential** of **Tio-B**, **Tio-Y** and **Tio-Z**, searching for a possible correlation between their scavenging activity and the aflatoxin inhibitory effect. In an *in vitro* test (DPPH reduction assay) both **Tio-Z** and **Tio-Y** displayed a strong anti-oxidant activity, higher than Tio-B (about 70%) and **cuminaldehyde** (no detectable anti-oxidant activity) ***(Figure 19- C)***. However, these results did not highlight any correlation with the trend of the antiaflatoxigenic capacity previously observed: in fact, the *ortho*-isomer **Tio-Z** was almost unable to inhibit aflatoxin production even if it showed a very high anti-oxidant potential. This apparently inconsistent result could rely on the well-known observation that recorded *in vitro* antioxidant activities frequently are not confirmed in *in vivo* systems, probably due to the complexity of the cellular environment. We thus performed *in vivo* tests taking advantage of the use of *S. cerevisiae*: the antioxidant activity was measured as the beneficial effect that compounds exerted in contrasting the oxidative stress induced by the administration of a strong ROS inducer, such as hydrogen peroxide (***Figure 19 – A and B***). The results of the two in vivo tests confirmed the absence of correlation between antioxidant and antiaflatoxigenic power.

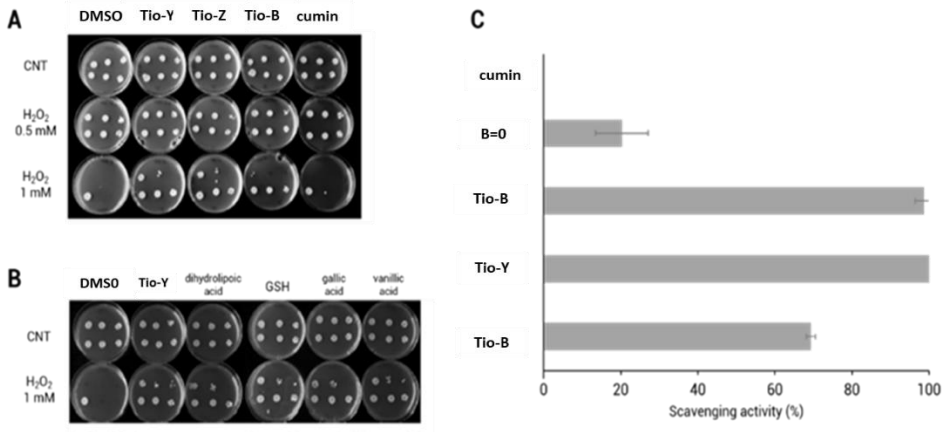


Figure 19: Anti-oxidative response. Yeast spot assay. *Three serial dilutions of yeast cultures were spotted at two different hydrogen peroxide concentrations and in presence of 50 µM samples of* ***Tio-B****,* ***Tio-Z*** *and* ***Tio-Y****. or 0.5% DMSO as control* ***(A)****.* ***Tio-Y*** *(50 µM) anti-oxidant potential was compared to that of different anti-oxidant standards, (dihydrolipoic acid, gallic acid, vanillic acid, and glutathione (GSH)) in presence of H_2O_2 (1 mM). Yeast cultures were incubated for 7 days at 28 °C* ***(B)****.* ***In vitro anti-oxidant assay (DPPH assay).*** *The values of the scavenging activity of the compounds (75 µM) are expressed as percentage of inhibition respect with ascorbic acid 30 mM (scavenging activity = 100%* ***(C)****. Figure from Degola et al. 2017.*

Since the investigation of the correlation between the differential effects of **Tio-B** isomers and their scavenging potential was not resolutive, we decided to get closer to the Tio-Y cellular target through the analysis of changes in the *A. flavus* proteome induced by the TS exposure. A two-dimensional electrophoresis (2D-PAGE) **proteomic approach** was then adopted to individuate which gene products were affected in fungal cultures treated with 50 µM **Tio-Y**. Gel reporting the 2D map of differentially expressed protein spots is showed in ***Figure 20***.

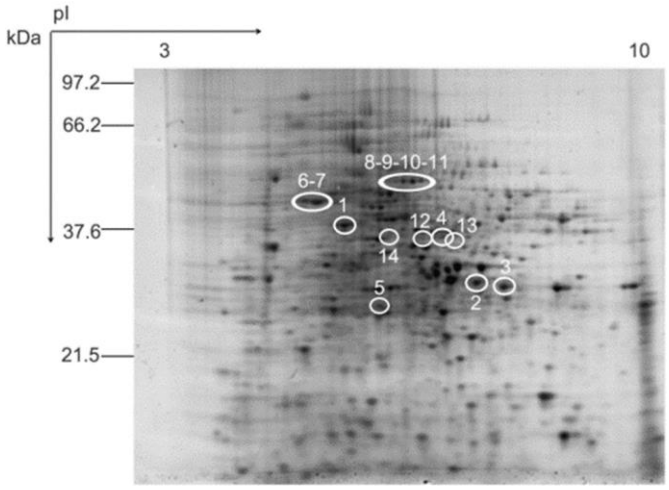


Figure 20: Gel showing the localization of the differentially expressed spots in A. flavus cultures treated with 50 µM solution of Tio-Y. *Circles show the position of the identified spots. Protein extracts were separated on 7-cm-long IPG strips with a pH gradient from 3 to 10, followed by 12% SDS-PAGE. The protein spot number refers to the numbers with which are indicated proteins in Table 11. Figure from Degola et al. 2017.*

We obtained 434 spots per gel and 28 of them were differentially expressed (16 spots up-regulated and 12 down-regulated). We were able to recover 14 of these differential spots whose proteins were subsequently identified using LTQ-Orbitrap mass spectrometry, and SEQUEST protein mass database. Among the 16 recovered spots, 9 corresponded to unique proteins. Four spots matched with the same polypeptide (the aldehyde dehydrogenase (*aldA*) - B8N8T4), even if they showed different isoelectric points, suggesting that the relevant proteins were interested by different post-translational modifications (such as phosphorylation). Similarly, two spots were identified as ATP-synthase beta subunit (B8NWC9) and two spots as zinc-binding oxidoreductase (B8NWT2). The remaining spots were identified as alcohol dehydrogenase 1 (*adh1*; P41747), transaldolase (I7ZW89), two malate dehydrogenase gene products (B8ND04 and I8TTW0), an *A. flavus* uncharacterized protein (B8NSV9), and dimethyl sterigmatocystin 6-O-methyltransferase (Q9P900). More detailed information have been published in *Degola et al. 2017.*

This last protein resulted particularly interesting because it belongs to the aflatoxin biosynthetic pathway; the down-regulation of *OmtB* in the treated culture well correlate with the observed antiaflatoxigenic effect of **Tio-Y.** In addition to a predictable down-regulation of some enzymes belonging to the AF biosynthetic pathway and directly involved in the toxin synthesis, proteins expected to intervene along the carbon flow (like *adh1*) and the energy metabolism (such as the ATP-synthase β-subunit) were also found to be altered, suggesting that **Tio-Y** could interfere with other crucial cell functions. For example, the expression of secondary metabolism and fungal differentiation regulators was also affected by the TS treatment (Real-time PCR, Spectrophotometric determination of alcohol dehydrogenase enzyme activity).

Results obtained with the proteomic analysis were validated by analysing the expression of some genes encoding some proteins resulted up- or down-regulated (*aflO* and *adh1*). We added to the study a subset of genes involved in the regulation of aflatoxin biosynthesis (*aflS*, whose product is an inherent regulator of many aflatoxin cluster genes, and *aflD, that* encodes for an early AF biosynthetic pathway enzyme) or more widely in the primary and secondary metabolism (*veA*, *laeA*, *nsdC*, and *nsdD*) ***(Figure 21)***. Using the Quantitative Real Time Polymerase Chain Reaction (qRT-PCR), we examined the gene expression level in *A. flavus* cultures grown in the presence of **Tio-Y**. As obtained in the proteomic analysis, both *aflO* and *adh1* were down-regulated in **Tio-Y** treated cultures; similarly, the other genes belonging to the aflatoxin cluster (*aflD* and *aflS*) showed a decrease in the gene expression level. Also *nsdD* and *adh1* were down-regulated in Tio-Y treated cultures. *nsdC, veA* and *laeA*, instead were up-regulated. Figure from Degola et al. 2017.

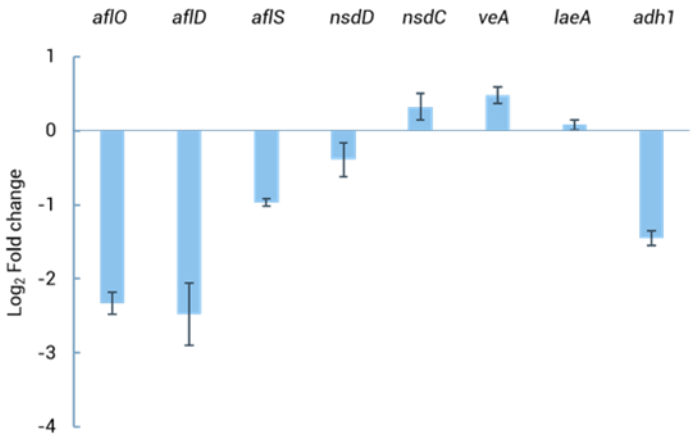


Figure 21: Fold change of gene expression in response to the treatment with Tio-Y.

Even if these data give evidence that **Tio-Y** probably influence directly the AFs production by modulating the transcription of genes belonging to the cluster (and therefore the level of the relevant proteins), it cannot be excluded the possibility that the target of **Tio-Y** is located upstream of the regulatory genes located in the aflatoxin gene cluster. In this sense, it should be noted that **Tio-Y** not only inhibits the aflatoxin production, but also impairs sclerotia development and the differentiation of proteins involved in primary metabolic functions.

nsdC and *nsdD*, are gene involved in sclerotia development. For this reason, Tio-Y was also tested on **sclerotia development** on *A. flavus* conidia were point spotted in Czapek solid medium. Tio-Y drastically reduced the number of sclerotia as compared to untreated control cultures (to <10% of control culture).

Data reported showed that Tio-Y is an extremely effective compound. But where does this compound act? So we wanted to investigate if one among the possible **Tio-Y** molecular target could be involved in the energy generation/carbon flow and in the redox state control of the cell. We wanted to investigate if **Tio-Y** interfered at the mitochondrial level. In fact, fungi secondary metabolism (and in particular mycotoxins biosynthetic pathways) require a well-defined regulation of oxidative intracellular balance, triggering the redox homeostasis needed for both fungal development and differentiation *(Roze at al. 2015)*. Because of this pivotal role, destabilization of oxidative-linked systems such as mitochondrial electron flow has been proposed as an effective way to prevent the production of aflatoxins in Aspergillus toxigenic species *(Dallabona et al 2019; Sun et al. 2016)*. We thus designed a set of experiments addressed at defining if, and how, **Tio-Y** was able

to interfere with the mitochondrial functionality. In fact, Sakuda and coworkers *(Sakuda et al. 2014)* recently showed that the administration of mitochondrial inhibitors (such as Na-azide, rotenone, oligomycin and antimycin A) that specifically affect different steps of the respiratory chain resulted in an inhibition of the aflatoxin accumulation in *A. flavus*, without significantly interfere with fungal growth. However, no correlation between the inhibitor target along the respiratory chain and the level of inhibition activity on the toxin accumulation was demonstrated yet, suggesting that a block of respiration "tout court" may be the cause of toxin biosynthesis inhibition. We took advantage of the use of yeast *Saccharomyces cerevisiae* as model system, since, even though yeast does not possess the secondary metabolism machinery responsible for the aflatoxin biosynthesis, it shares with other fungi all basal pathways for energy production. The long-term exposure effect of **Tio-Y** in yeast cells was evaluated at different levels: more specifically, the effect on the oxidative growth, on the oxygen consumption and on the enzymatic activity of mitochondrial respiratory complexes was measured.

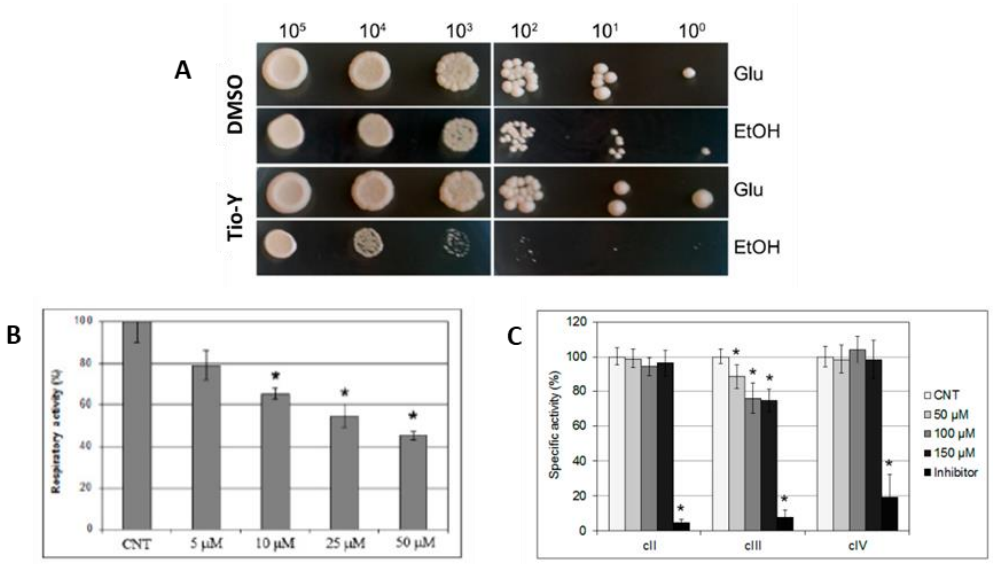


Figure 22: Yeast dilution bioassays showing the effect of Tio-Y under oxidative growth (12 A). *Cells of W303-1B strain serially diluted and spotted on YP medium supplemented with glucose or ethanol and added with Tio-Y 50 µM or 0.5% DMSO (CNT).* ***Oxygen consumption rate (B)****. W303-1B grown in the absence (CNT) or in the presence of Tio-Y at different concentrations (from 5 to 50 µM Values significantly different from CNT were indicated with an asterisk (p ≤ 0.05).* ***Chain complexes activity (C).*** *Biochemical activities of succinate quinone DCPIP reductase (cII), NADH-cytochrome c oxidoreductase (cIII) and cytochrome c oxidase (cIV) were measured on a mitochondrial enriched fraction. Tio-Y was added at increasing concentrations (from 50 to 150 µM) and compared to DMSO treated cells (CNT). Specific inhibitors for complex II (malonate), complex III (antimycin A) and complex IV (Na-azide) were used as control. Figure from Dallabona et al. 2019.*

We found that the Tio-Y treatment heavily affected the yeast cells growth in presence of a respirable carbon source (ethanol or glycerol), when no effects were recorded in presence of fermentable carbon source such as glucose ***(Figure 22-A)***. This pointed at an alteration at the respiratory level, that we demonstrated to be due to a decrease of oxygen consumption in treated cultures ***(Figure***

22-B). In order to clarify if the TS activity could be attributed to a specific step of the respiratory chain, we tested its effect on single mitochondrial complexes, respectively succinate-dehydrogenase (complex II), ubiquinol-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV). We found that treating isolated mitochondria with increasing concentration of TS induced a lowering in complex III specific enzymatic activity, in a dose-dependent manner. On the contrary, complexes II and IV resulted not affected *(**Figure 22-C**)*. Additionally, the dosage of proteins COR1 and COR2 (core subunits 1 and 2 respectively of the mitochondrial complex III), COX2 (subunit II of the mitochondrial complex IV) and ATP2 (beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase, the mitochondrial complex V) in Tio-Y treated yeast cultures revealed an up-regulation of ATP2 in yeast cells: this result reproduces what was observed in *A. flavus*, in which the analysis of the preoteome highlighted an increase of the ATP synthase β-subunit in response to TS exposure *(Degola et al. 2017*) *(**Figure 23**)*.

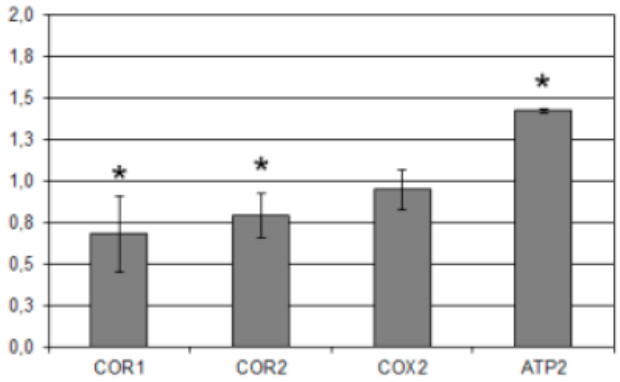


Figure 23: Western Blot analysis of mitochondrial extracts from yeast cells grown in presence of 50 µMm Tio-Y. *Quantification of immunoreactive luminescence signals was achieved by densitometric scanning of the respective bands. Signals were normalized to the 0.5% DMSO treated cultures (control); POR was used as a loading control. Quantification was performed on three independent blots and reported as mean of values (± S.D.). * $p \leq 0.05$ vs. control.*

To support, and at the same time to verify, the hypothesis of a defect in the electron flow along the respiratory chain induced by **Tio-Y,** with specific regard to an inhibition of the complex III, a structure-based docking study was performed to investigate the intermolecular interactions between the thiosemicarbazone and the ubiquinol-cytochrome c reductase ***(Figure 24)***. A comparison with antimycin A (AMY), a specific complex III inhibitor, was conducted. Since the inhibitory mechanism of AMY relies on the binding at bc1cytochrome level in the Qi site, that causes the disruption of the Q-cycle and the entire enzyme turn over, we calculated the theoretical positioning of Tio-Y in the same pocket. ***Figure 24*** shows the best pose of TS into the Qi site: as we

reported in *Dallabona et al. (2019),* the hydrophobic environment formed by Phe^{221}, Ile^{28}, Tyr^{225}, Ala^{24}, Trp^{32} and Leu^{201} allows the formation of a cavity in which the aromatic ring of Tio-Y can be efficiently stabilized. In addition, a strong hydrogen bond (2.001 Å) between thiosemicarbazone N^{2} and the carboxyl group of Asp^{229} is predicted, being convincingly responsible for the maintenance of TS into the binding site. The same proximity of AMY to heme b_H (3.376 Å), that is considered fundamental for the inhibition mechanism (due to the involvement of b_H heme in the oxidation of ubiquinol to ubiquinone), was found also for the TS. The interaction between the compound and bc1 cytochrome in the active site of mitochondrial complex III, validated by the comparative docking.

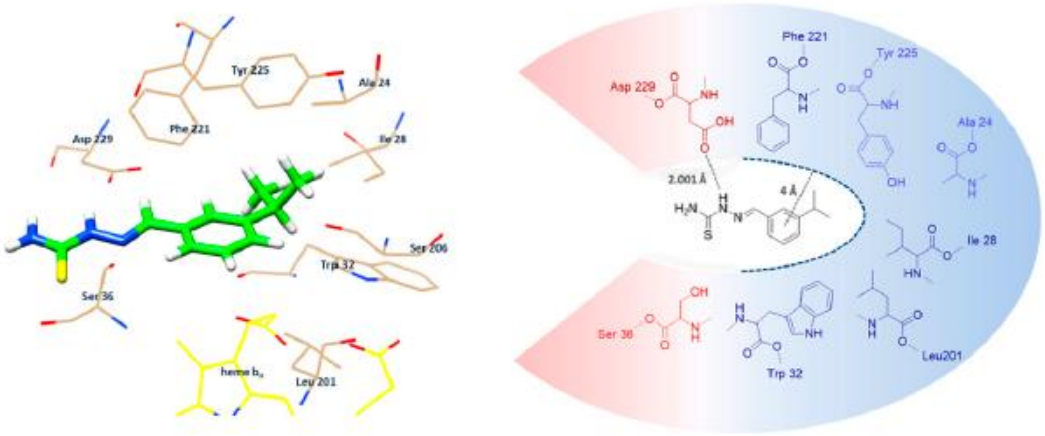


Figure 24: Representation of the binding mode of Tio-Y in the cytochrome bc1 AMY binding site as obtained with the Gold v5.5 software. *On the left: best docked pose of Tio-Y into the 3BCC binding pocket. On the right: schematic positioning of Tio-Y into the 3BCC binding pocket (in red: the hydrophilic area; in blue, the hydrophobic surface). Figure from Dallabona 2019.*

3.2. Cinnamaldehyde derivatives

Cinnamaldehyde is a natural volatile compound responsible for the smell of cinnamon and sweet taste. For long time, cinnamaldehyde has been used as a flavoring agent in chewing gums, ice creams, beverages, and sweets. Also, it has been widely used to give a cinnamon flavor to medical products, cosmetics, perfumes and food preservatives, with extensive applications and no side effects; traces of its use to preserve crops were found in many ancient Egyptian documents (*Shreaz S, et al. 2016)*. Cinnamon is a spice obtained from the inner bark of several trees from the genus *Cinnamomum*, native to South India and Srilanka; the name refers to the eternal tree of tropical medicine and its bark. The flavor of cinnamon is due to the presence of aromatic essential oils *(Jakhetia et al. 2010)*. Cinnamon and its derivatives has been reported to possess antifungal, antibacterial, antiviral, anti-oxidant and anticancer properties. Xie and coworkers in 2004 reported that treatment of *A. flavus* with cinnamaldehyde showed a decrease in spore germination rate, along with significant changes in the morphology and ultrastructure of both hyphae and spores *(Xie et al. 2004)*. As a consequence, cinnamaldehyde and its derivatives were screened against several pathogenic fungi, and proved to possess an antifungal activity against several strains that was suggested to be due to an inhibition of ATPase activity, a cell wall biosynthesis alteration and a membrane structure disruption (*Shreaz S, et al. 2016)*.

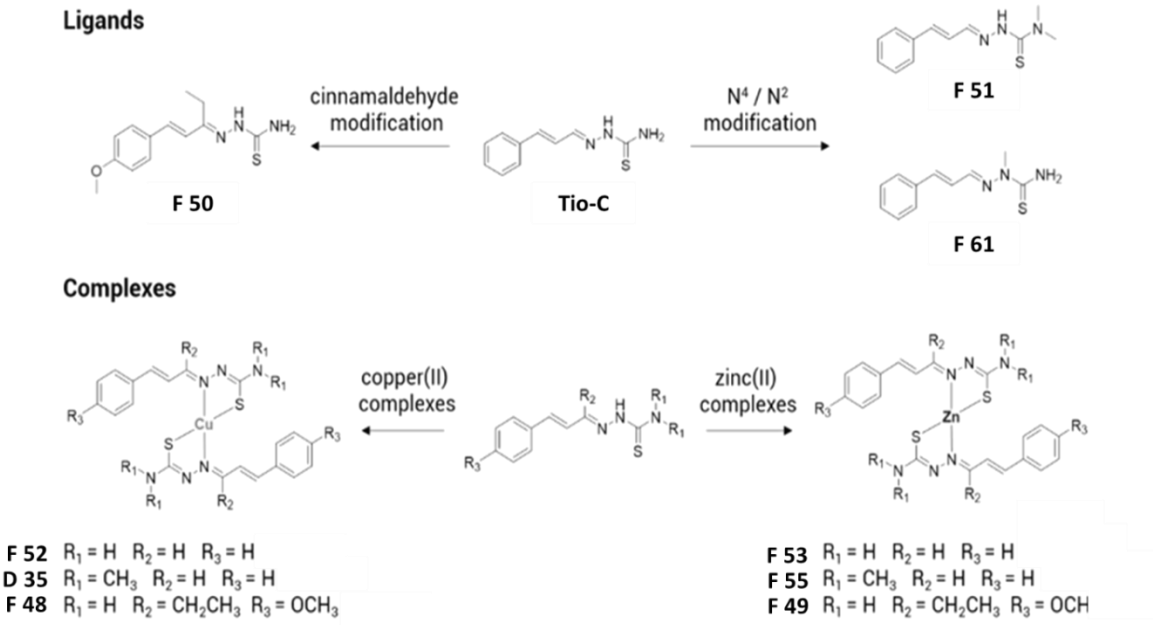


Figure 25: List of components of the cinnamaldehyde family.

We thus decided to include cinnamaldehyde in the panel of Aflatox® Project tested compounds, synthesizing the corresponding TS (namely **Tio-C**) and exploring its ability to inhibit *A. flavus* growth and aflatoxin production. The group of derivatives contained structural modifications of the parent compounds *(**Figure 25**)*: one functionalized in the N^4 **(F51)**, one modified in the N^2 **(F61)** and one modified in the cinnamaldehyde portion **(F50)**. The iminic bond of the N^2 methyl derivative (**F61**) was also reduced **(F62)**. In addition, each TS able to be deprotonated in N^2 was used as ligand to synthesize bis-TS complexes of Cu(II) **(F52 D35 F48)** and Zn(II) **(F53 F55 F49)**.

3.2.1. Effect on *A. flavus* growth and aflatoxin accumulation

As already observed for the Cuminaldehyde, the Cinnamaldehyde did not prove to possess any effect neither on fungal growth nor on AFs accumulation *(**Figure 26**)*. On the contrary, **Tio-C** resulted to be a very promising compound, showing very interesting activity against aflatoxin biosynthesis and fungal growth (79 and 56% respectively). The two metal complexes of Tio-C (**F52** and **F53**) displayed extremely different behaviors: **F52** resulted less fungistatic than **Tio-C**, but also less effective in inhibiting aflatoxin accumulation, while **F53** was able to completely abolish the AFs production overcoming by the 20% the fungistatic effect of the parental TS.

The screening highlighted that in **F50** the modification in the aldehyde portion led to raise the fungistatic effect of the parental molecule Tio-C. However, due to interferences in the aflatoxin determination (probably caused by fluorescence quenching phenomenon or by its ability to partially absorb the fluorescence emission of aflatoxin B_1, which therefore resulted impossible to be quantified correctly), the antimycotoxigenic activity of this compound was impossible to assess. The same was observed for its zinc(II) complex **F49**. The N^2 methylation (**F61**) did not improve the aflatoxin inhibition, but resulting completely ineffective on fungal growth it could be considered a good candidate for further studies focused on AFs metabolism. The functionalization of N^4 led to more interesting results: in fact, **F51** showed a total inhibition of aflatoxin accumulation that was accompanied by a lower effect on fungal growth respect with the parental compound (**Tio-C**). The relevant zinc complex (**F55**) showed similar properties, while the copper complex (**D35**) could not be tested because it precipitated immediately after dissolution in the culture medium used for the *A. flavus* assays. The same problem was encountered with complex **F48**.

The concern about TSs solubility in culture media is crucial, because it can affect significantly the results of the biological assays; additionally, the insolubility of a compound can be only occasionally

clearly visible, as in the case of **F54** and **F48**, whether in many cases it can represent a very slight effect, not directly observable during the experiment.

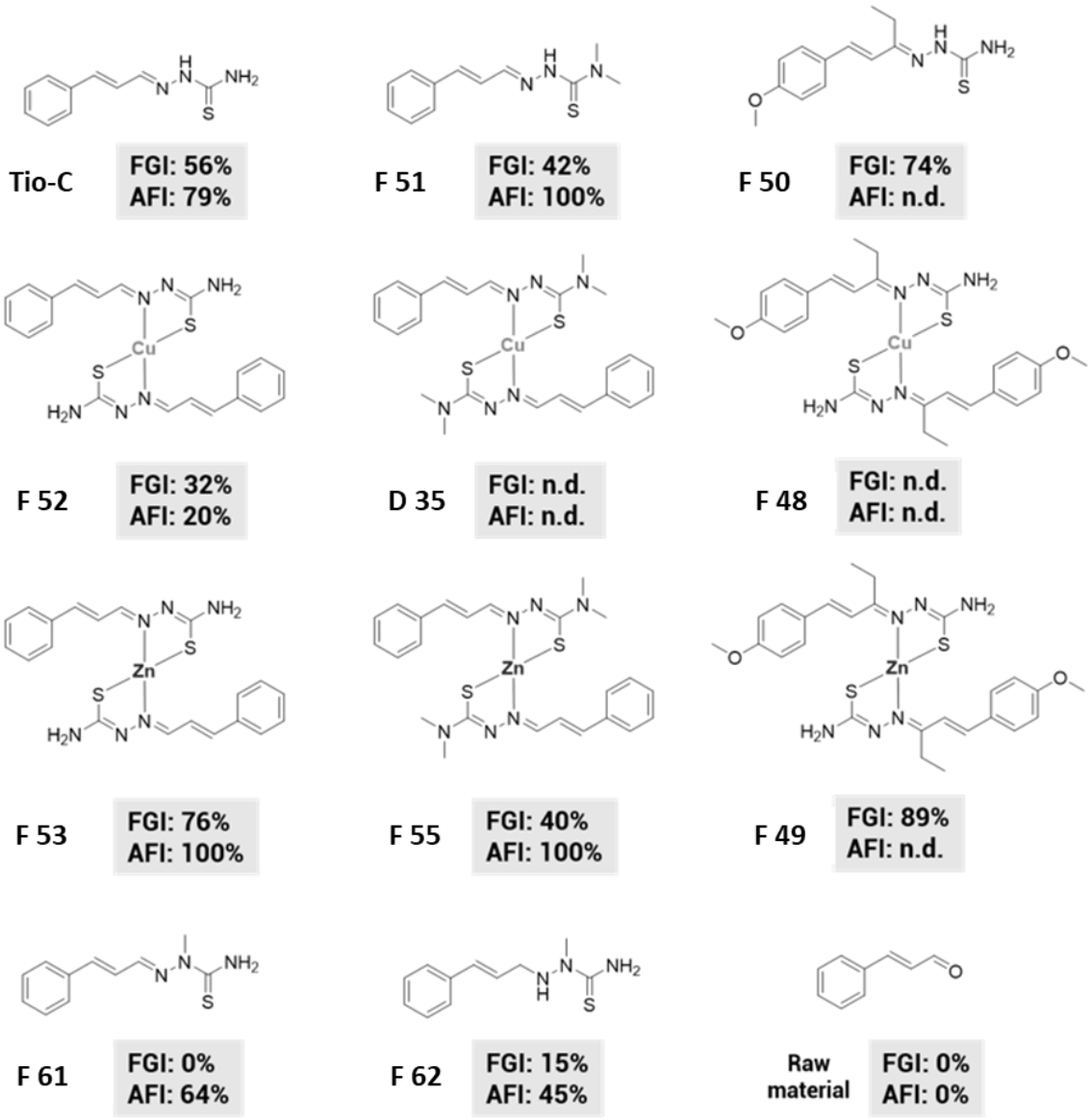


Figure 26: Effects of the cinnamaldehyde derivatives at 100 µM concentration on A. flavus. *FGI indicates fungal growth inhibition percentage at 100 µM and AFI indicates aflatoxin production inhibition percentage.*

3.3. Benzaldehyde derivatives

Another family here presented and therefore discussed includes the **benzaldehyde** and its derivatives. Benzaldehyde (**Benz**) is the simplest aromatic aldehyde, an organic compound consisting of a benzene ring in which one of the hydrogen atoms was replaced by a formyl group; it is one of the most industrially common aldehydes, mainly used in cosmetics as denaturant, flavoring agent and fragrance (PUBCHEM). Benzaldehyde and Cinnamaldehyde are similar molecules, differing for the length of the carbon chain that, in the Cinnamaldehyde (**Cinn**), presents two carbon covalently and double-bonded ***(Figure 27)***. We compared the effects of both compounds and some derivatives, trying to define if these apparently slight differences could affect their biological activities. The thiosemicarbazone of Benzaldehyde (**FG17**) was then synthesized and compared to thiosemicarbazone of Cinnamaldehyde (**Tio-C**). As for Cinnamaldehyde, we obtained structural modifications of the parent compound, modifying by the addition of a methyl group on the terminal nitrogen (N^4) and obtaining compound **D34** (***Figure 27***).

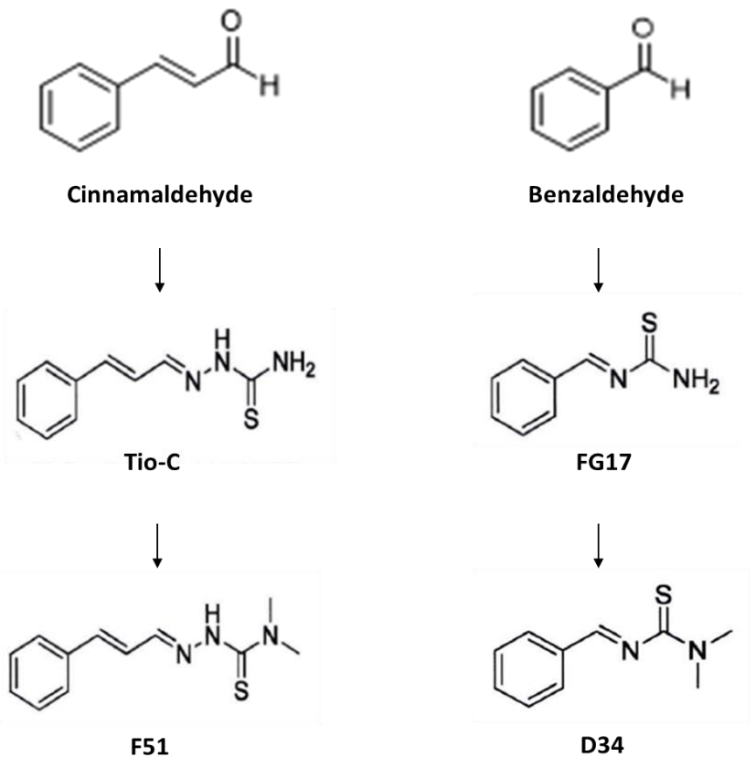


Figure 27: Structures of Benzaldehyde and Cinnamaldehyde derivatives.

3.3.1. Effect on *A. flavus* growth and aflatoxin accumulation

Cinnamaldehyde, that was described as a compound with an antiaflatoxigenic and antifungal activity by *Dandan Liang et al. (2015)*, when tested at 100 µM in our system did not show any effect; Benzaldheyde also showed biological activities at a trascurable level. On the contrary, the two thiosemicarbazonic derivatives (FG17 and Tio-C) proved to affect both the toxin accumulation and the mycelium growth; however, when compared, **FG17** resulted less effective than **Tio-C, that** at the concentrations of 50 and 100 µM determined an inhibition of aflatoxin accumulation of 70% and 90% respectively,***(Figure 28)***. It was conceivable that such increase in the biological activity was attributable to the addition of thiosemicarbazide to the aldehyde's scaffold, and, in particular, to the presence of the sulfur atom. In fact, similar results were observed (and reported) for cuminaldehyde and its semicarbazone derivatives (3.1. section).

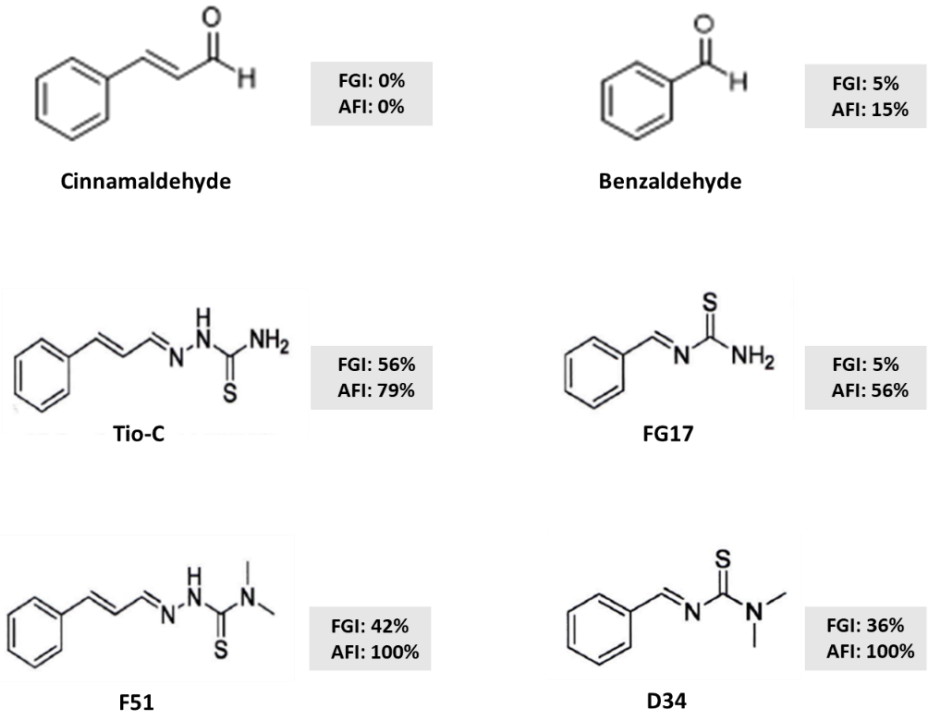


Figure 28: Effect of the Benzaldehyde derivatives compared to Cinnamaldehyde derivatives at 100 µM concentration on A. flavus. *FGI: fungal growth inhibition; AFI: aflatoxin accumulation inhibition.*

It was speculated that the differential role of sulfur and oxygen in determining the antiaflatoxigenic activity depended on the respective redox potential. In fact, due to the lower electronegativity the sulfur forms weaker hydrogen bonds compared to oxygen and is more subject to oxidation, through the formation and destruction of disulfide bridges in a tautomerismotione-thiol, therefore the thiosemicarbazonic form in thiol form tends to behave as a reductant and scavenger of reactive

oxygen species (ROS). With regard to the di-methylated derivatives of FG17 and Tio-C (**D34** and **F51** respectively), it could be observed that both resulted highly effective in lowering the toxin, reaching the 80% of inhibition at the concentration of 100µM. If at the lowest concentration D34 proved to be as effective as its ligand (FG17), at higher concentrations the effect was greater: this suggests that the presence of the di-methyl group in N^4 might implements the antiaflatoxigenic potential of this molecule. On the contrary, the modification of the cinnamaldehyde thiosemicarbazone did not lead to an analogous result: in fact, F51 did not showed any difference in efficacy with respect to Tio-C, even seemed to e exert a slight decrease (albeit not significant) at the concentration of 100 µM. Finally, the direct comparison between D34 and F51 did not highlight any difference between the two molecules at any concentration tested. From a structure-activity point of view, the addition of di-methyl groups on N^4 therefore seems to lead to an increase in the abatement capacity of aflatoxin and, especially in the case of D34, also an increase in the specificity of action. This difference could be a function of the lipophilicity of the di-methylated compounds, a characteristic generally correlated with the ability of molecules to penetrate cell membranes.

Compounds were also evaluated for their effect on the growth of the mycelium, estimated as a variation of OD after 46-48 hours from the inoculum ***(Figure 28)***. No effects of **Benz** and **Cinn** on the germination of conidia were observable at the concentration of 25 µM, but while in the first case it remains negligible even at the highest concentrations, in the second there is a dose-dependence that leads cinnamaldehyde to an inhibitory activity of 25% (data not shown). In contrast with what observed for the toxin accumulation, the treatment with the two ligands does not follow the same dose-dependent effect on the fungal growth: the inhibitory potential of FG17 remains negligible at all concentrations, indicating that the activity responsible for the inhibitory effect on the secondary metabolism is probably exerted after the first stages of the hypae development. On the other hand, the effect of Tio-C on germination followed what obtained in AF accumulation assay, seeming to increase as the stage of fungal development proceeds. In fact, at the concentration of 100 µM, we observed an inhibitory potential up to 50% on conidia germination, which turns into a 90% inhibition of aflatoxin ***(Figure 28)***. It is therefore clear that whatever the target of Tio-C is, the effect of its interaction with thiosemicarbazone continues throughout the growth and development of the mycelium. Once again, the comparison between D34 and F51 showed an interesting difference: **D34** determined a dose-dependend inhibition, on conidia germination, of 38% at the highest concentration, but the effect of this molecule on the development of mycelium seems to take place mainly in the initial phases of the process and mainly affects the germination, then undergoing a progressive loss of efficacy in the subsequent phases. Then, the extent of its inhibitory effect on the

accumulation of aflatoxin (which exceeds 80% at the concentration of 100 µM) cannot be completely attributed to a direct activity on the growth of *A. flavus*, pointing instead at a more probable specificity on the AFs biosynthesis. On the other hand, the germination inhibition induced by **F51** can explain the effect on the accumulation of the toxin, that is considerably greater and reaches the levels of both its ligand (Tio-C) and its corresponding di-methylated analog (**D34**). This suggests that the molecule initially exerts biological activity during germination and that this activity is maintained throughout the development of the fungus (as observed in the treatment with Tio-C), although it only partially explains the containment effect on the aflatoxin production (as for D34).

3.3.2. Effect on sclerotia biogenesis

A sclerotia biogenesis test was also conducted using *A. flavus* ipersclerotigen strain CR10+: it was cultivated on Czapek medium, the standard medium for the induction of the biogenesis of these structures, added with the molecules at the concentration of 50 µM. Medium added with DMSO 0.5% (v/v) was used as control. The quantity of sclerotia found in the cultures treated with Benz and Cinn did not differ from the control treatment ***(Figure 29-A and -B)***.

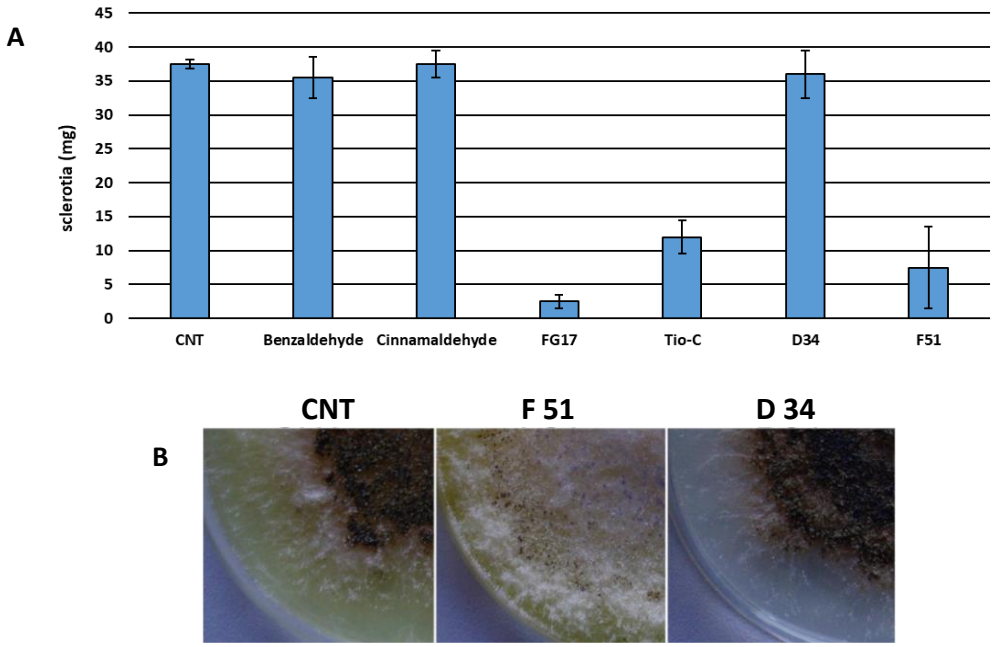


Figure 29: Effects of Benzaldehyde and Cinnamaldehyde derivatives, at 50 µM concentration, on sclerotia production. *FG17 completely inhibits the production of sclerotia, while Tio-C and F51 showed a slightly lower effect (**A**). Cinnamaldehyde, Benzaldehyde and D34 did not differ from the control (**A**). Pics of CZ cultures treated with F51, D34 and DMSO (CNT).*

On the contrary, the influence of FG17 and Tio-C was significant: in particular, FG17 almost completely inhibits the production of sclerotia, while Tio-C showed a slightly lower effect. Comparing this data with the effect that cinnamaldehyde thiosemicarbazone exerts on the fungal growth *(**Figure 29**)*, it was proposed that the interference of the compound at the early stages of development would impress a delay in the differentiation process that propagates up to the biogenesis of sclerotia.

Once again, the effect of D34 and F51 resulted differential: in fact, while the quantity of sclerotia produced in the F51-treated cultures was comparable to that obtained with its ligand (Tio-C), the amount found after the treatment with D34 was heavily different from its own ligand (FG17), but did not differ from the control. In this context, the addition of the di-methyl group on the FG17 ligand leads to the loss of its effect on the process of differentiation of the fungus *(**Figure 29**)*.

3.3.3. Gene expression analysis

Evidences previously obtained in our laboratory demonstrated how, in *Aspergillus flavus*, the treatment with some cuminaldehyde thiosemicarbazone derivatives could determine alterations in the expression level of genes involved in both primary and secondary *metabolism (Degola et al. 2017)*; some of them, were more or less directly related to both the biosynthetic metabolism of aflatoxins and the biogenesis of sclerotia.

Since, due to the apparent specificity of action on aflatoxin metabolism and sclerotia biogenesis, D34 and F51 turned out to be interesting, we decided to investigate their differential effects in terms of gene expression changes. The analysis was carried out on CCM-microplate cultures of the hypersclerotigen CR10+ strain, treated with D34 and F51 at the concentration of 50µM; 0.5% DMSO culture was used as control. The expression of the *aflR* and *omtB* (aflatoxin gene cluster), *dmtA* and *mtfA* (expression of chromatin genes), *NsdD* and *NsdC* (regulator of sclerotia production) and *VeA* (regulator of secondary metabolism) genes was then analyzed; housekeeping *tub1* gene was selected as internal amplification reference.

