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Résumé

Les mycotoxines sont des molécules toxiques produites par de nombreuses espèces fongiques. Les seules mycotoxines avérées aujourd'hui cancérigènes pour l'homme sont les aflatoxines. Elles sont produites par le genre *Aspergillus* principalement et sont retrouvées tout au long de la chaine alimentaire (champs, stockage, transformation, etc.). A cause du réchauffement climatique, la France devient de plus en plus exposée à la présence de ces mycotoxines. Afin de limiter l'exposition des consommateurs, de nombreuses stratégies de prévention ou de décontamination sont développées. Dans ce contexte, nous avons recherché à mettre au point un système de lutte biologique permettant de prévenir la production d'aflatoxines sur le maïs au champ. Pour cela, nous avons choisi des bactéries issues du sol et déjà connues pour être commercialisées pour la lutte biologique, les actinomycètes. Nous avons étudié l'interaction *in vitro* sur boites de Pétri entre *Aspergillus flavus*, principal producteur d'aflatoxines, et certains actinomycètes. Nous avons démontré que l'interaction peut réduire la concentration en aflatoxines mesurée par HPLC. De plus, certains isolats bactériens sont aussi capables de réduire, en culture pure, la concentration d'aflatoxine B1 dans le milieu. Des premiers tests d'adsorption ont été réalisés pour comprendre la nature de ce mécanisme. Par ailleurs, une étude approfondie via RT-qPCR sur 6 souches bactériennes du genre *Streptomyces* sp. a montré que celles-ci étaient capables d'impacter l'expression de différents gènes impliqués dans la voie de biosynthèse chez *A. flavus* et *A. parasiticus*. Enfin, nous avons complété les données déjà existantes sur l'impact de facteurs environnementaux (température, disponibilité en eau et du temps d'incubation) sur la production d'aflatoxines.

Abstract

Mycotoxins are toxic contaminants of foodstuffs produced by a wide range of fungal species. Aflatoxins are the only mycotoxins carcinogenic for humans. They are mainly produced by the *Aspergillus* genus and can be found at each step of the agrofood chain (e.g. field, storage, process). Due to climate changes, France is starting to be exposed to aflatoxins. In order to limit the consumer exposure, many prevention or decontamination techniques have been developed. To this aim, we started the development of a biocontrol against aflatoxins accumulation for maize field application. Actinomycetes, are soil-borne bacteria that has already been commercialized as biocontrol. In Petri dishes, we studied the *in vitro* interaction between some actinomycetes and *Aspergillus flavus*, the main aflatoxins producer. We revealed that the interaction reduced the aflatoxins content (monitored by HPLC). Moreover, some bacterial isolates were able to reduce pure-aflatoxin B1 added in the medium. To understand this mechanism, adsorption tests has been conducted. Otherwise, RT-qPCR methodology was used to study the impact of *Streptomyces-Aspergillus* sp. on aflatoxin gene expression. Finally, the current knowledge of the impact of environmental factors (temperature, water activity and incubation time) on aflatoxins production was supplemented.

When the going gets tough,

the tough get going.

Je souhaite dédier cette thèse ainsi que l'intégralité du travail qu'elle représente à ma grand-mère Pierrette Semper.

"Les souvenirs que tu m'a laissé valent plus que tout ce que je possède au monde"

List of abbreviations:

A: Adenine

A.: *Aspergillus* sp.

AFB1: Aflatoxin B1

AFB2: Aflatoxin B2   AFB: AFB1 + AFB2

AFG1: Aflatoxin G1   AFG: AFG1 + AFG2

AFG2: Aflatoxin G2   AFT: AFB1 + AFB2 + AFG1 +AFG2

AFM1: Aflatoxin M1

AFP1: O-demethylation of AFB1

AFQ1: 3α-hydroxylation of AFB1

AF-alb: Aflatoxin albumin adducts in blood

AVF: Averufin

AVN: Averantin

A_w: Water Activity

BHA: 2(3)-*tert*-Butyl-4-Hydroxyanisole

bw: body weight

C: Cytosine

c: conidies

cd: conidiophores

CEM: Corn Extract agar

CPA: Cyclopiazonic Acid

CYA: Czapek Yeast Agar

DH: Dehydratase domain of PKS

DHDMST: Dihydrodemethylsterigmatocystin

DHOMST: Dihydro-O-Methylsterigmatocystin

DHST: Dihydrosterigmatocystin

DMST: Demethylsterigmatocystin

D.O.N.: Deoxynivalenol

E.: *Emericella* sp.