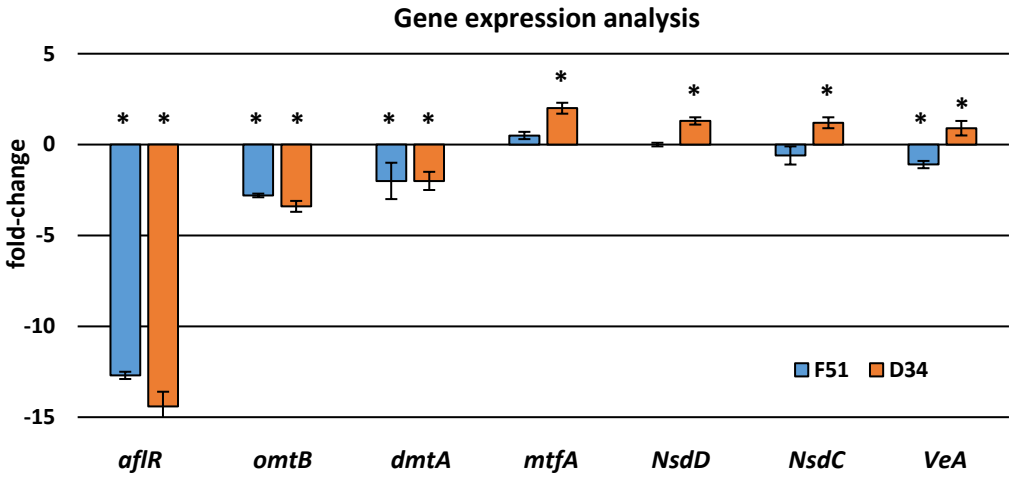


Figure 30: Analysis of gene expression. *The expression of the aflR, omtB, dmtA, mtfA, NsdD, NsdC and VeA genes was analyzed. aflR, omtB, dmtA, NsdC and VeA were down-regulated. Instead, mtfA and NsdD were up-regulated.*

Results, reported as fold-change, highlight that both molecules dramatically reduced the transcription level of the aflatoxin cluster regulator *aflR (**Figure 30**)*: a decrease of about 13 fold was recorded for the F51 treatment, while adding D34 to cultures induced a dcrease of about 14 fold. In this case, it can be stated that the two TS resulted in a similar effect. Data were perfectly consistent with the reduction of about 60% in the aflatoxin accumulation observed. The same consideration can be made for the aflatoxin structural gene *omtB*, which appears down-regulated of about three fold in both treatments. Transcription of *dmtA* gene is also slightly suppressed; as previously reported, the deletion of this gene leads to a negative regulation of aflatoxin biosynthesis, therefore a decrease in expression is in agreement with the low levels of aflatoxin found in the accumulation test. This gene is also involved in the negative regulation of sclerotia production, since its deletion quadruplies their quantity *(Kunlong Yang et al. 2016)*; however, the slight decrease in the level of its transcription observed after treatment with both TS did not promote an increase in sclerotia biomass. On the contrary, sclerotia produced after D34 administration was comparable to control, while cultures exposed to F51 showed almost a total inhibition of their biosintesis, indicating that the molecular target could be located in the metabolism at a level not directly controlled by this gene, or that multiple targets may be present. The control of sclerotia formation is in fact very complex, and involves other genes besides *dmtA*: therefore, we hypothesized that a two-fold decrease in the transcription level may not be sufficient to observe an effect similar to the gene's deletion.

Treatment with F51 did not induce any change in the expression of *mtfA, NsdD* and *NsdC* genes, while, on the contrary, with D34 all three genes were significantly up-regulated. The increase of the two regulators of sclerotia biogenesis, *NsdD* and *NsdC*, triggered by *mtfA* protein up-regulation as reported by *Yu Li et al. (2017)*, was confirmed by our data ***(Figure 30)***. Also the last gene considered, *VeA*, differently responded to the treatment with the two di-methylated thiosemicarbazonic variants: the general regulator of secondary metabolism was in fact down-regulated by F51, but up-regulated by D34.

3.3.4. Effect of F51 and D35 on *A. flavus* proteome

To deep findings discussed above, the effect of **D34** and **F51** on the *A. flavus* proteome was investigated through a two-dimensional electrophoretic analysis followed by the characterization of differentially expressed proteins. In this type of analysis not only the "control vs treated" conditions were compared, but also the two treatment (D34 vs F51). Total proteins extracts were obtained from 96 h CCM cultures of *A. flavus* treated with D34 and F51 (50 µM). At first, due to the absence of preliminary indications, the first dimension (isoelectrofocusing of protein species according to pI) was run with on a 3-10 pH range, ensuring the coverage of entire proteome. After having observed that most of the spots were concentrated in the central area of the pH range, investigation was implemented by focusing the extracts on pH 5-8 ranged strips. The analysis of 2D maps returned an average of 300 spots in each gel; differentially displayed spots detected in the various comparisons, at both pH ranges, is reported in ***Table 4***. The relatively small number of differential spots confirmed by the comparison within the control (about 17) suggests that the possible target(s) of both molecules could be located downstream the majority of essential growth and development pathways; in fact, if the interference occurred at a sufficiently upstream point in the primary metabolism, a heavier variation of the proteome should be expected. At the same time, the even lower number of differential spots found by the direct comparison between the two di-methylated variants seems to underline how the "distance" between their targets could actually be very short. The hypothesis that D34 and F51 have at least one different target is supported by the observation that, in several cases, the same differential spot results differently regulated in the two different treatment ***(Table 4)***.

A.	pH 3-10	pH 5-8
CNT vs D34	15	8
CNT vs F51	17	6
D34 vs F51	7	6
B.	**# spot (pH 3-10)**	**# spot (pH 5-8)**
D34 / F51	**4208** ↓ / =	**5202** = / ↑
	4515 ↑ / =	**5204** ↑ / ↓
	5311 = / ↑	**3209** ↓ / =
	5508 = / ↓	**6202** ↑ / =
	7710 ↓ / ↑	**7507** ↓ / =
	6609 ↓ / =	

Table 4: Differential spots identified at 3-10 and 5-8 pH ranges. *Number of spots found by comparing each single treatment vs control and by compating the two treatments are listed* ***(A)*** *The symbols ↓ ↑ indicate a down- and up-regulation of the protein species with respect to the control, respectively. The = symbol indicates no difference with respect to the control* ***(B).***

3.4. Jasmonic acid derivatives

Cis-jasmone is a compound well-known to be involved in plant defense system. Released from flowers and leaves of some species (i.e. jasmine, neroli, and bergamot) as a repellent for pests and herbivores, *cis*-jasmone and derivatives (or other similar molecules) are produced by wounded plants to suppress the infection of fungi including *Aspergillus*, *Fusarium* and *Helicoverpa (Christensen et al. 2015*). *Cis*-jasmone is an oxylipin obtained from α-linolenic acid through two possible multi-step biosynthetic pathways involving lipoxygenases *(Matsui, R. et al. 2017) (Schaller et al. 2009) (**Figure 31**).* Some natural metabolic routes for the α-linolenic acid oxidation were identified, and several final products and intermediates have shown to exert specialized roles in plant defense, so that this class of compounds are called "death acids", since they can promote cytotoxicity and cell death in the biotic agents.

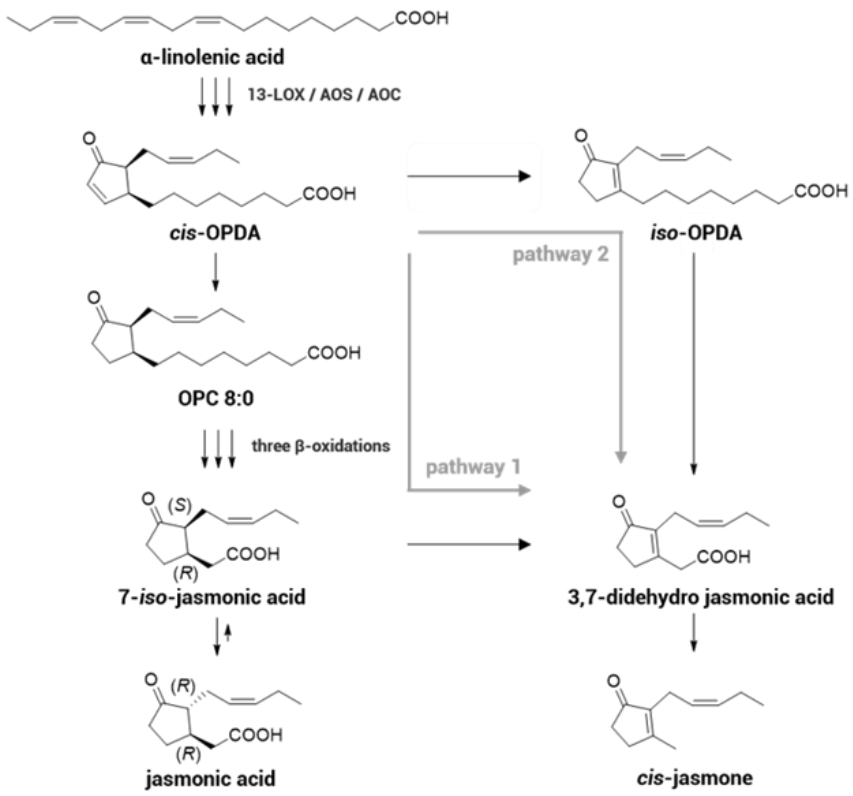


Figure 31: Cis-jasmone and jasmonic acid biosynthetic pathway.

Interestingly, recent evidences highlighted the *cis*-jasmone biosynthesis also in fungi *(Matsui, R. et al. 2017)*: for example, some phytopathogens such as *Fusarium oxysporum* and *Aspergillus flavus* were found to produce *oxilipins*, including mimics of plant jasmonic acid *(Fischer et al. 2016)*. The biological role of this compound in such organisms is still debated; however, it was proposed that

some fungi produce jasmonic acid (an intermediate of the *cis*-jasmone biosynthetic pathway) as a phytohormone to evade (or suppress) the host immune response *(Patkar et al. 2017)*. This suggests that *cis*-jasmone and its derivatives could be key molecules in the host-pathogen communication/signaling, becoming therefore remarkable for agricultural applications and, for our purposes, to be addressed to studies about the potential effect on *A. flavus* growth and development. We firstly compared the biological activity of the natural *cis*-jasmone (**J**) with those of the corresponding TS (**F36**). To gain deeper understanding of this class of molecules we synthesized and tested also di-hydrojasmone (**di-J**) and the TS of the di-hydrojasmone (**F31**), a natural analogue of the *cis*-jasmone hydrogenated in the aliphatic C^5 chain. Finally, we tested the corresponding copper complexes (**F43** and **F44**). The general scheme of the synthesis of all the derivatives is reported in ***Figure 32***.

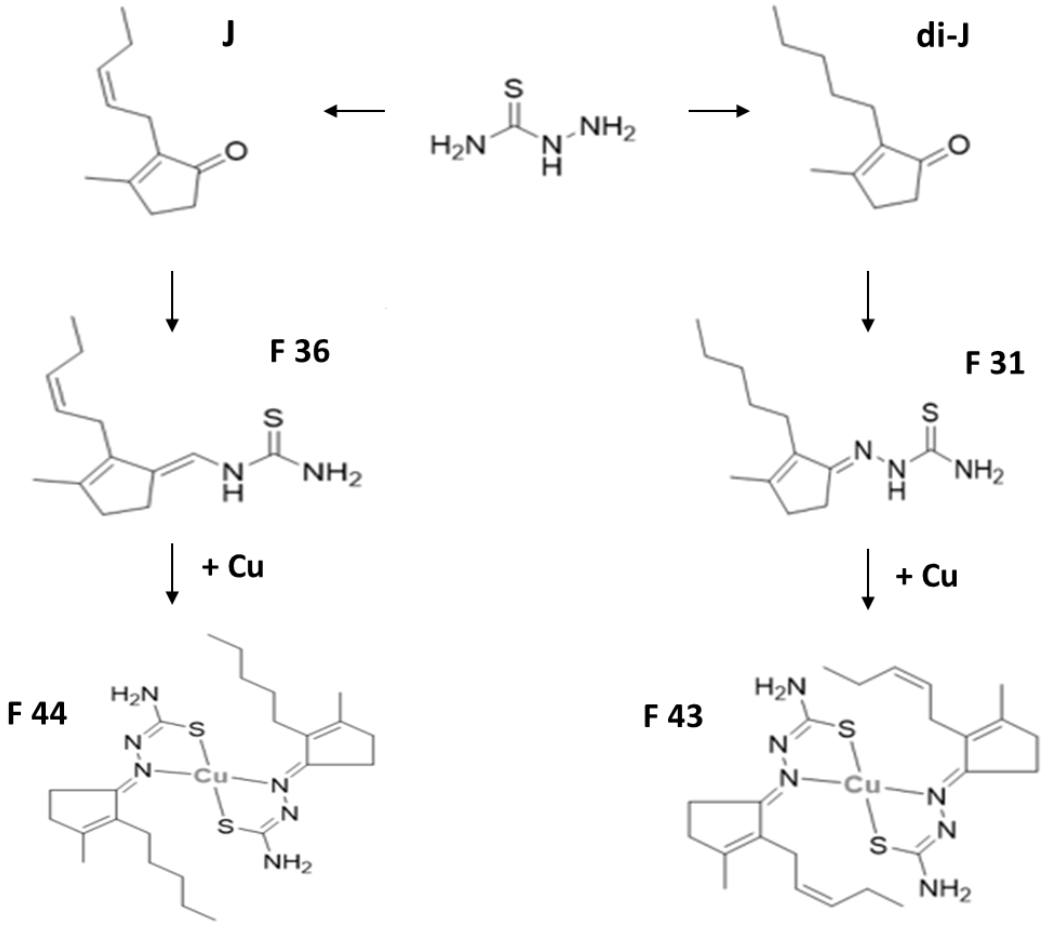


Figure 32: List of components of the jasmone family. J: *cis-jasmone;* ***di-J:*** *di-hydrojasmone;* ***F31:*** *di-hydrojasmone TS;* ***F36:*** *cis-jasmone TS;* ***F43:*** *di-hydrojasmone TS (Cu);* ***F44: :*** *cis-jasmone TS (Cu);*

3.4.1. Effect on *A. flavus* growth and aflatoxin accumulation

The effects of compounds on *A. flavus* were reported in ***Figure 33***. Some interesting differences between the derivatives were found: the natural ketone (**J**) did not showed any fungistatic effect, whereas induced a mild inhibitory effect on the aflatoxin production (30% at 100 µM). The di-hydrojasmone (**di-J**) reported the same effect on fungistatic activity and aflatoxin of natural ketone. **F31** and **F36** showed a higher fungistatic and antitoxigenic activity respect with their natural ketones (**J** and **di-J**). In particular, **F36** resulted to reduce the aflatoxin production up to 93% at 100 µM (88% at 50 µM; data not shown). However, even more interesting were the two copper complexes **F43** and **F44**; in fact, in contrast with their free ligands, they showed different activity trend: the complexation with copper induced an increase of the fungistatic effect only for **F44**, and the containment effect on aflatoxin accumulation seems to be due mainly to this inhibition of growth. On the other hand, **F43** did not produce different effects respect with **F31** on aflatoxin accumulation, but the presence of the Cu nucleus almost deleted its fungistatic effect.

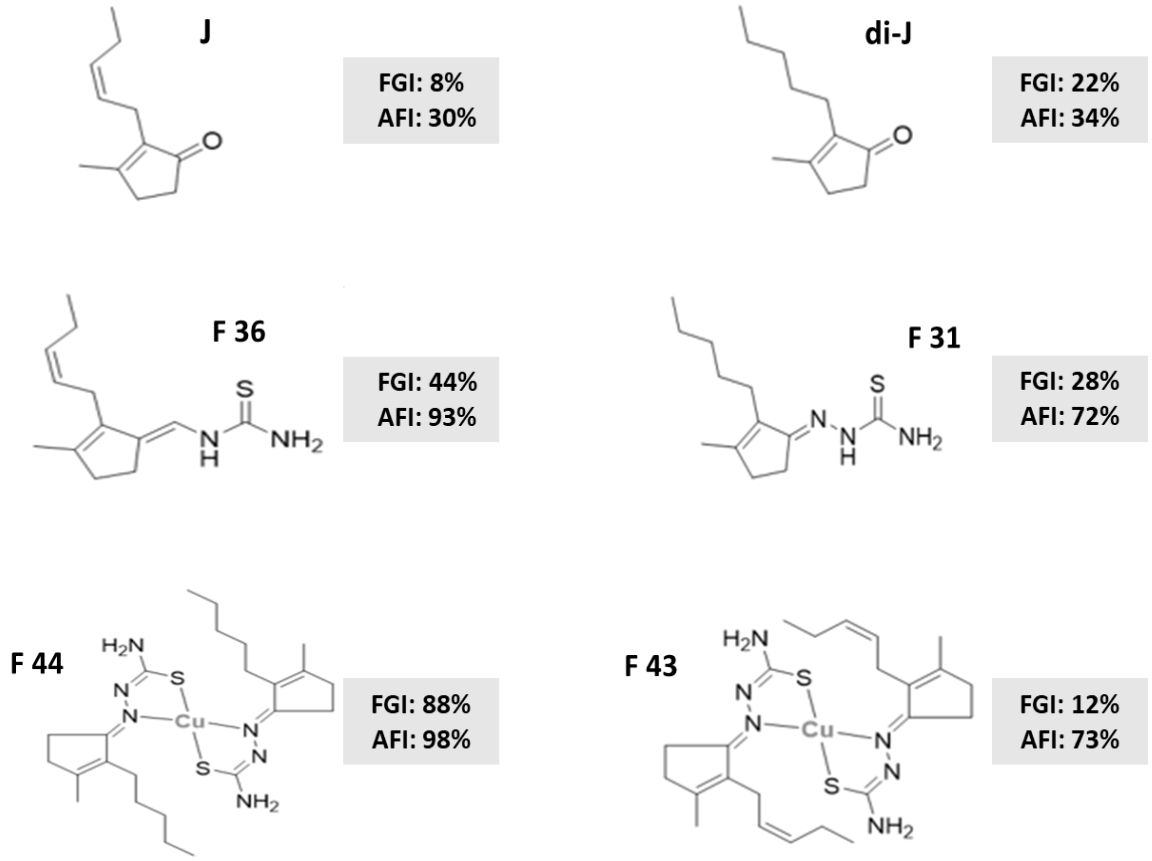


Figure 33: Effects of the cis-jasmone derivatives on A. flavus. *Fungal growth and aflatoxin production inhibition percentages correspond to a 100 µM treatment with the desired sample*

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3.4.2. Anti-oxidant activity compared to sclerotia biogenesis

In many *Aspergillus* species, the stress response is considered a prominent factor involved in the control of secondary metabolism, and several synthetic and natural compounds that modulate secondary pathways - such as AF biosynthesis and sclerotia biogenesis – proved to possess a scavenging activity against ROS. In particular, the sclerotia metamorphosis in filamentous fungi seems to be triggered by oxidative stress *(Georgiou, C. D. et al. 2006)*, according to the scheme reported in ***Figure 34*****.**

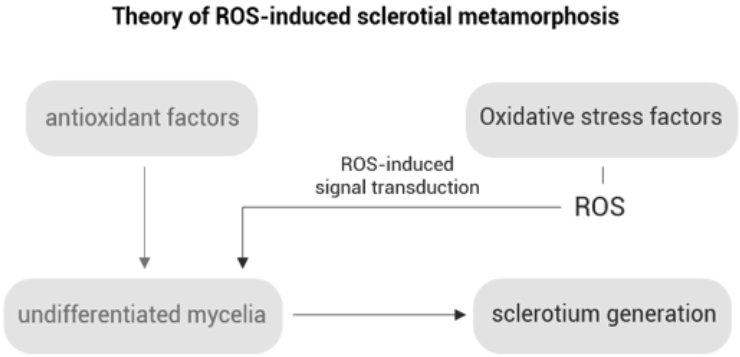
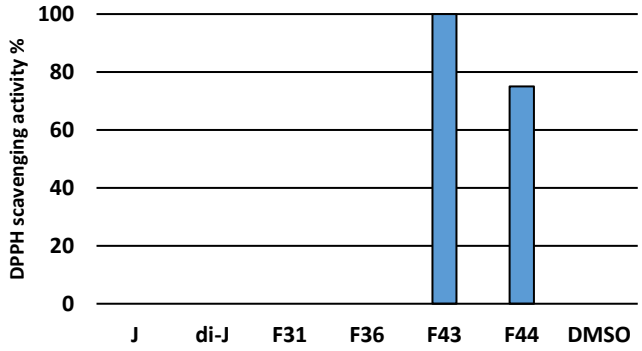


Figure34: Schematic representation of the effect of ROS on sclerotia development (Georgiou, C. D. et al. 2006).

In this context we tried to unravel the hypothetical correlation between the predicted antioxidant scavenging ability of compounds, as determined *in vitro* with the DPPH test, and their effect on the secondary metabolism, with particular attention to the sclerotia biogenesis. In ***Figure 35*** the antioxidant *in vitro* capacity and the effect on the sclerotia development are compared. The sclerotia production differed significantly depending on the molecule examined: *cis*-jasmone and di-hydrojasmone had no effect respect with the control, as it is also possible to observe in the relevant picture (***Figure 35 - C***). **F31** and **F36** lowered significantly the number of sclerotia per colony area, even if an high variability of values could be noticed; the effect is clearly evident in the pictures of the treated cultures (***Figure 35 – B***), where only the white mycelium is visible. Surprisingly, **F43** and **F44** showed a completely different behaviour: in fact, **F43** did not have any effect on sclerotia production, whereas **F44** stopped completely their biogenesis. This difference was consistent with results about the inhibition of the mycelium growth: since the area occupied by the *A. flavus* treated colony is extremely reduced if compared with the control (***Figure 35 - C***).

A



B

Compound	Sclerotia (mg)/colony area (cm^2)
J	1.70 ± 0.55
di-J	1.60 ± 0.45
F31	0.60 ± 0.50
F36	0.45 ± 0.30
F43	1.60 ± 0.40
F44	0.05 ± 0.10
DMSO	1.60 ± 0.35

C

Figure 35: Antioxidant scavenging activity of jasmon derivatives and their effect on sclerotia biogenesis. *DPPH scavenging activity of compounds **(A)**. Weight of sclerotia (mg) per colony area (cm^2) (**B**). Pictures of treated A. flavus cultures **(C)**. Molecules were tested at the concentration of 50 µM.*

The antioxidant activity *in vitro* was estimated as the percentage of DPPH reduced by a certain concentration of the sample. Result showed that the two ligands **F31** and **F36** (tested at 50 µM of concentration) have no antioxidant activity, whereas the two metal complexes are both extremely effective if compared to the control *(**Figure 35 - A**)*; this is probably due to the presence of copper, which being a redox active metal can reduce the DPPH radical itself. In consideration with observations on aflatoxins accumulation and sclerotia production, the hypothesis of a correlation between the scavenging potential *in vitro* of these TSs and their biological effect *in vivo* on *A. flavus* has to be confuted. The anti-aflatoxigenic effect of **F31** and **F36** seems to be associated with the inhibition of sclerotia biogenesis, suggesting that their target(s) could be involved in biochemical processes shared by both the aflatoxin and the sclerotia metabolism. On the contrary, the highly significant anti-aflatoxigenic effect of **F44** seems to be unspecific and due to the inhibition of the fungal primary metabolism, that in turn results in a strong inhibition of aflatoxin and sclerotia biosynthesis as a consequence. **F43** resulted the most interesting compound: the outcomes suggest a specificity of action whose target, differently from **F31** and **F36**, might be located in a metabolic knot closely related to aflatoxin biosynthetic pathway. This hypothesis is supported by the observation that the high inhibitory effect of this compound on aflatoxin accumulation is not accompanied by significant effect on sclerotia and fungal growth.

3.5. Anthraquinone derivatives

Anthraquinones are natural products, deriving from the polyketide and found in many organisms including bacteria, fungi, plants and insects *(Ehrlich et al. 2010)*. In addition, they are precursors of aflatoxins in their biosynthetic pathway: for example, in *Aspergillus flavus*, anthraquinone-derived polyketides are synthesized by iterative type I polyketide synthases (PKSs), and the expected initial product anthrone. Oxidation is required for formation of the first stable intermediate the anthraquinone norsolorinic acid in aflatoxin biosynthesis. During the fungal development of aflatoxigenic strains is possible to distinguish an antraquinonic phase, in which the anthraquinones were synthesized turning in yellow the white mycelium. Since these compounds are intermediates of aflatoxins biosynthesis, we hypothesized that providing a structural analog to the fungus (such as TS-antraquinone) it might block the toxin pathway. Hence, we decided to explore the effect of anthraquinone derivatives on the fungus growth and aflatoxin accumulation. Starting from the raw material 2-hydroxy-methyl-anthraquinone (**F47**) we obtained 2-formyl-anthraquinone-TS (**F46**) and the corresponding Cu and Zn complexes (**F58** and **F64** respectively). We also tested other two F46 derivatives, namely 2-formyl-anthraquinone (**F57**) and 2-tosyl-anthraquinone (**F100**). We also synthesized 2-hydroxy-formyl-anthraquinone (**F66**) and 2-hydroxy-formyl-anthraquinone-TS (**F65**). Finally, also the raw material 2-carbonyl chloride-anthraquinone (**F99**) was tested (***Figure 36***).

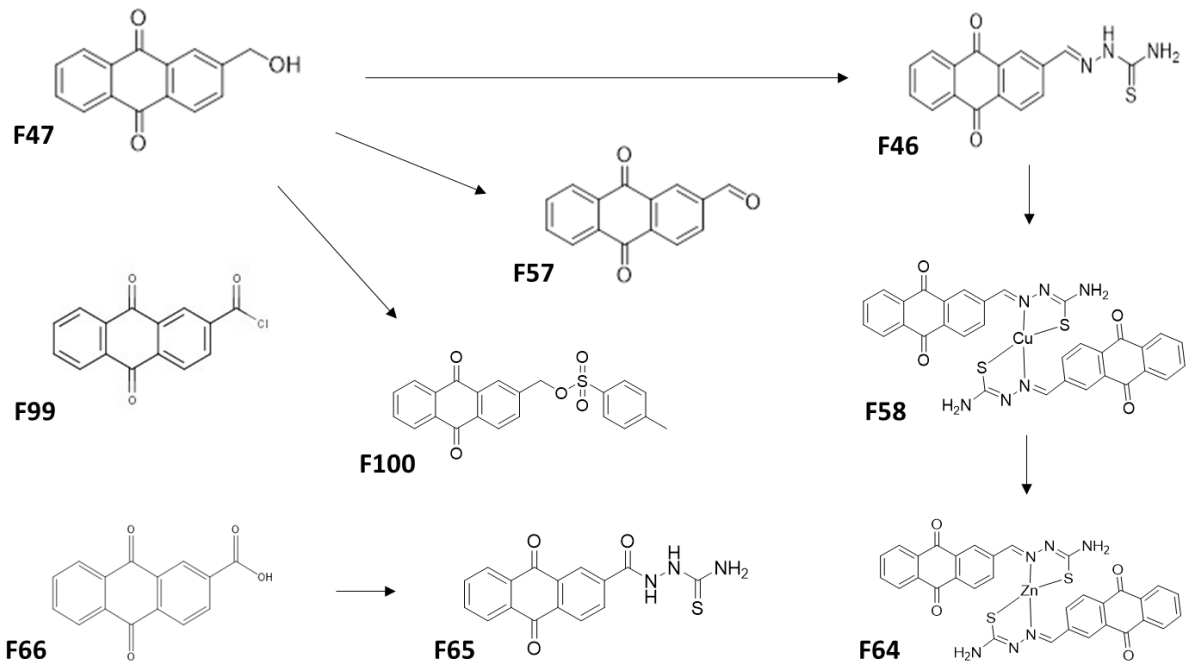


Figure 36: List of components of the anthraquinone family.

3.5.1. Effect on *A. flavus* growth and aflatoxins accumulation

Results about the fungal growth were reported in ***Figure 37.*** The analyses showed that, amongst the anthraquinone raw materials (F47, F66 and F99), only **F47** at 100 µM inhibited the germination spores at a significant level (57%); otherwise, **F66** and **F99** resulted in 14 and 11% of inhibition respectively. **F46** (thiosemicarbazone of F47) did not show any effect on *A. flavus* growth (2% inhibition). Only **F65** (thiosemicarbazone of F66) showed a slight increase in the growth inhibition if compared to F66 (from 14% to 19%). Anthraquinone derivatives **F57** showed the same inhibitory effect of F47, while **F100** possessed an high efficacy (87.2% inhibition). Unfortunately, both the Cu and Zn complexes (**F58** and **F64**) resulted inadequate to be tested: in fact, they were poorly soluble in the media used for the biological assays. The other TS belonging to the family (**F66, F99** and **F65**) did not induced the same phenomenon, allowing then the evaluation of the anti-mycotoxigenic potential. Results were reported in ***Figure 37.*** The 2-hydroxy-formyl-anthraquinone (**F66**) inhibited the aflatoxin accumulation up to 80%, while TS (**F65**) decreased the inhibitory effect of its parental from 80% to 22%. The aflatoxin's accumulation containment of compound **F99** reached the value of 47%. Amongst the 9 anthraquinone derivatives, only thiosemicarbazone **F66** full fit the specific requirements for being addressed, on the basis of its biological activity on *A. flavus*, to further analyses for citotoxicity and genotoxicity.

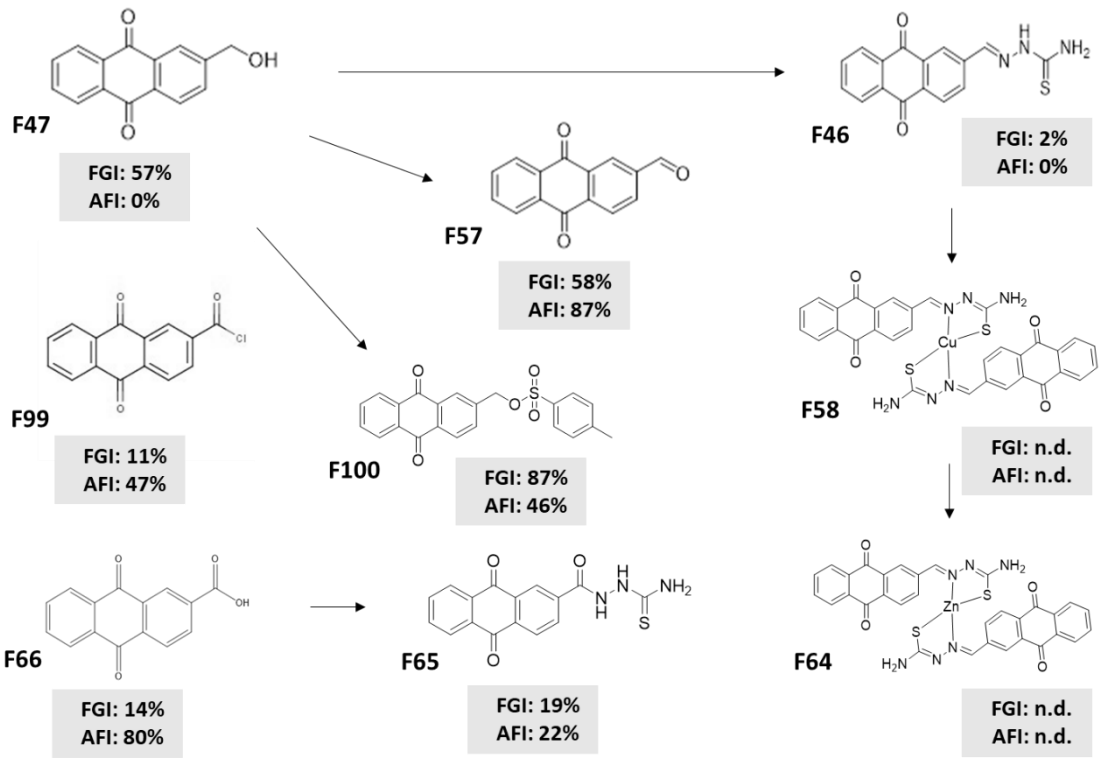


Figure 37: Effects of the anthraquinone derivatives on A. flavus. *Fungal growth and aflatoxin production inhibition percentages correspond to a 100 µM treatment with the desired sample. Data detected of Afla+ strain.*

However, during the analysis of the antimycotoxigenic properties of anthraquinone derivatives, a completely unexpected effect was observed: in fact, when added to the non-producing BS07 strain (as required by the assay, in order to exclude undesirable artifacts due to aspecific effects), a fluorescence emission in the wave-length characteristic of aflatoxin B (465 nm) was detected in the CCM culture medium *(**Figure 38**)*.

Due to the fact that for the incapacity to accumulate AF is genetically determined in BS07 (since it possesses a point mutation that causes a pre-termination in the sequence of PKSA, the first enzyme of the biosynthetic pathway (*Yu and Ehrlich 2011*)), we supposed that the fluorescence could be caused by the accumulation of a side-product of the AF pathway, or even an intermediate: we thus hypothesized that, due to their structural similarity with the precursor of the toxin, the compounds can enter the biosynthetic pathway and, being used by one of the down-stream enzymes, results in a sort of by-pass of the metabolic blockage. As a consequence, the accumulation in the medium of a product with similar chemical-physical characteristics of aflatoxins occurred.

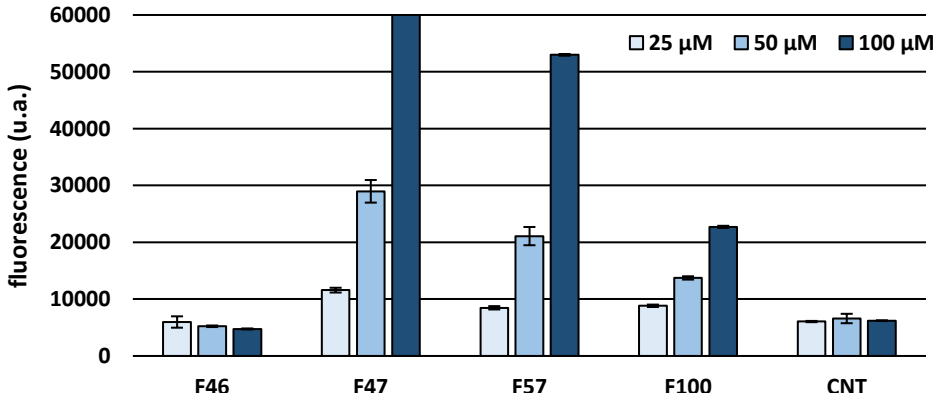


Figure 38: Effects of F46, F47, F57 and F100 anthraquinone derivatives on atoxigenic A. flavus strain BS07. *Fluorescence emission is expressed as arbitrary units (u.a.)*

3.5.2. Characterizing the fluorescent product:

Anthraquinone is ubiquitous in the environment, being detected in the air, water, soil, plants, and animal tissue, but the major sources of environmental exposure are both natural and anthropogenic; various natural compounds belonging to this family are widely used as preservatives and additives in reason of their antioxidant properties. Among the major uses, the application as pesticides, bird repellent (especially for geese), and additive in processes for paper and pulp industry

can be found. Additionally, such compounds are also used as a seed dressing or in seed treatments. In this panorama, and considering the Aflatox® aim that actually was to identify promising molecules to be proposed as new generation pesticides and, when possible, to be applied in field to contain aflatoxins contamination of crops, we decided to investigate and characterize the fluorescent product released in the culture medium by *A. flavus* atoxigenic strains treated with **F47**, **F57** and **F100**.

Due to the impossibility of our fluorescence-based method to discriminate different molecules emitting at the same wave-length in a mixture, we perform a TLC (Thin Layer Chromatography) analysis to verify if the compound responsible for the fluorescence was actually AFB_1. Culture broth samples were pipet off from CCM microplate and loaded in an inert solid phase; an aflatoxin B_1 standard was used as reference. The mobile phase (80% toluene, 15% methanol, 5% acetic acid) allows, in a closed and saturated system, a slow migration of molecules driven by capillarity, on the basis of their polarity. As showed in ***Figure 39***, it emerged that the fluorescent product did not have the same mobility of AFB_1 standard, but remained clearly visible at the inoculation point on the start line.

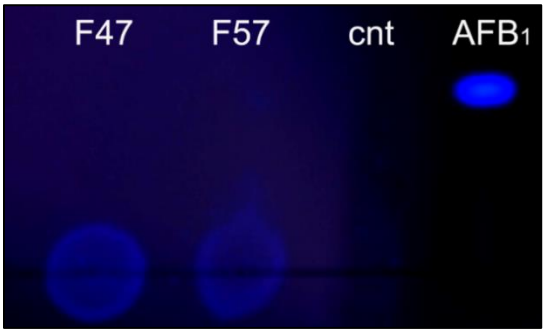


Figure 39: Separation of CCM broth from atoxigenic BS07 strain treated with F47 and F57 (at 100 µM) through Thin Layer Chromatography. *Control (cnt) corresponds to 1% DMSO treated cultures. On the left, AF B_1 as a standard.*

This results demonstrated that the unknown compound synthesized by the atoxigenic strain wasn't aflatoxin B, but a metabolic product which shares the same fluorescence properties with it.

However, it could not be excluded that, if not aflatoxin, the compound might be an intermediate of the same biosynthetic pathway; in order to clarify if (and eventually how) its accumulation was associated with the aflatoxins metabolism, different conditions regulating AF production were tested.

Temperature:

In response to the primary metabolism of the fungus, aflatoxins production reaches its optimum at 28°C, then gradually decreases until being completely blocked at 37 °C, temperature at which the transcription factor *AflR* results non-functional. In fact, in this condition, the zinc finger weakly binds the specific promoter regions, resulting incapable to trigger the transcription of different genes in the aflatoxin cluster *(O'Brian G.R. et al. 2007)*. Therefore, the accumulation of the unknown compound was assessed by administrating **F47**, **F57** and **F100** to BS07 CCM cultures, and evaluating the fluorescence emission after 6 days of incubation at 28 and 37 °C. Data below showed that the *A. flavus* afla- strain did not produce the unknown compound in presence of F47, F57 and F100 at 37°C, the same effect reported by afla+ strains that not produce aflatoxin when growth at 37°C *(**Figure 40**).*

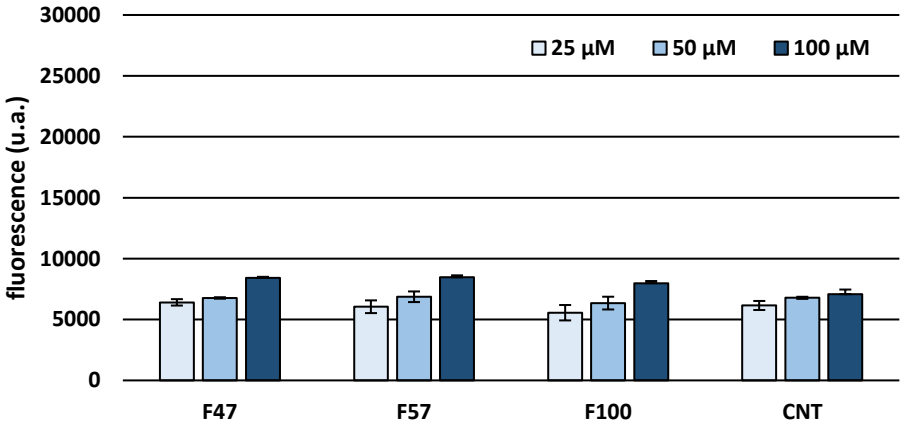


Figure 40: Effects of the F47, F57 and F100 anthraquinone derivatives on aflatoxin accumulation of A. flavus at 37°C. *Aflatoxin accumulation as fluorescence arbitrary unity (u.a.) at 25 - 50 and 100 µM when incubated at 37°C in BS07 non-aflatoxigenic strain. Data at 25°C were reported in figure 30.*

Aflatoxin's production inhibitors:

The accumulation of the fluorescent product by *A. flavus* atoxigenic BS07 treated with F47 was also evaluated in presence of **Tio-Y**, that was previously described to be a very selective inhibitor of AF biosynthesis that poorly interfere with the mycelium growth. Tests were conducted at 28 °C, the optimum for aflatoxins production. Both F47 and Tio-Y were administrated at the concentration of 50 µM each; control conditions were set with DMSO at the relevant volume. Obtained results showed how, in presence of the AF inhibitor, a decrease of fluorescence emission was recorded (***Figure 41**).*

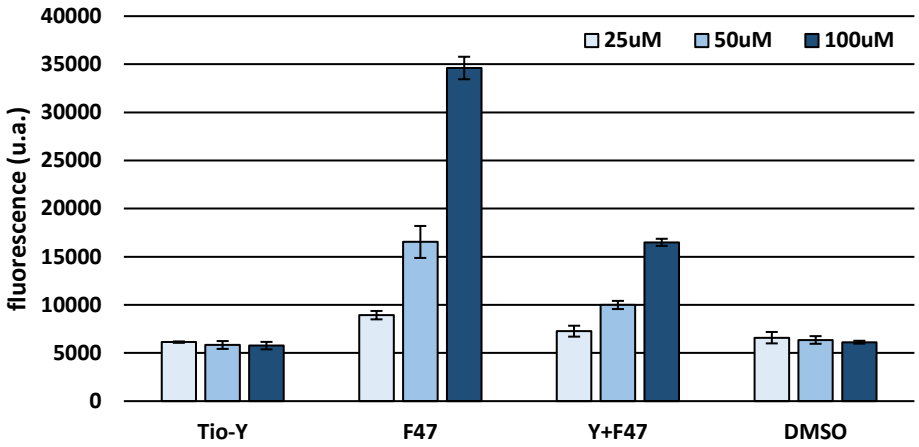


Figure 41: Effect of Tio-Y on fluorescence emission in atoxigenic of A. flavus cultures treated with F47. *Incubation was conducted in CCM at 25 °C for 6 days. Combined effect of F47 and Tio-Y was assayed in atoxigenic strains BS07. Tio-Y and F47 were tested individually at 25-50 and 100 µM. The same concentration for DMSO (CNT). When F47 was tested in combination of Tio-Y, Tio-Y was added at 100 µM.*

The fluorescent product was then demonstrated to respond to stimuli that, directly or indirectly, specifically affect the aflatoxins metabolism. Such evidence, together with the observed chemical properties shared (or nor) with AFB and the fact that the structure of F47 is similar but not identical to the precursor of the toxin, was considered encouraging respect to the hypothesis that the compound could be a sort of aflatoxin/intermediate "analog". Further experiments are needed to learn more about this unknown product; overall considering that, until the structure will not be resolved and the molecule will be purified, no information about its potential toxicity on other organisms could be acquired. In this sense the finding that commonly used molecules can "switch on" the production of an aflatoxin-like compound by non-aflatoxigenic strains (and in particular by a specific strain patented and applied as a biocontrol agent on maize crops) rises important concerns about the hypothesis of release for new molecules, even for environmental sustainability purposes, and confirm the necessity to test *in vitro* such molecules. This, in our opinion, represents the best validation of the importance of projects such as Aflatox®.

3.6. 2-acetylthiophene and 2-acetylfuran derivatives

The parental molecule of this family is **F20** (2-acetylthiofene thiosemicarbazone). Its derivatives included two metal complexes with Cu(II) and Zn(II) (**F34** and **F35** respectively), the 2-acetylfuran (**F21**, an analog of 2-acetylthophene in which the sulphur atom is replaced with an oxygen in the 5-membered ring) and its own metal complexes (namely **F25**, with a Cu(II) nucleus, and **F27**, with a Zn(II) nucleus)(***Figure 42***).

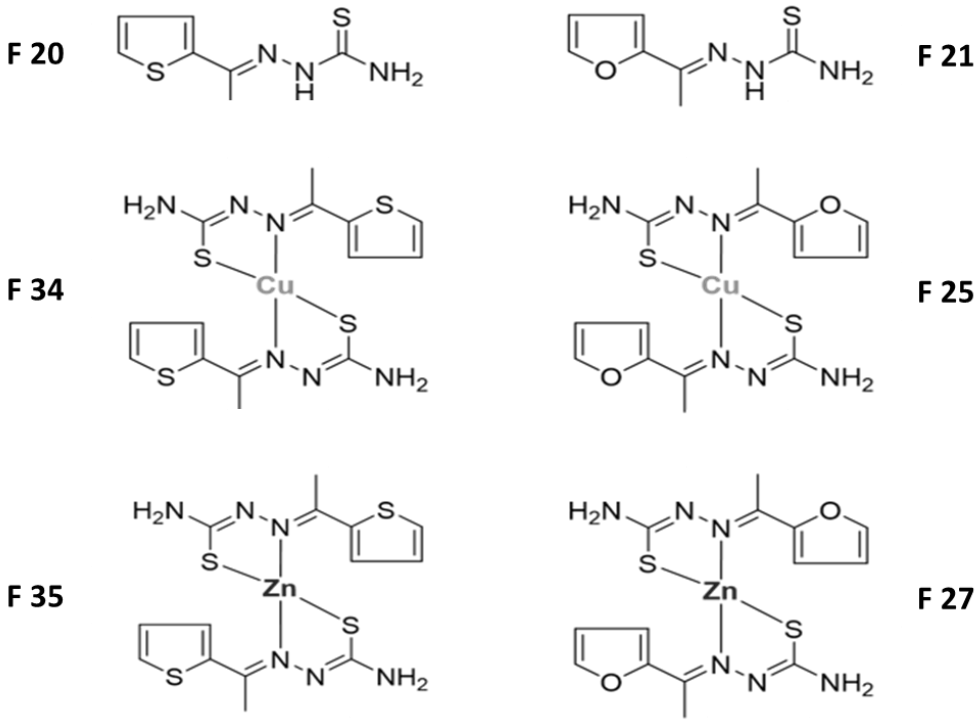


Figure 42: List of compounds of the 2-acetylthiophene and 2-acetyfuran family

3.6.1. Effect on *A. flavus* growth and aflatoxin accumulation

Both the ligand **F20** and its Cu complex (**F34**) resulted to possess an anti-aflatoxigenic, whereas **F35** (the Zn complex) showed just a mild inhibitory effect on aflatoxin accumulation. On the other hand, all the three molecules showed a negligible effect on the fungal growth: the highest fungistatic effect was registered for **F34**, reaching the 30% of inhibition at the concentration of 100 µM ***(Figure 43)***.

The 2-acetylfuran derivatives provided different results: **F21** (ligand) and **F27** (Zn complex) were almost inactive on both aflatoxin and growth; on the contrary, **F25** (Cu complex) revealed higher effects in both tests. In general, the entire series resulted less effective if compared with results

obtained with 2-acetylthiophene derivatives. We found that the effect of **F20** and **F21** on the fungus growth is low, and, in the latter, the substitution of sulfur results in a loss of the activity against AF accumulation. However, an increase of fungistatic and anti-toxigenic effect was observed when both the ligands were complexed with copper **(F34**, **F25)**; on the contrary, zinc addition **(F35**, **F27)** did not furnish a significant improvement of biological activities if compared to copper *(**Figure 43**)*.

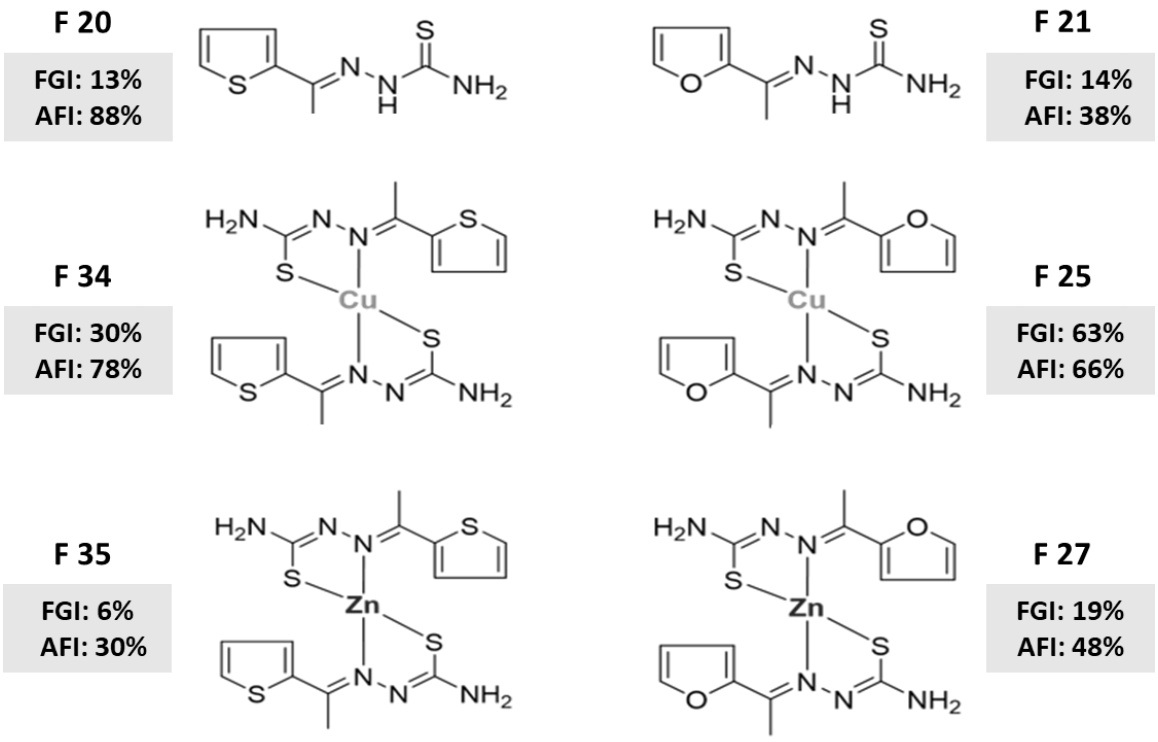


Figure 43: Effects of the 2-acetylthiophene and 2-acetyfuran derivatives on A. flavus at 100 µM concentration.

3.6.2. Gene expression analysis

F20, the most interesting compound (low inhibition of fungus growth and high inhibition of aflatoxin production), was then evaluated for its specific effect on the expression of selected genes involved in the secondary metabolism. In particular, the response of two genes belonging to aflatoxins gene cluster (***OmtB*** and ***AflS***) and two genes linked to sclerotia biogenesis (***NsdC*** and ***NsdD***) were analyzed in *A. flavus* CCM cultures exposed to F20 100 µM treatment. As reported in ***Figure 44***, a down-regulation of all genes was observed: the decrease of expression level for the two aflatoxins cluster's genes can explain the strong inhibitory effect of F20 on AFs production (88.4% inhibition at 100 µM). At the same time, the down-regulation of ***NsdC*** and ***NsdD,*** points to possible interference in the

process of sclerotia formation also, as the two secondary metabolisms are know to share many metabolic knots with the aflatoxin metabolism *(Chang et al. 2002)*.

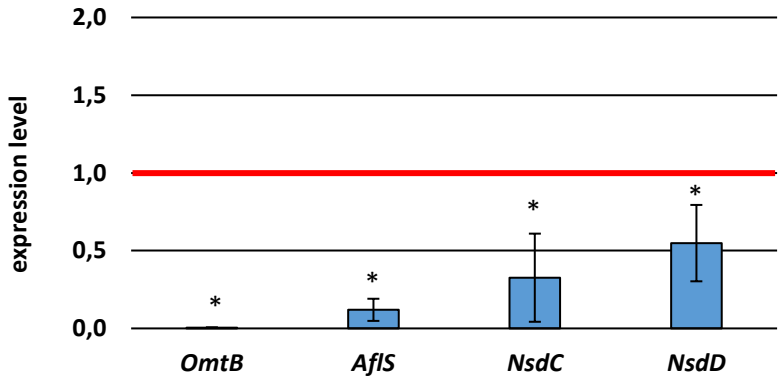


Figure 44: Effect of F20 on gene expression. Real time PCR analysis were conducted on mycelium from 96 h CCM cultures treated with F20 (50 µM).

This study on the 2-acetylthiopnene derivatives led to discover very interesting properties of the ligand **F20**: in fact, this molecule resulted extremely effective in inhibiting the aflatoxin production (88% of inhibition at 100 µM) without affecting the fungal growth (13%).

3.7. Phenone derivatives

During previous studies already published *(Degola et al. 2017; Rogolino et al. 2017)*, the presence of the C=S group was demonstrated to be essential for the anti-aflatoxigenic activity of thiosemicarbazones, that deeply depends on the substituents on the phenyl ring: an increase in the lipophilicity of the compound was in fact followed by an increase of the aflatoxin inhibition effect, probably due to a higher ability of the molecule in penetrating through the cell membrane *(Veldman et al. 2004; Price et al. 2011)***.**

We was therefore interested in investigating these hypotheses in a newly synthesized family of TS: the phenone derivatives.

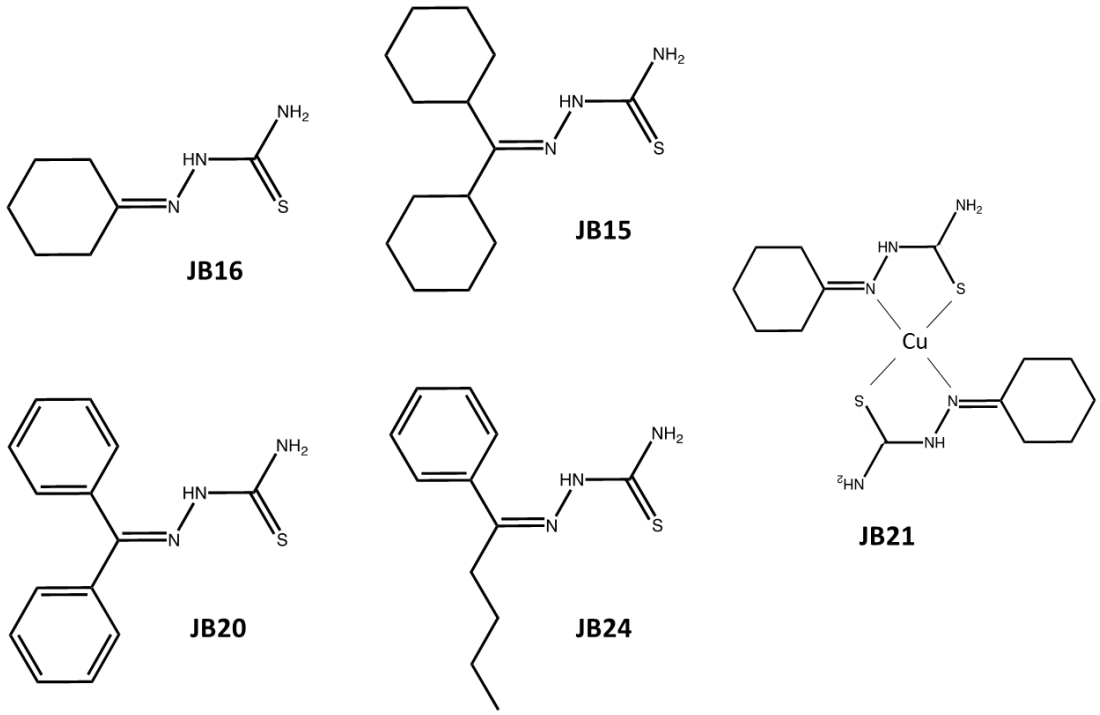


Figure 45: Schematic representation of Phenone thiosemicarbazones. *Cyclohexanone-TS* ***(JB16)****. Dicyclohexylketone-TS* ***(JB15)****. Benzophenone-TS* ***(JB20)****. Valerophenone-TS* ***(JB24)*** *and Copper (II) Cyclohexanone-TS complex* ***(JB21)****.*

3.7.1. Effect on *A. flavus* growth and aflatoxin accumulation

Compounds belonging to the phenone family ***(Figure 45)*** have been evaluated for their effect on the secondary metabolism, in terms of aflatoxins biosynthesis and sclerotia biogenesis. Results previously acquired (*Rogolino et al. 2017*) suggested that the metal chelation can lead to an improvement of the inhibitory effect of parental molecules against AFs accumulation, and in particular copper complexes. Whose coordination with the metal nucleus was proposed to improve

its bioavailability, facilitating the penetration into lipid membranes and thus resulting in a higher anti-aflatoxigenic activity. In fact, it should kept in mind that copper salts have been largely used in agriculture as pesticides against a wide range of plant pathogens. In order to explore this aspect, we also synthesized compound **JB21**, the Cu(II) complex of **JB16**.

As shown in ***Figure 46,*** **JB16** weakly inhibited AFs accumulation (11%), whereas **JB15, JB20** and **JB24** determined an increasing activity, ranging from 38% to 56% of inhibition at 100 µM. Surprisingly, the effect of **JB21** as anti-mycotoxigenic compound was only slightly higher than the ligand alone, confirming that for these molecules the complexation with copper wasn't as ameliorative as expected on the basis of other evidences. The fungus growth evaluation revealed that among all derivatives, only **JB15** induced a notable reduction of fresh weight (over 80% of inhibition at the highest concentration ***(Figure 46).*** Out of an antimicrobial perspective, a low antifungal activity combined with high anti-mycotoxigenic activity is extremely desirable, since this can assure the preservation of the environmental mycobiota while ensuring the protection from noxious secondary metabolites; therefore, due to their ability to halve AFs accumulation without significantly affecting biomass production, **JB20** and **JB24** were chosen for further analyses.

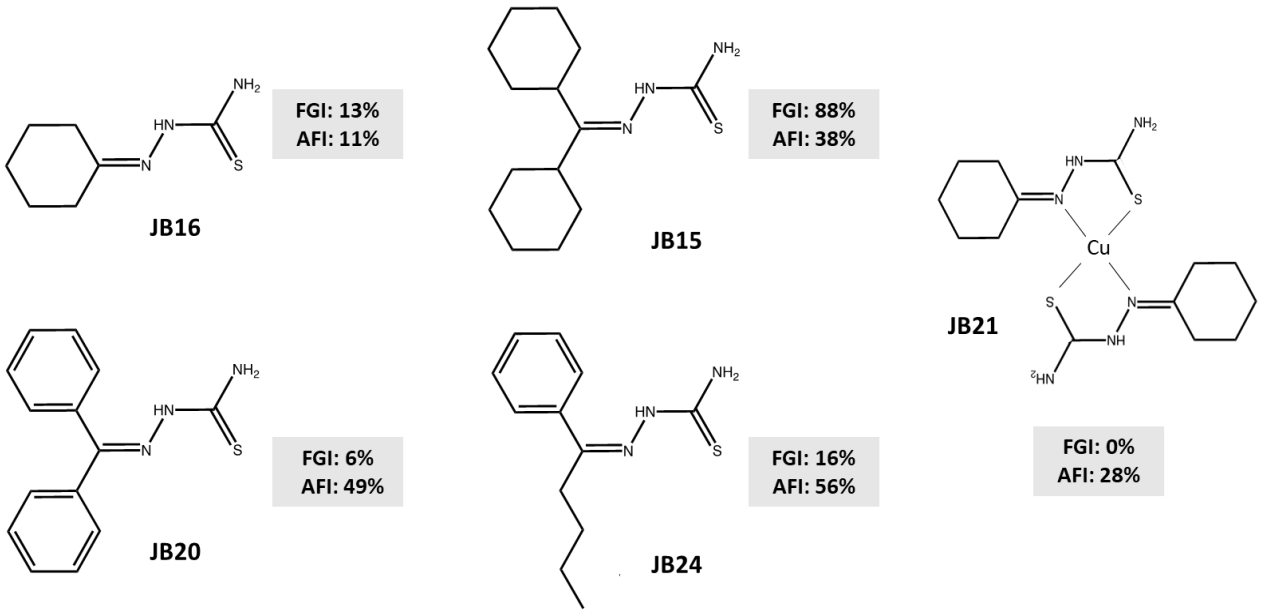


Figure 46: Effects of the Phenone derivatives on A. flavus. *Fungal growth and aflatoxin production inhibition percentages correspond to a 100 µM treatment with the desired sample.*

3.7.2. Effect on sclerotia biogenesis

Since aflatoxin biosynthesis is known to share several regulatory steps with other developmental processes belonging to the secondary metabolisms such as sclerotia biogenesis *(Amare et al. 2014)*, the effect on the production of these structures was assessed. Sclerotia formation was induced by culturing an aflatoxigenic and sclerogenic *A. flavus* strain in CZ solid medium, amended with 100 µM of **JB20** and **JB24** (and 1% DMSO as control). The presence of the TS-derivatives in the culture medium reduced the formation of sclerotia, while, as expected, no mycelium growth inhibition was observed. With respect to the control, the exposure to **JB20** and **JB24** limited the sclerotia biogenesis to 30% and 86% respectively ***(Figure 47)***. These results confirm our previous observations obtained with other thiosemicarbazones, effective in containing AFs production but with a slight effect on fungal growth, that severely impair sclerotia development in *A. flavus (Zani et al. 2015).*

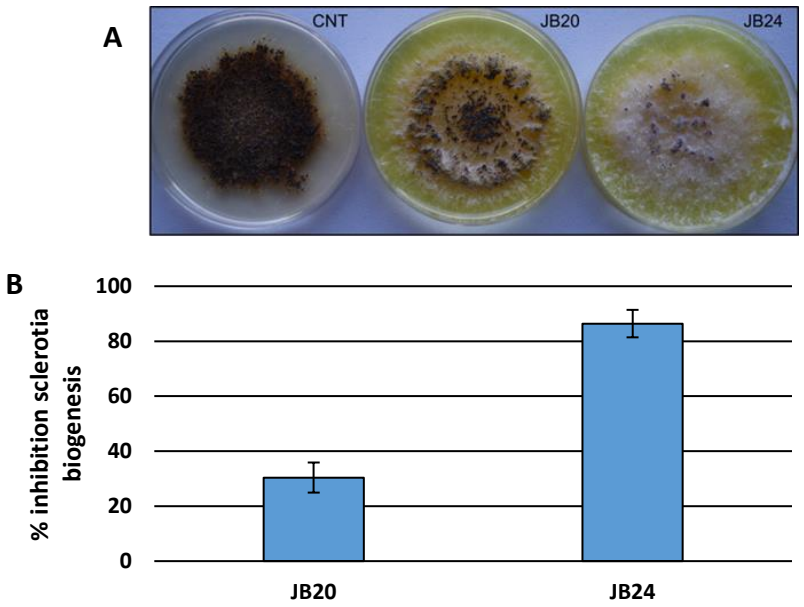


Figure 47: Effect of the JB20 and JB24 ad 100 µM on sclerotia development. *Pictures of treated A. flavus cultures* ***(A)****. % inhibition of sclerotia biogenesis* ***(B)****.*

In conclusion, as we were focused on finding specific inhibitors of aflatoxin biosynthesis rather than inhibitors of fungal growth, benzophenone **(JB20)** and valerophenone thiosemicarbazones (**JB24**) seemed interesting in this sense.

These results were published in *Journal Food and Agricolture Chemistry in 2019 (Bartoli et al. 2019).*

3.7.3. Nanoformulation of most promising compounds

Among the new strategies aimed to control phytopathogenic fungi, applications of nano-sized particles were recently proposed to be utilized in the management of plant diseases, mainly relying on silver nanoformulations *(Jo et al. 2009, Kim et al. 2012).* In fact, due to their high antimicrobial activity, nanoparticles (NPs) are widely used for various purposes, including materials for medical devices and water sanitization, and are being considered as an alternative to antibiotics that, in certain cases, can effectively prevent microbial drug resistance *(Beyth et al. 2015)*. During last decades nanoparticles have been developed for agronomical application to target bacteria, fungi, and viruses: recent researches demonstrated that nanoparticles might be used to limit the use of pesticides, whose abuse is causing a detrimental ecological and environmental impact, as well as harmful effects on human health. As technological advances allowed a more economical production, their use as antimicrobial agents has become more common, but to date there have been relatively few studies on nanoparticles applicability against *Aspergillus* species and their capacity to accumulate AFs *(Kovač et al. 2018; Jo et al. 2009; Kim et al. 2012; Beyth et al. 2015).*

In this study, poly-(ε-caprolactone) was used by Nanomater Srl. (Alghero, Italy) to produce nanoparticles (**NPs**), due to its biocompatibility and biodegradability properties; poly-(ε-caprolactone) NPs were formulated using poly(vinyl alcohol) (**PVA**) or Pluronic® (**F127**) as non-ionic surfactants (named as **PVA-NP** and **F127-NP**). NPs were then loaded with **JB20** and **JB24**, obtaining **PVA-20, PVA-24, F127-20**, and **F127-24** respectively, with the purpose to overcome TSs low water solubility, to modulate their delivery inside the cell and to prevent/limit the atmospheric degradation. Effect on *A. flavus* growth, aflatoxins accumulation and sclerotia biogenesis was determined and compared.

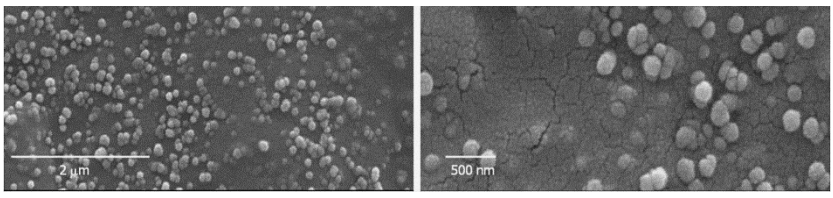


Figure 48: Unloaded F127 NPs at different magnifications (a, x20000 and b, x50000), as observed with Scanning Electron Microscopy, chosen as example.

3.7.4. Antifungal activity of NPs loaded with TSs

The antifungal potential of raw materials and unloaded NPs was firstly assessed *(**Figure 49**)*. Neither Pluronic® F127 nor PVA showed any effect on the hyphal growth; on the contrary, **PVA-NP** determined a 28% of growth inhibition at the concentration 50 µM, while at lower concentrations did not significantly differ from PVA *(**Figure 49-A**)*. These results suggested that the high surface/volume *ratio* of nanoformulation would enhance their direct interaction with the fungal cells, probably improving fungal wall and membrane penetration. The addition of **JB20** and **JB24** did not increase the efficacy of **PVA-NP** *(**Figure 49-A**)*: both **PVA-20** and **PVA-24** induced less than 15% of inhibition on fungal growth at 10 and 25 µM concentration, reaching the maximum at 50 µM (33.1% and 24% for PVA-20 and PVA-24, respectively). **F127-NP** resulted less effective in limiting the mycelium development of *A. flavus* than **PVA-NP** *(**Figure 49-B**)*; as for **PVA-NP**, no significant differences between loaded and unloaded **F127-NPs** were detected.

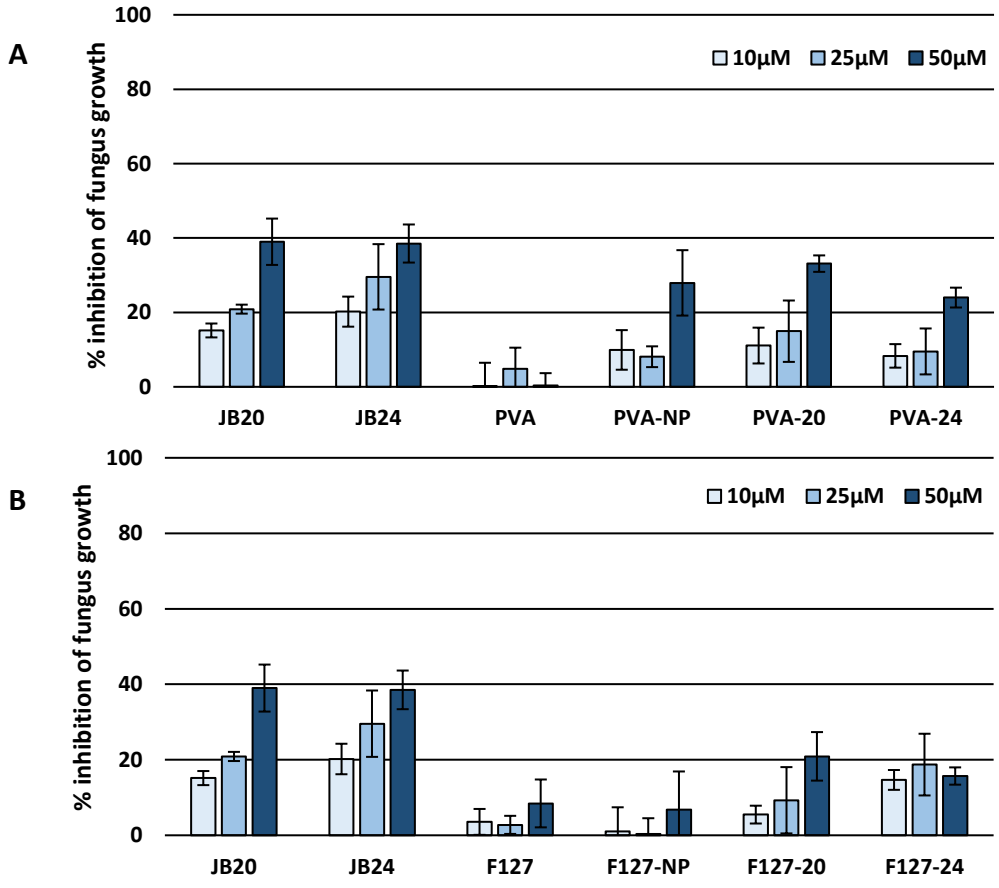


Figure 49: NPs effect on growth of mycelium in A. flavus. *Fungus growth Inhibition when A.flavus was treated with JB20, JB24 and loaded JB20 and JB24 PVA-NPs* ***(A)*** *and F127-NPs* ***(B).*** *The data were expressed as mean percentage inhibition respect to control.*

Preliminary results suggested that the activity of PVA-NPs and F127-NPs on *A. flavus* growth is strictly correlated with the type of surfactant: in fact, PVA seemed to promote a higher fungistatic effect than Pluronic® F127. This finding could be attributed to the presence of hydroxyl groups in PVA chains, able to establish electrostatic interactions or hydrogen bonds with major components present in the cell wall's outer layer, such as β-1,6-branched and β-1,3-glucan cross-linked to chitin, and amorphous α-1,3-glucan *(Yoshimi et al. 2016)*.

3.7.5. Aflatoxin inhibition of NPs loaded with TSs.

The evaluation of NPs anti-aflatoxigenic potential revealed that no one of **PVA-NPs** was able to inhibit AFs accumulation by more than 17%, at all concentrations tested ***(Figure 50-A)***. The addition of thiosemicarbazones (PVA-20 and PVA-24) did not enhance the effect of PVA-NPs. Otherwise, **F127-NPs** showed an increasing, dose-dependent inhibition on the toxin's biosynthesis. Amongst the F127-NPs, **F127-20** proved to be significantly more effective than both **F127-NP** and **F127-24** at each tested concentration ***(Figure 50-B)***; more interestingly, the addition of TS **JB24** determined an improvement of the biological activity with respect to the unloaded particles at the highest concentration.

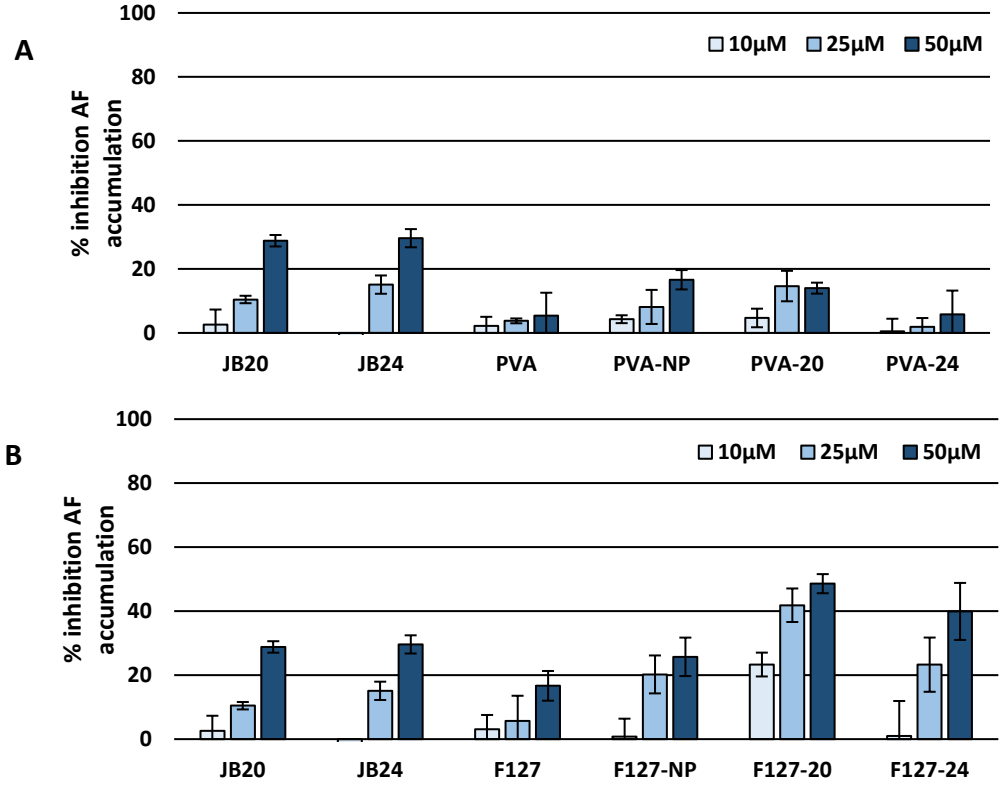


Figure 50: Anti-aflatoxigenic effect of NPs in A. flavus. *PVA-NPs **(A)** and F127-NPs **(B)** loaded with JB20 and JB24 at 10, 25 and 50 µM were compared to unloaded NPs and JB20 and JB24. Toxin accumulation inhibition was expressed as mean percentage respect to control.*

Within the huge microbial population associated with polymer degradation, *Aspergillus ssp* are prevalent; *A. flavus* in particular is highly effective in the degradation of aliphatic polyesters (e.g., PCL, PGA, PLGA and PLA) as well as PVA, polyethylene and polypropylene *(Pathak et al. 2017, Chiellini et al. 2006)*, and the polymer degradation is accomplished via different enzymatic activities with a side production of oxidized metabolites (CO_2, CH_4, H_2O) *(Pathak et al. 2017)*. Aflatoxin biosynthesis is influenced by many environmental factors including carbon and nitrogen source, water activity, temperature, pH as well as CO_2 and O_2 level *(Yu 2012, Giorni et al. 2008)*. On the basis of these considerations, we cannot exclude that the higher antimycotoxigenic activity observed for **F127** with respect to **PVA** might be related also to the different and peculiar degradation pathways of these polymers, that in turn may produce metabolites with different capacities to interfere with the aflatoxin biosynthesis.

3.7.6. Interference with sclerotia development.

The effect of NPs on the sclerotia biogenesis was assessed. Sclerotia production was induced by culturing the *A. flavus* strain in CZ solid medium with 50 µM PVA, Pluronic® F127 and relevant NPs. As expected (and consistently with previously obtained results) PVA and Pluronic® F127 treated cultures did not show any difference with respect to the control; the same was observed in PVA-NP treatment, while a slight effect (less than 10% inhibition) was recorded for F127-NP ***(Figure 51)***.

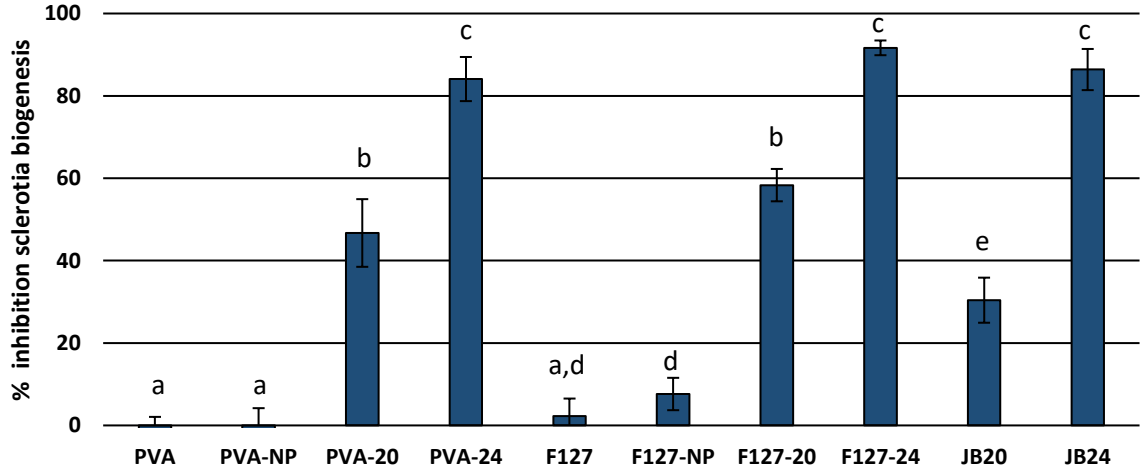


Figure 51: Effects of 50 µM NPs on sclerotia biogenesis in CZ solid medium. *NPs loaded with JB20-TS and JB24-TS were compared with unloaded NPs and the two thiosemicarbazones. Dry weight of sclerotia biomass was compared with control and reported as mean percentage inhibition.*

If compared with TS JB20, the effect of PVA and F127 loaded NPs resulted only slightly improved; on the contrary, no advantages, in terms of increases activity, was obtained by loading JB24 on NPs, since the TS showed *per se* the highest level of sclerotia inhibition. Looking at the nanoformulations, it was possible to observe that the addition of thiosemicarbazones **JB20** and **JB24** dramatically increased the inhibitory effect of both **PVA-** and **F127-NP**, resulting in a strong impairing of sclerotia biogenesis. Interestingly, PVA and F127-NP loaded with the same TS showed a similar effect: 46.7 and 58.3% of inhibition in sclerotia formation were obtained with **PVA-20** and **F127-20** respectively, while the addition of JB24 to both NPs (**PVA-24** and **F127-24**) determined a percentage of inhibition of 84.1 and 91.7% ***(Figure 51)***.

The comparison between the effects of **PVA-** and **F127-NPs** highlighted that the biological activity in *A. flavus* strongly correlates with the type of surfactant: in fact, PVA tended to promote a higher fungistatic effect with respect to Pluronic® F127, which, on the contrary, showed the best anti-mycotoxigenic activity. Overall, the most interesting results were obtained towards the inhibition of sclerotia production: as survival structures of many phytopathogenic fungi, sclerotia grant persistence in soil and resistance to abiotic stress factors such as heat, drought, and chemicals *(Coley-Smith 1979)*; on the other hand, AFs biosynthesis is known to share several regulatory steps with other developmental processes belonging to the secondary metabolisms, being triggered by various intra- and extra-cellular inputs that have been found involved also in the control of sclerotia biogenesis *(Amare et al 2014)*. Thus, the observation that **F127-24** possess a high activity against the biogenesis of sclerotia in *A. flavus* despite of a moderate (nevertheless interesting) anti-aflatoxigenic potential, seemed argue with the most frequent findings obtained with modulators/disruptors of *A. flavus* secondary metabolism. However, even not so ameliorative with respect to the non-nanoformulated thiosemicarbazones, the heavy impairment of sclerotia formation indicates that **JB20** and **JB24** NPs may be proposed as potential agents of control against the proliferation of mycotoxigenic sclerotigenic fungi, through the limitation of their dissemination and persistence in the environment. Even if other parameters, such as phytotoxicity, must be evaluated before any practical applications, this study supports the possible use of thiosemicarbazone nano-formulations as an alternative method to usual pesticides for the eradication of *Aspergillus*-derived aflatoxins contamination. Results were reported in a scientific publication currently submitted for publication.

3.8. Fenchone derivatives

Fenchone is a Ketone organic compound, used as a flavor in foods, fragrance personal care products, including cosmetics, shampoos, perfumes, soaps, lotions, toothpastes, sexual wellness and chemicals in electronic cigarettes *(PubChem)*. It was a component of essential colorless oil liquid widespread in plants in particular in fennel (*Foeniculum vulgare*). Recently, its role as a plant metabolite like antifungal drug has been discovered *(Zuzarte et al. 2012)*; for this reason, we included fenchone derivatives, such as raw material (**F81**), its thiosemicarbazone (**F80**) and two metallic complexes (Cu(II) **F87** and Zn(II) **F88**) in the battery of biological assays on *A. flavus.* Compounds were reported in ***Figure 52***.

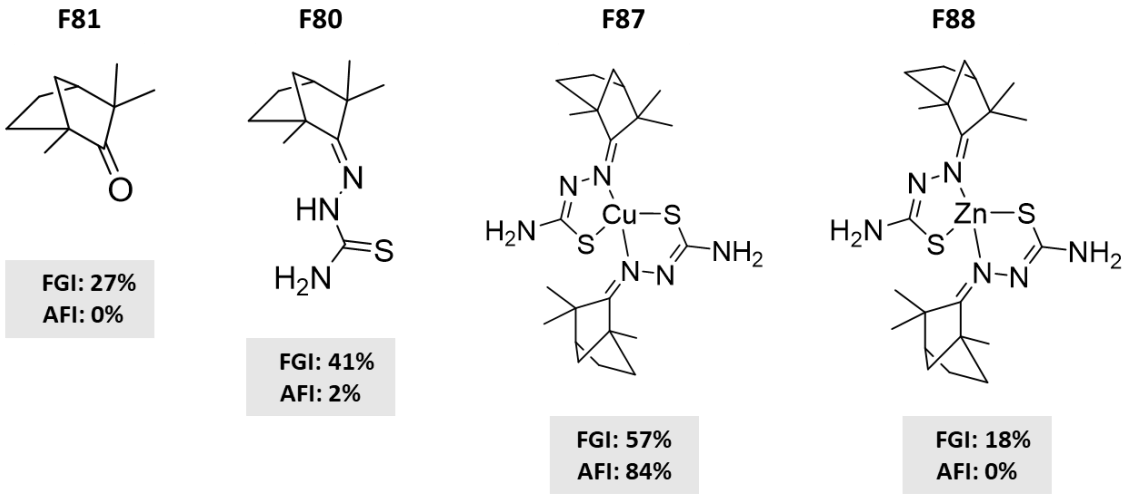


Figure 52: Schematic representation of fenchone derivatives and the effects on A. flavus at 100 µM sample concentration on fungus growth and aflatoxin accumulation.

3.8.1. Effect on *A. flavus* growth and aflatoxins accumulation

As shown in ***Figure 52***, the raw material **F81** induced a low inhibition on the fungal growth (27%), while the TS-derivative (**F80**) reached the 41% of inhibition. Complexation of TS with Cu(II) (**F87**) determined an additional increase of antifungal activity up to 57%. On the contrary, Zn(II) complex did not showed any significant effect.

With regard to aflatoxin accumulation, fenchone derivatives generally did not displayed any interesting activity, except for **F87**: in fact, Cu(II) complex was able to contain the aflatoxin accumulation up to 84% (***Figure 52***). Therefore, on the basis of these results, only **F87** could be proposed as anti-mycotoxigenic compound for future applications in the field.

3.9. Camphorquinone derivatives

Since fenchone TS did not reveal any promising on primary and secondary metabolism of *A. flavus*, we analyzed the effect of a similar molecule to evaluate whether a slight difference in the molecular scaffold could provide a better activity on the fungus. Camphorquinone was a popularly-used photosensitizer dentistry in composite resin restoration, with a similar structure to fenchone molecule *(Chang et al. 2015)*. From the raw material camphorquinone (**F67**) we thus obtained its TS-derivative, but this compound presented two thiosemicarbazones (**F63**). Also we wanted to test the effect of two different metal centres, copper(II) and zinc(II), on the fungus growth and on the anti-aflatoxigenic activity. We obtained Cu complex camphorquinone-bisTS (**F79**) and Zn complex camphorquinone-bisTS (**F82**). Compounds were reported in ***Figure 53*.**

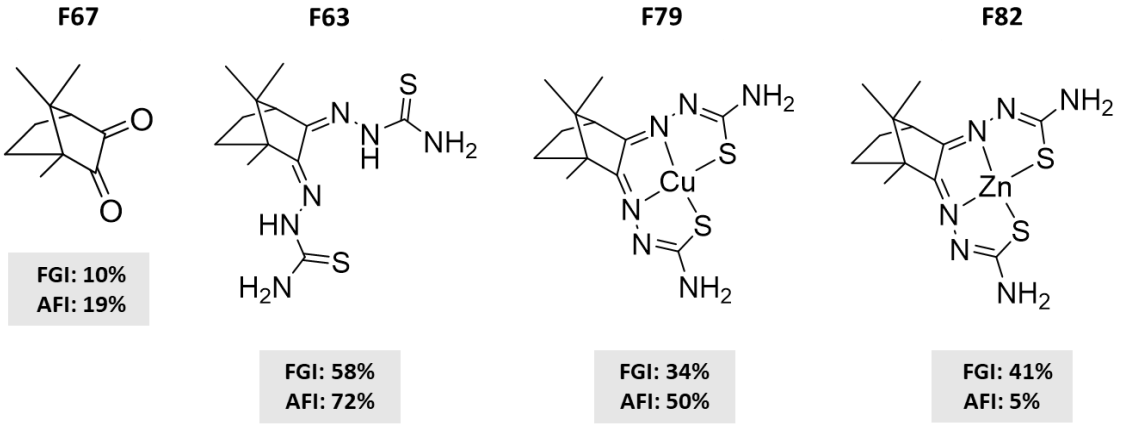


Figure 53: Schematic representation of camphorquinone derivatives at 50 µM.

3.9.1. Effect on *A. flavus* growth and aflatoxins accumulation

Camphorquinone derivatives have been evaluated for their ability to inhibit the *A. flavus* growth and the aflatoxin accumulation ***(Figure 53)***. The camphorquinone-bisTS (**F63**) inhibited the fungal growth more than raw material (F67) (58 vs 10% inhibition). Both Zn(II) and Cu(II) complexes showed an increased activity (34% and 41% respectively). However, the very low inhibitory potential against aflatoxin production of **F67** (19%) implemented when compound was provided with two thiosemicarbazide (**F63**) (72%). In this case, the complexation with the two metallic nuclei decreased the anti-aflatoxigenic activity (50 and 5% for Zn and Cu respectively). Considering the above discussed results, only **F63** could be proposed as anti-mycotoxigenic and fungicide compound.

3.10. Thiosemicarbazones derivatives with long hydrophobic chain

Aflatoxins are produced by a sequence of almost 15 enzymatic reactions, down-stream a complex system of cyclization and oxidations which lead to the formation of the poliketide, the first intermediate of the pathway. The enzyme involved in this process belongs to the class of polyketide synthases (PKSs), and catalyzes a specific cycle of the polyketide chain extension and/or β-keto processing *(Wilkinson C. J. et al. 2001).* Palmitic acid is the precursor of polyketide biosynthesis: is a C12 aliphatic chain with a terminal carboxylic acid function, produced by the first step of the fatty acid synthesis and used as precursor for longer fatty acids; once the acyl starter unit is selected, it is transferred to an acyl-carrier protein (ACP) and then to pksA, that represents the first enzyme of the aflatoxin biosynthesis pathway. Is has been demonstrated that different starters from alien sources could compete in the pksA palmitate-binding site for the specific precursor, triggering the synthesis of structural variations of aflatoxins and/or the blockage of the entire pathway *(Yolande, A. et al. 2009)*.

In this context, our Project partners designed a series of TSs with different aliphatic chain length, from 7 to 11 carbon atoms. The use of long aliphatic groups with these specific lengths was aimed at attempt to mimic palmitate and effectively inhibiting pksA enzymatic activity. All the thiosemicarbazones were obtained from the corresponding natural aldehydes, namely heptanal (**F37**), octanal (**F102**), nonanal (**F38**), decanal (**F103**) and undecanal (**F39**) condensed with thiosemicarbazide ***(Figure 54)***. Additional derivatives of these compounds were also synthesized to highlight more detailed structure-activity correlation.

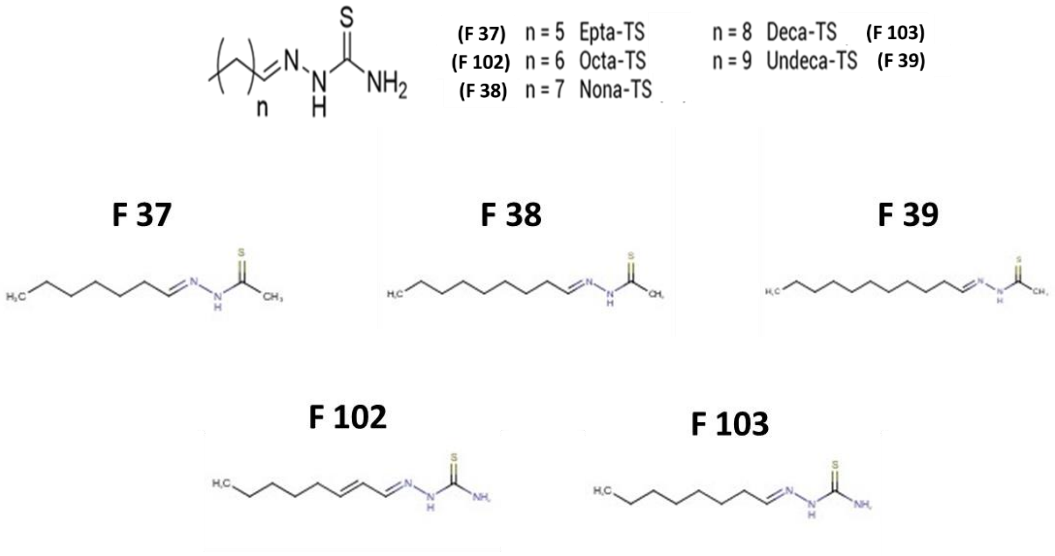


Figure 54: List of components of long hydrophobic chain thiosemicarbazones derivatives.

3.10.1. Effect on *A. flavus* growth, aflatoxin accumulation and sclerotia biogenesis

All compounds belonging to the long hydrophobic chain TS derivatives were tested on the fungus growth, aflatoxin production and sclerotia biogenesis. Also in this case, data were reported as percentage of inhibition ***(Figure 55)***. The analysis of the effect on fungal growth showed that the TS-derivatives **F102, F38, F103** and **F39** at 50 µM significantly inhibited the germination of conidia; **F37** was the less effective in limit the mycelium development, determining only the 42% of inhibition. As expected **F39** had a strong inhibition activity on aflatoxin accumulation (100%); also **F38** was reported to induce a noticeable effect (89%), probably due to its high fungistatic activity. Contrariwise, **F37** did not reduced at all the aflatoxin accumulation (4%) even in possess of a moderate effect on *A. flavus* growth (42%); this suggests that the growing hyphae produce much more aflatoxins if compared to the control. **F102** and **F103** repressed conidia germination (77% and 88%) but the same level of inhibition was not transferred on the aflatoxin production (31% and 62%). Even if at a lower rate, **F102** and **F103** shared the same activity of **F37**.
Results on sclerotia biogenesis showed that while **F37** reached in halving their number, all other molecules did not affect in any way the formation of these structures, demonstrating that the effect of TSs was heavier on the fungus growth than on specific pathways of secondary metabolism.