EFSA: European Food Safety Authority

ENS: Ecole Nationale Supérieure

ER: trans-acting Enoyl domain of PKS

EST: Short single-read Transcript sequences database.

EU: European Union

F.: *Fusarium* sp.

FB1: Fumonisin B1

FB2: Fumonisin B2

G: Guanine

G proteins: Heterotrimeric G protein

h: hyphae

HCC:Hepatocellular Carcinoma

HAVN: 5'-Hydroxy-Averantin

HBsAG positive: HBV-positives patients

HBsAG negative: HBV-negatives patients

HBV: Hepatitis B Virus

His-rich: section rich in Histidine in the *aflR* gene

HIV: Human Immunodeficiency Virus

HOMST: 11-Hydroxy-O-Methylsterigmatocystin

HR-PKS: Highly-reducing PKS

IARC: International Agency centre for Research on Cancer

I_D: Index of Dominance

KR: β-Ketoreductase domain of PKS

L: Large

LD50: Letal Dose to kill 50% animals

MA: Aerial Mycelium

MADE: Myxobacteria Aflatoxin Degradation Enzyme

NOR: Norsolorinic Acid

NAA: Norsolorinic Acid Anthrone or Noranthrone

NLD: Nuclear Localization Domain

NR-PKS: Non-reducing PKS

OAVN: Oxoaverantin

OMST: O-methylsterigmatocystin

OTA: Ochratoxin A

p: phialides

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDA: Potatoes Dextrose Agar

PKS: Polyketide Synthase

PR-PKS: Partially-Reducing PKS

psia: hydroxylated linoleic acids

psic: oleic acids

PYB: Peptone Yeast extract Broth

QTL: Quantitative Trait Loci

R: Radical group of trichothecenes

RA: *Retinaculum Apertum*

RASFF: Rapid Alert System for Food and Feed

RCM: Residual Concentration in the Medium

RF: *Rectus Flexibilis*

RT-qPCR: Reverse Transcription quantitative PCR

S: Small (chapter 1.2.4.)

S: *Spirales* (chapter 1.2.10 & 3)

SSR: Single Sequence Repeats

ST: Sterigmatocystin

T: Thymine

Tc: C Vps tethering start point

TDS: Total Diet Studies

tsp: transcription start point

TSP: Translational Start Point

v: vesicule

VAL: Versiconal

VCG: Vegetative Compatibility Groups

VERA: Versicolorin A

VERB: Versicolorin B

VHA: Versiconal Hemiacetal Acetate

ZEA: Zearalenon

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Communications:

Articles:

2014   **C. Verheecke**, T. Liboz, M. Darriet, N. Sabaou and F. Mathieu. *In vitro* interaction of actinomycetes isolates with *Aspergillus flavus*: impact on aflatoxin B1 and B2 production. *Letters in Applied Microbiology* **58**, 597-603.

2014   **C. Verheecke,** T. Liboz, P. Anson, R. Diaz and F. Mathieu. Effect of *Streptomyces* interaction on aflatoxin production by *Aspergillus flavus* and *A. parasiticus*. Submitted to: *Microbiology*.

2014   N. Azzoune, S. Mokrane, A. Riba, N. Bouras, **C. Verheecke**, N. Sabaou and F. Mathieu. Contamination of common spices by aflatoxigenic fungi and aflatoxin B1 in Algeria. Accepted in: *Quality Assurance and Safety of Crops & Foods*.

2014   **C. Verheecke**, T. Liboz, P. Anson, M. Darriet and F. Mathieu. *Streptomyces-Aspergillus* interactions: Impact on Aflatoxins B accumulation. Accepted with major modification in: *Food Additives and Contaminants*: special issue ISM2014 (Annex 1).

Communications during National and International symposiums:

2014   **C. Verheecke**, T. Liboz, P. Anson, M. Darriet and F. Mathieu. *Streptomyces-Aspergillus* interactions: Impact on Aflatoxins B accumulation. **Oral Presentation,** at ICM2014 Beijing, China and Journées Mycotoxines 2014, Montpellier, France.

2014   A. Yekkour, O. Toumatia, A. Meklat, **C. Verheecke**, N. Sabaou, A. Zitouni and F. Mathieu. First survey on the trichothecenes-producing *Fusarium culmorum* strains and their impact on infecting wheat and barley in Algeria. **Poster,** ISM2014, Beijing, China.

2014   **C. Verheecke** and R. Diaz, P. Anson, T. Liboz and F. Mathieu. Validation of reference genes for quantitative real-time PCR studies in *Aspergillus parasiticus.* **Poster,** ISM2014, Beijing, China.

2014   D. Caron, **C. Verheecke** and F. Mathieu. Aflatoxins on maize cobs inoculated with *Aspergillus flavus* in greenhouse. **Poster,** ISM2014, Beijing, China.

2013   **C. Verheecke**, M. Darriet, T. Liboz, N. Sabaou and F. Mathieu. Involvement of Actinobacteria in the reduction of Aflatoxin B1 & B2 biosynthesis by *Aspergillus flavus* **Oral Presentation.** ISM-MycoRed, Martina-Franca, Italy.

2013   **C. Verheecke,** K. Damak, T. Liboz and F. Mathieu**.** Impact de l'Aw et la Température sur la croissance et la production d'Aflatoxine B1 par *Aspergillus flavus*. **Oral Presentation,** Journée Mycotoxines, Brest.

2012   **C. Verheecke,** T. Liboz, M. Darriet, N. Sabaou and F. Mathieu. Développement d'un biocontrôle pour réduire la présence d'Aflatoxine B1 dans la filière maïs, **Oral Presentation**, Journée *Streptomyces*, Nancy.

2012   **C. Verheecke**, M. Darriet, T. Liboz, N. Sabaou and F. Mathieu. Involvement of Actinobacteria in the reduction of Aflatoxin B1 & B2 biosynthesis by *Aspergillus flavus*. **Poster,** World Mycotoxin Forum meets IUPAC, Rotterdam, Hollande.

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General Introduction

General Introduction

Mycotoxins are toxic substances produced by fungi that contaminate various food and feedstuffs. There are about a hundred different types of mycotoxins which are produced by a wide range of fungal species. The variety of their toxicity is linked to the diversity of their chemical structure.

Amongst them, aflatoxins (except for aflatoxin M1) are the only mycotoxins considered as carcinogenic for humans (Group 1, IARC). In addition to carcinogenicity, they are also highly hepatotoxic, nephrotoxic, immunotoxic, etc. These toxins often contaminate maize, peanuts, pistachios and brazil nuts, etc. In European Union (EU), they are regulated for food and feedstuffs. Aflatoxins accumulation is due to the colonisation of foodstuffs by fungi. The latter mainly belong to the *Aspergillus* genus and can be found all along the agrofood chain (e.g. field, storage, process).

The producers of aflatoxins present 29 genes regrouped in a cluster situated in a subtelomeric region (chromosome 3). They encode enzymes that convert MalonylCoA and AcetylCoA into aflatoxins. This pathway is regulated by specific (AflR, AflS) as well as general transcription regulators which are themselves triggered by many environmental parameters.

Those enviromental parameters can be abiotic or biotic. The former involve temperature, water activity (a_w), CO_2 concentration, etc. Optimal conditions for aflatoxin B1 production are 25 to 30°C and an a_w of 0.96-0.99. Due to climate changes, those environmental conditions are starting to occurr in France where sporadic aflatoxins content above the EU limits have been detected (e.g.: 2003). Interwoven with abiotic parameters, biotic parameters such as fungal or bacterial interactions can also impact aflatoxins accumulation.

Biocontrols (based on various organisms) have been developed to avoid aflatoxins accumulation at field. Currently, afla-guard[®]: a non-toxigenic *A. flavus* strain is the main biocontrol available on the market. However, other biocontrols based on bacteria are under development. Bacteria such as *Streptomyces* (actinomycetes) were identified as interesting producers of inhibiting metabolites.

In France, the main crop at risk of aflatoxins contamination is maize. Contamination could have major economic impacts (15.6 million tones of maize were produced in France in 2011). Thus, it is crucial to limit aflatoxins content below the legal limits.