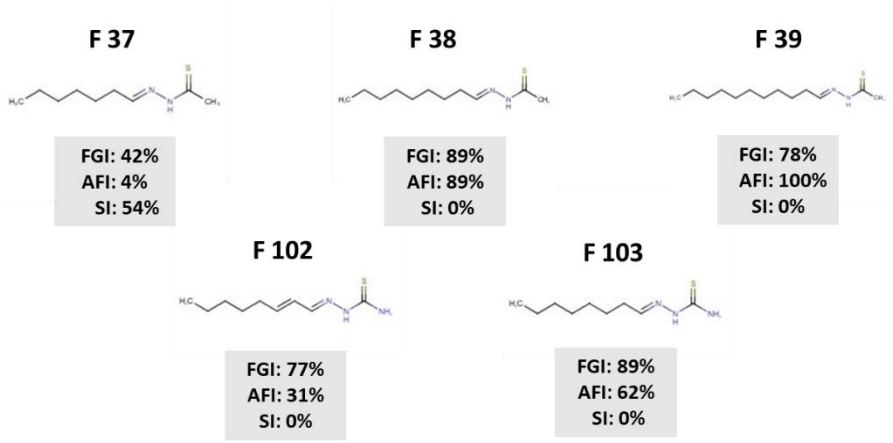


Figure 55: Effects of the long hydrophobic chain TSs on A. flavus at 50 µM. *Effects on fungal growth, aflatoxin production and sclerotia biogenesis. The data were plotted according to the carbon chain length.*

The effect on aflatoxin accumulation of long hydrophobic chain thiosemicarbazones derivatives seemed directly proportional to the chain length: longer is the carbon chain greater is the inhibitory activity.

3.10.2. Metal complexes and modification

Once identified an interesting structure-activity correlation between the length of carbon chain and the anti-aflatoxigenic effect for Hepta-TS **(F37)**, Nona-TS **(F38)** and Undeca-TS **(F39)**, we decided to synthesize and test the consistent Cu(II) complexes **(F41 F42 F45),** in order to evaluate a possible synergy with the presence of a redox metal center (***Figure 56***).

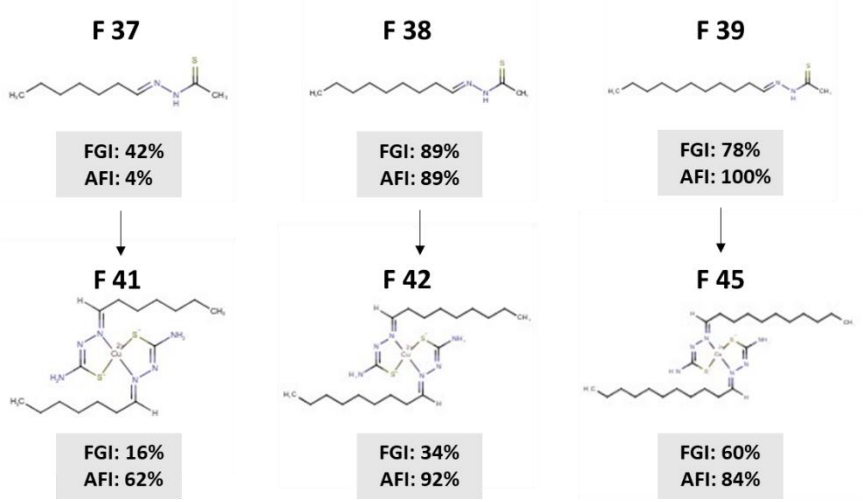


Figure 56: List of the long hydrophobic chain TS copper (II) complexes and its effects on fungal growth and on aflatoxin accumulation compared to free ligand on A. flavus at 50 µM.

Obtained results were quite conflicting with some previous findings: as reported in Figure 47, the Cu(II) nucleus resulted in a fungistatic effect of **F41 F42 F45** lower than the relevant free ligand **(F37 F38 F39)**. Aflatoxins accumulation assay demonstrated that the addition of copper did not enhanced the anti-mycotoxigenic activity of Nona-TS, as observed, instead, for Epta-TS (from 4% to 62%). Finally, Cu(II) complexation in **F45** resulted in a slight loss of effect of a free ligand **F39,** which was able to completely prevent AF production (84 vs 100%)**.** It was thus acquired that, in this family of compounds, copper improved the performances of the free TSs synthesized only in the case of Epta-TS, and limited to anti-aflatoxigenic activity.

We also explored a modification of the TS-fragment structure: the addition of a methyle group on the N^2 position lead to obtain **F59** (**F38** methylated). This substitution has the effect of removing the acidic proton involved in the thione–thiol tautomerism, and then to significantly modify the molecule hydrophilicity and its ability to chelate metals. Then **F59** was reduced on aliphatic chain (**F55).** We wanted also evaluate two similar compounds starting for two different reagents: **F102** (Octanal-TS) and **F101** (Octenal-TS) ***(Figure 57).*** Both methylation (**F59)** and chain's reduction **(F55)**

determined a decrease **in F38** activities, mostly on aflatoxin accumulation ***(Figure 57)***. On the contrary, a significant aumentation of anti-aflatoxigenic effect of TS F102 was recorded due to the modification of **F101** aliphatic chain (cis-trans).

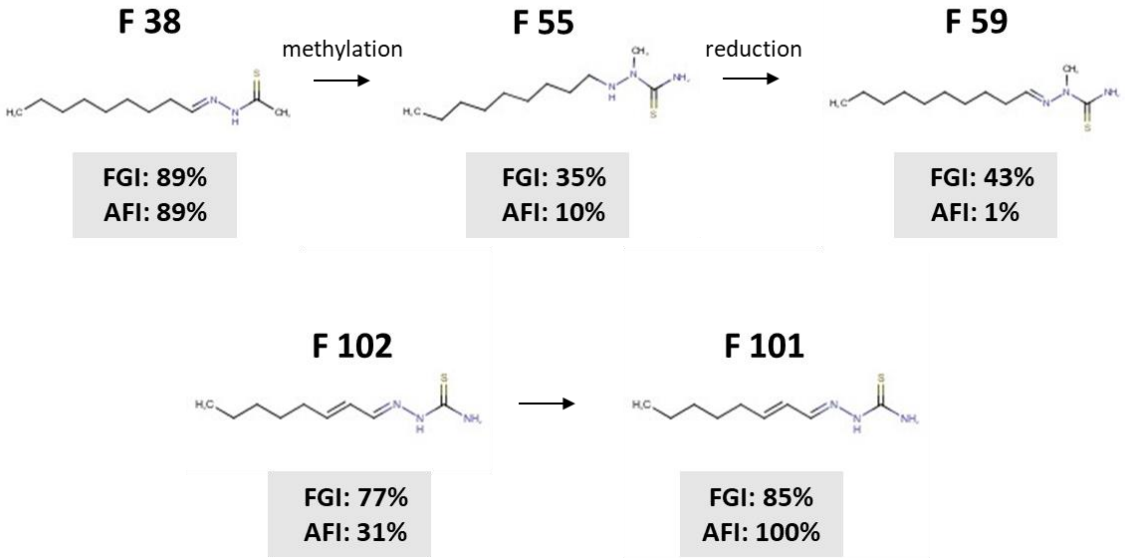


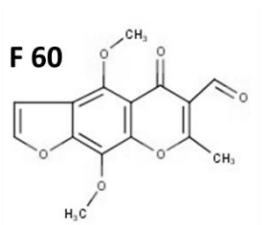
Figure 57: Modification of the long hydrophobic chain TS (F38 and F102) and its effects on fungal growth and aflatoxin accumulation on A. flavus at 50 µM.

3.11. Non effective derivatives

Not all the newly synthesized compounds (free ligands, modifications and metallic complexes) affected primary and/or secondary metabolisms of *Aspergillus flavus*. For these reasons, these compounds did not passed the second step of Aflatox® flowchart. Here we reported all these compounds along with their percentage of fungal growth (FGI) and (AFI) aflatoxin inhibition:

- **Kellinone derivatives**

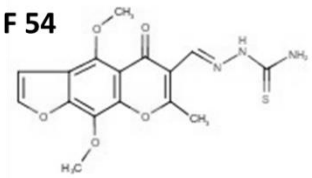
F 60



khellinone

FGI: 0%
AFI: 3%

F 54

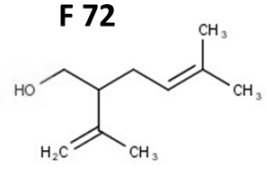


TS-khellinone

FGI: 5%
AFI: 17%

- **Lavandulol derivatives**

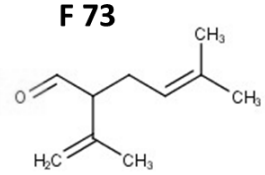
F 72



lavandulol

FGI: 29%
AFI: 26%

F 73

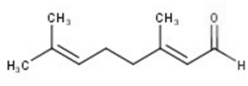


lavandulol

FGI: 34%
AFI: 0%

- **Citral derivatives**

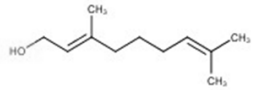
F 98



citronellal

FGI: 5%
AFI: 24%

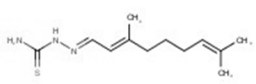
F 74



citral

FGI: 26%
AFI: 0%

F 75

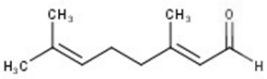


di-citral

FGI: 37%
AFI: 0%

- **Flavone derivatives**

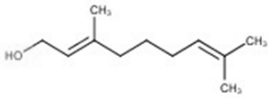
F 70



flavone

FGI: 83%
AFI: #%

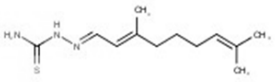
F 71



Flavone-TS

FGI: 100%
AFI: #%

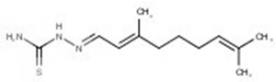
F 85



Zn complex flavone-TS

FGI: 26%
AFI: n.d.

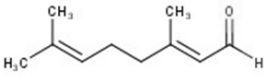
F 86



Cu complex flavone-ts

FGI: 65%
AFI: n.d.

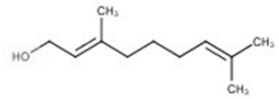
F 68



4',7-dihydroxy-flavone

FGI: 36%
AFI: 22%

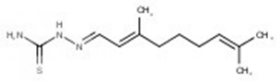
F 69



4',7-dihydroxy-flavone-TS

FGI: 26%
AFI: 15%

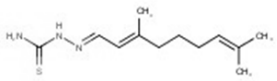
F 83



Zn complex 4',7-dihydroxy-flavone-TS

FGI: 19%
AFI: n.d.

F 84



Cu complex 4',7-dihydroxy-flavone-TS

FGI: 100%
AFI: 41%

4. The Aflatox® Q-SAR Database

The description of the structure (***Figure n°58***) and an overview (***Figure 59***) of the database are reported below wereas the extended description has been already reported in the PhD thesis of Marianna Pioli.

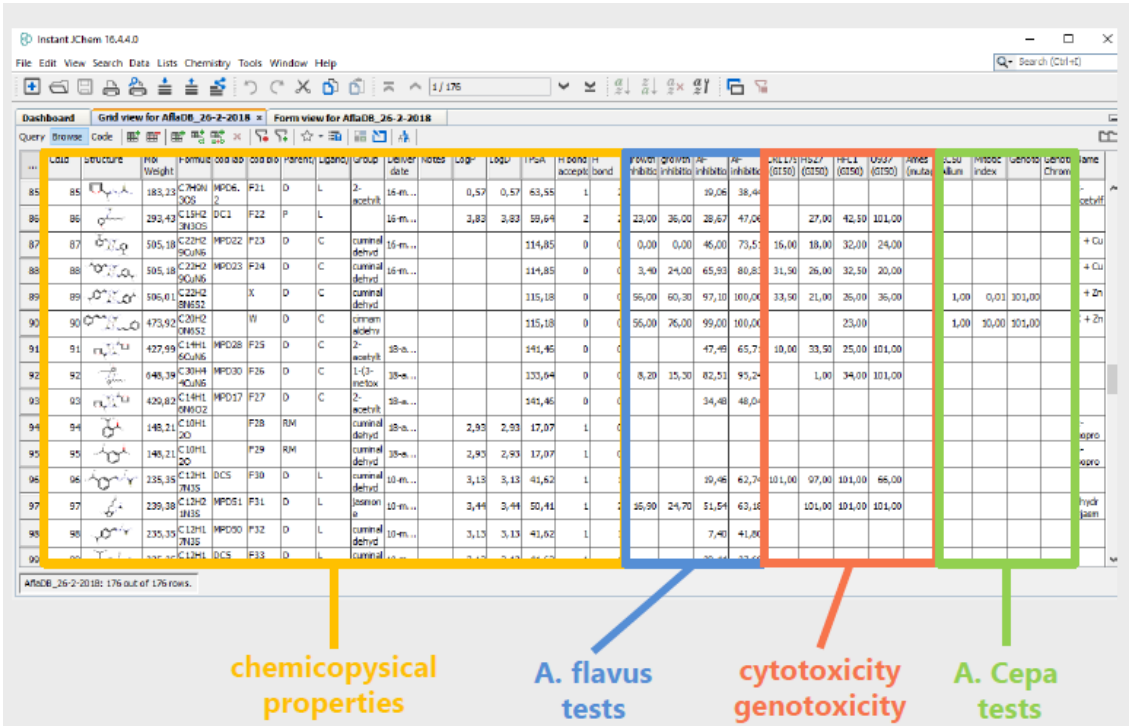


Figure 58: Database grid view.

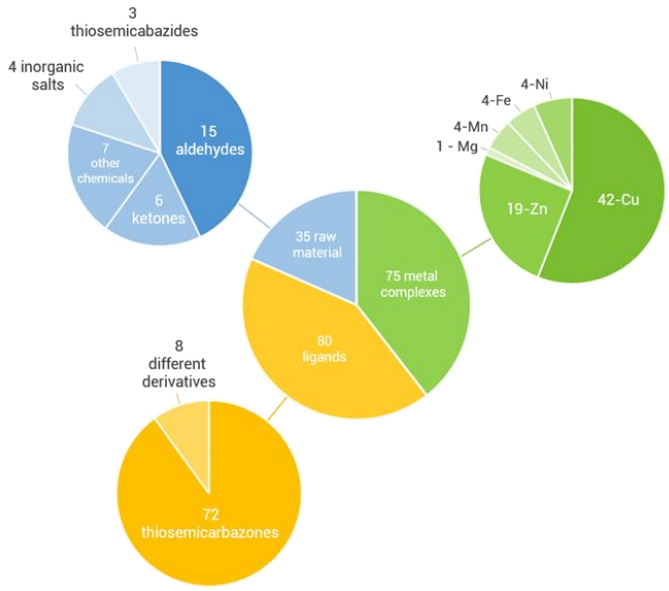


Figure 59 detailed description of the compounds collected in the Aflatox database.

5. Conclusions

The Topic discussed above describes the Aflatox® Project as it was conceived and carried out. The declared aim, that was essentially the synthesis and characterization of new compounds to be proposed for innovative strategies against aflatoxins contamination of cereals commodities, was pursued; significant “structural clues” have been provided by comparative scaffold-activity studies, underlining the importance of some chemical properties in the definition of the biological activity in A. flavus. Due to their cytotoxic and genotoxic effect, as assessed on pulmonary, epithelial and intestinal healthy cell lines (Hs27, Crl179 and HFL1), the 43 compounds that passed the fist step were reduced, since only 15 possessed an IG50 higher than the threshold value established (100 µM). Finally, 2-acetylthiophene (F20), 3-isopropylbenzaldehyde (Tio-Y) and cis-jasmone (F36) were the only three compounds that overcame all the project’s phases: these molecules, that showed an aflatoxin inhibition higher than 75% and a fungal growth inhibition lower that 25%, proved to do not possess cytotoxic or genotoxic effects neither on humal cells nor in A. cepa tests. Data thus suggests how they could be considered good candidates for the finalities of our project.

As well as this principal goal, a deeper analyses of those molecules found to possess some interesting biological activity were conducted; among all tested compounds, only 7 showed high anti-aflatoxigenic potential but low fungistatic effect: these molecules can be useful in further analyses aimed to understand the regulatory mechanisms of mycotoxins metabolism in toxigenic fungi. Therefore, compounds able to selectively inhibit the aflatoxin production and/or sclerotia biogenesis in A. flavus cultures were investigated, obtaining, in some cases, evidences about their cellular/molecular target.

In cosideration of what presented and argued, we believe that the research on TSs potentialities cannot be considered exhausted, but, on the contrary, this class of molecules may have in store for us new unexpected properties to be discovered and exploited in the future.

TOPIC 2:

The DIFESAMAIS® Project

Control of aflatoxin production by biocompetition

The DIFESAMAIS Project®

As already mentioned in the Introduction, aflatoxin B is the most toxic and carcinogenic mycotoxin that could contaminate food and feed commodities, but any impact in terms of grain production has been detected. Corn contamination by aflatoxins is one of the main issue that farmers of large areas of Northern Italy have to cope with. The relevant problem is that consumers could be exposed to the toxin both directly and by consuming foods of animal origin: for this reason, the concentration of such toxins in food for human and animal use is strictly regulated by national authorities. To date, different strategies have been implemented to try to reduce, if not eliminate, toxin contamination at the pre-harvest level to limit exposure to aflatoxins in both animals and consumers. However, a careful analysis of the costs/efficacy of each of the various inputs must be performed in order to provide an acceptable income for the farmers. On the other hand, there is an increasing market demand for food and feed commodities obtained with environmentally low-impact practices (organic farming) possibly through the experimentation of innovative and organic defense techniques. At present, it is largely accepted that minimization of mycotoxin risk requires a multifactorial approach since climatic, agronomic factors are found to interact each other in complex, local ecological realities.

The “DIFESA MAIS” Project, funded by the Emilia Romagna Agricultural Development Plan (PSR 2014-2020), was the second project I was involved in during my PhD. “DIFESA MAIS” intends to design and validate an innovative combined approach addressed to the development and the valorization of organic farming/integrated production of corn, with particular attention to the maintenance of biodiversity. It proposed an environmentally sustainable approach with low environmental impact and application costs, which main goals were: i) the development, validation and comparative analysis of the technologies for monitoring and controlling insect/larvae infestations; ii) the individuation of sustainable procedures to reduce aflatoxin contamination; iii) the development of a synergy of agricultural actions aimed at containing aflatoxin contamination in maize crops. The expected output of this project was 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. Advantages are expected on all steps of corn productive chain, improving its quality, while positive effects are expected on the conservation of biodiversity (for example controlling the evolution of microbial and insects population avoiding the use of phytochemicals). Beneficial effects on consumer’s health are also expected. The project was three years: The first year, analysis and study of the various agronomic practices and *in vitro*

validation of the various strategies to be adopted. In the second and third years, agronomic practices and containment strategies for aflatoxin have been validated in experimental fields. Both academic (University of Parma and University of Bologna) and industrial partners (PROGEO S.C.A. and AGRITES s.r.l.) were involved in the present Project. University of Bologna and PROGEO company partners were involved in the implementation of good agronomic and cultural practices, aimed at preventing plant stress (for example by mulching, irrigation, fertilization, crop rotation) together with innovative insect control procedures relying on the administration of a natural insect specific toxin (*Bacillus thurigiensis* toxin (Bt)) and the use of an interspecific bicompetitor (*Trichogramma brassicae*) to prevent insect (*Ostrinia nubilalis*) deterioration of grains, that, in turn, promotes fungal infection and aflatoxin contamination. The "DIFESAMAIS" Project may be described as the implementation of the above reported procedures individually validated, and of their interaction according to a multifactorial approach, in order to design and providing to farmers a "package" of procedures to be used to reduce aflatoxin contamination

Our main contribution to the Project relies on the characterization of an intraspecific (*Aspergillus flavus*) bio-competitor strain and the development of an intraspecific bio-competitive strategy to reduce aflatoxin contamination on maize crops. This approach, based on the application on the relevant crop fields of an atoxigenic *Aspergillus flavus* strain, has already been successfully used both in maize and cotton cultivations worldwide (*Degola et al. 2011; Accinelli et al. 2014; Lyna et al. 2009; Weaver et al. 2015*). For this purpose, a natural strain of *A. flavus* belonging to the fungal population colonizing the fields of the Po Valley was used *(Degola et al. 2011; Degola et al. 2012)*.

1. BACKGROUND

1.1. Modern agriculture: problems and solutions

In recent decades, we saw the evolution of classical agriculture in a modern form, through the introduction of new cultivation techniques, a growing use of mechanization and agronomic inputs, and the strong diffusion of crop rotations, up to monoculture. However, while the adoption of the new production system was perfectly in line with market demands, on the other it raised concerns regarding long-term environmental sustainability. The intensification of production and the use of chemical inputs have led to disastrous environmental consequences. Consequences such as the loss of intrinsic field fertility and its erosion, the reduction and simplification of biodiversity in the agroecosystem, as well as the pollution of aquifers and the phenomena of eutrophication of fresh and salty water *(Vasileiadis et al. 2011)*. Moreover, the widespread use of synthetic pesticides has led over time to the development of organisms resistant to the main active ingredients used, given the high selective pressure to which they have been subjected, creating the need to periodically find new formulations to bypass the adaptation of harmful organisms *(Meissle et al. 2010)*. In response to the need to review the agricultural production system, since 2007 the "ENDURE" (European Network for the Durable Exploitation of Crop Protection Strategies), a commission composed of more than 300 European researchers, has set itself the goal of identifying and define crop protection strategies that depend as little as possible on pesticide use (http://www.endure-network.eu). In 2009, the European Parliament issued Directive 2009/128/EC on the sustainable use of pesticides, in view of the implementation of IPM (Integrated Pest Management), which became mandatory within the European Union in 2014. This Directive says: "Careful consideration of all the methods available for crop protection and the consequent integration with appropriate measures aimed at avoiding the development of populations of harmful organisms and at the same time maintaining the use of pesticides below economically and ecologically justifiable levels, minimizing risks to human health and the environment. The IPM aims to grow healthy crops by minimizing the alteration of the agro-ecosystem and stimulating biological control mechanisms." (Directive 2009/128 / EC, art. 3) ***(Figure 60)***.

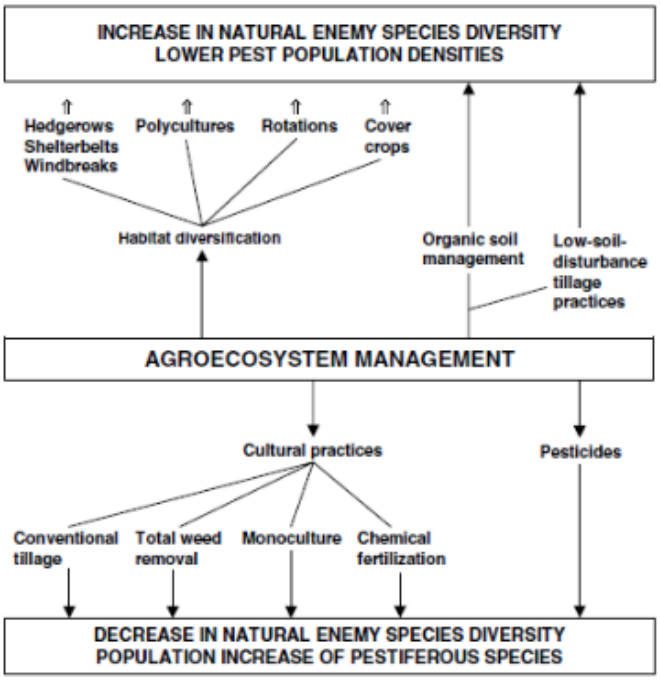


Figure 60: Effect of management of cultural practices on the biodiversity of natural limiters and the abundance of harmful pests *(Altieri and Nicholls 1998).*

The harmful organisms that usually attack the corn benefited by the continuous presence in the field of the same plant species. The temporal continuity and the possibility of wintering in the crop residues allow the harmful organisms to maintain a stable source of inoculation between one season and another and to develop in the absence of natural limiters, given the simplicity of agro-ecosystems. Furthermore, the control of phytophagous that are most damaging to maize, such as the *Ostrinia nubilalis*, the *Diabrotica virgifera* and several species of elateridae (*Agriotes spp.*) is particularly complicated due to the cryptic nature of these insects. The larvae develop inside the plant (stem, inflorescences) or at the level of the root system, limiting the effectiveness of insecticides by contact to short phenological phases or making the use of systemic insecticides unavoidable. The adoption of methods to monitor populations of pests damaging for maize and specific phenological models, together with the identification of economic damage thresholds would allow a rational use of insecticides and a more effective use of biological control techniques, according to IPM guidelines. The reintroduction of crop rotations, the search for cultivars resistant to harmful and competitive organisms against weeds, and the choice of varieties suitable for different cultivation areas are some of the measures aimed at reducing production losses and obtaining a product of quality, both for human and animal consumption *(Vasileiadis et al. 2011)*.

2. Main lepidoptera harmful to maize in Italy

The presence of a high variety of lepidoptera such as *Sesamia cretica* (Lederer) and *Ostrinia nubilalis* (Hübner) in maize fields negatively affects the quality of the grain ***(Figure 61)***. The grain of corn, as well as the entire plant, after be attacked by these species of lepidoptera is more susceptible to the attack of filamentous fungi such as *Aspergillus flavus*. A grain of corn no longer intact, or the stem of the perforated plant, allow the fungus to colonize them. The spore can attach more easily to the surface of the plant or the corn grain. The spore can to recover more easily nutrients and water and under favorable condition, such as humidity 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 environment colonizing surrounding crops and plants. An excessive proliferation of toxigenic fungi could therefore lead to a high amount of aflatoxin produced above the limits allowed by law. Therefore, an indirect method of preventing aflatoxin contamination is to monitor and reduce the presence of this *lepidoptera spp*.

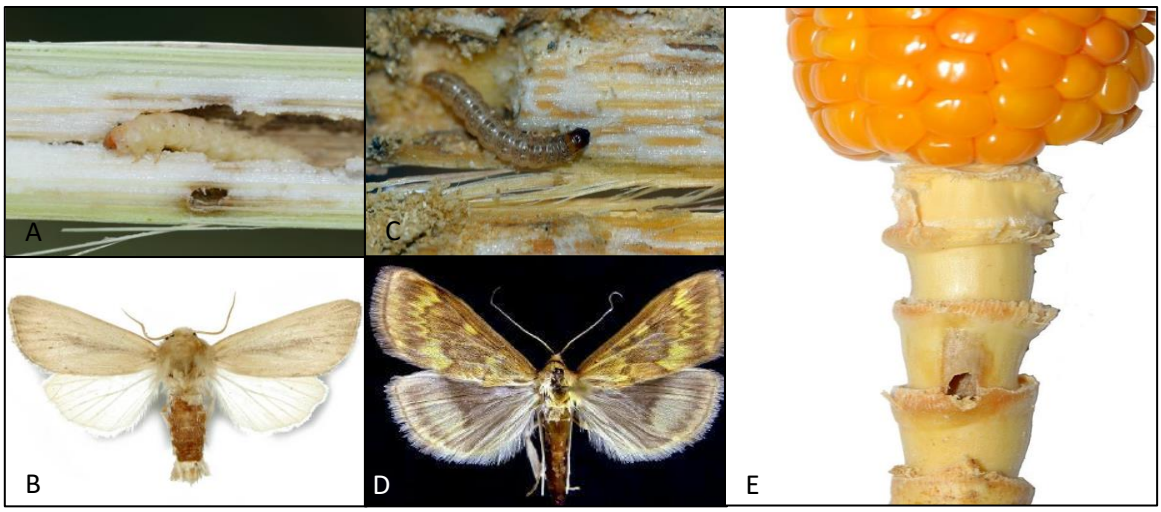


Figure 61: *Larva (**A**) and adult (**B**) of Sesamia cretica; Larva (**C**) and adult (**D**) of Ostrinia nubilalis; Typical holes in the peduncle of the corncob (**E**).*

2.1. Impact of borers and biological control techniques

Sesamia cretica and *Ostrinia nubilalis* are widely distributed in Mediterranean areas. In Italy, it is prevalent mainly in the central-southern regions, but in recent years, the progressive increase of temperatures allowed to spread also in the northern regions. The first damages are on the leaf, and

with the unfolding of the leaves, we can see a series of linear and overlapping holes. Later, the larvae penetrate in the stem and in the axis of the male inflorescence, leaving at the entrance of the holes the typical gnawed resulting from the penetration activity. The numerous tunnels in the stem destabilize the plants and the vascular system, causing a grain yield reduction up to 15-20%. The larvae of the second generation preferentially attack the maize ears, penetrating from the peduncle, dig tunnels in the cob, nourishing the caryopses from the inside and generating sites of infection for mycotoxigenic saprophytic fungi such as *Fusarium* and *Aspergillus* spp. (Pollini 2013).

In *2005, Alma et al*. showed a positive correlation between the number of corn spike tunnels and fumonisins contamination, underlining the importance of increasing the efficacy of treatments against the *O. nubilalis*. However, the simple increase in applications would hardly be economically sustainable and could lead to a decrease in *O. nubilalis* competitors and to the increase of other phytophagous ones *(Alma et al. 2008)*. In particular, the use of pyrethroids such as permethrin can cause mite-onset phenomena from *Tetranychus urticae Koch (Higley et al. 1996)*. The choice of insecticides with low environmental impact and selective for useful insects is therefore a fundamental factor to limit the key phytophagous causing the slightest alteration to the trophic balance of the agro-ecosystem. Defined by DeBach (1964) as the action of parasitoids, predators and pathogens in maintaining the population density of another organism at lower levels compared to their absence. From the application point of view, it consists in the use of natural enemies to reduce the damage caused by harmful organisms and bring it below the tolerance threshold. Regarding the limitation of lepidopterans that are harmful to maize, in particular *O. nubilalis*, the main strategies of biological control concern the use of tolerant maize cultivars and pheromones for confusion and sexual distraction, the application of *Bacillus thuringiensis* (Berliner) or the use of Bt-maize and the launch of oophagous parasitoids such as *Trichogramma spp*. *(Razinger et al. 2015)*.

2.2. *Bacillus thuringiensis*: mode of action and biotechnological application

Gram + sporogenic entomopathogenic bacterium, which, during the stationary phase of its biological cycle, forms parasporal crystals. These protein crystals are composed of different endotoxins, encoded by specific genes (Cry) that determine their variability and, consequently, the different orders of insects for which they are toxic. The first toxins detected mainly affected the lepidopterans, but with further studies and the use of plasmids for the transfer of genetic material, genes coding for toxins active also on beetles, dipterans and nematodes have been identified. The

intense selectivity that characterizes the Bt makes it a particularly suitable product for biological control, preserving the natural enemies already present in the agro-ecosystem and facilitating the management of acquired genetic resistance. In this case, the formulation that we used in sperimental field was Turex®. This product contained 46.2% of spores of two different *Bacillus thuringiensis spp.* (25% of *B. thuringiensis kurstaki* and 25% of *B. thuringiensis aizawai*) and 3.8 % of Δ-endotoxin, and medium. The toxicity of Bt occurs after the ingestion of protein crystals by the target insect, usually in larval stage. These are solubilized during digestion, releasing the active toxin (Cry) that binds to receptors of the peritrophic membrane of the mesenteron and performs a neurotoxic action, causing paralysis of the intestinal musculature and of buccal apparatus. The insect cannot feed and do damage. The Bt shows most of the Cry genes coding for endotoxins inside the plasmids, making it particularly suitable for the use of the same plasmids for the transfer of genetic material. In 1996, the first cultivars containing Cry genes modified for the production of toxins in plant tissues were placed on the market: being produced continuously, the proteotoxins exert a protective action against the target insects, reducing the number of necessary interventions and thus determining a savings in terms of costs and environmental pollution. Nevertheless, further studies have shown the possibility that target insects develop an increasing tolerance to the toxin when subjected to continuous exposure, pointing out the inescapability of using different fighting and prevention techniques in a synergistic manner *(Schnepf et al. 1998)*.

2.3. *Trichogramma brassicae*: Employment in agriculture

The *Trichogrammatidae* family includes 89 genera and more than 800 species distributed throughout the world. They are primary endoparasitoids (solitary or gregarious) of insect eggs, with a range of hosts that includes the orders *Hemiptera, Coleoptera and Lepidoptera*. The genus *Trichogramma*, with over 200 species described is the most representative genus within the family thanks to its widespread use as a biocontrol agent of harmful pests. The species belonging to this genus have proved to be particularly suitable for mass breeding due to flooding in agriculture, with the possibility of using eggs of *Ephestia kuehniella* (Zeller) as a breeding substrate. *T. brassicae* is one of the species bred in biofabric and widely used in agriculture for the control of harmful lepidopterans, in particular *O. nubilalis* and *Helicoverpa armigera* (Hübner), on corn, tomato (*Solanum lycopersicum L*.) and pepper. After mating, the female lays its eggs inside eggs of

Lepidoptera: the embryonic development is very rapid and, once shelled, the larva of *T. brassicae* feeds on the larva of pepidoptera inside the egg. At maturity, the larva grows inside the egg and emerges as an adult. Mating occurs shortly after flicker, and the female can go on to parasitize up to 50 eggs before dying. From the fertilized eggs both male and female individuals are formed, while in the case of failed fertilization it has only the peeling of males (*parthenogenesis arrenotoca*). The duration of development from egg to adult varies from 7 to 20 days depending on temperature and diet, a factor that also influences the size of adults to flicker and their fertility (BCPC 2004). In several cases the effectiveness of flood throwing with *T. brassicae* for the control of *O. nubilalis* and the reduction of damage on maize crops has been reported. The dispersion of *T. brassicae* in the field is carried out with the launch of cellulose spheres containing parasitized *E. kuehniella* eggs, with a density of 12,000 - 500,000 eggs / ha. In recent years, with the implementation of precision technologies in agriculture, the possibility of launching *T. brassicae* using drones equipped with a tank, a timed dispenser and a GPS system for georeferencing (BCPC 2004) was born.

3. *Aspergillus flavus* as intraspecific bio-competitor (BC)

Several approaches have been identified to control aflatoxin in crops. In addition to agronomic techniques for the control of aflatoxin contamination, different species of bacteria, yeasts and fungi have been evaluated for their possible biocompetition activity *(Dorner 2004; Yin et al. 2008)*. Biocontrol by competitive inhibition using *Aspergillus flavus* atoxigenic strains has been shown to be an effective method to control the production of aflatoxin in peanuts, corn and cottonseed. Successful examples have been reported in this field. In particular, the use of *A. flavus* (afla-) strains to reduce aflatoxin contamination is well documented in cotton seeds *(Cotty, 1994)* and the related *A. flavus* strain used for this purpose (AF36) were recorded in the United States by the EPA *(Dorner, 2004)*. While many atoxigenic strains are effective in reducing in vitro contamination, field effectiveness can be influenced by various environmental conditions *(Cotty and Mellon, 2006)*. The natural populations of atoxigenic strains are considered reservoirs from which to select the strongest biocompetitors. The mechanisms by which the afla strains interfere with the accumulation of aflatoxin have not yet been definitively established. The prevailing opinion is that it depends on the competitive exclusion of the aflatoxin producer (afla+) strains from the substrate as a result of a physical shift (of success) and of the competition for nutrients by the afla strains. However, several hypotheses can still be considered *(Mehl and Cotty, 2010)*. Controversial evidence has been reported of the existence of "diffusible factors" released by the afla competitor strain *(Cotty and Bayman, 1993; Horn et al., 2000)*. Degola and co-workers, in the first decade of 2000, isolated from field of Torino a *Aspergillus flavus* atoxigenic strain such an excellent bio-competitor. In *Degola 2011,* they had answered some questions such as: 1) does the injection time of each strain offer a competitive advantage to both strains? 2) What is the lowest inoculation ratio between the two strains that is still effective in containing toxins? 3) Does the concentration of spores in the initial inoculum affect the effectiveness of the competition? 4) Do the factors mentioned above, and others not yet discovered, synergistically interact in the mycotoxin accumulation process? The selection of biocontrol strains is not easy, as it is difficult to assess suitability for the activity without costly field tests. Reconstruction experiments have generally been conducted under laboratory conditions to study the biological mechanisms underlying the efficacy of atoxigenic strains in preventing aflatoxin production and / or to provide a preliminary indication of the performance of the strains when released in the field.

3.1. How was the bio-competitor strain discovered?

In 2011, Degola and co-workers conducted a study to develop a procedure to analyze a natural population of *A. flavus* isolated from corn grain. The aim was to identify atoxigenic strains that could be used as bio-competitors to deal with the problem of aflatoxin contamination of the cornfields of our region. To evaluate the efficacy of various atoxigenic strains in reducing mycotoxin concentration when co-inoculated with aflatoxin positive strains, they used a simple fluorescence method for quantitatively estimating the level of aflatoxin in solid culture medium. The use of a microplates to speed up the process of evaluating high numbers of pairings of afla–/afla+ strains. It was an economic test because it uses small volumes (200 µl/well) and was relatively fast. In fact, four days of incubation at 28 °C were sufficient to detect aflatoxin production even with a "slow" mycotoxin producer (*Degola et al., 2009*). The test procedure wasreported in the materials and methods section. The very fact that these particular atoxigenic strains were isolated, should be an indication of their ability to persist together with aflatoxigenic strains in the relevant crop environment. This is, at least, one of the features requested (of afla– strains) for efficient biocontrol of aflatoxin contamination. On the other hand, the producers of aflatoxins that have been isolated on the same screen are the most likely competitors against which the afla strains, used as biocompetitors, must fight for the employment of ecological niche *(Brown et al., 1991)*. They found that the times of inoculation of afla as well as the ratio of inoculation between the afla and afla+ strains are crucial parameters to be taken into consideration with regard to the effectiveness of the competition. The interaction between inoculum concentration and afla-/afla+ ratio inoculation on aflatoxin production was evaluated. Several afla- / afla+ ratios were tested (1: 1 1: 2 and 1: 4). The data reported in Degola 2011 confirmed that by reducing the number of spores of the bio-competitor strain, the amount of aflatoxin produced by the afla + strain increases. A preliminary assessment of the competitive capacity of the different strains can be performed in vitro and the relative importance of the different factors that could interact with the mechanisms influencing aflatoxin production during coinfection can be evaluated under laboratory conditions. However, the efficacy of the bio-competitor depends on several factors such as the association with the culture, temperature, humidity and stress resistance, intraspecific and interspecific competition with other fungi or microorganisms. For this reason, the effectiveness of a strain identified as a bio-competitor must be evaluated in the field. Large-scale studies and annual repetitions are therefore needed to evaluate the effectiveness of the bio-competition strategy in the field *(Dorner, 2009)*.

4. Preliminary assessments

Many years ago, Degola and co-workers detected the best atoxigenic *A.flavus* bio-competitor strain (BC). In vitro, this strain was extremely effective in reducing the aflatoxin accumulationfrom many afla+ strains. This BC has been used in the DIFESAMAIS project to understand if it was extremely effective even in the field because in the field there are many variables that cannot be controlled with in vitro experiments. (Humidity, temperature, other microorganisms, number of spores).

The final scope of this project was to evaluate if:

1) The ratio of the fungal population (afla-/afla +) resident changed after the treatment;
2) The administration of the BC was effective on aflatoxin accumulation compared to the control;
3) This new methodology adopted was more effective than the chemical treatment with Coragen®;
4) The synergism of the three biological treatments lowered more the aflatoxin accumulation;
5) The persistence of the BC in the field one month after treatment;
6) The abundance of the BC one month after treatment was proportional to the reduction of aflatoxin;

4.1. Co- treatment with formulation Turex®

Among the various strategies, the DIFESAMAIS Project proposed to treat a parcel of land by exploiting the synergism of two microorganisms: the BC, *and Bacillus thuringiensis* and its Δ-endotoxin. The hypothesis predicted that synergism could significantly reduce the finding of aflatoxin B_1 in the grain. For this reason, in vitro we wanted to test if this co-treatment was irrelevant or penalizing for the BC. A 96 multi-well plate was set up in which $5*10^2$ spores were inoculated in YES5% medium in the presence of Turex® but also in the presence of only Δ-endotoxin at concentrations of 50 µg and 100 µg for a final volume of 200 µL. The plate was incubated at 28°C for 48 hours. Below, in ***Figure 62***, was reported the fungus growth at 48 hours. The fungus growth was slightly delayed in the presence of Turex® formulation (*Bacillus thuringiensis* and its Δ-endotoxin). Instead, only Δ-endotoxin did not seem to interfere of fungus growth.

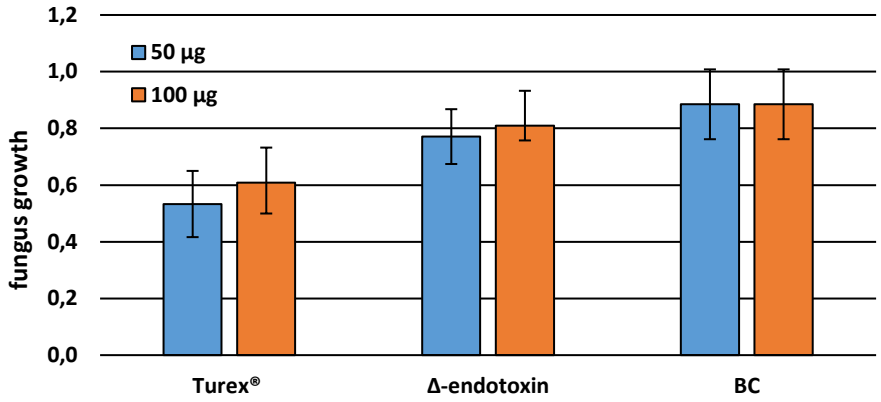


Figure 62: Fungus growth in presence of Turex® formulation. *The fungus growth was performed in presence of formulation of Turex® (B. thuringiensis and its Δ-endotoxin), of only Δ-endotoxin and BC alone adding H_20 as control. Data are reported as increase of optical density OD.*

A bio-competition test was also conducted between the BC and the Turex® formulation on YES5% solid synthetic medium in a Petri dish. The Turex® has been added in two different ways. In the first test, the Turex® was added to the medium while the *Aspergillus flavus* strain was spotted after that the medium was solid. Also in this case, the Turex® was tested at two different concentrations: 50µg / mL and 100µg / mL. Three spots (10 µL of 10^4 spores/mL) of BC were then inoculated. In the second test TUREX was spotted at the same way with bio-competitor strain. Also in this case, two Turex® concentrations were tested. The same tests were performed testing the co-treatment between bio-competitor and Δ-endotoxin alone. In ***Figure 63*** were reported the bio-competitions in Petri dish.

Figure 63: The Turex® formulation has been spotted with spores of BC (A). The Turex®formulation has been added *to the culture medium* ***(B)****. Only Δ-endotoxin has been spotted with spores of BC* ***(C)****. Only the Δ-endotoxin has been added to the culture medium* ***(D).***

A test on food matrix was also performed treating the maize kernels with the BC and the Turex® formulation in the Petri dish. Maize was previously sterilized. For each plate 30 g of corn were added, corresponding to about 90-92 seeds. Also in this case the Turex® was added at two different concentrations: 100 µg/mL and 1000 µg mL. The maize kernels were washed in 18 mL of distilled H_20 and 2 mL of Turex® formulation. 360 µL of the spores suspension (10^6 spores/mL) of BC was added. The corn kernels were left in solution for 5'. Then they were dray and incubated in Petri dishes. They were left for 6 days at 28 °C.

The infected seeds were then counted and the infection rate calculated. In ***Figure 64*** was reported the data of this experiment. Δ-endotoxin did not seem to have any effect on fungus growth. For these reasons, we could to test this newly strategy in experimental field.

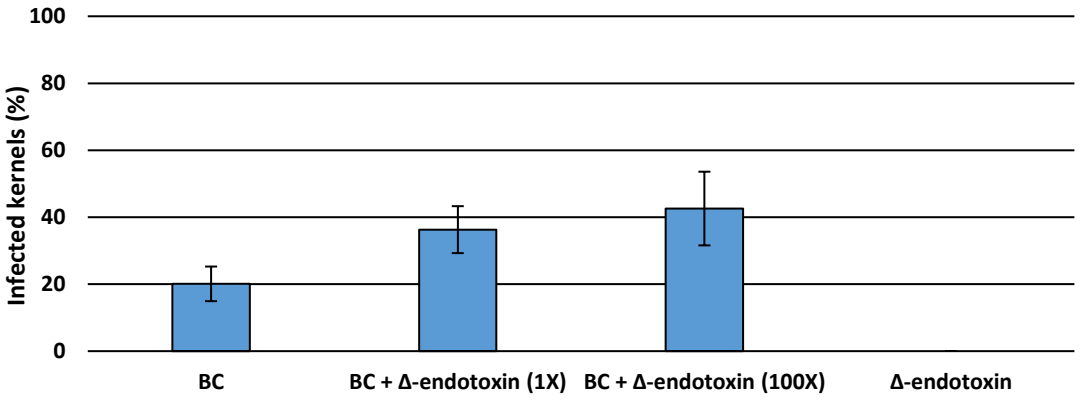


Figure 64: Percentage infection of corn kernels. *Δ-endotoxin did not seem to have any effect on fungal growth.*

5. Experimental field

The project lasted three years: The first year we analyzed and studied of the various agronomic practices to adopt in field to prevent the contamination of aflatoxin. We also validated in vitro various strategies to be adopted, such as bio-competition and other treatment using *Bacillus thuringiensis* and *Trichogramma brassicae*. In this year, we also tested the validity of co-treatment of *A. flavus* atoxigenic strain with the formulation of *Bacillus thuringiensis* and its Δendotoxin. In the second and third years, agronomic practices and containment strategies for aflatoxin have been validated in experimental fields. Each year two experimental field were treated. The experimental field were located in province of Bologna. The first company was "Marabini Aurelio" in Castel San Pietro Terme ***(Figure 65)***. The second company was "Cà Selvatica Società Agricola" in Crespellano. The area dedicated to experimentation represented a part of the total extension of the field (2.5 ha of 6 ha in total). The following year the treated fields were located in the same companies but changing the plot of land (adjacent land).

Figure 65: Experimental field. Crespellano *(44 ° 28'37.1 "N 11 ° 28'55.2" E)* ***(A)***. ***Castel S. Pietro Terme*** *(44 ° 28'37.1 "N 11 ° 28'55.2" E) (B). The experimental fields of season 2017 are indicated in blue. Instead, experimental fields of season 2018 in red.*

5.1. Microbiological analyses for the isolates characterization

Post-harvest analyses were conducted in order to isolate the population of *A. flavus* resident on the treated maize fields after bio-competitor administration. *Aspergillus* strains were isolated and molecularly characterized in order to evaluate the persistence of the relevant bio-competitor.

For each treatment were obtained 30 plates, for a total of 120 plates for thesis. More than 2000 strains of *A. flavus*, both Afla+ and Afla-, have been isolated as a result of the two years experimentation here reported. After that, each isolated strain was classified as afla- or afla- strains. In all treatments, afla- strains (between 70 and 80%) prevailed over toxigenic strains (20-30%). This afla+ prevalence was consistently observed in all four experimental field and in each of the two years of the project, with an exception: Only in the first year of experimental field, in both field, the ratio of the fungal population (afla-/afla+) resident after Coragen® was different respect all other treatments (afla- strains ~60% and afla+ strains ~40%). Below, in ***Figure 66***, was reported the data of microbiological analyses.

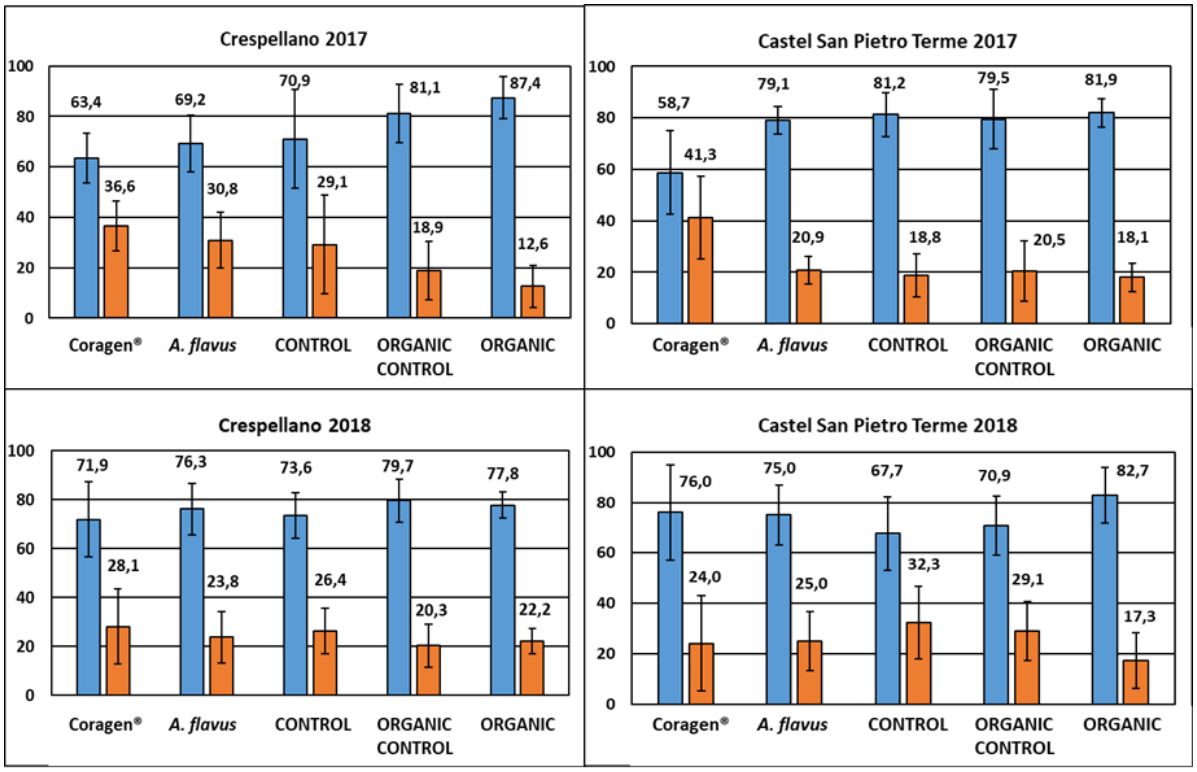


Figure 66: Af-/Af+ strains recovered from the treated maize fields. *A prevalence of non-toxigenic strains (about 70%) was detected in all treatments.* ***Five plots*** *corresponding to the relative strategies:* ***Chemical treatment*** *with Coragen®;* ***Biocompetitor treatment*** *using the conidial suspension of A. flavus biocompetitor strain (BC); In the* ***Control treatment*** *Absence of any chemical or biological treatment for the defense of maize;* ***Organic control*** *with only T. brassicae and Turex® formulation;* ***Organic treatment*** *with the conidial suspension of A. flavus biocompetitor (BC) in combination with T. brassicae and B. thuringiensis. Field scheme on page 172.*

5.2. Molecular characterization of *A. flavus* afla- population

Finally, the atoxigenic strains were genotyped by RAPD-PCR to identify and quantify BC presence in the afla- population. Few years ago, the DNA of the BC and of several afla- strains was analyzed and a unique RAPD-PCR profile was identified for the relevant BC. For this reason, this molecular technique was used to identify the persistence of BC after one mouth of treatment.

In ***Figure 67,*** the RAPD-PCR profile was shown. Sample reported in line n°1 showed the same amplification profile of the bio-competitor sample (BC), while in all the other lines the distribution of the bands was different.

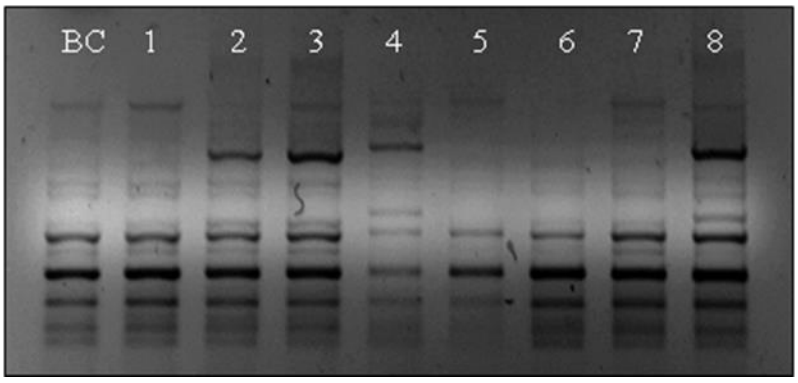


Figure 67: The RAPD-PCR profile. *The afla- strains were genotyped by RAPD-PCR to identify and quantify bio-competitor in all afla- population. Sample reported in line n°1 shows the same amplification profile of the biocompetitor (BC) sample, while in all the other lines the distribution of the bands is different.*

5.3. The presence of the biocompetitor strain in the field one month after treatment

To evaluate the presence of the BC in the field, one month after the relevant treatment, we analyzed the amount RAPD-PCR profiles of afla- and calculated the percentage of biocompetior stain. In ***Figure 68*** were reported the numbers of all strains that had a characteristic profile of BC.

In **Crespellano 2017,** 8 strains in BIOCOMPETITOR TREATMENT presented a characteristic profile of bio-competitor only in H replicate. In other three replicates (E-F and G) nothing has been found. No one afla- strain presented a RAPD biocompetitor profile in the CONTROL strategy. A different distribution of the BC was found in ORGANIC TREATMENT (21 in E; 15 in F; 3 in G and 0 in H).

In **Castel San Pietro Terme 2017** all replicates of BIOCOMPETITOR TREATMENT presented a large number of profiles identified such as BC (6 in A; 19 in B; 15 in C and 20 in D). In CONTROL strategy, 6 strains presented a characteristic profile of BC only in C replicate. Other three replicates (A-B and D) nothing has been found. These contaminations in control strategy were probably due to wind

and insects moving spores. In ORGANIC TREATMENT, instead, only 7 strains were identified in B replicate and only 4 strains in D replicate.

In **Crespellano 2018** all replicates of BIOCOMPETITOR TREATMENT presented a large number of profiles identified such as BC (16 in E; 8 in F; 12 in G and 20 in H). No one afla- strain presented a RAPD bio-competitor profile in the CONTROL strategy. In ORGANIC TREATMENT, instead, a different distribution of the BC was found: 12 in E; 12 in F; 20 in G and 20 in H.

In **Castel San Pietro Terme 2018** all replicates of BIOCOMPETITOR TREATMENT presented a large number of profiles identified such as BC (20 in A; 31 in B; 20 in C and 33 in D). No one afla- strain presented a RAPD biocompetitor profile in the CONTROL strategy. In ORGANIC TREATMENT, instead, 16 strains were identified in A replicate, 18 in B, 0 in C and 11 strains in D replicate.

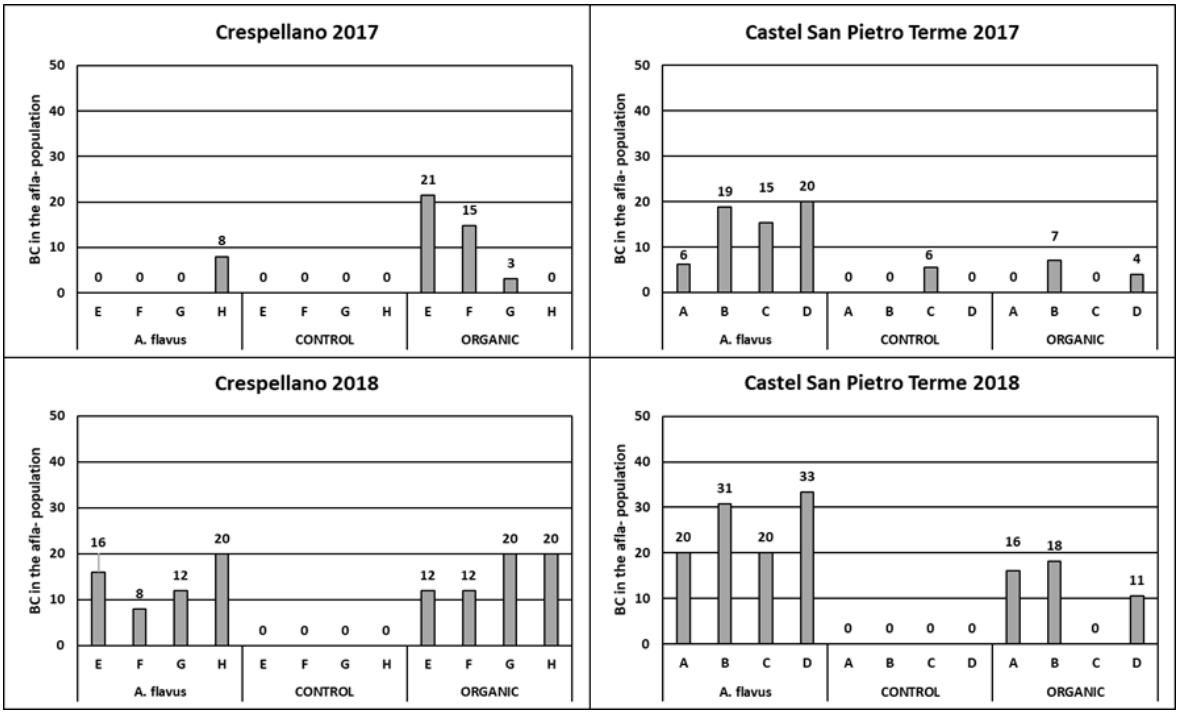


Figure 68: Number of A. flavus biocompetitor strain presented in each replicate of each plot. Biocompetitor treatment *using the conidial suspension of A. flavus biocompetitor strain (BC); In the* ***Control treatment*** *Absence of any chemical or biological treatment for the defense of maize;* ***Organic treatment*** *with the conidial suspension of A. flavus biocompetitor (BC) in combination with T. brassicae and B. thuringiensis.*

We also wanted analyzed the percentage of persistence of BC in all strategy analyzed. In ***Figure 69*** were reported the data as percentage of BC respect to all afla- population:

In **Crespellano 2017,** the percentage of BC found in BIOCOMPETITOR TREATMENT was of 3.3%. No strain was detected with the characteristic profile of BC in the CONTROL strategy, and 10.1%in ORGANIC TREATMENT.

In **Castel San Pietro Terme 2017** was detected 14.7% of BC in BIOCOMPETITOR TREATMENT, 1.4% in CONTROL strategy and 2.6% in ORGANIC TREATMENT.

In **Crespellano 2018** was detected 14% of BC in BIOCOMPETITOR TREATMENT, 0% in CONTROL strategy and 16% in ORGANIC TREATMENT.

Totally unexpected was the persistence of the BC in **Castel San Pietro Terme 2018**. In BIOCOMPETITOR TREATMENT the persistence of BC was very high: 25.9%. 16.7% in ORGANIC TREATMENT and 0% in CONTROL strategy.

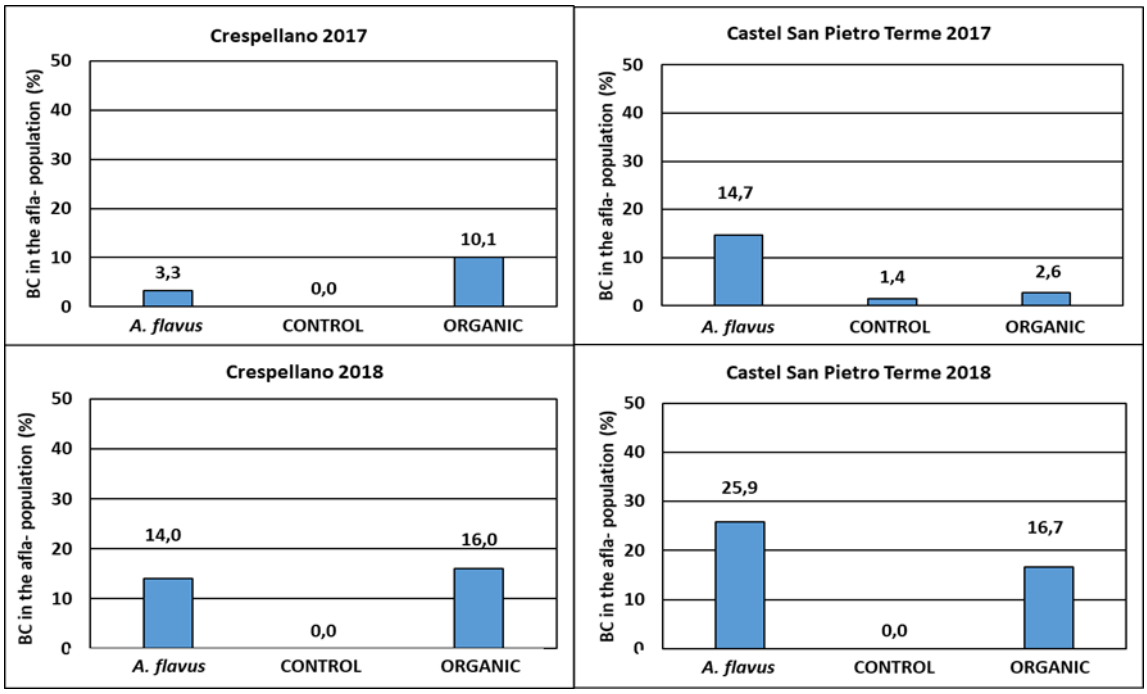


Figure 69: Presence of the biocompetitor one month after the treatment. Biocompetitor treatment *using the conidial suspension of A. flavus biocompetitor strain (BC); In the* ***Control treatment*** *Absence of any chemical or biological treatment for the defense of maize;* ***Organic treatment*** *with the conidial suspension of A. flavus biocompetitor (BC) in combination with T. brassicae and B. thuringiensis.*

5.4. Aflatoxin determination and merging of data

For each experimental field the grain of corn was analyzed to assess the quantity of aflatoxin B_1. The concentration of aflatoxins, reported as µg/kg (ppb), was detected using ELISA kits with antibodies specific for aflatoxin B1. Additionally, a HPLC-based assay for aflatoxin B_1 was performed. The box-plots reported in ***Figure 70*** show the levels of aflatoxin accumulation in each treatment in each experimental field.

The line at 0.02 ppm indicate maximal concentration allowed of aflatoxin B_1. The **year 2017** was particularly hot and dry; in fact, the quantity of aflatoxin B_1 was considerably higher than the legal limits allowed (see the control in Crespellano and Ozzano 2017).

However, in **Crespellano 2017** both the chemical treatment (Coragen®) and the biocompetitor treatment considerably reduced the amount of aflatoxin B_1 even if slight above the threshold. Similarly, the synergism of the three products (BC, *B. turigiensis* and *T. brassicae*) lowered the production of aflatoxin B_1 respect to the single treatment with only BC.

The level of aflatoxin B_1, In **Ozzano 2017** was lower respect to that of **Crespellano 2017**, but always above the legal limits. Also in this case, both the chemical treatment and the biocompetitor treatment reduced the amount of aflatoxin B_1 bringing them exactly to the threshold. Only the organic treatment did not reduced the quantity of aflatoxin B_1.

The year 2018, at variance with what reported of year 2017, was particularly mild and rainy; consequently, the level of aflatoxin B_1 in the control was considerably below the accepted threshold.

In **Crespellano 2018** the biocompetitor treatment did not modify the level of aflatoxin contamination when compared to the control; this output may be related to the fact that the amount of aflatoxin detected in the control grains was already very low. The chemical treatment, instead, considerably reduced the amount of aflatoxin B_1 contamination. Also in this case, the organic treatment did not reduce the quantity of aflatoxin B_1.

In **Castel San Pietro Terme 2018** both the chemical treatment and the biocompetitor treatment considerably reduced the amount of aflatoxin B_1. However, it should be noted that control levels of aflatoxin B_1 were already below the allowed limits. In the same way, the contemporary treatment with the three products (BC, *B. turigensis* and *T. brassicae*) lowered the production of aflatoxin B_1 respect to the control.

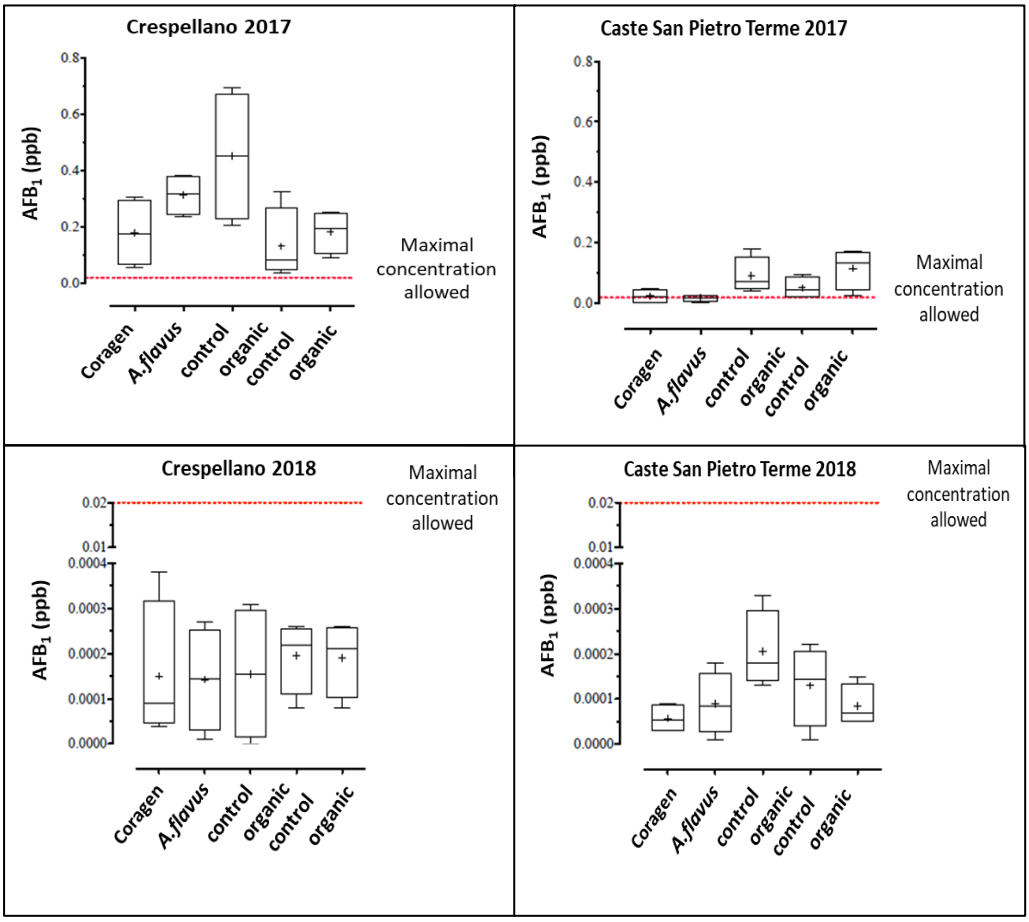


Figure 70: Quantity of aflatoxin detected in each strategy.

We wanted to merge the data of chemical analyses on quantity of aflatoxin B_1 and the presence of BC in experimental field after one month from treatment. We wanted understand if there was a correlation between them. In ***Figure 71***, in each box-plot were reported the percentage of BC presence.

In **Crespellano 2017** and **Castel San Pietro 2018** seemed to be a correlation between the data. The greater was the presence of the BC, lower was the amount of aflatoxin B_1 detected by chemical analyzes. Instead, in **Ozzano 2017**and **Crespellano 2018** there was a correlation between the data only for biocompetitor treatment. No correlation in the organic treatment in which was administrated the BC with *B. thuringiensis* and *T. brassicae.*

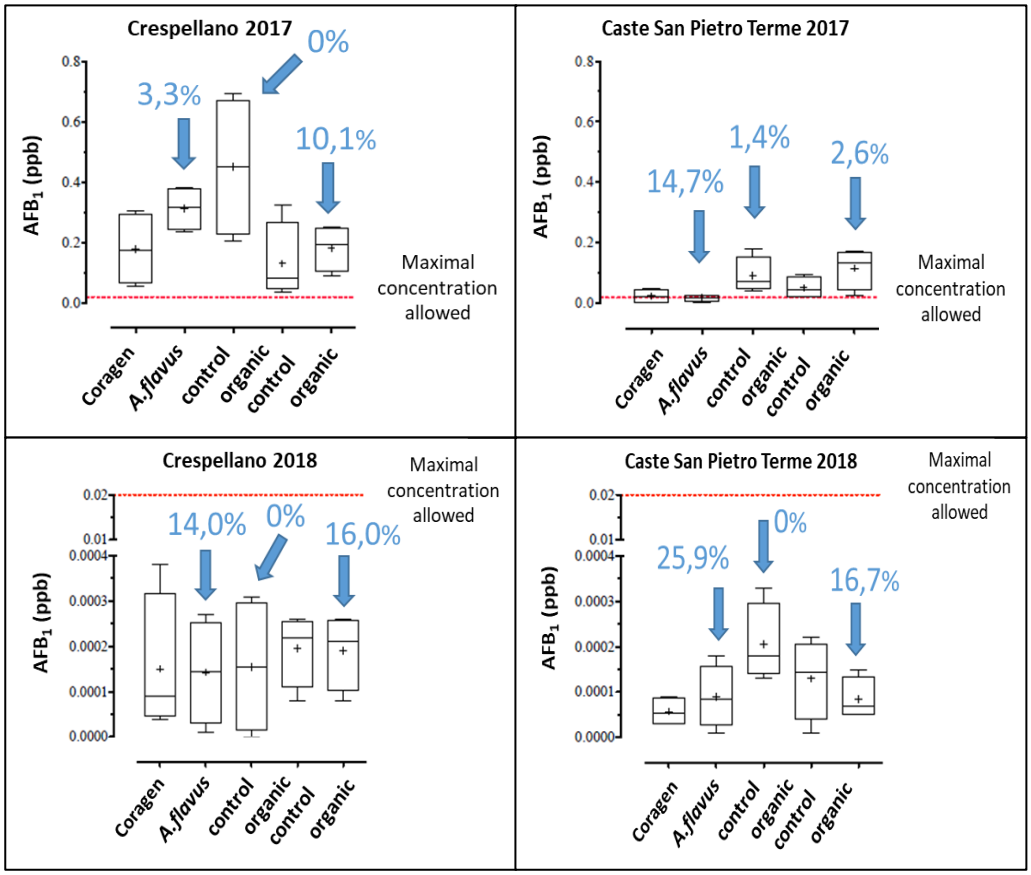


Figure 71: Merging the data of chemical analyses on quantity of aflatoxin B_1 and the presence of biocompetitor in experimental field after one month from treatment.

6. The biocompetitive potential of the *A. flavus* isolated population

During two years of experimentation, over 2000 afla+ and afla- *A. flavus* strains have been isolated from maize kernels sampled in the treated fields. A set of experiments were performed on a representative group belonging to this collection of strains to get hints on the BC responsiveness of the "endemic" population of *A. flavus*. Analyses were set following a preliminary conducted assay aimed at assessing *in vitro* the effectiveness of an atoxigenic strain against AFs accumulation by toxigenic isolates (*Degola et al. 2012)*. Conidial suspensions of both afla+ and afla- strains were co-inoculated in same wells of a CCM microplate, then the production of aflatoxin was fluorimetrically recorded in the medium as for the aflatoxin accumulation assay; biocompetiton potential of afla- strain, estimated by comparison with control wells (inoculated with afla+ strain only), was expressed as percentage of inhibition *(**Figure 72**)*.

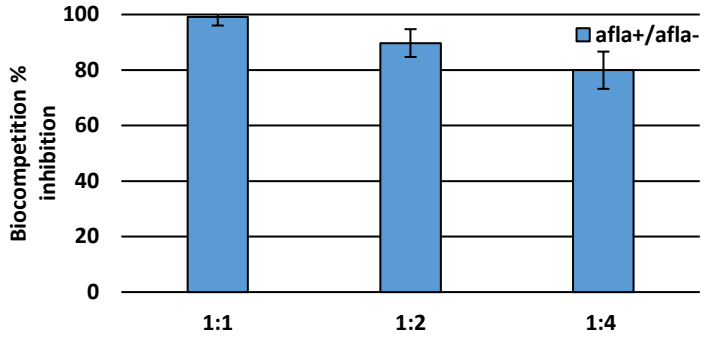


Figure 72: Biocompetition activity of afla- strain on aflatoxin accumulation by a toxigenic strain. *Conidia of afla- and afla+ strains were co-inoculated in an increasing afla-/afla+ ratio (1:1, 1:2 or 1:4) in CCM medium, and incubated at 25 °C for 6 days in the dark.*

At first, a selection of 40 *A. flavus* strains were selected in order to perform a "global" biocompetition assay: 20 afla+ strains were chosen among those that accumulated high amounts of aflatoxin, whereas 20 afla- strains were randomly selected. Each toxigenic strain was co-inoculated with each afla+ isolate, and compared with the BC. The rational was to ascertain if the failure or the limited efficacy of the BC, observed in some experimental condition in the treated fields, was the consequence of the specific "virulence" of the afla+ population challenged by the BC or if the biocompetition did not occurred in the field owing to other unknown environmental factors.

An example of results is reported in ***Figure 73***. As a general consideration, the inhibitory performance of BC was confirmed as the best, since it was effective in lowering the aflatoxins accumulation of all the afla+ strains; on the other hand, if separately considered, some afla- isolates resulted in an higher inhibition rate against specific afla+ strains, as in the case of OZ10- that lowered aflatoxin accumulation by OZ6+ more efficiently that the BC.

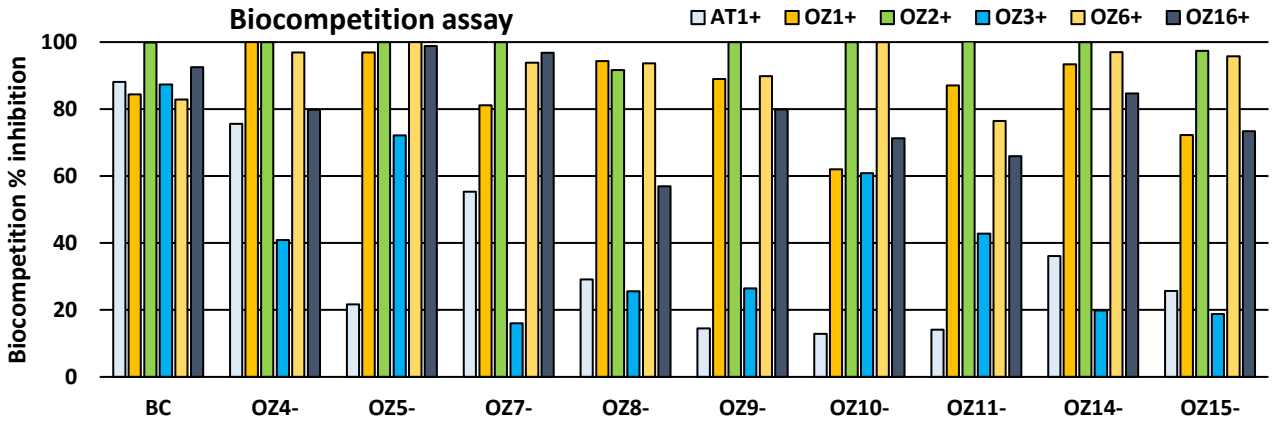


Figure 73: An example of global biocompetition assay of A. flavus population recovered on grains sampled in the experimental fields. *Here, the aflatoxins containment efficacy of 9 afla- strain and BC were tested respect to 6 toxigenic strain. Conidia of afla- and afla+ strains were co-inoculated in an afla-/afla+ ratio of 1:1, in CCM medium, and incubated at 25 °C for 6 days in the dark. Inhibition of isolated afla- strains is compared with the BC activity, whereas the afla+ strain AT1+ is added as internal control.*

A second test was conducted to compare the effectiveness of the BC in inhibiting the AF production with afla- endogenous isolates, even in a disadvantageous proportion: thus, a conidial suspension of the toxigenic isolate CR24+ was singularly co-inoculated with 10 afla- endogenous strains in an afla+/afla- 1:1 and 1:10 ratio.

We found that a ten-fold augmentation of the afla+ conidial concentration did not affect the biocompetition efficacy of the BC and of the majority of endogenous afla- isolates: in fact, as shown in ***Figure 74***, only strain OZ18- diminished in aflatoxin containment ability when its presence in the co-culture was one-tenth of the total inoculum.

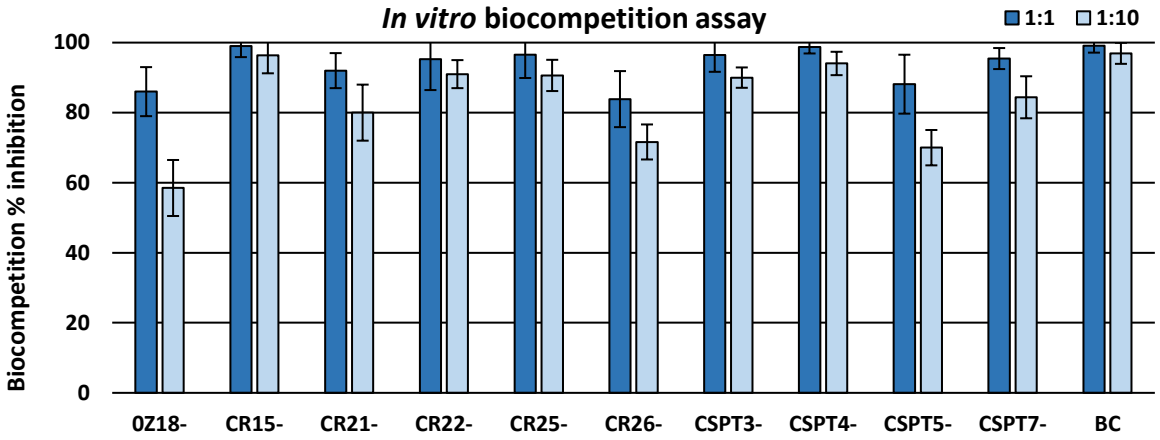


Figure 74: Dose-dependent biocompetition efficacy of endogenous A. flavus afla- and BC. E*fficacy of 10 afla- strains is tested in 1:1 and 1:10 afla-/afla+ ratio against CR24+ strain. Inhibition of isolated afla- strains is compared with the BC activity.*

Data evidenced a certain variability among *A. flavus* strains in their ability to prevent (for afla-) aflatoxin contamination or to overcome the intraspecific inhibitory effect (for afla+) of biocompetitors, underscoring the existence of different mechanisms that may or not act synergistically;

in consideration that in a cooperative mycelial network, such as in a colonizing inoculum, the efficacy of the interference on aflatoxin production could be determined by the strength of the vegetative compatibility *(Wicklow et al. 2007)*, and that population of *A. flavus* colonizing a single field commonly include diverse *VCGs (Bayman et al. 1991; Ehrlich 2008),* we explored a multi-competition approach to investigate the efficacy of the use of a combination of afla- strains to ameliorate the interference with aflatoxin production by one or more afla+ strains. A microplate

biocompetition assay was built, comparing the aflatoxin containment of the BC with the inhibitory effect of a pool of 10 endogenous afla- isolates *(**Figure 75**)*.

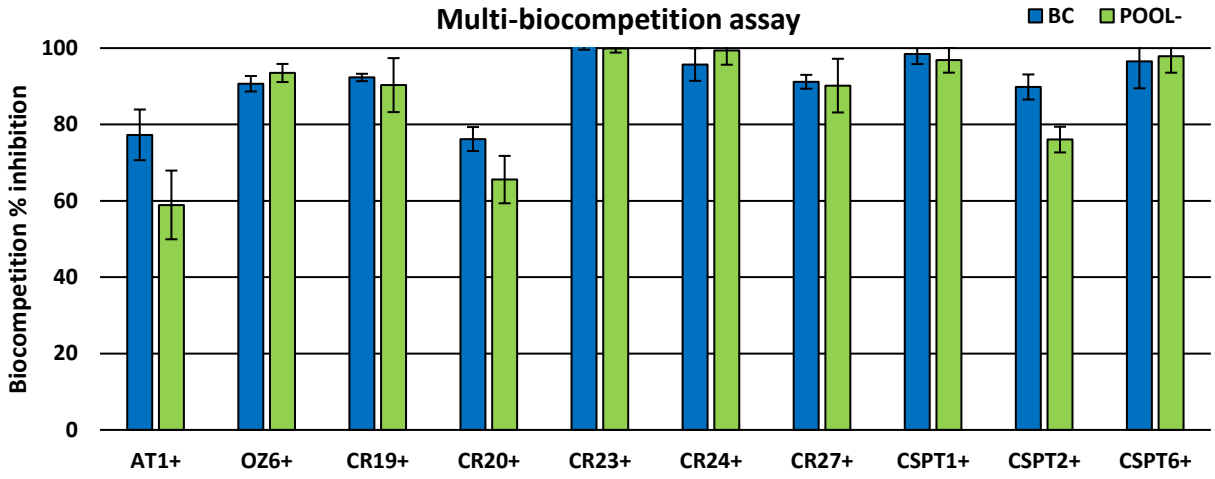


Figure 75: Comparison between the biocompetitive efficacy of an endogenous afla- pool and the BC. *The aflatoxin containment efficacy of a conidial suspension of pooled 10 afla- strains is tested against 9 afla+ endogenous strains. Inhibition of the pool is compared with the BC activity, AT1+ strain is used as reference.*

Results, presented in ***Figure 75***, did not report significant differences between the two conditions: in terms of aflatoxin inhibition, the biocompetitive advantage in using the pool of atoxigenic strains did not increase the containment obtained with the single BC. As well as confirmatory of the reliability of the latter against the resident population of *A. flavus*, this observation seemed to exclude the hypothesis of a synergism between afla- isolates; however, the further characterization of the VCG of atoxigenic isolates pooled would be helpful for clarify this aspect.

7. Conclusions

The atoxigenic *A. flavus* strain used as a bio-competitor proved to be as effective as the chemical pesticide (Coragen®) treatment in reducing aflatoxin contamination in the field, being therefore highly promising for a new, green perspective strategy for the containment of aflatoxins contamination. Interspecific biocontrol on corn borers resulted less effective. Additionally, the proved persistence of the BC one month from the application has to be considered encouraging for an efficient, cost-effective procedure for mycotoxin management.

Thanks to the isolation of more than 2000 strains of *A. flavus*, both afla+ and afla-, as a result of the two years experimentation, the present investigation confirmed both the anti-aflatoxigenic activity of our bio-competitor strain and a comparable activity of atoxigenic strains belonging to the resident *A. flavus* population. The possibility to test, through the microplate biocompetition assay, the ability of one or more strains to reduce aflatoxin production by the "real time" residents *A. flavus* colonizing the relevant field is an additional value that might enhance the effectiveness of such intraspecific biocontrol strategy. As final consideration, it would be worth to test if a pool of afla- strain, recovered from the field during the previous season, could be able to effectively reduce the aflatoxin production by the afla+ population colonizing the field during the following season(s). *In vitro* experiments to challenge this hypothesis are now in progress.

TOPIC 3:

On the molecular characterization of *A. flavus* strains

Molecular tools for the characterization of *Aspergillus flavus* strains

Ecological theories were developed essentially for the ecosystems present on the soil surface, neglecting for a long time all those life forms present inside it, in particular microorganisms, and that represent an enormous quantity of "*invisible life of fundamental importance for the entire life on earth (Wardle and Giller, 1996)*. The mycoflora represents a relevant part of the soil biomass, being the one that most influences its biological properties, regulating all the degradative biochemical processes and determining its nutritional value *(Bloem et al. 2003)*. The different species of microorganisms present in the soil (such as bacteria and fungi) have, in fact, priority roles in energy transformations and biogeochemical processes, intervening in the decomposition of organic material through biodegradation processes and in the recycling of essential elements such as carbon, phosphorus, nitrogen and others *(Alexander et al. 1977)*. The diversity of microorganisms within an ecosystem is therefore a key element also for maintaining a qualitatively healthy state of agricultural soil *(Borneman et al 1996)*.

Tropical biotas provide excellent settings in which to explore mechanisms of evolutionary diversification; in particular, various researches on biodiversity patterns and diversification processes has recently been implemented by studies conducted in Madagascar. Several researchers have examined the diversity models and the diversification mechanisms of this island, proposed not only for fauna but also at microbiological level on fungi and bacteria. Madagascar has a diverse biota that has evolved in isolation, establishing a promising system for the study of pattern and process in species diversification *(Vences et al. 2009)*. For this reason Madagascar is an excellent landscape for the study on biodiversity and all phenomena taking part of it. The theme of regionality is linked strictly linked to the concept of biodiversity; microorganisms adapt to the soil and respond to stimuli that lead them to evolve towards a direction, while other microorganisms subjected to different stimuli evolve in different directions. This occupies a particular relevance in the case of biological control strategies relying on microbial intraspecific biocompetitors, as the effectiveness of such strategies depends on how the biocompetitor can adapt to the application area and reach to biocompete it with other fungi.

One of the most important contributors to the occurrence of mycotoxin contamination of crops and commodities is probably represented by fungal biodiversity, since phenotypic and metabolic plasticity definitely empowered mycotoxigenic fungi to adapt to a wider range of environmental conditions and, in turn, to successfully colonize a broad variety of agriculturally important plants.In

the 2011 our group had the possibility to isolate the microbial population from maize kernels sampled in the Lokobe Strict Reserve, a nature reserve located on southeastern side of Nosy Be, an island off the coast of Madagascar in northwestern Madagascar. Famous for its black lemurs and the beautiful Nosy Be's panther chameleon, in our perspective its relevance relies on the exceptional biodiversity of its microbial communities, that remain relatively unexplored. Thus, the possibility to provide the first (even partial) survey of Malagasy *Aspergillus flavus* diversity could establish a baseline for a wider characterization of the aflatoxigenic microbial population, aimed at the design of bio-control strategies against aflatoxin contamination and based on the intraspecific competition. The collection, that counted over 70 strains belonging to *Aspergillus* section Flavi, was initially analyzed for the species attribution, for the aflatoxigenicity and, in the case of atoxigenic individuals, for the biocompetitive potential in terms of aflatoxin containment. About 65 strains were classified as *A. flavus*, while other 5 were attributed to *A. tamarii* species. One strain in particular, the atoxigenic Lok18- isolate, was found to be an excellent biocompetitor, since it proved to be highly effective in preventing/lowering aflatoxins accumulation by various *A. flavus* toxigenic strains both in culture medium and on natural substrate such as corn seeds (data not published).

With the aim to individuate genetic markers reliably applicable for the detection/traceability of desirable *A. flavus* strains, we took advance of the collaboration of Prof. Dr. Olivier Puel, at the "*Toxalim laboratory*" of the *INRA Research Center for Food Toxicology* (*Institut National de la Recherche Agronomique,* Toulouse, France), and of Prof. Ludwig Niessen, at the *Technical University of Munich* (Freising, Germany).

1. Molecular characterization of strains

1.1. Background

Universally recognized as the main species associated with aflatoxin contamination of agricultural crops, *A. flavus* and A*. parasiticus* have been joined by additional species of section Flavi responsible for AFs production in certain geographical area *(Varga et al, 2011)* (*A. arachidicola* and *A. nomius*), and recently other new species were reported as aflatoxigenic, such as *A. mottae*, *A. sergii* and *A. transmontanensis (Soares et al. 2012).* In the soil populations of *A. flavus,* phenotypic differences or similarity amongst individuals typically depend on the vegetative compatibility groups (VCGs), that could be more than hundreds in the same population *(Leslie et al. 1993).* Even if VCG was generally considered as a strong barrier to genetic exchange, recent findings seemed to demonstrate that outcrossing is possible, leading to the genesis of new groups that, in turn, contribute to an increasing of biodiversity *(Olarte, et al. 2012).* This deserves a special consideration, since most of variations in genetic characteristics (but, overall, in mycotoxin production) can be attributed to the attribution to different VCGs.

Due to the economical relevance of toxigenic strains, extensive researches have been conducted both at the specimen attribution and the toxigenic potential level; in this sense, genetically based characteristics were long time individuated as the most important contributor to qualitative differences in mycotoxins production, and, among them, one of the most studied is the variability in mycotoxin biosynthetic genes. In fact, due to its proximity to the telomeric region of chromosome III, AFs cluster seems to be highly subjected to genomic rearrangements such as recombinations, gene losses, DNA partial deletions, translocations or sequence inversions, becoming then responsible for the majority of the genetic difference in mycotoxin production *(Chang et al. 2005-2006; Carbone et al 2007).* Major concerns about the variation of AFs production by *Aspergillus* section *Flavi* strains has recently increased because the use of atoxigenic *A. flavus* as biological control agents to reduce the risk of aflatoxin contamination *(Degola et al. 2011)*. Thus, since the choice of a good biocontrol candidate could not be based only on the phenotypic characteristic of atoxigenicity but also on its genotypic condition, the understanding of genetic variability in aflatoxin cluster of atoxigenic isolates is important for the selection of safe and effective non-producing strains functional to biocontrol strategies for the aflatoxin containment. In addition, on the genetic background rely other factors involved in a better understanding of natural diversity of *A. flavus* populations in agricultural soil and, in turn, in the,successful application of a intraspecific biocontrol strategy. For instance, the ability of the introduced biocompetitor to recombine with the pre-

existing aflatoxigenic strains, the adaptation of *A. flavus* isolates to effectively colonize the relevant crop and the potential production of other toxic metabolites (in addition to aflatoxins) which could affect animal health *(Ehrlich et al. 2014).*

Unfortunately, studies characterizing the molecular diversity of European Aspergillus populations are still scarce *(Gallo et al. 2012; Mauro et al. 2013).*

Genetic and biochemical analyses conducted over years on toxigenic fungi have elucidated that specific variances are directly responsible for intra- and inter-specific differences between toxigenic and toxigenic-related species, taking in account that toxigenic and non-toxingenic aspergilla belonging to same species are phenotypically undistinguishable. Various are the methodologies employed for this purpose, considering that many studies showed the usefulness of DNA markers for analysis of genetic variability between different individuals in fungal, plants and animal populations *(AIAT 2006),* and, specifically to *Aspergillus*, also thanks to the fact that genomes of several species have recently been sequenced. At a genomic DNA level, the molecular diversity of different strains has been achieved for *A. flavus* and *A. parasiticus* by using random amplified polymorphic DNA (RAPD) markers *(Lourenco et al. 2007; Tran-dinh et al. 1999).* Also, major Aspergillus section Flavi members were demonstrated to could be identified by their Inter Simple Sequence Repeat (ISSR) fingerprinting, through sequence characterized amplified regions (SCARs) *(Priyanka et al. 2014).*

Non-aflatoxigenic isolates can be also characterized and identified by PCR using primers targeting both structural and regulatory genes of AF gene cluster and its flanking regions, as demonstrated by Chang and colleagues *(Chang et al. 2005),* demonstrating that deletion of a part or the entire cluster is not rare, and results in different patterns. In closely relates species such as *A. oryzae* and *A. sojae*, in which the synthesis of AFs is not possible, they were also found defective for several AF biosynthesis pathway genes *(Chang et al. 2004; Takahashi et al. 2002; Kusumoto et al. 2000).* Together with single nucleotide polymorphisms (SNPs) in the sequence of single genes, more or less extended deletions in the AF cluster were used to categorized isolates *A. flavus* in different groups *(Chang et al. 2015; Ehrlich et al. 2004).* 20 strains were analyzed on *NorB-CypA* region to identify the deletion that prevents *A. flavus* to produce AFG like *A. parasiticus (Ehrlich et al. 2004)*. 9 of these strains were also analyzed on the *samA-rosA* region to understand if these strains belonged to the *oryzae* section (*Chang et al. 2015).*

1.2. Aflatoxin production determination and sclerotia biogenesis

The aflatoxigenic potential of every strains was assessed with an accumulation assay: $5*10^3$ spores were inoculated in each well of a multiwell plate, in CCM liquid medium. After six days, the fluorescence emitted by aflatoxin B_1 released in the medium was evaluated. On this basis, the strains were classified as producers (Afla+) and not producers (Afla-) of toxins. Among the African strains, only **Lok11, Lok26, Lok 30, Lok32, Lok46, Lok65, Lok68, Lok70** proved to be aflatoxigenic strains, while all the others were classified as Afla- strains ***(Figure 76)***. The aflatoxin accumulation assay was conducted as control on Italian strains AT1+, Fri2+, Pie1- and TOϕ , previously characterized in our laboratory *(Degola et al 2011; 2012)*. All strains have also been tested on secondary differentiation: the biogenesis of the scerozia, as already widely anticipated, are differentiative structures of resistance to environmental stresses. Only **LOK11, LOK16, LOK26, LOK30**, were sclerotigen strains.