In order to limit aflatoxins content, management of biotic and abiotic parameters can mostly prevent aflatoxins accumulation. For maize, at seed level, Bt hybrids decrease aflatoxin B1 contamination by 6.2 fold. At field, agricultural practices focusing on irrigation, fertility and massive insects prevention can reduce aflatoxins accumulation. During maize storage, temperature management is the most commonly used technique to monitor grain conservation. At that step, chemical and natural compounds (BHA, PP, essential oils) can prevent aflatoxins production.

In terms of decontamination techniques, tortilla production can reduce up to 84% of the initial aflatoxin B1 content. Chemicals (e.g.: ammoniation or ozone) or degrading organisms can reduce aflatoxins content. Adsorbents and binding bacteria can also prevent aflatoxins absorption by animals.

Within this context, we developed a project to prevent aflatoxins occurrence in the maize foodchain called: Aflafree. To this aim, our work was divided into 2 axis. Firstly, we developed a biocontrol able to reduce aflatoxins contamination at field without impacting the maize microbial ecosystem and to understand the associated mechanisms involved. Secondly, we investigated the impact of *A. flavus* and its associated aflatoxins production on the local ecosystem (*Fusarium* sp.) and on D.O.N. production during the sensible step of maize pre-storage.

This work is a first step towards the development of a biocontrol agent (or/and its enzymes & metabolites) against aflatoxins accumulation in maize. It is also the first milestone in the understanding the impact of *A. flavus* on the mycotoxigenic fungi already present in the maize ecosystem in France.

Hereafter, Chapter 1 focuses on bibliography. We will start with a brief introduction on mycotoxins, followed by the state of research on aflatoxins. We will specially focus on the prevention of their production and decontamination of food and feedstuffs thanks to bacteria.

Chapter 2 presents the different techniques developed to monitor the impacts of interactions and abiotic parameters on *A. flavus* (and *A. parasiticus*).

Chapter 3 focuses on the results and discussions. They are divided into 3 different parts:

(i)   Impact of environmental parameters on aflatoxin B1, aflatoxin B2 and D.O.N. production;

(ii)   Study of actinomycetes and *A. flavus* interaction;

(iii)   Characterisation of the mechanisms involved by RT-qPCR and adsorption tests;

Finally, we discusses results, drawing up conclusions and introducing future perspectives.

1. Bibliographic review

Bibliographic review

1.1. Mycotoxins

1.1.1. Definition

According to the Collins dictionary, mycotoxins are "any of various toxic substances produced by fungi, some of which may affect food and others of which are alleged to have been used in warfare". The word mycotoxin comes from the ancient Greek word "mykes" which means mushroom and the Latin word "toxicus" which means poison (Online Etymology Dictionary, 2014). Bennett & Klich (2003) elaborated a more precise definition of mycotoxins:

(i)   low molecular weight molecules;

(ii) secondary metabolites produced by filamentous fungi;

(iii) which can cause death or disease to human being or animal at a low concentration.

The 3 main mycotoxins producers are *Aspergillus, Penicillium* and *Fusarium* genera. Currently, 300 to 400 mycotoxins are known, among which 30 have been studied for their toxic and/or disturbing impacts for human and animal (Bennett & Klich, 2003; Boudergue *et al.*, 2009).

1.1.2. Classification

Due to their diverse chemical structures and origins, mycotoxins are very hard to classify. They can be arranged according to their chemical structure, toxicity, biosynthetic origin and/or producing fungi.

Among the different biosynthetic origins, the best-known involve the polyketides.

The Polyketide Synthases (PKS) are a family of multi-domains enzymes largely found in bacteria, fungi and plants. There are 3 types of PKS:

Type I: large enzymes with multiple functional domains only active once during the biosynthesis (bacteria and fungi);

Type II: a complex of several single module proteins with separated enzymatic activities, acting iteratively to produce a polyketide (bacteria);

Type III: a single active site enzyme which acts repeatedly to form the final product; they function as homodimers and do not include a Acyl-Carrier Protein domain (mainly in plants).

As we studied fungi, this study will solely focus on Type I PKS with a special attention to the following ones:

Fungal PKS (mainly Type I iterative PKS) can be divided according to their reducing functions depending on the absence or the presence of some or all domains of β-Ketoreductase (KR), Dehydratase (DH) and Enoyl Reductase (ER). The different types of PKS and their associated mycotoxins are represented in table 1.

The Non-Reducing PKS (NR-PKS) AflC is essential for aflatoxins production (Watanabe & Townsend, 2002). This chemical family of mycotoxins includes 18 compounds based on 3 furans and 1 coumarin structure (Table 1).

The 6-methylsalicylique synthase, e.g. in *Penicillium expansum,* is a PKS without the ER domain. This PKS is called Partially-Reducing PKS (PR-PKS) and is essential for patulin production (Gallo *et al.*, 2013).

The genes *aoks1* of *A. westerdijkiae* and *otapksPN* of *P. nordicum* encode 2 different PR-PKS without the ER and DH domains. Their encoding PR-PKS are essential for the production of Ochratoxin A (OTA): an isocoumarin coupled with a l-phenylalanin (Bacha *et al.*, 2009; Gallo *et al.*, 2013).

The production of fumonisins and Zearalenon (ZEA) relies on Highly-Reducing PKS (HR-PKS), a specific type of PKS with the following 3 reducing domains: KR, DH and ER (Gallo *et al.*, 2013). Fumonisins are based on a linear chain of 18 carbons. The fumonisin B1 (FB1) and fumonisin B2 (FB2) are the most commonly known. FB2 is the C10 deoxy analogue of FB1. On the contrary, ZEA is an acid resorcyclic lactone.

Table 1 - The main mycotoxins originating from polyketides synthases. Based on Gallo *et al.*, (2013).
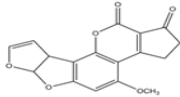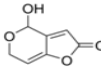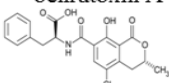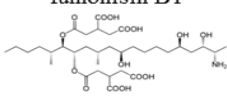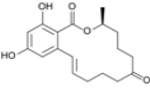
Mycotoxins	PKS type	Structure	Known metabolites	Example
aflatoxins	NR-PKS	3 furans and 1 coumarin	18	aflatoxin B1
patulin	PR-PKS	polyketide lactone	1	
ochratoxins	PR- PKS	isocoumarin coupled with an L-phenylalanin	6	ochratoxin A
fumonisins	HR-PKS	20 carbons chain with acid esther and an acetyl amino	15	fumonisin B1
zearalenon	HR-PKS	acid resorcyclic lactone	1	
HR: Highly-Reducing, NR: Non-Reducing, PR: Partially Reducing, PKS: Polyketide Synthase.				

The second best known biosynthetic origin is the terpene cyclase trichodiene synthase (e.g.: Tri5). The latter is essential for the cyclisation of the farnesyl pyrophosphate which itself induces the production of trichothecenes (Hohn & Vanmiddlesworth, 1986). There are more than 200 trichothecenes with a common 12-13 epoxytrichothec-9-ene core structure (Table 2). They are classified in 4 groups from A to D, according to their attached radical group (R). The group at the C-8 position is the differentiating element between groups A and B. Table 2 represents the structure of those groups. For example, the T-2 toxin (Table 2 a.) has an ester function at C-8 whilst all Type B trichothecenes have a C-8 keto (carbonyl) function. These 2 types of trichothecenes are the most alarming in terms of occurrence and toxicity (McCormick *et al.*, 2011).

Table 2 - Structure of trichothecenes: type A and B (Inchem, 1990).

The third biosynthetic origin is the dimethylallyltryptophan synthase. The latter is essential for the conversion of l-tryptophan and dimethylallyl diphosphate into tetracyclic ergoline ring. This ring is the main core of ergots alkaloids which are toxins produced by fungi and plants. Ergots have been extensively reviewed in Wallwey & Li, (2010).

1.1.3. Toxicity

1.1.3.a Brief history

Ergotism is supposedly the oldest human illness linked to mycotoxins, with major outbreaks in the Middle Age. Ergotism was the result of eating bread polluted by "ergot". The most severe symptom of this illness was leg-necrosis and delirium. "Ergot" comes from *Claviceps purpurea* which can contaminate rye and can be transmitted to humans through bread consumption (van Dongen & de Groot, 1995).

At the end of 1959, peanuts from Brazil were imported in England as protein supplements in farming feeds. Soon afterwards, young turkeys began to die and other animals such as pigs fell ill. 100,000 turkey poults were killed by the so-called "turkey X disease",

"X" referring to its supposedly viral origin (Cole, 1986). Shortly after, aflatoxins were identified as the source of this intoxication (Nesbitt *et al.*, 1962).