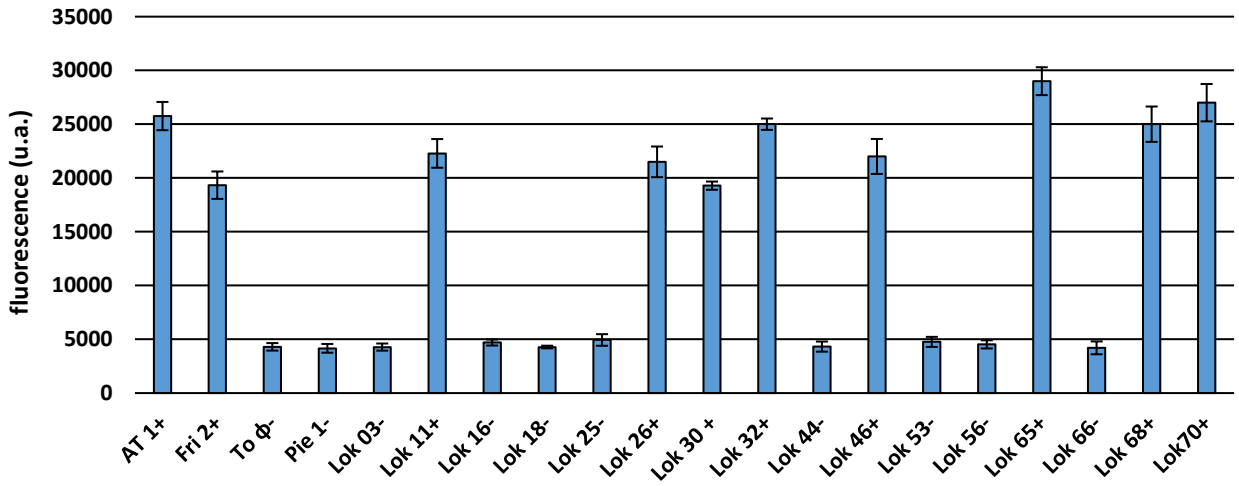


Figure 76: Aflatoxin accumulation of Aspergillus strains.

1.3. Mating Type Analysis

In filamentous ascomycetes a single non-allelic locus (idiomorph) contains one of two highly divergent mating type genes, either *MAT1-1* or *MAT1-2*, that occupy the same chromosomal position in strains of opposite mating type but are not related by structure or common descent *(Metzenberg and Glass, 1990)*. The size of an idiomorph varies among heterothallic ascomycetes and can contain more than a single MAT open reading frame *(Mandel et al. 2007)*. *MAT1-1* encodes a protein with a α-box motif and *MAT1-2* codes for a protein of the high mobility group (HMG) *(Coppin et al. 1997; Turgeon and Yoder, 2000)*. A single *MAT1-1* or *MAT1-2* gene was detected in the genomes of *A. flavus*, *A. parasiticus* and *A. oryzae.* The sequenced-genome strain of *Aspergillus flavus* contains a homolog of the *MAT1-1* mating-type gene. Genome sequencing of *Aspergillus oryzae*, which is closely related to *A. flavus (Rokas and Galagan, 2008)*, also revealed a *MAT1-1 α-box* gene on chromosome 6. The single idiomorph in these genomes is consistent with a potential heterothallic organization of mating-type genes and the possibility of mating in *A. flavus* and *A. oryzae (Galagan et al., 2005; Machida et al., 2005)*.

In this work we used oligonucleotide primers targeting conserved regions for several reported *MAT1-1 α-box* and *MAT1-2 HMG* genes. Our primer pair for the *MAT1-1 α-box* amplifies a 396 bp fragment, which contains 52 bp of a conserved intron. Instead, the *MAT1-2 HMG* primers target a 270 bp segment, which includes 51 bp of the second of two conserved introns *(Ramirez-Prado el al. 2008)*. Results from our mating type diagnostic PCR revealed that all Italian and African strains resulted positive to Mat1-1 gene, while Italian strains AT1+ and Fri2+ resulted to possess a Mat1-2 gene.

1.4. Analysis of the sequence GAP in the *norB-cypA* region

The comparison of the aflatoxin gene cluster sequence from various strains revealed that all *A. flavus* strains share a deletion of the genomic sequence upstream from the polyketide synthase gene (*pksA*) at the level of *cypA* and *norB* genes (also known as *aflF* and *aflU*), that encode for two enzymes involved in the conversion of the relevant intermediate in aflatoxins G_1 and G_2 *(Ehrlich et al. 2004)*. Such deletion makes *Aspergillus flavus* isolates unable to accumulate the G form of AFs, while other aflatoxigenic species belonging to the section Flavi (*Aspergillus parasiticus* and *Aspergillus nomius*, for instance) produce both aflatoxins B and G *(Klich and Pitt 1988)*. Multiple alignment of the farthest upstream region of aflatoxin gene cluster from isolates of two *A. flavus*

strains (belonging to S and L type) and three aflatoxin B- and G-producing Aspergillus species (*BN008R*, *A. nomius*, and *A. parasiticus*) highlighted the presence of a 0.8- to 1.5-kb gap in the *A. flavus* sequence (*Ehrlich et al. 2004)*. The gap is approximately located from 0.4 to 0.6 kb from the translational stop codon of *norB*, which is the putative 5' terminus of the gene cluster ***(Figure 77)***. Deletions of at least three different length were identified: the first, of 1.0 Kb, corresponding to L genotype; the second, of 1.5 Kb, corresponding to S genotype; the third (2.5 Kb) corresponded to *BN008R* strain (isolated in Kenya). Even if the presence of the sequence's gap resulted shared in all *A. flavus* strains, its length was demonstrated to be highly variable. Thus, we used this molecular character as a molecular marker: primers AP1729 and AP3551 were used for amplifying the *norB-cypA region (Ehrlich et al. 2004),* and, on the basis of dimension of PCR amplification products, we characterized and grouped our strains.

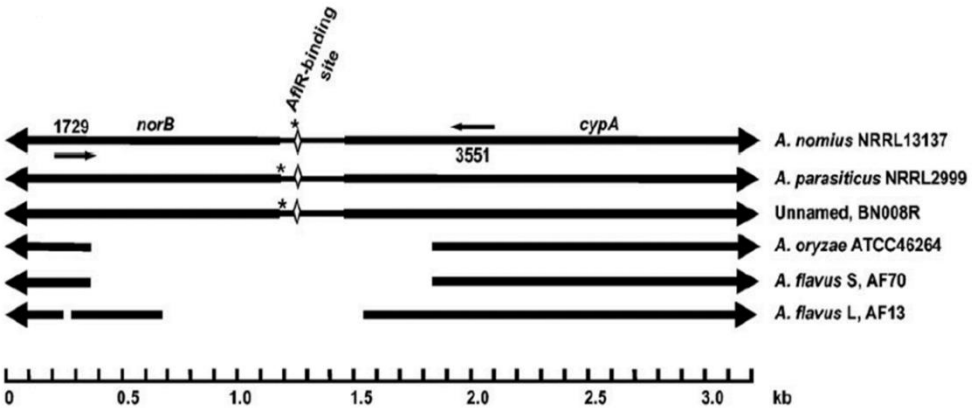


Figure 77: Characteristics of the norB-cypA region in different Aspergillus species. *Schematic diagram of the norB-cypA region of different aflatoxin biosynthesis gene cluster homologs. Thick arrows indicate coding regions and direction of transcription of norB and cypA. Gaps represent deletions of 32 and 854 bp in A. flavus NRRL3357 and AF13, and 1516 bp in A. flavus AF70 and A. oryzae ATCC46264 when the sequences are compared to the sequence of A. parasiticus in this region. Additional smaller deletions or insertions are marked by asterisks (11 bp in A. parasiticus NRRL2999 at bp 1166, 13 bp in BN008R, and 4 bp in A. nomius NRRL13137). Small arrows indicate the positions of primers AP1729 and AP3551.*

An example of different size PCR products is reported: in ***Figure 78,*** **Fri2+** and **Lok18-** amplification product resulted compatible with S genotype, while **Pie1-** product's size was comprised between genotype's S and L length. **All the other strains** showed a profile compatible with the L genotype. Only **Lok3-** showed no amplification, this showed that the deletion is much larger than the others.

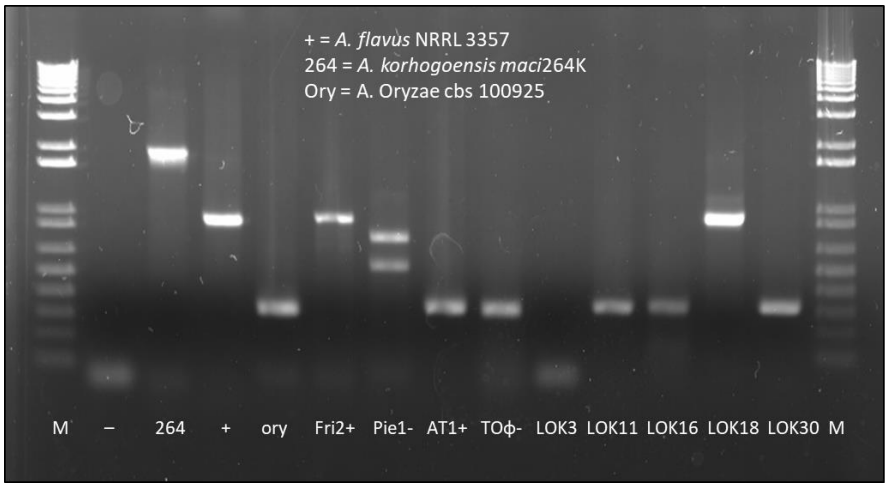


Figure 78: Electrophoretic profiles of NorB-CypA region PCR amplification. *M: molecular marker 1 kb DNA Ladder SIGMA; - : negative control; 264: A. korhogoensis maci264K, A. flavus NRRL3357 and A.oryzae: positive controls. Fri2+. Pie1-, AT1+, TOφ-: Italian strains; LOK3, LOK11, LOK16, LOK18 and LOK30: Lokobe strains.*

Through the combined use of AP1729 and AP3551 oligos with specific primers designed on the sequence of *aflF* and *aflU (Degola et al 2006),* we achieved a more precise drawing of the GAP size/position in each strain. In ***Table 5*** were reported all possible combination of primers used and their size and in ***Figure 79*** were reported the GAP size/position that we detected after the PCR amplification analysis on owr afla+ and afla- strains in study.

Pairs of primers	Amplification product length (bp)
AP1729 -AP3551	1822 bp
AflF-Fw - AflF-ReV	359 bp
AflU-Fw - AflFU-ReV	365 bp
AflF-Rev - AflU-ReV	1744 bp
AP3551 - AflF-Rev	1561 bp
AP1729 - AflU-Rev	2005 bp
AP1729 - AflF-Fw	620 bp

Table 5: Combination of primers to design best precise the GAP size/position in each strain. Also reported the size of each amplification.

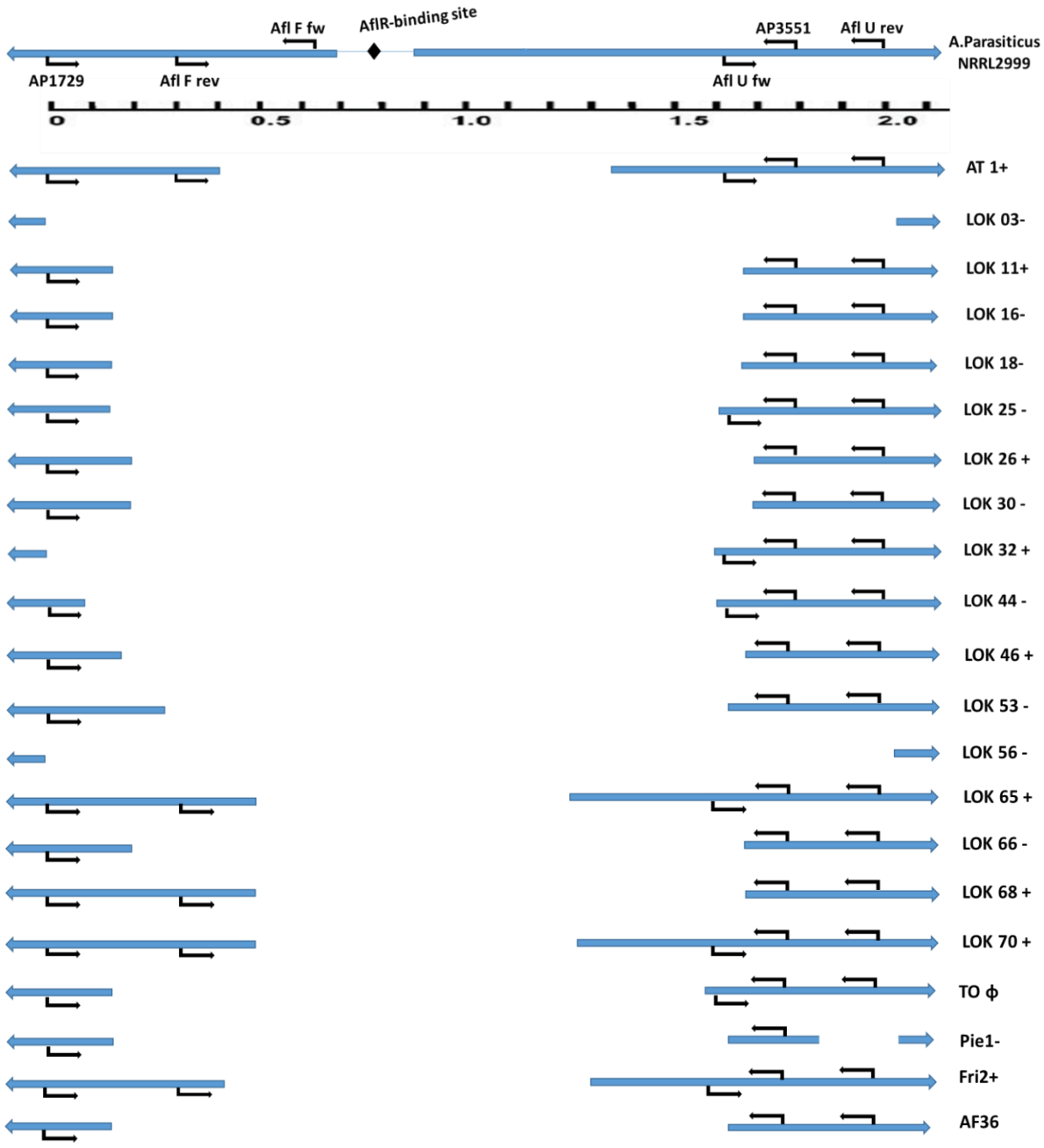


Figure 79: Schematic representation of GAP size/position in NorB-CypA region of Italian and Madagascar afla+ and afla- strains of A. flauvs. *Black arrows indicate the direction of transcription and the positions of primers AP1729, AP3551 AflF and AflU.*

Analyses provided evidences confirming that the isolates actually differ for the size of the deletion in the *norB/cypA* region, being therefore distinguishable from each other. These data also showed that there was no correlation between afla+ and afla-, as afla- strains, and also the afla+ strains, did not behave together, but the size of the deletion was random. Furthermore, no correlation was showed between the African and Italian strains.

1.5. Phylogenetic analysis

In order to obtain some additional information about the phylogenetic relation between strains, some of them were subjected to sequencing analysis for nine genes (***βtub***, ***cmdA***, ***mcm7***, ***rpb1***, ***ITS***, ***amdS***, ***preA***, ***preB*** and ***ppgA***), chosen on the basis of results previously speculated by *Carvajal-Campos el al. (2017)*. ***Calmodulins*** are proteins particularly abundant in eukaryotic cells (up to 1% of total proteins), particularly important for the intracellular signaling processes. The ***mcm7*** gene encodes for one of the highly conserved micro-chromosome (mcm) maintenance proteins; these proteins are essential for the initiation of eukaryotic genome replication. The ***rpb1*** gene encodes for an RNA 2 polymerase subunit. The RPB1 subunit, in combination with other polymeric subunits, forms a binding domain between DNA and RNA polymerase. The RPB1 subunit strongly interacts with the RPB8 subunit. ***ITS*** (Internal transcribed spacer) refers to the DNA spacer located between the ribosomal RNA gene (rRNA) and the large subunit units of the rRNA in the chromosome or in the corresponding region transcribed in the polistronic rRNA precursor transcript. The genes ***preA***, ***preB*** and ***ppgA*** are related to the sexuality of the fungus. ***Tubulin***, generally used as reference, is a globular protein weighing about 55 kDa, which is the fundamental unit of cytoskeletal structures called microtubules. In the cytoplasm, tubulin is present in the form of α and β dimers. The phylogenetic tree was created starting from a concatenated alignment. The sequencing results were analyzed with the alignment program BioEdit/ClustalW (http://www.mbio.ncsu.edu/bioedit/bioedit.html), being blasted before on NCBI. Evolutionary models for both genes were tested using JModelTest. For concatenated data, the best-fit nucleotide substitution models and partitioning scheme were chosen using PartitionFinder v2.0.0 *(Lanfear et al. 2017)* under BIC. To search for the best-fit scheme, a greedy algorithm with linked branch lengths of alternative partitions was used. Partitions obtained consisted of a set that corresponded to a specific model: this set included *βtub* and *cmdA*. 382 bp in 49 sequence of *βtu*b (K80+G) and 463 bp in 50sequence of *cmdA* (K80+G). For *βtu*b: AICc: 19 (TrNef + G). BIC: 11 (K80+G) and DT: 1 (K80+G). For *cmdA*: AICc: 60 (TIMef + I + G). BIC: 20 (TrNef + I + G) and DT: 20 (TrNef + I + G). Bayesian inference statistical methods were used to obtain tree phylogenetic relationships for concatenated data, using the best-fit substitution models listed above. For Bayesian analyses, MrBayes v3.2 *(Ronquist et al. 2012)* was used. Topologies for both genes were obtained. After that, both genes were concatenated using Mesquite v3.2 *(Maddison, 2017)* to have a robust results. The preliminary results settled of strains as *A. flavus*. Phylogenetic trees were visualized and edited with FigTree v1.4.2 *(Rambaut et al. 2014).*

At first, only a small number of African strains (Lok03-, Lok11+, Lok16-, Lok18- and Lok30+) were subjected to the PCR analyses on four genes **β*tub, cmdA, mcm7* and *rpb1***. Italian strains AT1+, Fri2+, Pie1- and TOϕ- were used as control. PCR amplifucation products were then send to a commercial sequencing using forward and reverse PCR primers. The raw sequences were transferred into the BioEdit Sequence Alignment Editor. The sequences were aligned and designed *A. flavus* Phylogenetic tree. The figTree is reported below in ***Figure 80***.

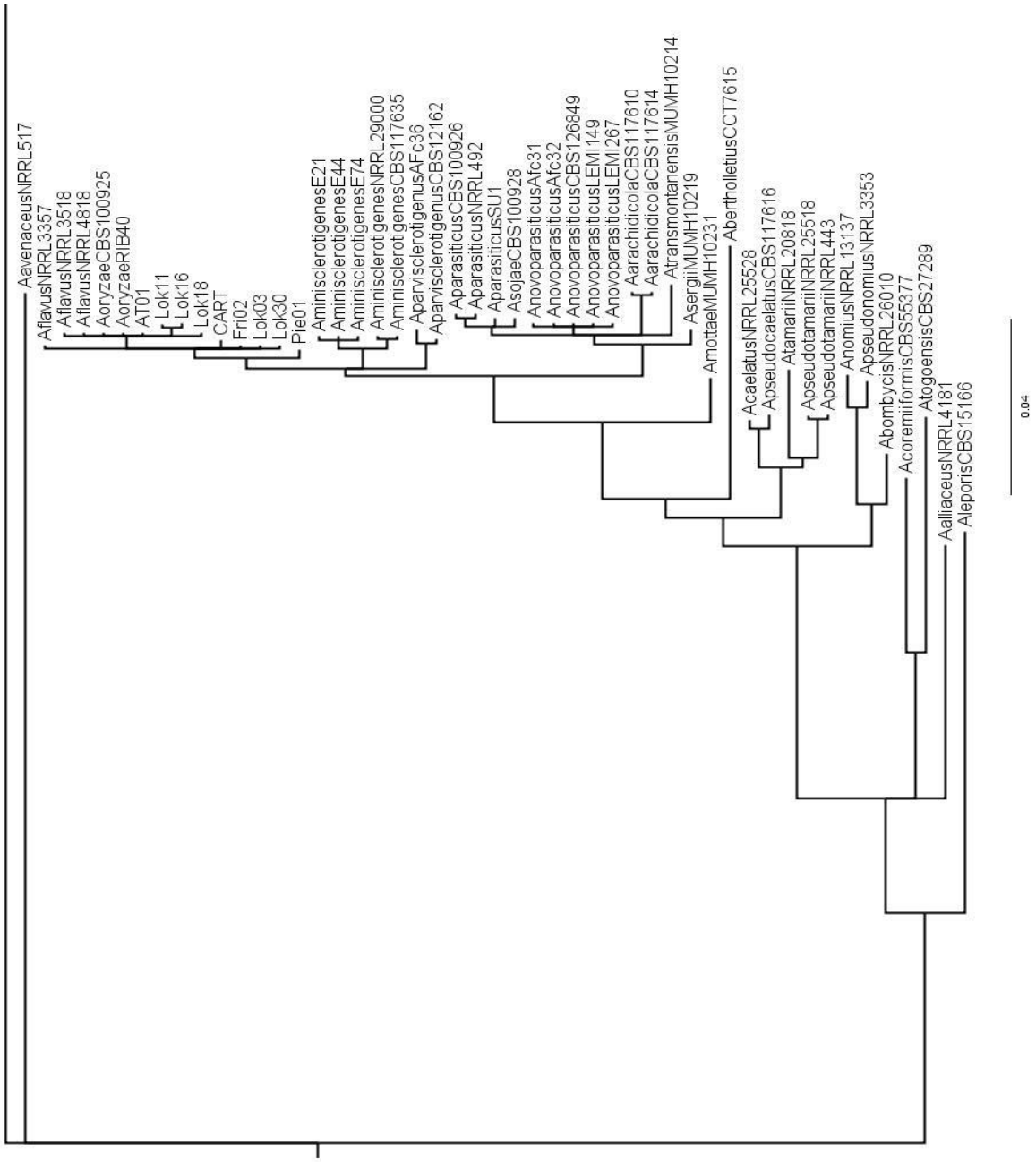


Figure 80: Phylogenetic tree. *Phylogenetic tree of Aspergillus section Flavi based on concatenated sequences from two genomic loci (βtub, cmdA). Bayesian tree was calculated from 49 strains, and includes the type strain for most species. A. avenaceus NRRL517 was used as the outgroup taxon.*

The phylogenetic tree inferred from 2 concatenated genes (*βtub* and *cmdA*), obtained from Bayesian analyses, yielded topologies particularly congruent for *A. flavus* clade. *Aspergillus avenaceus* was used as the outgroup taxon. The topology then split in two robust groups, one formed by the *A. tamarii* clade, which included *A. caelatus, A. pseudocaelatus* and *A. pseudotamarii*, and a second monophyletic group, which included the *A. parasiticus* and *A. flavus* clades, as well as *A. mottae*. This latter was placed as the ancestral taxon of the group including *A. parasiticus* and *A. flavus* clades. *Aspergillus parasiticus* clade was consistent with *Soares et al.* results and included *A. parasiticus, A. sojae, A. arachidicola, A. novoparasiticus, A. sergii* and *A. transmontanensis. A. sergii* and A*. transmontanensis* are basal taxa, respectively. The *A. flavus* clade included *A. flavus, A. oryzae, A. minisclerotigenes, A. parvisclerotigenus* and all our strains. The *A. flavus* clade is comprised of two main groups: one that includes A*. flavus*, its domesticated species *A. oryzae* and *A. minisclerotigenes*, and the other group including *A. parvisclerotigenus* and *A. parasiticus* spp. The nine isolates (Lok03-, Lok11+, Lok16-, Lok18-, Lok30+ and AT1+, Fri2+, Pie1-, TOϕ-) putatively identified as *A. flavus* were tightly clustered, suggesting they were a distinct species from *A. parvisclerotigenus* and *A. parasiticus.* Our isolates clustered together even if were isolated from two different and extremely distant areas. Lok18- seemed to cluster with the *A. oryzae* group and for this reason, we wanted to deep its belonging by analyzing of the *samA-rosA* region.

1.6. Analysis of the *samA-rosA* region

Same strains used for the phylogenetic analysis were then subjected to a screening for the characterization of genomic *samA-rosA* region. This portion of gDNA, that in *A. nidulans* was demonstrated to be highly variable *(Chang et al. 2015)*, contains the *samA* gene, that encodes for a protein involved in cellular morphogenesis which may play a role in the mitogen-activated kinase cascades, and the *rosA* gene, encoding for a $Zn(II)_2Cys_6$ DNA-binding protein that acts as a repressor of sexual development. Genomes comparison between *A. flavus NRRL3357* (L strain) and *A. flavus 70S* (S strain) demonstrated that specific variations in the *samA-rosA* sequence could be associated with the two morphotypes; a third group was identified, found to be characteristic of *A. oryzae RIB40* strain. The three *samA-rosA*-based groups were later discovered to be commonly present in *A. flavus* L strain populations, while *A. oryzae* strains resulted to possess only the two 70S and RIB40 phenotypes. While the *samA-rosA* region of *A. flavus* RIB40 and *A. flavus* 70S was 99% similar to *A. flavus NRRL3357*, *A. parasiticus* and *A. nomius*, known to be phylogenetically distant, had similar

only for 93 and 83% respectively. Schematic representation of conserved region is reported in ***Figure 81***.

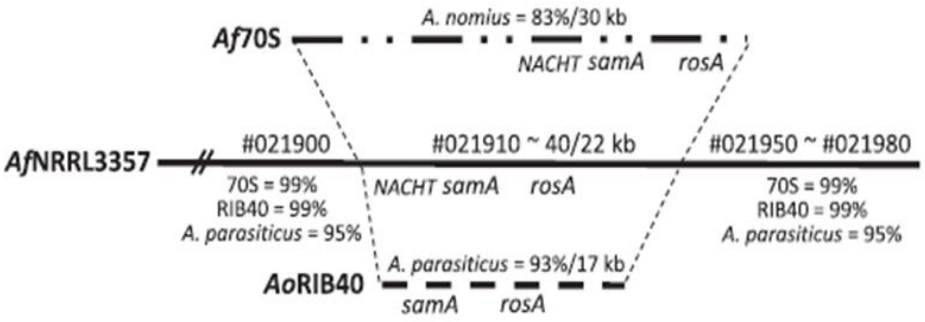


Figure 81: Scheme of conserved highly variable regions containing samA and rosA genes in A. flavus and A. oryzae. *The relevant position of genes in the genome of 70S, NRRL3357 and RIB40 strains were reported. Figure from Chang et al. 2015.*

PCR amplification products of 9 strains, detected on electrophoretic gel, were reported in ***Table 6*** and showed that strains Fri2+, Pie1-, Lok03- and Lok30+ shared the NRRL3357 phenotype; the contrary, strains AT1+, Lok11+, Lok16- and Lok18- possess the 70S phenotype. RIB40 phenotype was found only in the case of TOϕ- strain.

STRAIN	*NRRL3357 phenotype (838 bp)*	*70S Phenotype (575 bp)*	*RIB40 Phenotype (933 bp)*
Lok 3 -	X		
Lok 11 +		X	
Lok 16 -		X	
Lok 18 -		X	
Lok 30 +	X		
AT 1 +		X	
Fri 2 +	X		
Pie 1 -	X		
TO ϕ -			X

Table 6: Data of PCR amplification products of strains revealed the characteristic phenotype: NRRL3357 Phenotype, 70S Phenotype and RIB40 Phenotype. *Fri2+, Pie1-, Lok03- and Lok30+ strains have NRRL3357 Phenotype (838 bp fragment); AT1+, Lok11+, Lok16- and Lok18- strains have 70S Phenotype (575 bp fragment); only CART have RIB40 Phenotype (933 bp fragment).*

1.7. Conclusions

As reported in *Chang et al. 2015,* merging all data obtained we could identify the relative species of belonging of the nine *A. flavus* strains subjected to analysis. For this reason, we have grouped all data obtained in the ***Table 7*** below. Merging the data of AFs production, Mating Type, *norB-cypA* sequence gap size, the *samA-rosA* type and results of phylogenetic tree and analyzing them, were confirmed that the majority of analyzed strains belong to *A. flavus* species. However, the characteristics of **Lok18-** seemed to link the strain to *A. oryzae* species.

Strain	AFs production	Sclerotia	Mating Type	*βtub/Cmd* phylogenetic tree	*norB-cypA* deletion size	*samA-rosA* type	Species ID
Fri2	+	no	Mat1-2	*A. flavus*	S	NRRL3357	*A. flavus*
Pie1	-	yes	Mat1-1	*A. flavus*	S < X < L	NRRL3357	*A. flavus*
AT1	+	yes	Mat1-2	*A. flavus*	L	70S	*A. flavus*
TOϕ	-	yes	Mat1-1	*A. flavus*	L	RIB40	*A. flavus*
Lok03	-	no	Mat1-1	*A. flavus*	-	NRRL3357	*A. flavus*
Lok11	+	yes	Mat1-1	*A. flavus*	L	70S	*A. flavus*
Lok16	-	yes	Mat1-1	*A. flavus*	L	70S	*A. flavus*
Lok18	-	no	Mat1-1	*A. flavus*	S	70S	***A. oryzae***
Lok30	+	yes	Mat1-1	*A. flavus*	L	NRRL3357	*A. flavus*

Table 7: All data obtained were merged to obtain the relevant species of each strain. *Were reported data of AFs and sclerotia production, βtub/Cmd phylogenetic tree, norB-cypA deletion size and samA-rosA type. All strains seemed belong to A. flavus species. LOk18- seemed to link the strain to A. oryzae species.*

2. Development of a LAMP assay for the differentiation of atoxigenic strains

The control of aflatoxin is a complex problem that requires the development of a wide range of control strategies along the food chain, both in the field (pre- and post- harvest stages) and at the consumers level. For this reason, in agricultural-based feeds and foods the regular monitoring of toxigenic mycobiota is a crucial pre-requisite in the development of strategies aimed at containing (even preventing) mycotoxin exposure of feed animals and human population. At the same time, intraspecific biocontrol approaches that take advantage of using atoxigenic *A. flavus* strains rely on the stability and the persistence of the biocontrol agents. In this sense, rapid and reliable methods for the detection of specific strains are highly desirable, since they could provide the opportunity to monitor the occurrence of the biocontrol agent, over time, on target crops in target regions.

This aspect assumes a particular importance for one of our research topics, that was the development of an atoxigenic strain-based technology: driven by the need to obtain a fast method of investigation able to detect Afla- strains (and, possibly, one specific strain) in cereal grains and feed commodities, we addressed to the LAMP assay; if valid, this method would considerably reduce the experimental time to evaluate the persistence of the atoxigenic bio-competitor after the field treatments. With this purpose we tried to develop a LAMP assay build and validated on 27 *A. flavus* Italian strains (11 strains atoxigenic and 16 toxigenic), isolated from maize grains collected in the Po valley, and 15 *A. flavus* Malagasy strains, from Lokobe strict reserve (10 atoxigenic and 5 toxigenic).

2.1. Background

Amongst the various molecular-based methods, **LAMP** (Loop-mediated Isothermal Amplification) was suggested to be a promising tool in the prediction of a potential aflatoxin risk in food and food raw materials, being therefore suitable for high throughput analysis in the food industry. This technology amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions, employing a DNA polymerase from the thermophilic *Geobacillus stearothermophilus* (*Bst* DNA polymerase) and a set of four primers that hybridize to six different parts of the target DNA sequence *(Notomi et al. 2000)*. Additional primers (loop-primers) can be used to increase the efficiency of DNA amplification. Due to its processivity, *Bst* DNA polymerase can synthetize vast amounts of high molecular weight DNA within short time *(Niessen et al. 2014)*. For these reasons, LAMP assays are nowadays frequently applied as an alternative to PCR-based analysis *(Notomi et al.*

2000), and were described in literature as suitable for the detection of bacteria, viruses, fungi and a variety of protists, animals and plants.

This method relies on auto-cycling strand displacement DNA synthesis performed by a DNA polymerase with high strand displacement activity, and on the use of a set of specifically designed two inner and two outer primers. All four primers are used during the initial steps of the LAMP reaction, but later, during the cycling reaction, only the inner primers are employed for strand displacement DNA synthesis. The inner primers (called forward inner primer, FIP, and backward inner primer, BIP, respectively) contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA: one for priming in the first stage and the other for self-priming in later stages. For ease of explanation, the sequences (typically 23–24 nt) inside both ends of the target region for amplification in a DNA are designated F2c and B2, respectively *(Figure 7) (Notomi et al. 2000)*.

Two inner sequences (typically 23–24 nt), located 40 nt from the ends of F2c and B2, are designated as F1c and B1, while two sequences (17–21 nt) outside the ends of F2c and B2 are designated as F3c and B3. Given this structure, the sequences of FIP and BIP were designed as follows:

- FIP contains F1c, a TTTT spacer and the sequence (F2) complementary to F2c.;
- BIP contains the sequence (B1c) complementary to B1, a TTTT spacer and B2.

The two outer primers consist of B3 and the sequence (F3) complementary to F3c, respectively. The mechanism and expected reaction steps of LAMP are illustrated in ***Figure 82***. Inner primer FIP hybridizes to F2c in the target DNA and initiates complementary strand synthesis ***(Figure 82)***. Outer primer F3, which is a few bases shorter and lower in concentration than FIP, slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which can form a looped out structure at one end (structure 4). This single-stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis, leading to the production of a dumb-bell form DNA (structure 6), which is quickly converted to a stem–loop DNA by self-primed DNA synthesis (structure 7). This stem–loop DNA then serves as the starting material for LAMP cycling, the second stage of the LAMP reaction. To initiate LAMP cycling, FIP hybridizes to the loop in the stem–loop DNA (structure 7) and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem–loop DNA with an additional inverted copy of the target sequence in the stem and a loop formed at the opposite end via the BIP sequence (structure 8). Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem–loop DNA (structure 10) and one gap repaired stem–loop DNA with a stem elongated to twice as long (double copies of the target

sequence) and a loop at the opposite end (structure 9). Both these products then serve as template for a BIP-primed strand displacement reaction in the subsequent cycles, a part of which is designated the elongation and recycling step, illustrated in the right half of ***Figure 82***. Thus, in LAMP the target sequence is amplified 3-fold every half cycle.

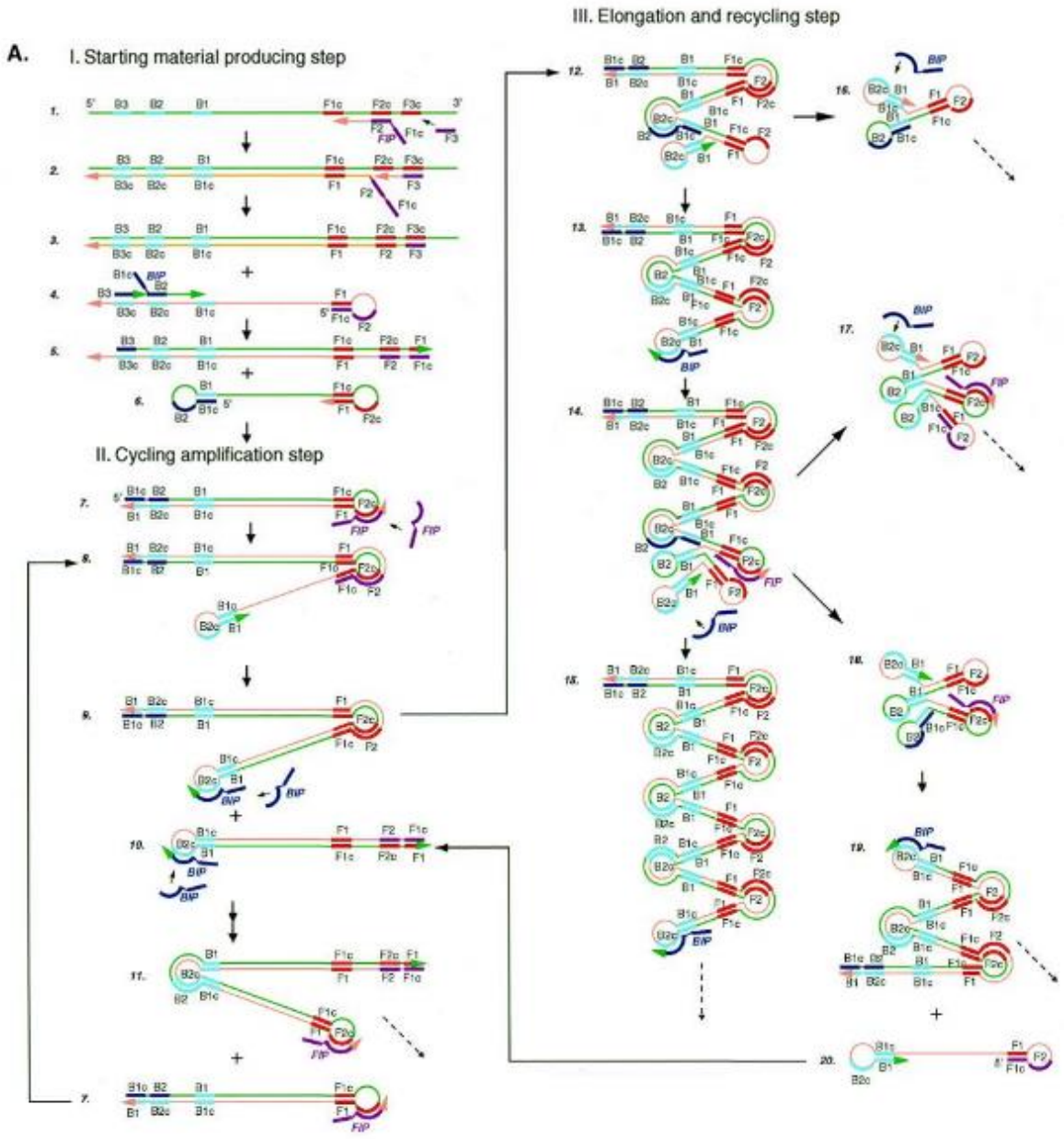


Figure 82: Schematic representation of the mechanism of LAMP. *Steps in the LAMP reaction. This figure shows the process that starts from primer FIP.*

The final products are a mixture of stem–loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the

target sequence in the same strand *(**Figure 82**, structures 16–18)*. The use of four primers (recognition of six distinct sequences) in the initial steps of LAMP and two primers (recognition of four distinct sequences) during the subsequent steps ensures high specificity for target amplification. Moreover, in LAMP four primers (six distinct recognition sequences) are simultaneously used to initiate DNA synthesis from the original unamplified DNA to generate a stem–loop DNA for subsequent LAMP cycling, during which the target is recognized by four sequences. Therefore, target selectivity is expected to be higher than those obtained in PCR and SDA *(Notomi et al. 2000)*.

Signals detection can be attained directly by agarose gel electrophoresis stained with ethidium bromide, and observed as the typical ladder-like pattern of DNA concatemers, resulting from LAMP-mediated DNA amplification *(Gill and Ghemi 2008)*. Recently, alternative 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)*. Calcein was also demonstrated to be affective as a visual indicator, under UV light, as well as neutral red is effective under daylight conditions *(Niessen et al. 2014; 2017)*.

2.2. Individuation of a possible target for the LAMP assay

Aflatoxins are polyketide-derived secondary metabolites produced via the following conversion pathway: acetate → polyketide → anthraquinones → xanthones → aflatoxins. As molecular target, we chose a gene belonging to the aflatoxin cluster gene. The *HexB* gene (also known as *aflB* and *fas-2*), annotated in the genome of *A. flavus* as a fatty acid synthase beta subunit (syn. hexanoate synthase beta subunit), encodes for an enzyme involved in the conversion of acetate to norsolorinic acid, one of the first steps of aflatoxin biosynthetic pathway ***(Figure 83)*** . It is located downstream the *aflD* gene on the aflatoxin gene cluster *(Yu and Chang, 2004)*.

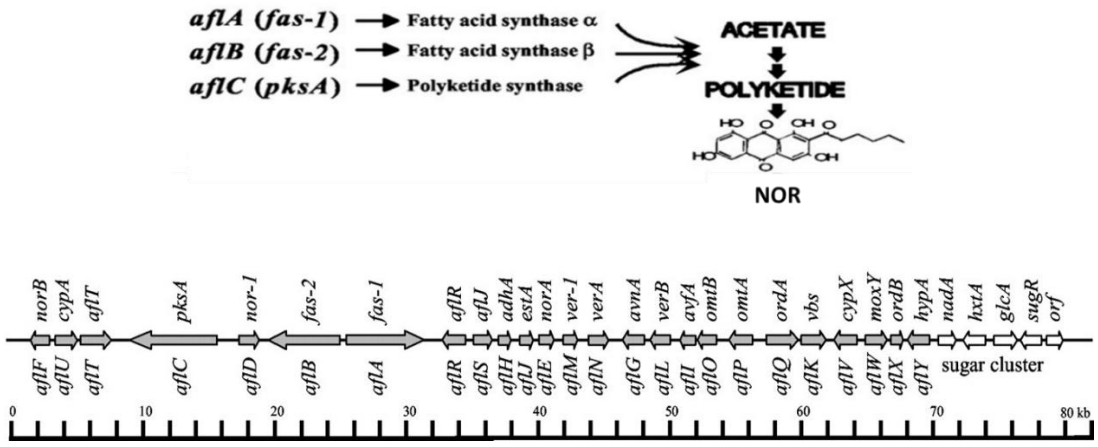


Figure 83: The HexB gene, also known as aflB and fas-2, is annotated in the genome of A. flavus as a fatty acid synthase beta subunit, and encodes for an enzyme involved in the conversion of acetate to norsolorinic acid.

Comparison of *HexB* sequences of various strains, were revealed two nucleotide polymorphisms (SNP). Two SNPs in particular, at nucleotide positions 4167 and 4182 ***(Figure 84)***, were found to be conserved in most *A. flavus* aflatoxin producing and non-producing strains, suggesting the hypothesis that the integration of both SNPs in one of the primers used in the LAMP assay could allow the selective detection of afla- strains.

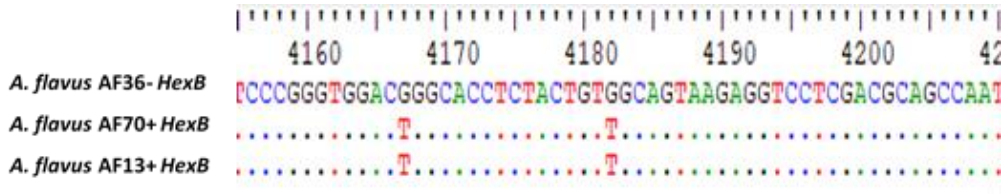


Figure 84: Comparison of the HexB sequences of AF36- strain with the homologs in the aflatoxin producing strains A. flavus AF70 and A. flavus AF13 revealed several single nucleotide polymorphisms (SNP).

2.3. Primers design for LAMP assay and optimization of reaction's conditions

The Work described in the following chapter was performed in order to verify the underlying hypothesis. At first, six primers were designed on the *HexB* gene sequence of *A. flavus* AF36 (see GenBank accession no. AY510455, nt 24822-30667) using the PrimerExplorer software (online version, Eiken Genome site at http://primerexplorer.jp/e/).

The LAMP assay was firstly tested with both neutral red and calcein-based detection methods: reactions were incubated with a temperature gradient ranging from 60°C to 70°C, in order to assess the optimal temperature to discriminate Afla- from Afla+ strains. For this purpose, gDNA from the atoxigenic BS07 and the toxigenic AT1+ *A. flavus* strains was used as template in the assay. The optimal temperature for neutral red-based detection was 69.2 °C. While, for the LAMP reaction with calcein solution-based detection the optimal reaction temperature was 68 °C ***(Figure 85)***.

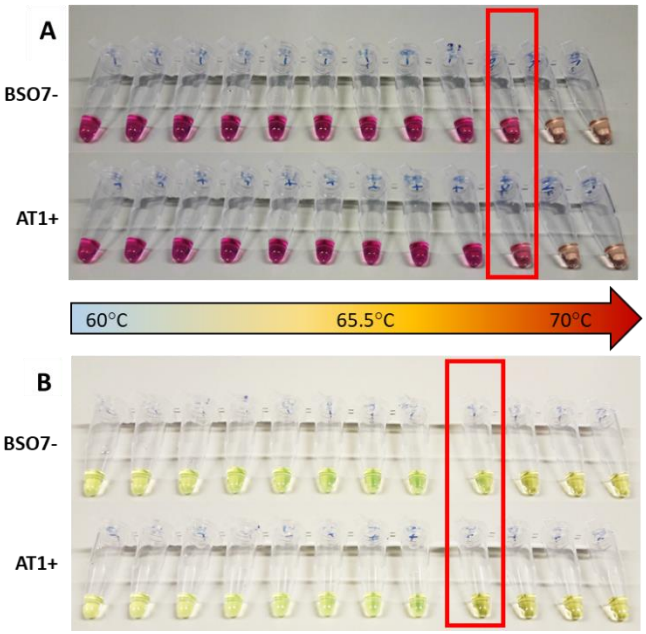


Figure 85: LAMP assay temperature gradient ranging from 60°C and 70°C. *LAMP assay using neutral red to detection (A), and calcein detection solution (B).*

Sarkar in 1990 published that to increase the specificity of PCR reaction we could add DMSO or formamide. In PCR analysis, DMSO or formamide bind to the DNA at the Cytosine residue and changes its conformation which makes the DNA more labile for heat denaturation. This is the reason for the lowering of Tm and increased GC region, since most of the primers are GC rich; DMSO or

formamide indirectly facilitates the annealing of primers to the template this enhances the amplification. These reagents, in particular formamide, can be used also in LAMP Assay because formamide is a simple and inexpensive method for increasing the reactions of LAMP amplification *(Niessen et al. 2017).* Therefore, the addition of formamide was tested.

Formamide could not be used with neutral red-based detection due to an inhibition phenomenon, even with the smallest amount of formamide. Instead, the optimal conditions to discriminate afla+/afla- with LAMP based on calcein signal detection were 0.50 µL of formamide ***(Figure 86)***.

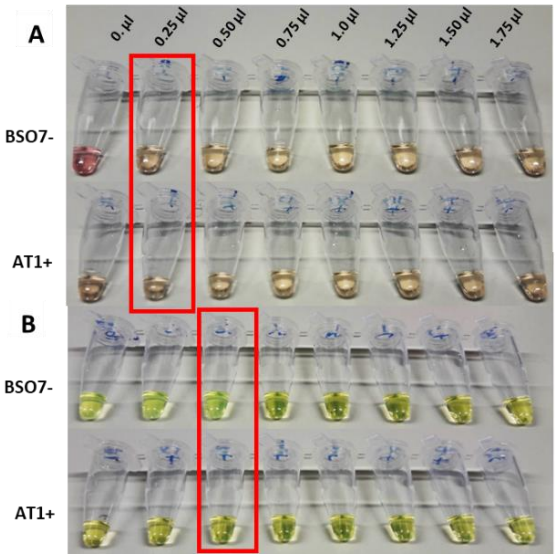


Figure 86: LAMP assay with increasing quantity of formamide. *LAMP assay using neutral red to detection at 69 °C (A), and calcein detection solution AT 68 °C (B).*

Then, we wanted to re-test the LAMP reaction based on calcein signal detection and 0.50 µL of formamide at temperature gradient ranging from 60 °C and 70 °C to identify the optimal reaction temperature that allowed to discriminate afla+/afl-. We identified that the optimal temperature for these conditions was 65.5 °C. Therefore, for LAMP reaction with calcein-based detection the optimal reaction temperature was 65.5 °C with addition of 0.5 µL of formamide per reaction. Instead, for the LAMP neutral red-based detection, the optimal reaction temperature remain 69 °C without formamide addition.

2.4. *HexB* gene analysis

Once the LAMP reaction was optimized for temperature and mixture composition, it was validate on the *A. flavus* strains of the collection. Genomic DNA of all strains was extracted using the method described by *Cenis et al. (1992)*, and the *HexB* sequence was partially checked for the presence of relevant SNPs. A 579 bp fragment of *HexB* gene was amplified with a classical PCR reaction. Obtained fragments, subjected to sequencing, were then checked and aligned: it was observed that almost all afla+ strains possessed a thymine in both the 4167 and 4182 positions, as the reference sequence corresponding to AF70 toxigenic strains; in contrast, afla- strains showed a guanine in the corresponding positions ***(Figure 87)***. Only the two afla- strains TO- and LOK16- shared the two thymines with afla+ strains. To explain this discrepancy, we speculated that these two atoxigenic strains probably were originally aflatoxin producers, but they lost their ability to synthesize aflatoxin due to deletion/mutations at the level of other genes in the aflatoxin cluster (*Degola et al. 2011).*

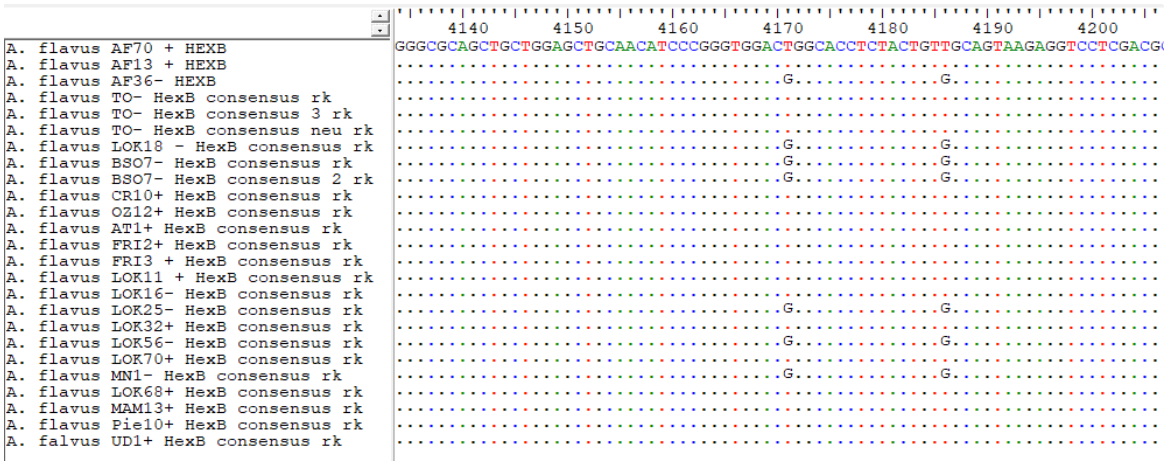
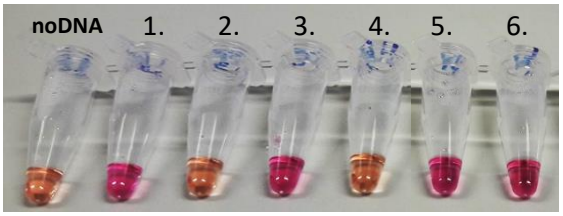


Figure 87: Multiple alignment of partial HexB sequences of afla- and afla+ strains as well as the HexB sequences of the reference strains.

Then, the optimized LAMP assay was validated on all strains (example in ***Figure 88***). All tested afla- strains gave a positive result; positive signals were indicated by a color change of reactions, from yellow to pink, as a result of acidification during DNA biosynthesis. Four afla- strains (Pie1-, CR15-, CSPT7- and MAM03-) resulted negative in the LAMP assay, event expected positive according to their chemotype; this was due to the absence of a HexB gene, as confirmed by a classical PCR reaction. Amongst the twentytwo afla+ analyzed strains, only nine provided a negative assay, as

expected, while 13 gave a positive LAMP result (***Table 8 B1-B2***). This result suggested that the reaction conditions were still not fully optimized for a fully reliable discrimination of afla- strains.

Figure 88.



1. BS07-
2. AT1+
3. OZ18-
4. FRI3+
5. LOK25-
6. MN1-

B1

afla -	HexB gene	LAMP n.red	LAMP calcein
BSO7-	X	X	X
TO -	X	X	X
Pie 1-	-	-	-
CR 15-	-	-	-
OZ 11-	X	X	X
OZ 18-	X	X	X
CSPT 7-	-	-	-
MN 1-	X	X	X
LOM 6-	X	X	X
Fri 7-	X	X	X
MAM 03-	-	-	-
LOK 16-	X	X	X
LOK 18-	X	X	X
LOK 21-	X	X	X
LOK 25-	X	X	X
LOK 30-	X	X	X
LOK 44-	X	X	X
LOK 53-	X	X	X
LOK 56-	X	X	X
LOK 66-	X	X	X

B2

afla +	HexB gene	LAMP n.red	LAMP calcein
SF 2+	X	?	-
Pie 10+	X	?	?
AT 1+	X	-	-
CR 10+	X	?	?
CR 20+	X	?	?
CR 23+	X	?	?
OZ 6+	X	?	?
OZ 12+	X	?	?
CSPT 2+	X	?	?
VEN 8+	X	-	-
UD 1+	X	?	?
Fri 2+	X	?	?
Fri 3+	X	-	-
TN B+	X	-	-
TN G+	X	-	-
MAM 13+	X	?	?
LOK 11+	X	-	-
LOK 26+	X	-	-
LOK 32+	X	-	-
LOK 65+	X	?	?
LOK 68+	X	?	?
LOK 70+	X	-	-

Table 8: Example of positive and negative results of LAMP assay neutral red-based detection (A). Complete list of all A. flavus strains tested in LAMP assay (B). *The A. flavus strains were divided in two table: one for Afla- strains (B1) and one for Afla+ strains (B2). In each table HexB gene expression, sequencing, LAMP assay with both ways detection were reported.*

2.5. LAMP assay tested on potential aflatoxin producers

To determine further the specificity of the afla-LAMP assay built on *A. flavus* strains, reactions were performed under the assay conditions previously described using purified DNA of other other species belonging to Aspergillus generum. A total of fourty-eight species were tested ***(Table 9)***.

n.	SPECIES	LAMP n.red
1	A. *aculeatus*	-
2	A. *arachidicola* +	X
3	A. *auricomus*	-
4	A. *awamori*	-
5	A. *bombycis*	-
6	A. *bridgeri*	-
7	A. *caelatus*	-
8	A. *candidus*	-
9	A. *carbonarius*	-
10	A. *clavatus*	-
11	A. *elegans*	-
12	A. *ellipticus*	-
13	A. *flavus*	-
14	A. *flavus*	X
15	A. *flavus*	X
16	A. *flavus*	X
17	A. *flavus*	X
18	A. *foetidus*	-
19	A. *fresenii*	-
20	A. *fumigatus*	-
21	A. *helicotrex*	-
22	A. *heteromorphus*	-
23	A. *insulicola*	-
24	A. *japonicus*	-

n.	SPECIES	LAMP n.red
25	A. *minisclerotigenes*	-
26	A. *niger*	-
27	A. *niger*	-
28	A. *nomius*	-
29	A. *ochraceus*	-
30	A. *ochraceoroseus*	-
31	A. *oryzae* -	X
32	A. *parasiticus*	X
33	A. *parasiticus*	-
34	A. *parasiticus*	X
35	A. *parasiticus*	X
36	A. *parasiticus*	X
37	A. *parvisclerotigenus*	-
38	A. *penicillioides*	-
39	A. *petrakii*	-
40	A. *pseudotamarii*	-
41	A. *rambelli*	-
42	A. *sojae* -	X
43	A. *tamarii* -	-
44	A. *terreus*	-
45	A. *toxicarius*	X
46	A. *tubingensis*	-
47	A. *usamii*	-
48	A. *versicolor*	-

Table 9: Positive and negative results of LAMP assay. *LAMP assay with neutral red-based detection (A). Complete list of Aspergillus spp. tested (B).*

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. Some results can be discussed: for example, strains such as *A. oryzae* that is conspecific with *A. flavus* and typically does not produce aflatoxins whereas *A. sojae* is conspecific with *A. parasiticus* and is also supposed to be a non-producer of aflatoxins (the domesticated species) were positively detected by the LAMP. Unfortunately, as in the case of *A. flavus*, we obtained positive results also for strains belonging to aflatoxigenic species. Further analysis of the results showed that *A. arachidicola, A. parasiticus* and *A. taoxicarius* are typical producers of aflatoxins and it can be assumed that their *HexB* genes are highly similar to the *HexB* gene in *A. flavus*, hence they show a cross reaction with the primers used.

2.6. LAMP assay tested on *Fusarium* and *Penicillium genera*

Besides Aspergillus spp., also DNA isolated from 16 *Penicillium* spp. and 13 *Fusarium* spp. was tested for cross-reactions in the afla- LAMP assay. It turned out that all tested species of *Penicillium* and *Fusarium* gave no signal in the afla- LAMP assay ***(Table 10)***.

n.	*Penicillium spp.*	LAMP n.red
1	P. *aurantiogriseum*	-
2	P. *camemberti*	-
3	P. *chrysogenum*	-
4	P. *commune*	-
5	P. *corylophilum*	-
6	P. *digitatum*	-
7	P. *expansum*	-
8	P. *glabrum*	-
9	P. *griseofulvum*	-
10	P. *nalgiovense*	-
11	P *nordicum*	-
12	P. *olsonii*	-
13	P. *roseopurpureum*	-
14	P. *rugulosum*	-
15	P. *verrucosum*	-
16	P. *verrucosum*	-

n.	*Fusarium spp.*	LAMP n.red
1	F. *armeniacum*	-
2	F. *armeniacum*	-
3	F. *camptoceras*	-
4	F. *cerealis*	-
5	F. *culmorum*	-
6	F. *equiseti*	-
7	F. *graminearum*	-
8	F. *graminearum*	-
9	F. *langsethiae*	-
10	F. *oxysporum*	-
11	F. *poae*	-
12	F. *sporotrichioides*	-
13	F. *sulphureum*	-

Table 10: Positive and negative results of LAMP assay. *LAMP assay with neutral red-based detection and complete list of Penicillium spp. tested and Fusarium spp. tested.*

2.7. LAMP assay using conidia

The LAMP reaction was also tested using conidia as template that were added directly to the master mix without sample preparation. Results showed that conidia must be washed three times before addition to the assay in order to remove compounds from conidia or from the growth medium that interfere with the assay by turning the assay color to pink even before the LAMP reaction had run. The concentration of conidia/mL was calculated using optical density and a calibration curve. Washed conidial suspensions were serially diluted in water and added to the LAMP assay. All in all, results showed that the afla-LAMP assay based on neutral red signal detection can be used directly with conidia of *A. flavus* pure cultures to discriminate afla+/afla-. Analysis of serially diluted conidia showed that the limit of detection for conidia was $5*10^3$ spores per reaction. The LAMP assay based on calcein signal detection could not be used directly on spores of *A. flavus* to discriminate afla+/afla- in pure cultures ***(Figure 89)***.

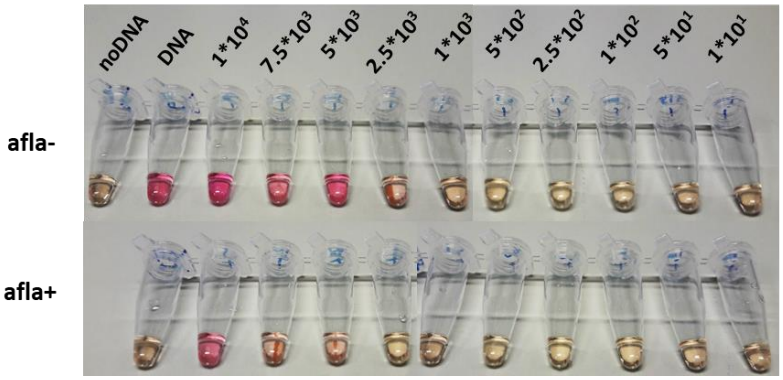


Figure 89: Sensibility of the HexB-specific LAMP assay and different dyes for signal detection using conidia serial dilutions as template. *The LAMP reaction with neutral red-based detection.*

3. Conclusions

The main objective of stage was to design six primers for a LAMP assay and test them for their ability to differentiate between *A. flavus* aflatoxin producers (Afla+) and *A. flavus* aflatoxin non producers (Afla-). The primer-mix was also tested on other Aspergillus species, also on species representing other fungal genera (*Penicillium* and *Fusarium* species).

Results obtained during the current study have showed that the developed LAMP assay could generally discriminate afla- strains from afla+ strains of *A. flavus*. However, the assay needs some further optimization of the reaction conditions in order to exclude false positive results. Moreover, the *HexB* gene sequences of afla- and afla+ strains should be analyzed on the protein level in order to determine whether the disability to produce the toxin is due to the SNPs found during this study or whether there are other reasons for this.

The future step would have been to try to optimize the primers for only two non-toxigenic *Aspergillus flavus* strains (TOϕ and Lok18-), considered excellent bio-competitor strains in the toxin containment strategy.

TOPIC 4:

A. flavus populations and their mycovirome

The attempt to comprehend all the possible biological variables involved in the aflatoxin biosynthesis, and to possibly evaluate their interplay with the intraspecific biocompetition between toxigenic and atoxigenic strains, the ecological perspective should be considered. Amongst these, biotic factors have been highlighted as mostly crucial: the observation that multi-level interaction between microrganisms, through the involvement of both molecular and biochemical targets, are essential to control the success of spreading and survival in the environment by providing adaptive advantages, has long-time been proven. Many reports have demonstrated that the presence of viruses could modulate various physiological parameters in some relevant fungal genera, such as the growth rate, the virulence against the host and the biosynthesis of secondary metabolites. Thanks to the availability of two different population of *A. flavus* strains (that, due to their belonging to very far and different ecological niches and thus being adapted to different environmental biodiversity, might provide a wider opportunity of recording and acquiring the most manifest differences) we investigate the mycovirome's composition that characterize our collection of *Aspergillus flavus* isolated from maize grains sampled in the Po valley (Italy) and the Lokobe strict Reserve (Madagascar).

1. Background

Like all other living organisms, fungi are susceptible to viral infection. Among fungal genera, *Aspergillus* is probably the most investigated due to its clinical, ecological and economical relevance *(Rokas, 2013)*. Viruses infecting fungi are known as *mycoviruses*, and since 50 years ago, when they have been detected in *Aspergillus* species, the research continues to be active, leading to revolutionary discoveries and enhancing the understanding of viral diversity and the significance of virus infection/symbiosis in fungi. The variety of mycoviruses has increased rapidly over the last few years, and this trend is expected to continue, especially due to the development and the widespread use of RNA deep sequencing techniques *(Kotta-Loizou, 2017)*. To date, the International Committee for the Taxonomy of Viruses (ICTV) officially recognizes 17 taxa, 16 families and one genus that does not belong to a family; these accommodate (exclusively or not) mycoviruses with double-stranded RNA linear genomes (dsSNA), positive-sense, single-stranded RNA linear genomes (ssRNA) including reverse transcribing RNA linear genomes, negative-sense ssRNA linear genomes or ssDNA circular genomes ***(Figure 90)***. No mycoviruses with dsDNA genomes have been fully characterized at the molecular level yet. Since the majority of mycoviruses do not have an extracellular phase in their

replication cycle, often their genomes are unencapsidated or non-conventionally encapsidated, and true virions, when present, are proteinaceous in nature. Enveloped virions have not been individuated yet *(Kotta-Loizou, 2017).*

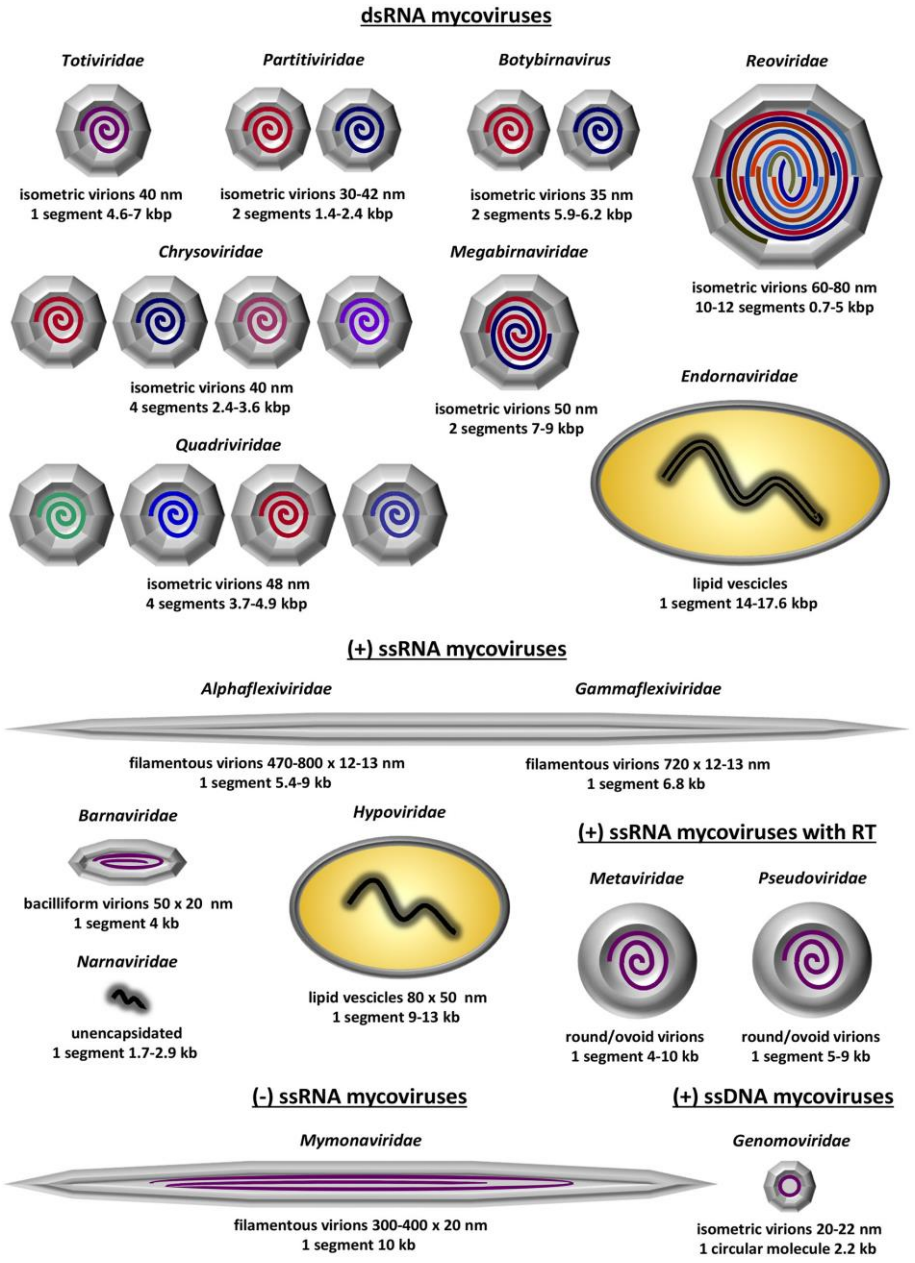


Figure 90: Graphical representation of the 13 established mycovirus families *from Virus Taxonomy: The Classification and Nomenclature of Viruses—The Online (10th) Report of the ICTV; https://talk.ictvonline.org/ictvreports/ictv_online_report/.* ***6 families with dsRNA, 5 families with ssRNA and 2 families containing ssRNA that is reverse transcribed. T****he virion structure and the number of genomic segments for each family are depicted.*

1.1. Evolution and transmission of mycoviruses

Being based on the proposal that RNA mycoviruses do not have any extracellular transmission routes, hypotheses about their origin agree that they should be ancient and coevolved together with fungal hosts. This could also explain why individual mycoviruses are generally limited to a single host species, and hardly show to be transmitted among different fungal species. This lack of extracellular transmission route is attributed to the physical barrier of the fungal cell wall, which generally does not allow the direct uptake of viral particles. Contrariwise, the majority of known mycoviruses spread horizontally via hyphal anastomosis and heterokaryosis, and vertically within the production of fungal spores; in particular, *van Diepeningen (1997)* and *Varga (1998)* reported that in Aspergillus spp. the transfer of dsRNA elements to vegetative conidia is very efficient. On the other hand, how the viruses diffuse from one fungal strain to another and the role of heterokaryon incompatibility between fungal isolates as a barrier to mycovirus spread, is of great interest. *Varga et al. (1994)* demonstrated that viruses can be transferred between isogenic or vegetative compatible *A. niger* strains using polyethylene-glycol protoplast fusion, but occasionally not all the dsRNA elements from the donor were successfully transferred to the recipient. Similar results were obtained by *van Diepeningen et al. (1997),* confirming the restrictions posed by vegetative incompatibility and application of mechanical disturbance of mycelia during the virus transfer via the released cytoplasm between incompatible strains. It was suggested that, when hyphae of two incompatible fungal strains fuse, they recognize each other as non-self, causing the fusion cells death, a type of Programmed Cell Death (PCD). However, in some cases, mycoviruses can transmit to genetically incompatible fungal species under natural conditions. The rejection of heterokaryon is formed mildly with the presence of mycoviruses. It's was believed that some mycoviruses may counteract PCD via suppression of genes involved in PCD activation, preventing the fail of cytoplasmic exchange and resulting in a possible horizontal transmission.

1.2. The incidence of mycoviruses in *Aspergillus spp*. and effects on the fungal host

The presence of mycoviruses in *Aspergillus* species was reported for the first time in 1970, when the *Aspergillus foetidus* strain IMI 41871 and in *A. niger* strain IMI 146891 were found to harbor spherical VLPs containing dsRNA *(Banks et al., 1970)*. These *A. foetidus* dsRNA elements only recently were fully sequenced and characterized, but since the 1980 a large screening of Aspergillus isolates panel revealed the presence of dsRNA elements in other various species. The presence of mycoviruses interests all the main section of Aspergillus genera, and normally cover the 10% of

incidence: for example, when *Elias and Cotty in 1996* screened 92 isolated of *Aspergillus* section Flavi discovered that the 11% was infected by viruses. *Varga et al in 1994* found that, among the 300 isolates of *Aspergillus* section niger collected in Indonesia, Tunis and India, 32 carried mycoviruses (12%). Again, *Van Diepeningen et al. in 2006* demonstrated that in 668 isolated of *Aspergillus* section Nigri collected in worldwide 64 were infected (10%). Over 366 strains of *Aspergillus* section Fumigati were collected in United Kingdom and screened by *Bhatti et al in 2012*, and in 25 of them the presence of viral particles was assessed (8%).

Only few and slight evidences have been provided on the effect that mycoviruses can exert on the fungal host: for example, the infection is proposed to reduce the fungal growth, or modulate the secondary metabolites biosynthesis, or interfere with the sclerotia biogenesis *(Ejmal et al. 2018)*. Recently, *Nerva et al. (2019*) observed for the first time an up-regulation of ochratoxin A in a toxigenic isolate of *Aspergillus ochraceus* transfected with purified mycoviruses from a different *A. ochraceus* strain and from *Penicillium aurantiogriseum*.

On the relation between mycoviruses and aflatoxigenic species, evidences are scarce and contrasting: aflatoxigenic strains were as likely to be infected by dsRNA as non-toxigenic strains, but curing strains infected with dsRNA did not result in altered aflatoxin-producing ability, as occasionally suggested *(Schmidt et al. 1983; Schmidt et al. 1986; Silva et al. 2011).* Due to the high frequency of *A. flavus* in the ecosystem, its serious implications to human and animal health and the limited data on the biological meaning of its interaction with mycoviruses, additional investigations are desirable.

1.3. Detection of Mycoviruses

To identificate mycoviruses, their genomes must be fully sequenced and annotated. This information is crucial for assessing the diversity of the virome in Aspergillus species, since the electrophoretic profiles of the dsRNA elements on agarose gels cannot be reliably used to infer evolutionary relationships with other viruses. *Nerva et al* in their work of *2015* reported how detection techniques evolved over time, from electrophoretic gel to NGS techonolgy. The classic method for the detection of mycoviruses consists of the purification of dsRNA using CF11 cellulose; this simple and cheap technique is able to detect the most of viruses with dsRNA genomes (and, with some extent, also dsRNA replication intermediates from ssRNA genome viruses), and this is probably the reason why the majority of the so far described mycoviruses have a dsRNA genome (Nerva et al. 2015). Another common way of detection is through virus particle's purification using

differential centrifugation protocols combined with observation by transmission electron microscopy (TEM). This approach allows the detection of viruses with different kind of genomes, but requires a traditional virological expertise and expensive equipment, which are not always available in the most common laboratories working on fungal and molecular biology; in addition, this technique is unsuitable for “naked” viruses, a fairly common feature for mycoviruses. The identification of new mycovirus taxonomic groups therefore strongly requires new approaches of diagnosis and detection. In this sense, a new impulse to this research field came from the even more diffused availability of next-generation sequencing (NGS) dedicated protocols; in fact, as reported in a wide number of publications, NGS techniques proved di be able to detect the presence of viral sequences in samples with very low viral titer. For example, *Al Rwahnih et al.,(2011) and Espach, (2013)* analyzed the total RNA extract from plant using NGS techniques, reaching not only to detect the plant viruses, but also to characterize at the same time the whole virome of the fungal endophytes. If applied to total RNA extract, NGS allows the detection of all the different viral taxa because, independently from the DNA or RNA genome, all of them must produce mRNA during their replication cycle. NGS can be applied also to small RNA (sRNA) libraries: *Kreuze et al. (2009)* showed the possibility to assemble new and unknown viral sequences from RNA libraries obtained from plants. To our knowledge, this approach has been used very recently for *de novo* assembly of viral genomes from fungi *(Vainio et al., 2015)*. Nerva et al (2015) reported a complete method of RNAseq.

2. Definition of the mycovirome inside *A. flavus* population from Italy and Madagascar

A selection of 62 *A. flavus* strains, isolated from maize grains collected in the Po valley (35 strains from Emilia-Romagna, 9 from Friuli-Venezia Giulia, 6 from Piemonte, 6 from Veneto, 3 from Lombardia and 3 from Trentino Alto-Adige), and 36 *A. flavus* strains and 5 strains of *A. tamarii* from maize grains collected in the Lokobe nature reserve in Madagascar, was subjected to the analysis of the mycovirome *(**Figure 91**)*.

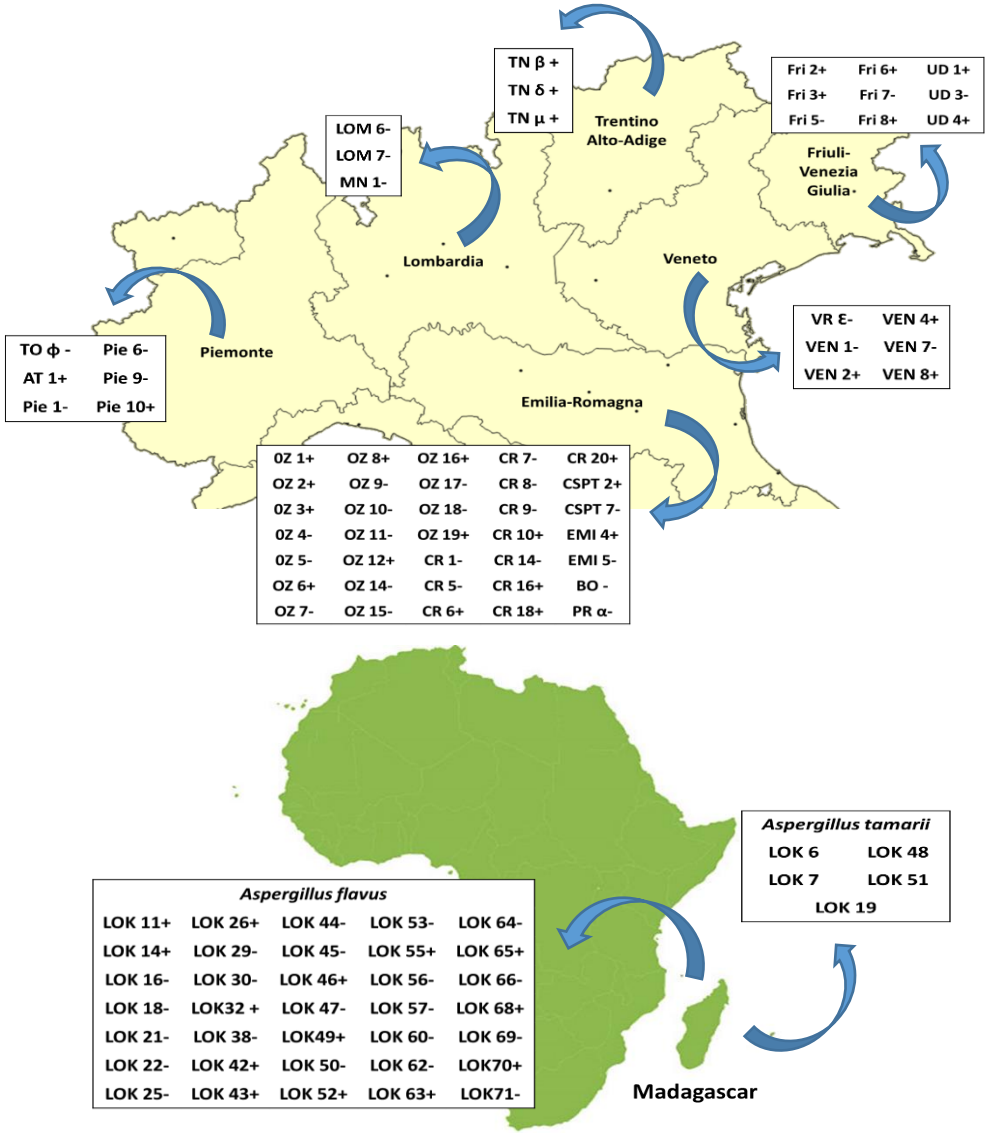


Figure 91: Complete list of Italian and Madagascar strains.

Strains were grown in YES 5% medium in multi-well plates; the total RNA was extracted from single mycelia before conidiation, then put together at the same concentration (1ng) for each sample. RNA mix was subjected to RNAseq analysis. As results of sequence alignment in specific databases, viral dsRNAs have been identified. The presence of strain-specific viruses was assessed in each single

strain subjecting the relevant RNA to reverse transcription and then to Real Time PCR analysis. T***ables 11 and 12*** report the complete list of *A. flavus* strains with the relevant mycoviruses.

MADAGASCAR			
STRAIN	**MYCOVIRUS**	**STRAIN**	**MYCOVIRUS**
LOK 16 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 62 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 18 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 64 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 21 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 66 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 38 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 69 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 44 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 71 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 47 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 30 +	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 50 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 49 +	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 56 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 65 +	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 57 -	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2	LOK 68 +	Aspergillus flavus negative single-stranded RNA 1 Aspergillus flavus negative single-stranded RNA 2
LOK 6	Aspergillus flavus RNA virus 1		
LOK 7	Aspergillus flavus RNA virus 1		
LOK 19	Aspergillus flavus RNA virus 1		
ITALY			
STRAIN	**MYCOVIRUS**	**STRAIN**	**MYCOVIRUS**
CR 1 -	Aspergillus flavus polymycovirus 1	OZ 7 -	Aspergillus flavus flexivirus 1
CR 5 -	Aspergillus flavus ourmia-like virus 1 Aspergillus flavus ourmia-like virus 2 Aspergillus flavus flexivirus 1	OZ 15 -	Aspergillus flavus ourmia-like virus 2 Aspergillus flavus ourmia-like virus 3 Aspergillus flavus flexivirus 1
CR 8 -	Aspergillus flavus ourmia-like virus 2	OZ 11 -	Aspergillus flavus ourmia-like virus 2
CR 9 -	Aspergillus flavus polymycovirus 1		Aspergillus flavus ourmia-like virus 3
VEN 7 -	Aspergillus flavus narnavirus 2	LOM 7 -	Aspergillus flavus ourmia-like virus 2
Pie 6-	Aspergillus flavus narnavirus 2		Aspergillus flavus narnavirus 1
Fri 7 -	Aspergillus flavus partitivirus 2	MN 1 -	Aspergillus flavus narnavirus 1
Fri 3 +	Aspergillus flavus partitivirus 2		Aspergillus flavus clostero-like virus 1

Table 11: Complete list of Italian and Madagascar A. flavus strains with the relevant mycoviruses.

ITALIAN STRAINS VIRUS FREE					
Afla+			**Afla-**		
OZ 1 +	CR 6 +	UD 1 +	Fri 5 -	OZ 14 -	OZ 10 -
0Z 2 +	Fri 6 +	OZ 16 +	UD 3 -	MAM 13 -	EMI 5 -
OZ 3 +	CR 10 +	Fri 8 +	BO -	OZ 17 -	OZ 4 -
OZ 6 +	TN μ +	Pie 10 +	VEN 1 -	TO ϕ -	OZ 5 -
OZ 8 +	CR 16 +	UD 4 +	VR Ɛ -	PR α -	CR 7 -
EMI 4 +	CR 18 +	AT 1 +	Pie 1 -	OZ 18 -	CR 14 -
OZ 19 +	Fri 2 +	VEN 2 +	Pie 9 -	GR -	OZ 9 -
VEN 4 +	TN δ +	TN β +			
VEN 8 +	LOM 6 +				
MADAGASCAR STRAINS VIRUS FREE					
Afla+			**Afla-**		
LOK 11 +	LOK 42 +	LOK 55 +	LOK 22 -	LOK 48	LOK 53 -
LOK 14 +	LOK 43 +	LOK 63+	LOK 25 -	LOK 45 -	LOK 60 -
LOK 26 +	LOK 46 +	LOK 70 +	LOK 29 -	LOK 51	LOK 72 -
LOK 32 +	LOK 52 +				

Table 12: Complete list of Italian and Madagascar A. flavus strains virus free.

As general observation, it could be commented that the 20% of Italian strains and 50% of Malagasy strains resulted infected by mycoviruses. The characterized viral population proved to be heterogeneous, but overall completely different between the two groups of isolates belonging to the two different geographical areas. While in the African group only Aspergillus flavus negative single-stranded RNA 1 and Aspergillus flavus negative single-stranded RNA 2 were detected, Italian strains were found to be infected by several different viruses (polymycovirus 1 ourmia-like virus 1, 2 and 3, flexivirus 1, narnavirus 1 and 2, clostero-like virus 1, partitivirus 2, RNA virus 1, negative single-stranded RNA 1 negative single-stranded RNA 2). *Aspergillus tamarii* strains, included as outgroup, showed to carry only Aspergillus flavus RNA virus 1. Interestingly, data showed that *A. flavus* strains infected by viruses were predominantly non-toxigenic strains: in fact, amongst the 60 isolates only one afla+ resulted infected, whereas 12 afla- were positive to mycoviruses detection. The same also repeats for African isolates: 3 afla+ strains versus 15 afla- were infected.

3. Future perspectives

Once characterized the virome of these two *A. flavus* populations, in the next phase:

1) We will investigate 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. The hypothesis would be to extract RNA from the first 5 generations and test the persistence of the virus. It would also be effective to test its persistence by isolating a single spore and assess its infection;

2) Furthermore, we would like to evaluate parameters such as growth, aflatoxin production and sclerotia biogenesis in naturally infected strains, using as a control the curing virus-infected strain or by introducing the virus into a virus-free strain. Two afla+ strains were already artificially transfected with two different viral strains. The same strains were transfected with H_2O as control. Tests on fungal growth, on aflatoxin accumulation and on the sclerotia production are still ongoing.

3) On transfected strains, we are also evaluating the persistence of specific viruses along successive 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 will be check by Real Time PCR.

Materials and methods

1. Strains, culture conditions and biological assays

1.1. Media for isolation, cultivation, maintenance and biologic assays of *A. flavus* strains

- **Yeast Extract Sucrose medium (YES) 5%:** [2% (w/v) yeast extract (Sigma), 5% (w/v) sucrose (Sigma)]. Components are dissolved in distilled water and the medium is autoclaved at 120°C for 20'. A solid version is obtained by adding 2% (w/v) agar (Difco) before autoclaving.

- **Dichloran-Rose Bengal Chloramphenicol agar medium (DRBC):** this medium is used for the isolation and enumeration of *A. flavus* strains from maize kernels. Each component (10 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 20 g/L agar, 0.025 g/L Rose Bengal, 0.002 g/L dichloran, 0.1 g/L chloramphenicol) is dissolved individually in distilled water. The medium is autoclaved at 120°C for 20'.

- **Clarified Coconut Medium (CCM):** This particular medium allows to detect aflatoxins directly in the growth medium, during the growth of the fungus, as a fluorescence emission when exposed to an UV source at the specific wavelength of 365nm (*Davies et al. 1987*). In 2012, *Degola et al.* developed the CCM, a modified medium based on the use of commercial coconut cream for food use: 400 mL of coconut cream ("Coconut cream", VITASIA, Lidl Stiftung & Co., Neckarsulm) are brought to a volume of 1.2 L with double distilled water. Once the mixture is mixed, a short sterilization in autoclave is carried out (10' at 120 °C). The autoclaved medium is maintained at 4 °C for at least 24 hours. The intermediate (liquid) fraction is recovered in sterility and centrifuged for 15' at 3500 rpm to remove the residual corpuscular fraction.

- **Czapek medium (CZ):** This particular culture medium, poor in nutrients, is used to induce the sclerotia production. Each component (3 g/L $NaNO_3$, 0.5 g/L $MgSO_4 \cdot 7H_2O$), 0.5 g/L KCl, 1 g/L K_2HPO_4, sucrose 30 g/L, agar 1.5%) is dissolved in double distilled water. The pH is adjusted to 6.2. The medium is autoclaved at 120°C for 20'.

1.2. Newly synthesized molecules

- **Ligands and Modifications**

The desired TSs were obtained mixing an equimolar amount of thiosemicarbazide with the appropriate aldehyde in absolute ethanol. A small amount of acetic acid was added to catalyse the condensation. The mixture was refluxed under stirring for 8 hours and left overnight at 0 °C. The precipitate was filtered out, washed with cold ethanol and dried under vacuum.

- **Metal complexes**

The appropriate ligand was mixed with the appropriate metal salt in ethanol using a metal to ligand ratio of 1:2. The mixture was left under stirring at room temperature for 2 hours. Usually a change in the solution colour was observed during the reaction. Finally, the solvent was removed under reduced pressure and the product was washed twice with diethylether, then dried under vacuum.

1.3. Fungal collections

During the last decades, a high number of *A. flavus* strains were isolated from maize kernels collected in different Countries, becoming part of the fungal collection conserved in our laboratory. In particular, various strains were isolated from corn samples belonging to the Po Valley, in Northern Italy, and to the Lokobe Nature Strict Reserve, in the Nosy Be island of Madagascar. The collections include both *A. flavus* aflatoxigenic and non-aflatoxigenic strains. Some of them were previously used and described in various publications *(Degola el al. 2007; 2009; 2011)* Fungal cultures were conserved as sporal suspension and stored on YES-agar slants at −20 °C.

1.4. Preparation of conidial suspension

A. flavus conidial suspensions were obtained from cultures grown on YES 5% agar medium: sections of agar medium were inoculated and incubated for 10-14 days at 28 °C in the dark, inside sterile glass tubes with 500 µL of double-distilled water. When cultures were completely conidiated, the spores were recovered by adding 2 mL of a sterile aqueous solution (0.01% Tween20) and vortexing for 20''; recovered suspensions were filtered with sterile nylon filters (porosity of about 32 µm) to remove fragments of hyphae and residual of medium. The concentration of spores in the conidial

suspension was determined by measuring the optical density (OD_{600}) and using the calibration curve previously built. ***(Figure 92)***.

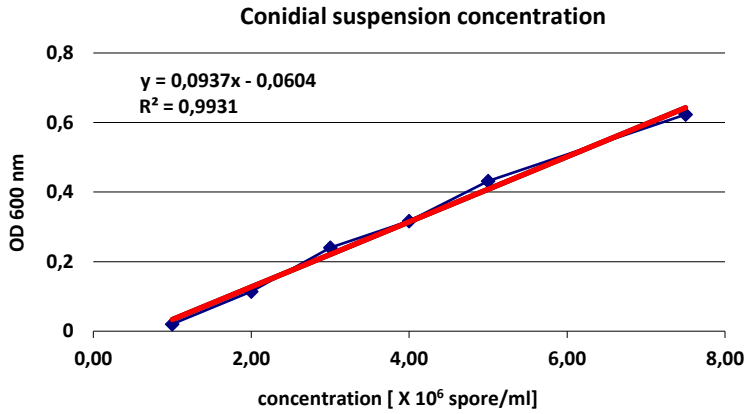


Figure 92: Calibration curve to determine spectrophotometrically the concentration of A. flavus conidial suspension.

1.5. Determination of emission/shielding properties of thiosemicarbazones

Before they could be used in biological assays based on fluorescence, compounds have been evaluated for their possible interference with the detection of AFs in culture medium: molecules must do not emit or absorb fluorescence at excitation (360 nm) and emission (465 nm) wavelengths of aflatoxins. For this purpose, compounds were added, at the final concentration of 50 and 100 µM, to both fresh CCM medium and CCM medium from afla+ cultures (therefore contaminated by aflatoxin); samples were evaluate to determine if the presence of the molecule induced an increase of fluorescence in the first case (emission), or a decrease in the second case (shielding). The fluorescence was measured using a fluorescence microplate reader (SPECTRAFluor PLUS, TECAN). Compounds that proved to emit or absorb fluorescence more than 20% in the test conditions were discarded. However, for promising compounds, a semi-quantitative dosage of aflatoxin was conducted by Thin Layer Chromatography (TLC).

1.6. Germination/early growth assay

The first stage of mycelium development was evaluated using a multi-well plate system (Tissue Colture Plate 96-wells Flat Bottom with Lid, Sarstedt, USA). *A. flavus* cultures were prepared inoculating in each well $5x10^3$ spores in a final volume of 200 µL of YES 5% liquid medium, amended (or not) with the molecules test; in control cultures, DMSO was used at the same volume added in cultures treated with thiosemicarbazones. The inoculated microplates were incubated at 28 °C in stationary conditions, in the dark. The optical density was measured after 46-48 hours from the inoculum at the wavelength of 620nm, using a microplate reader (MULTISKAN EX, Thermo Electron Corporation, Finland). For each well, the OD values of treated cultures recorded immediately before incubation were subtracted to OD values recorded after incubation; then, such normalized values were compared to the untreated cultures (control). Molecules were tested at increasing concentration ranging from 10 to 100 µM; four replicates were performed for each condition, and experiments were conducted in triplicate. Data were expressed as percentage of growth inhibition respect to control.

1.7. Aflatoxin accumulation assay

A high-throughput method for the detection and the quantitation of aflatoxin accumulated in the culture medium was used. This assay, based on the emission of the natural fluorescence of AFs when excited by an UV source at a specific wavelength, was described in 2012 by *Degola et al*. Spores suspensions ($5x10^2$ spore/well) of a producing (afla +) and a non-producing strain (afla) were inoculated, in a 96-well microplate, in a final volume of 200 µL of CCM medium supplemented with the different compounds at increasing concentrations (10-100 µM). In control cultures, DMSO was used at the same volume added in cultures treated with thiosemicarbazones. Plates were incubated in static conditions at 25 °C in the dark for 6 days. The amount of aflatoxin released in the medium was detected by a fluorescent microplate reader (SPECTRA Fluor PLUS, TECAN), set according to the following parameters: λ_{ecc} = 365 nm; λ_{em} = 460 nm; gain = 83; bottom reading; n ° flash = 3; integration time = 200 µsec. The fluorescence values corresponding to afla+ cultures were normalized by subtracting the emission values of the corresponding afla- cultures. Four replicates were performed for each condition, and experiments were conducted in triplicate. Data were expressed as percentage of aflatoxin inhibition respect to control.

1.8. Biogenesis of sclerotia

Effect on the development of mycelium was measured through the analysis of sclerotia biogenesis. Sclerotia are vegetative structures formed by undifferentiated hyphae and produced by some strains under specific environmental conditions, being strategic for the diffusion of the fungus in the environment *(Cotty, 1989; Abbas et al. 2005)*. Czapek medium (CZ) was poured in Petri dishes (Ø = 5 cm) amended with compounds at the final concentration of 50 µM (DMSO 0.5% v/v in the control plates). Spots of 5 µL of a 10^4 spore/mL of conidial suspension from an *A. flavus* hypersclerotigen strain CR10+ were inoculated on the surface of the solid medium; after incubation at 28 °C in the dark for 7-10 days the sclerotia were manually recovered from the surface of the cultures, washed once with a solution of H_2O and Tween (0.1% v/v) and then twice with 70% ethanol, to remove residues of mycelium, medium fragments and conidia. Finally, sclerotia were dried at 40-50 °C for 2-3 days, then weighted. Data were expressed as percentage of inhibition respect to control.

2. Molecular analyses

2.1. DNA extraction

Total DNA was extracted from four-day-old mycelia using DNAzol™ reagent (Invitrogen™). A total of 200 mg of mycelium from YES-liquid culture were frozen in liquid nitrogen and ground to a powder with an Amalgamator model TAC 200/S (Linea TAC s.r.l., Asti, Italy; oscillation frequency: 4200 strokes/min). Genomic DNA was extracted according to the manufacturer's instructions: 300 µL of DNAzol™ were added to the samples and grounded three times. After 5' at room temperature, 300 µL of chloroform were added, then samples were vortexed 10'', incubated at room temperature for 10 min and centrifuged at 12000 rpm 4 °C for 10'. gDNA was precipitated by adding 225 µL of EtOH 96% to the recovered supernatant. Then stay on ice for 5' and centrifuge at 7000 rpm 4 °C for 5'. The gDNA, precipitated in the pellet, was washed two times using 300 µL of a mix of EtOH 96% and DNAzol™ (0.75:1). Samples were dried it completely, then resuspended in 50-100 µL of TE + RNAse. Genomic DNA of samples is quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The quality of extracted DNA was checked via electrophoresis in an 1% agarose gel.

2.2. Classic PCR analysis

Standard PCR analyses were performed in 20 µL of PCR Flexi Buffer 5X (Promega) containing 1 ng of gDNA as template, 25 mM $MgCl_2$, 0.25 mM of each dNTP, 10 µM of each primer and 0.5 U of Taq DNA-polymerase (GoTaq®, Promega). The cycling parameters were set as follows: 4' at 94 °C; for 5 cycles: 1' at 94 °C, 1' at 60 °C and 1' at 72 °C; for 30 cycles: 1' at 94 °C, 1' at 55 °C and 1' at 72 °C; final extension at 72 °C for 6' *(Degola et al 2009)*. Primers used were reported in ***table 14***. Amplification products were visualized on a 2% agarose gel in TAE buffer The dimension of amplification products was determined by comparing signals with a molecular weight marker.

2.3. RAPD-PCR analysis

RAPD-PCR (Random Amplification of Polymorphic DNA) analysis is a polymerase chain reaction conducted at a low stringency in which the same oligonucleotide is used in the reaction as forward and reverse primer. Typically, the result of this amplification is a multi-signal profile as it can be visualized on an agarose gel. In this work, the degenerated oligonucleotide 5'-GAGAGAGAGAGAGAGAYG-3' was used.

GoTaq® DNA polymerase Kit was used and its protocol was followed. PCR was performed in 20 µL of PCR Flexi Buffer 5X (Promega) containing 1 µL of gDNA diluted 1:50 as template, 25 mM $MgCl_2$, 25 mM of each dNTP, 10 µM of primer and 0.5 U of Taq DNA-polymerase (GoTaq®, Promega). The cycling parameters were: 4' at 94 °C; for 35 cycles: 1' at 94 °C, 20'' at 44 °C and 2' at 72 °C; final extension for 6' at 72 °C. The RAPD-PCR pattern of afla- isolates were visualized on an 1% agarose gel.

2.4. LAMP assay

LAMP (loop-mediated isothermal amplification) is a molecular technology that 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)*. Six primers were designed on the *HexB* gene sequence of *A. flavus* AF36 (see GenBank accession no. AY510455, nt 24822-30667) using the PrimerExplorer software (online version, Eiken Genome site at http://primerexplorer.jp/e/) (***Table 13***).

PRIMER	primer sequence (5'→ 3')
FIP (Forward inner primer)	5'-GAGGGAAACACGTGCGTAGGATCGGGCACCTCTACTGTGG-3'
BIP (Backward inner primer)	5'-CCTTTGCGCGATACGCTGGTCGGCAATTCTGCGCATCA-3'
F3 (Forward)	5'-GCTGGAGCTGCAACATCC-3'
B3 (backward)	5'-GTCTGCATCTCCGATGGC-3'
LF (loop-primer Forward)	5'-GCGTCGAGGACCTCTTA-3'
LB (loop-primer Backward)	5'-CTCGGGTCCCATTGTCCAT-3'

Table 13: Complite list of LAMP primers.

The LAMP master mix for neutral red-based detection was set up containing the following components per 25 µL of reaction volume: 2.5 µL of 10x ammonium sulfate buffer, 1 µL of $MgCl_2$ 200 mM, 3.5 µL of dNTP-mix (10 mM each GACT), 2.5 µL of primer-mix (80 µL FIP and 80 µL BIP, 20 µL

LF and 20 µL LB, 10 µL F3 and 10 µL B3), 8.5 µL of sterile deionized water, 1 µL of *Bst* DNA polymerase and 1 µL of neutral red 2.5 mM. Five nanogram of genomic DNA in 5 µL aqueous solution per reaction. Reactions for calcein-based detection contained the same ingredients in the same concentrations but were prepared with 2.5 µL of 10X MOPS buffer (200 mM MOPS, 100 mM KCl, 100 mM (NH4)2SO4)) and 1 µL of 3mM calcein detection solution was added to the master mix from a 50% glycerol stock (-20 °C). LAMP reactions were incubated in a mastercycler Gradient thermal cycler for 60 min (*Niessen, 2017*).

2.5. RNA extraction and cDNA synthesis

Mycelium of *A. flavus* cultures incubated in wells of a microplate at 25 °C for 72 hours with substance and DMSO as a control was used. Three mycelia (approximately 50 mg) per sample were frozen with liquid nitrogen and pulverized with a micro pestle directly in Eppendorf. The TRIzol® protocol (Life Technologies, Invitrogen) was followed. After the addition of 0.5 mL of TRIzol® reagent and a incubated for 5' at room temperature (RT), 140 µL of chloroform is added to the samples and vortexed for 15''. After 10' of incubation at RT, samples were centrifuged at 12000 rpm for 15' at 4 °C. The RNA present in the supernatant aqueous phase of the centrifuged samples is then recovered, transferred to a clean eppendorf and precipitated with 0.334 mL of isopropanol. 10' of incubation at RT and centrifuge at 12000 rpm for 15' at 4 °C. The supernatant is discarded and 0.670 mL of 75% ethanol are added to the pellet. We proceed to detaching the pellets from the bottom of Eppendorf to allow a better washing and then, we perform a spin at 7500 rpm for 5' at 4 °C. The excess ethanol is pipet off and the whole is dried at room temperature and finally suspended in 75-100 µL of water treated with DEPC followed by an incubation for 10' at 55-60 °C. RNA concentration is estimated using NanoDrop 2000 (ThermoFisher scientific). The quality of the extracted RNA is checked on 1% agarose gel (250 mg of agarose powder dissolved in 0.5 mL of TAE 50X + 25 mL of DEPC water; running buffer: 8 mL of TAE 50X diluted in 400 mL of sterile two-distilled H_2O). 2 µL of RNA samples are added to 6 µL of H_2O DEPC and 1µL of EtBr. Heat the mix for 5 minutes at 65 °C, transfer in ice and briefly centrifuge. Finally add 1µL of RNA Loading Buffer (LB) and load 10 µL in gel electrophoresis. Run at 100V for about 40' and check with UV chamber.

DNAse treatment: To purify RNA from genomic DNA contamination, the samples were treated with DNase I (Amp Grade, Invitrogen), as described by the manufacture. 2 µg of RNA were incubated with 1 µL of 10X DNase I Reaction Buffer and 1 µL of DNase I, Amp Grade, 1U/µL and H_2O DEPC up

to 10 µL total. Incubate tube for 15’ at room temperature. DNase I was inactivated by the addition of 1 µL of 25 mM EDTA and heating 10’ at 65 °C. The RNA samples were ready to use in reverse transcription, prior to amplification. The inactivation of the enzyme is guaranteed by the chelating activity of EDTA against the bivalent ion, but the same activity could then interfere with the subsequent amplification reaction by DNA polymerase. Therefore, the chelator was removed from samples treated by precipitation in ethanol. To each samples were added: H_2O DEPC up to 50 µL, 1 µL of Glycogen, 5 µL of CH_3COONa 3M and 150 µL of Ethanol 96%. Samples were immersed in liquid nitrogen and stored at -80 °C for one hour or -20 °C. Samples were centrifuge at 10’ at 7500 rpm, supernatants were pipet-off and discarded and the pellets were washed with 200 µL of EtOH 70%. After centrifuge, dry the pellets at RT. Then, pellets are suspended in 50 µL of H_2O DEPC.

Reverse-transcription: The synthesis of cDNA, starting from RNA extracted, was obtained using GoScript™ Reverse Transcription Mix, Oligo(dT) (Promega, Madison, WI, USA) according to the manufacturer: 2 µg of total RNA for each sample were pre-heated 5 min at 70 °C and were added to 10 µL of GoScript™ Reverse Trascription Mix [GoScript™ Reaction buffer, oligo(dT) and GoScript™ Enzyme Mix] and H_2O (diethyl pyrocarbonate treated) to 20 µL. The reverse transcription reaction was run in a DNA thermal cycler, in the following conditions: 25 °C for 5 min, 42 °C for 60 min, and then at 70 °C for 15 min. Obtained cDNAs were diluted 1:10 then used in Real Time amplification reactions.

2.6. Real Time PCR (qPCR)

SYBR® green BIORAD Kit was used and its protocol was followed. Real Time PCR reactions were performed in 10 µL of final volume of reaction in Sybr green BIORAD® 2X. 12.5 µM of primers Forward and Reverse were added to the mix solution. 1 µL of cDNA diluted 1:50 in reaction was added as template. The cycling parameters were: 2’ at 50 °C; 10’ at 95 °C; for 40 cycles: 15’’ at 95 °C, 1’ at 60 °C; dissociation curve 15’’ at 95°C; 1’ at 60 °C, 15’’ at 95 °C; and final hold at 4 °C. List of primers in ***Table 14*** and ***15***.

2.7. Primer lists

Primers used for the analysis of *A. flavus* (Topic 1, 2 and 3) ***(Table 14)***.

primer code	gene	accession number	primer sequence (5'→ 3')	PCR product size (bp)	cDNA PCR product size (bp)
Nor-F (a)	***AflD***	AY510455	ACGGATCACTTAGCCAGCAC	986	
Nor-R (a)			CTACCAGGGGAGTTGAGATCC		
AflF-F	***AflF***	AF139440	ATGCACGCCCTGAACGATTTG	359	
AflF-R			AACAGCGGCACACCCTTAGAG		
OmtB-F (a)	***AflO***	AF159789	GCCTTGACATGGAAACCATC	1304	1131
OmtB-R (a)			CCAAGATGGCCTGCTCTTTA		
AflF-F (c)	***AflR***	L32576	ATGCACGCCCTGAACGATTTG	1032	300
AflR-R (b)			CCGTCAGACAGCCACTGGACACGG		
AflJ-gF (c)	***AflS***	AF077975	GAACGCTGATTGCCAATGCC	1399	1256
AflJ-giR (c)			CGGTCAGGATGTTACTAAGC		
AflJ-cR (c)			GACTGGGCGCCCACCGTTGC		598
AflU-F	***AflU***	AF139430	GCAAGTACGGTCCCATTGTTC	365	
AflU-R			CCGCGAGATGGTTCAAGTTCG		
dmtA-F (e)	***dmtA***	AF139220	AAGAGGCGGCGACAGTATA		213
dmtA-R (e)			GATAGGTAGAGGTTGCGTGTTC		
mtfA-F (e)	***mtfA***		GCATCGACATATGCTGCAAAG		256
mtfA-R (e)			GGCGCAAAGGTGGAGTAGG		
NsdD-F (e)	***NsdD***	AF020210	GTCACGACTCAAGATTCGC		168
NsdD-R (e)			ATGCTGCCCGCAGAAGTAG		
NsdC-F (e)	***NsdC***	AF051390	CGAGAAGCAACAACTAAATACAAT		154
NsdC-R (e)			TTGCAACTGTGGAGGTATTC		
VeA-F	***VeA***	AF066460	CGAGACGGAAGCCTCCGT		358
VeA-R			TGGAGGATCGACTGGACGA		
Tub1-F (a)	***Tub1***	M38265	GCTTTCTGGCAAACCATCTC	1406	1198
Tub1-R (a)			GGTCGTTCATGTTGCTCTCA		
MAT1.1 F			ATTGCCCATTTGGCTTTGAA	636	
MAT1.1 R			TTGATGACCATGCCACCCGA		
MAT1.2 F			GCATTCATCCTTTATCGTCAGC	636	
MAT1.2 R			GCTTCTTTTCGGATGGCTTGCG		
AP1729 (d)	***NorB-CypA***		GTGCCCAGCATCTTGGTCCACC	1822	
AP3551 (d)			AAGGACTTGATGATTCCTC		

Table 14: *Primers marked as 'a', 'b', 'c', 'd' and 'e' were selected from those used in previous works ('a', Schermet al. 2005; 'b', Criseo et al. 2001; 'c' Degola et al. 2006; 'd', Ehrlich et al. 2004 and 'e' Li el al. 2017).*

Primers used for the *A. flavus* mycovirome characterization (Topic 4) ***(Table 15)***.

Primer name	nucleotide sequences
Aspergillus flavus polymycovirus 1	Fw: 5'- CGGCGTGCTCTGGACTCCTG -3' Rev: 5'- TCACACATCATGGCGCG -3'
Aspergillus flavus ourmia-like virus 1	Fw: 5'- TCTTAACCCAGCCCGCCGTG -3' Rev: 5'- GTTTTGCGAGACGAGGCGGT -3'
Aspergillus flavus ourmia-like virus 2	Fw: 5'- GGGCTGGAGGGGCGAAACAA -3' Rev: 5'- GGTCGGCGGGATCTTCGGAC -3'
Aspergillus flavus ourmia-like virus 3	Fw: 5'- CCTCGGGCTTCCTCGACTGG -3' Rev: 5'- GGTTGCCTGTCACAAAGGCGG -3'
Aspergillus flavus flexivirus 1	Fw: 5'- CGTCTGGAGAGGGACCCGGT -3' Rev: 5'- CCGCCCCTCAACCCCTTACG -3'
Aspergillus flavus narnavirus 1	Fw: 5'- AACAGCCCCTTCCAAGCCGG -3' Rev: 5'- GCCTCTCCCCGTTACGCAGG -3'
Aspergillus flavus narnavirus 2	Fw: 5'- GTCGCCTGCTCCCAATCCGT -3' Rev: 5'- GTGCCGCTAATCACGCTTGGA -3'
Aspergillus flavus narnavirus 3	Fw: 5'- AGCAGCTTCTCCGGAAAGCC -3' Rev: 5'- CCTCACGGCTAGGGATTGCT -3'
Aspergillus flavus clostero-like virus 1	Fw: 5'- GGTGGCTAACTGGTCGCGGT -3' Rev: 5'- GTACCGCCGGCCATTCGAGA -3'
Aspergillus flavus partitivirus 2	Fw: 5'- AAACAGATTATCACGGACGAGATG -3' Rev: 5'- TCCTCATGTGATTCGACCAGAT -3'
Aspergillus flavus RNA virus 1	Fw: 5'- CTCGGAGTCCCAGTACGCAG -3' Rev: 5'- GACGAAAGGTGCACGCGAAT -3'
Aspergillus flavus negative ssRNA 1	Fw: 5'- TTGGGGCCATCTTCTCCACG -3' Rev: 5'- CTCGTCTCTGAGCGCATCGA -3'
Aspergillus flavus negative ssRNA 2	Fw: 5'- CCCTCAACCAAAGCCGCTTC -3' Rev: 5'- CCAAGGAGCCGAGGAGACAG -3'

Table 15: Complete list of primers used in Mycoviroma Project.

3. Proteomic analysis

3.1. Extraction and dosage of total mycelial proteins

For the extraction of the total proteins, Mycelium of *A. flavus* is used after 96 hour of incubation in microplate cultures in CCM and treated with compounds. Equivalent cultures treated with 0.5% DMSO are used as a control. About 150 mg of mycelium were collected and frozen in liquid nitrogen and pulverized with a micropestle directly in Eppendorf. 200 µL of Extraction Buffer (80 mg of Tris HCl 50 mM, pH 7.5; 4.2 g of Urea 7 M; 1.52 g of thiourea 2 M; 100 mg of DTT1%; 200 mg of PVP 2%; 200 µL of Triton X-100 and H_20 to 10 mL. Before using: 2 µL/mL of β-mercaptoethanol and 40 µL/mL of PMSF 100mM) were added to each sample. Samples were centrifuged twice for 20' at 13000 rpm at 4°C, then the supernatant was recovered and transferred to a clean Eppendorf and immediately subjected to analysis or stored at -80°C. For the quantification of the total protein concentration of the extracts, the Bradford colorimetric method was used; the calibration curve was built on BSA stadards (1 - 0.5 - 0.1 mg/mL).

3.2. Two-dimensional analysis of the electrophoretic profile (2D-E)

Precipitation phase: The extracts obtained as described above are precipitated by adding 100 µL of TCA 45% every 300 µL of sample. Samples are vortexed for 10'' each one and incubated on ice for 10' and then centrifuged for 15' at 13000 rpm at 4°C and discard the supernatant. Pellet was washed with 300 µL of cold acetone (-20°C) and incubation of 15' at -20°C. After 15' of centrifuge at 13000 rpm at 4°C, pellets are finally brought to dry in SpeedVac concentrator (at least 30') and rehydrated with 70 µL Rehydration Buffer (Urea 8 M + CHAPS 2% + DTT 2%). The total proteins concentration was determined using the Bradford method.

Isoelectrofocusing (IEF): BioRad ReadyStrip®IPG linear strips (pH 3-10 and pH 5-8) of 7 cm were used. 120 µg of total proteins with 1.25 µL of ampholytes (BioLite Ampholyte, pH 3-10; Bio-Rad) and 1 µL of LB (Laemmli) were brought with Rehydration Buffer at a total volume of 125 µL. Once the entire volume of the sample has been distributed along the surface of the gel side of the strip, the whole strip is then covered with mineral oil and left in passive overnight rehydration (max 16 h) at 20 °C. The next day the strips are placed on the electrodes (Bio-Rad PROTEAN® IEF CELL) according to polarity for the separation of the protein species according to the pI. Focusing parameters were set according to the BioRad protocol for the type of strips used (50 µA / strip up to 14000 V / h). At

the end, the strips were incubated in agitation for 10’ in 2.5 mL of Buffer 1 (Equilibration Buffer [Urea 6 M, Tris HCl 0.375 M pH 8.8, SDS 2%, glycerol 20%] + DTT 2%) and for 10’ in 2.5 mL of Buffer 2 (Equilibration Buffer + Iodoacetamyde 2.5%) for a reduction/alkylation treatment.

Second dimension electrophoresis and staining: Strips were briefly washed in Laemmli Buffer 2X (Tris-Gly 2X + SDS 0.1%), positioned on the upper side of 12% denaturing polyacrylamide gels (Mini-PROTEAN® TGX ™ Precast Gels , Bio-Rad) and fixed to the gel with 1 mL of a 0.5% agarose solution and blue tracer. The run was conducted for 30' at 200 V in a Mini-PROTEAN Tetra Cell (Bio-Rad), following the manufacturer’s instructions. Separated gels were stained for 4-5 h in SYPRO® Ruby Protein Stain (Bio-Rad), then, acquired with Molecular Imager® VersaDoc™ (Bio-Rad).

Image processing for the identification of differentially expressed spots: 2D gels images were acquired and processed by PDQuest™ Advanced software (Bio-Rad) version 8.0.1. The volumetric normalization of the spots was obtained according to "total quantity in valid spots"; the comparison was carried out on 3 replicates (3 gels) per sample, considering significant volume variations between identical spots at least 2 fold with respect to the control, and only for the spots present in at least 2 of the 3 replicas. The Student's t-test ($p \leq 0.05$) was used for the statistical analysis. The total of the differential spots was therefore obtained from the Boolean intersection between the significantly more / less abundant spot category and the statistically different spot category. Once the differentially expressed spots have been identified, a map is constructed for their recovery.

Recovery of spots and identification of protein species: After the first coloring with SyproRuby®, allows a second coloring of the gel with Coomassie Blue, provided that the excess of the fluorophore is carefully removed. Therefore the gel is immersed in an abundant volume of Washing Solution (MetOH 10% + CH_3COOH 7%), left under stirring 30', rinsed with bidistilled H_2O and incubated overnight, in Coomassie Blue Staining (115 mg of Coomassie Blue R-250 in 100 ml of 30% MetOH solution [alternatively use EtOH] + 10% CH_3COOH). At the end of the incubation the gel must be transferred to a clean tray and, after removing the staining solution, it is immersed in a destaining solution (30% MetOH + 10% CH_3COOH) and left in agitation for the time necessary for the discoloration lead to a contrast. The recovery of the spots was carried out manually, cutting circular sections of gel using sterile and cut-to-size micropipette tips. The differential spots identified by the analysis of 2D maps were recovered from gels of each condition. Identification of proteins corresponding to recovered spots was obtained by MALDI-TOF / TOF mass spectrometry at an external service.

4. Chemical assay

4.1. Thin Layer Chromatography (TLC)

Thin Layer Chromatography is based on the principle of separation of different compounds and helps the visualization of separated compound spots easily. Broth samples from 6 days CCM-microplate cultures amended with compounds were centrifuged for 5' at 12000 rpm to remove spores and residual of mycelium. Aliquots of 2 µL from each samples were spotted on a aluminium silica gel plate, then the run was conducted in a closed chamber plate in which the plate was dipped; a mobile phase composed of 80% toluene, 15% methanol and 5% acetic acid was used. AFB_1 standard was used as control. Plates were exposed to a 360 nm transilluminator for the Uv detection of signals.

4.2. 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity assay

Measurement of scavenging activity of thiosemicarbazones against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was performed measuring the bleaching of a purple-colored methanol solution of the stable DPPH radical. 4 mL solution containing the TS was added to 1 mL aliquot of freshly prepared 85 µM DPPH. The resulting mixture was stirred and incubated at room temperature for 30'. Compounds were tested at the concentration of 75 µM. The decrease in absorbance measured at 518 nm depicts the scavenging activity of the compound against DPPH. As positive control ascorbic acid (30 mM) was used to determine the maximal decrease in DPPH absorbance *(Choi et al. 2002).* Values were expressed as percentage of inhibition of DPPH absorbance in relation to the control values without the test compound (ascorbic acid maximal inhibition was considered 100% of inhibition).

5. Statistical analysis

For statistical analyses, one-way analysis of variance (ANOVA) was used in the Past 3.x software *(Hammer et al. 2001)*. Results of mycelial growth, aflatoxin accumulation, sclerotia production and scavenging activity assays, when expressed as percentage of inhibition, were analyzed by Kruskal-Wallis test; differences were considered significant at $p < 0.001$. Other data were analyzed through Student's t test; differences were considered statistically significant at $p < 0.05$.

6. Experimental fields setting for biocompetition strategy

6.1. Preparation of spore suspension for experimental field treatment

A high amount of spores was required to treat two plots (biocompetitor treatment and organic treatment). For this reason, we had to find a method that would allow us to have a high quantity of spores of the biocompetitor afla- strain (BC), respecting the idea of the project (green and low environmental impact). For this reason, we used plastic bottles for food use. In two years, a total of 200 bottles was used (50 bottles per field). The Parmalat dairy company kindly donated the bottles. The plastic bottles were sterilized using a solution with sodium hypochlorite (NaClO) *(**Figure 93-A**)*. After 4 hours they were washed with autoclaved deionized H_2O. After that, a thin layer of agar solid medium was created inside the bottles: were added 100mL of still-liquid medium and the bottles were rolled on ice to allow rapidly solidifying on the inner walls of bottles *(**Figure 93-B**)*. The BC was inoculated on the thin layer of agar solid medium into the bottles. Bottles were incubated at 28 °C for 30 days, until the cultures were completly matured *(**Figure 93-C**)*.

Figure 93.

The day before the treatment, the spores were recovered using a sterile solution of H_20 and Tween20 0.1%, then filtered with sterile nylon filters (32 µm porosity) and concentrated in a final volume of 250 mL of sterile bidistilled H_2O, ranging the final concentration of $2.5*10^{11}$ spores *(**Figure 94**)*.

Figure 94.

6.2. Partitioning of the experimental fields

To evaluate the different defense strategies adopted for aflatoxin contamination each experimental field was divided into **five plots** corresponding to the relative strategies.

CHEMICAL TREATMENT. Treatment with Coragen®, a selective insecticide for *Ostrinia nubilalis* based on chlorantraniliprole.

BIOCOMPETITOR TREATMENT. Treatment with spraying the conidial suspension of *A. flavus* biocompetitor strain (BC) with a pesticide sprayer.

CONTROL. Absence of any chemical or biological treatment for the defense of maize.

ORGANIC CONTROL. Treatment with only *T. brassicae* and Turex® formulation (46.2 % of spores of two different *Bacillus thuringiensis spp.* (25% of *B. thuringiensis kurstaki* and 25% of *B. thuringiensis aizawai*) and 3.8 % of Δ-endotoxin, and 50% medium.

ORGANIC TREATMENT. Treatment with spraying the conidial suspension of *A. flavus* biocompetitor in combination with *T. brassicae* and *B. thuringiensis*. In this thesis, two *T. brassicae* treatments using a drone were performed at a distance of 15 days from each other and a treatment with Turex®, a biological insecticide based on *B. thuringiensis*.

4.000 m^2 was the area of each field thesis (200 m X 20 m). For each treatment, the plot was divided in four replicates of 1000 m^2 each one, obtaining a sufficient number of data to be able to statistically process. To prevent drift effects during insecticide treatments with Coragen®, that could to influence the results of biological control, a "buffer zone" of about 10 meters was set up between each plot. The experimental field diagram was showed in ***Figure 95***.

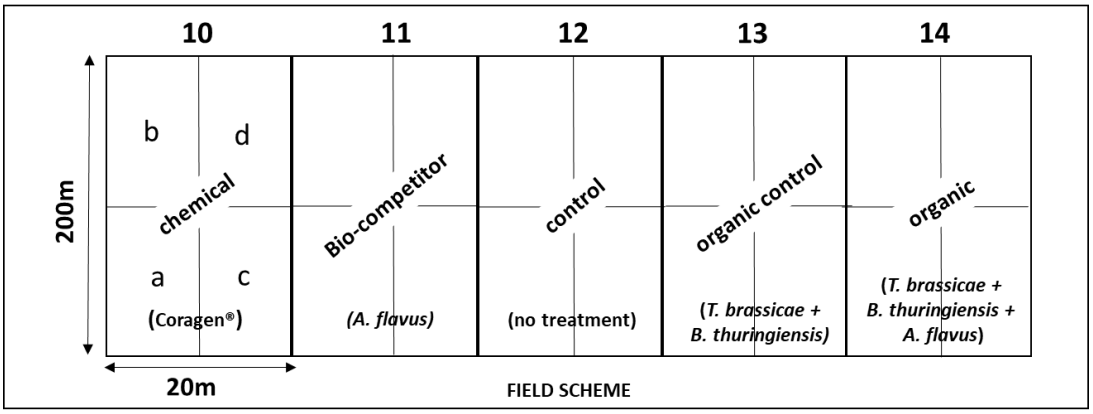


Figure 95: The experimental field diagram.

Treatment: Plots of the experimental fields of the two years of experimentation were treated with *A. flavus* biocompetitor conidial suspension in the first week of July, during the second flight of *O. nubilalis.* In the same time, some plots were treated with *B. thuringiensis.* Two treatment with *T. brassicae* were performed, the week before and after the BC's treatment, using a drone (***Figure 96 A and B***). The drone technology makes it to treat very quickly 4 ha of field in 10 minutes. During the flight the small balls, made of biodegradable material and containing the *T. brassicae* eggs, were released automatically at a precise distance each other. Once released, the biodegradable material melts, freeing the eggs. In a few hours, and no later than a day the eggs hatch. The *T. brassicae* adult, flying, can parasitize the corn borer eggs.

Harvest and sampling: In 21 and 22 August 2017, the grain was collected and sampled for various laboratory experiments. In 2018, the grain was collected and sampled in 2 and 3 September because during the summer there were many rains and the percentage of grain was not suitable for harvesting (***Figure 96 C and D***).

Figure 96: From treatment to harvest and sampling. *Treatment with biocompetitor sporal suspension and TUREX® formulation using a pesticides sprayer* ***(A).*** *Treatment with T. brassicae usind a drone* ***(B)****. Harvest and sampling of corn grains* ***(C and D).***

6.3. Grain sampling method

For the quantitative determination of AFB_1 contaminations in the field, a large number of grain subsamples were collected from each treatment. The subsamples of each treatment were then brought together, forming five main samples. From these samples, a single sub-sample of 1 kg was taken for laboratory analysis at PROGEO industry. The concentration of aflatoxins, measured in µg/kg (ppb), was detected using HPLC (High Performance Liquid Chromatography) analysis. The values expressed in ppb were then converted to ppm.

6.4. Evaluation of combination of two treatment in Vitro: Biocompetitor and Turex®

To evaluate if in a combination of two treatment, the formulation Turex® (*B. thuringinesis* and its Δ-endotoxin) negatively affected the BC, we performed a test in Petri dishes. 20 mL of YES5% agar medium were inoculated into Petri dishes. After the solidificacion of medium, formulation Turex® and 10^4 spores of BC were spotted in the middle of Petri. The same experiment was performed using only Δ-endotoxin. Another experiment was performed inoculating the formulation Turex® and also only Δ-endotoxin in the synthetic agar medium. Than, spores suspension was spotted on the agar medium. Petri dishes were stored at 28°C. The effect was evaluated after 10 days.

6.5. Post-harvest analyses

A. flavus strains isolation and identification

The resident population of *A. flavus* was isolated from maize kernels after sampling. Eight kernels from to 4 different replicates of plot treated were washed with 5 mL of a sterile washing solution (0.1% Tween20 in double distilled water), which was plated on selective medium DRBC (100 µL of a 1:10 dilution). Plates were incubated at 31 °C in the dark for three days. For each treatment were obtained 120 plates, 30 for each replicate. Colonies classified as *A. flavus* following a morphological identification according to *Pitt and Hocking (1997) and Samson et al. (2004*) were recovered and collected: from each plate, isolated 3 strains belonging to the *A. flavus* species using a phenotype method (color of spores, radial growth of mycelium). Strains were then assayed for the capacity to accumulate aflatoxins.

In ***Figure 97*** were reported the phases of isolation and identification of strain as afla- or afla+ strain.

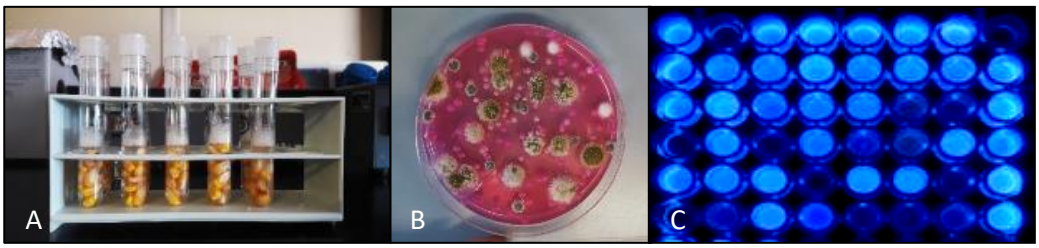


Figure 97: Phases of isolation and identification of strain. *Eight kernels in washing solution* ***(A).*** *A. flavus grouted in selective medium* ***(B)****. Aflatoxin assay in CCM medium to discriminate toxigenic from non-toxigenic A. flavus strains* ***(C).***

6.6. Molecular characterization of *A. flavus* atoxigenic (afla-) population

Rapid DNA extraction

A rapid DNA extraction was used for all afla- strains isolates to evaluate the persistance of biocompetitor afla- strain in plots treated after one month to the application of sporal suspension. the Total DNA was extracted from all mycelia of afla- strains, recovered from multiwall microplates of aflatoxin assay. Each mycelium was frozen in liquid nitrogen and, using a pestel, grounded. Then, 400 µL of lysis buffer (EDTA 50 mM and SDS 0.2% pH: 8.5) were added to the samples and gently mixed. After 10' at room temperature, samples were centrifuged at 15000 rpm 4°C for 15'. After transferred them on ice, the supernatant was pipet off and transferred in new Eppendorf. Then, 62.5 µL of CH_3COONa 3M were added to each samples, mixed by inversion, and maintained at 4°C for 60'. Centrifuge at 15000 rpm 4°C for 15'. The pellet is done with polysaccharides. The supernatant, containing the gDNA, is pipet off and transferred in new Eppendorf. gDNA was then diluted 1:50. Genomic DNA of samples is quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Stored at -20°C.

7. Yeast assay

7.1. Spot assay

To examine the anti-oxidant effect of the test compounds, a spot assay of *S. cerevisiae* (BY4741 strain) was performed *(Kim et al. 2005)*. 1×10^6 cells/mL cultured overnight in YPD at 28 °C were serially diluted from 10 to 100 fold in liquid synthetic medium (SD; 6.9% w/v YNB (Formedium™) supplemented with 2% (w/v) glucose and appropriated amino acids and bases for auxotrophy). Then, cells from each serial dilution were spotted adjacently on SD agar medium supplemented with 50 µM of each TS (0.5% v/v DMSO was used as control) and 0.5 or 1 mM hydrogen peroxide (H_2O_2). To examine alleviation of oxidative stress of vanillic, gallic, and dihydrolipoic acids; glutathione (GSH) and thiosemicarbazones (all supplemented at the concentration of 50 µM) on cells, 0.5 or 1 mM hydrogen peroxide (H_2O_2) was incorporated into the medium. Culture growth at 28 °C was monitored for 7 days. Experiments were performed in triplicate. Compounds were considered to have anti-oxidant activity if cell growth improved compared to cohorts exposed to H_2O_2 without the test compound.

7.2. Spot Assay for Yeast Oxidative Growth

Cells of W303-1B strain were serially diluted from 10^7 to 10^2 cells/mL. 10 µL of each dilution was spotted on YP agar plates supplemented with 2% ethanol or 2% glucose, amended with 50 µM of Tio-Y or 0.5% (v/v) DMSO as control, in order to obtain spots containing from 10^5 to 100 cell/spot. Plates were incubated at 28 °C.

7.3. Mitochondrial Respiratory Activity and Cytochrome Profiles

Clark-type oxygen electrode (Oxygraph System Hansatech Instruments) was used to measure the oxygen consumption rate at 30 °C using with 1 mL of air-saturated respiration buffer (0.1 M phthalate KOH, pH 5.0), 0.5% glucose. Yeast W303-1B cells were cultured at 28 °C in YP medium supplemented with glucose at the non-repressing concentration of 0.6%, until glucose exhaustion, in the presence of Tio-Y at different concentrations (5, 10, 25, and 50 µM). Oxygen consumption was normalized to the dry weight of the cells. Cytochrome profiles were determined spectrophotometrically at room temperature (Varian Cary300 UV-VIS Spectrophotometer), by recording reduced oxidized cytochrome spectra of yeast W303-1B cells cultured for oxygen consumption rate determination.

7.4. Isolation of Mitochondria, Gel Electrophoresis and Western Blot Analysis

Mitochondrial proteins for Western Blot analyses were obtained by suspending the cells in extraction buffer containing 0.6 M sorbitol, 10 mM imidazole, 0.5 mM EDTA, 0.1% BSA and 1 mM PMSF. Cells were broken by vortexing on ice using glass beads and mitochondrial proteins were obtained by centrifugation and re-suspended in the extraction buffer. Quantification of protein concentration was performed by Bradford's method *(Bradford 1976)*, using Bio-Rad protein assay following the manufacturer's instructions. For each sample, 15 g of mitochondrial total proteins were loaded on 12% polyacrylamide gel, subjected to SDS-PAGE, and Western Blot was performed. Gels were electroblotted onto nitrocellulose filters and successively immunostained with specific antibodies against CORE1 and CORE2, COX2 and ATP2. POR1 antibody was used as loading control. After incubation with the appropriate secondary antibodies, ECL Western Blotting Substrate (Clarity^{TM}, BioRad) was used for final detection. Signals were quantified through QuantityOne Software (Bio-Rad, Hercules, CA, USA). Enzymatic activities: Complex II (succinate dehydrogenase), complex III (cytochrome c reductase) and complex IV (cytochrome c oxidase) specific activities were measured spectrophotometrically according to Barrientos et al. *(Barrientos et al. 2009)* on a mitochondrial-enriched fraction prepared as previously described *(Soto et al. 2009)*. Tio-Y was added to the cuvette. As control, the same volume of DMSO was added.

Contributions

Analyses on the model organism *Saccharomyces cerevisiae* described in Topic 1 (Aflatox® Project) have been conducted in collaboration with (and with the supervision of) Proff. Tiziana Lodi and Cristina Dallabona from the ***Molecular Genetics and Biotechnology Lab*** (Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma).

Molecular docking and setting of Aflatox® Database described in Topic 1 have been performed in collaboration with Proff. Giorgio Pelosi and Franco Bisceglie from the ***Bioinorganic Chemistry Group*** (Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma).

Phylogenetic and metabolic analyses on Lokobe's *A. flavus* were performed in the laboratory of Prof. Olivier Puel at the *INRA* "*Toxalim Laboratory*" (***Research Center for Food Toxicology*** **of the** ***Institut National de la Recherche Agronomique*** in Toulouse (France), in collaboration with Dr. Amaranta Carvajal-Campos.

The development of LAMP assays for the differentiation of *Aspergillus flavus* afla-/afla+ strains and the characterization of HexB gene have been conducted in the laboratory of Prof. Ludwig Niessen at the ***Technical University of Munich*** (***TUM***) in Freising (Germany).

Characterization of *A. flavus* mycovirome have been conducted in collaboration with Prof. Walter Chitarra and Dr. Luca Nerva from CRA-VIT laboratories (***Research Center for Viticulture and Enology of The Council for Agricultural Research and Economics***) in Conegliano Veneto (TV).

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I ringraziamenti di un ricercatore

Eccomi qua, giunto alla fine del mio Dottorato di Ricerca. Sembra ieri quando ho varcato nuovamente la soglia del laboratorio di Genetica Vegetale ma questa volta da dottorando. Fin dal primo giorno ho vissuto questa esperienza come un'avventura. Mi rendo conto di aver scalato una montagna piena di ostacoli ma allo stesso tempo ricca di emozioni e soddisfazioni. In questi tre anni sono cresciuto non solo professionalmente ma anche moralmente e ho acquisito più consapevolezza di me, dei miei limiti e dei miei punti di forza. La scuola di dottorato mi ha permesso di fare tantissime esperienze diverse sia in Italia che all'estero. Ho cercato di comprendere e individuare i meccanismi molecolari che ruotano attorno alla vita, meccanismi che sembrano inusuali o inutili ma al mondo tutto ha un senso. L'autonomia professionale e le varie tecniche molecolari, biochimiche e microbiologiche che ho acquisito mi hanno permesso di realizzare questa tesi e di arricchire un bagaglio professionale non indifferente.

Mi mancherà l'avere una guida come Francesca che mi ha sempre lasciato libero di essere me stesso, senza imposizioni, che mi ha guidato e consigliato sia professionalmente che umanamente. Grazie per essermi stata amica leale e sincera, sempre presente e pronta ad aiutarmi, per non aver mai fatto pesare le mie incertezze, perché ad ogni mio dubbio avevi sempre una risposta.

Ringrazio il Prof. Restivo per avermi dato questa possibilità, per aver creduto in me fin dall'inizio e per la gentilezza, il tatto e la disponibilità che ha sempre dimostrato nei miei confronti. Uomo paziente e dalle mille risorse, mi ha trasmesso il suo amore inesauribile per la scienza. Il sorriso che leggevo sul suo volto erano per me punto di forza per continuare ad andare avanti e a non mollare.

Voglio ringraziare i miei tesisti Anna, Chiara, Azzurra, Fabiola, Simone, Damiana, Alessia e Martina. Spero di avervi trasmesso la mia passione per la ricerca e dato le basi per potervi realizzare in futuro. Ringrazio i tesisti magistrali Roberto, Gianluigi e Antonio per le mille risate.

Un ringraziamento ai dottorandi Giulia, Andrea, Sofia e Sonia, agli assegnisti e borsisti Serena, Camilla, Marco e Carla, e ai tecnici Silvia e Antonietta che avete fatto parte di questa esperienza.

Ringrazio di cuore Chiara, Gina, Cosimo, Mariagrazia, Carlo, Oriana, Luca, Gabriele, Adriana, Roberto e la micia, Cavalli, Andrea, Stefano, Paolo, Carla e la Pat per essermi amici sempre, per aver trascorso con me momenti unici. Grazie per essere stati al mio fianco. Un vostro sorriso, sguardo, gesto o graffio mi hanno sempre dato la giusta carica per andare avanti e non mollare mai!

Ringrazio Claudia, Davide, Elvira, Sandro, Corinna, Camillo, Manuel, Martina, Federica, Davide, Adriana, Ivan e Christian per esserci sempre!

Un ringraziamento speciale va mia sorella Francesca. Siamo sempre stati insieme e condiviso tutto. La distanza non ci ha allontanato ma ha trasformato il nostro legame in qualcosa di indistruttibile. Avrai sempre un posto nel mio cuore. Vado fiero del nostro rapporto di amore fraterno.

Ringrazio mamma e papà perché mi avete sempre appoggiato in questo viaggio. Mi avete sostenuto moralmente su ogni mia perplessità. Il vostro sorriso e la vostra forza mi hanno aiutato a non abbandonare la via e a credere in me. Come le colombe mi avete dato il volo, e se volo alto è perché voi avete permesso di farlo.

Voglio, infine, ringraziare tutte le persone che lungo questo cammino mi hanno supportato in ogni mia scelta e hanno sempre creduto in me, Grazie.