1.1.3.b Toxicities

Following this incident, toxicologists began to study the toxicity of mycotoxins. Humans and animals are exposed to mycotoxins through ingestion, skin contact and inhalation. For instance, moisture-damaged indoor environments are one of the greatest threats in terms of inhalation of mycotoxins (Täubel *et al.*, 2011). Nonetheless, until now, the highest risk of exposure remains ingestion.

Mycotoxins have a wide range of health impacts. This is due to the variety of their chemical structures. Table 3 draws a link between mycotoxins and their health impacts. Among those mycotoxins, aflatoxins (except for aflatoxin M1) are the only ones recognized as carcinogenic for humans (Group 1 (IARC, 2014)).

Table 3 - Toxicity of the main mycotoxins regulated by the EU. '+'= symptoms on animals; no sign = no evidence of symptoms on animals.

mycotoxins	genotoxicity	teratogenicity	cancerogenicity (humans)	cancerogenicity (animals)	oestrogenic	Anorexia, weight loss	neurotoxicity	growth	hepatotoxicity	diarrhea, vomiting	oesophageal defects	nephrotoxicity	abortion	infertility, vulvovaginitis	pulmonary edema	Immunotoxicity	neural tube defects
aflatoxin B1	+	+	+	+		+		+	+							+	
ochratoxin A	+	+		+		+		+	+			+				+	
patulin		+			+	+						+				+	
deoxynivalenol						+		+		+		+				+	+
zearalenon	+	+		+	+				+				+	+		+	
fumonisin B1		+		+		+		+	+	+	+	+			+	+	+
t-2		+				+		+	+							+	
citrinin	+	+										+					

Further details on this table are given below, except for aflatoxins' impact on human and animal health which will be further developed in chapter 1.2.2.

OTA is potentially carcinogenic for humans (Group 2B (IARC, 2014)). OTA is absorbed through the gastrointestinal tract, transported in blood vessels and accumulated in kidneys (Ringot *et al.*, 2006). For animals, OTA is genotoxic, teratogenic, carcinogenic, hepatotoxic, nephrotoxic and immunotoxic (Hayes *et al.*, 1974; Boorman *et al.*, 1992; Castegnaro & McGregor, 1998; Al-Anati & Petzinger, 2006; Palma *et al.*, 2007). OTA

exposure is supposedly linked to the Balkans human nephropathy (Petkova-Bocharova & Castegnaro, 1990).

Patulin is not carcinogenic for humans (Group 3 (IARC, 2014)). For animals, it is teratogenic and possibly immunotoxic (Osswald *et al.*, 1978; Paucod *et al.*, 1989; Llewellyn *et al.*, 1998). In addition, symptoms such as weight loss, intestinal and gastric problems, neurotoxicity and nephrotoxicity can occur (Pfohl-Leszkowicz, 1999).

Deoxynivalenol (D.O.N.) is not carcinogenic for humans (Group 3 ((IARC, 2014)). The symptoms (animals and humans) linked to D.O.N. exposure are weight loss, anorexia, nausea, diarrhea, nutritional loss and immune system modification (Pestka, 2007; Burel *et al.*, 2009; Sobrova *et al.*, 2010).

ZEA (Group 3 (IARC, 2014)) is genotoxic, teratogenic, carcinogenic, hepatotoxic, haematotoxic and immunotoxic for animals (Zinedine *et al.*, 2007). It is also an endocrine disruptor due to its close structure to 17 ß-oestradiol (Fitzpatrick *et al.*, 1989) leading to animal abortion and infertility. For humans, there is a presumed link between exposure to ZEA and premature puberty in Puerto-Rico (Sáenz de Rodriguez *et al.*, 1985).

Fumonisins (B1+B2) (Group 2B (IARC, 2014)) are not considered as genotoxic but are teratogenic (Voss & Riley, 2013) (Group 2B (IARC, 2014)). Its other health impacts include liver toxicity, cancer, leukoencephalomalacia and pulmonary edema (the entire list of impacts is available in table 3). For humans, fumonisins are supposedly linked to esophagus cancer (Rheeder *et al.*, 1992).

T-2 toxin (Group 3 (IARC, 2014)) is teratogenic, hepatotoxic and causes weight loss, decrease in blood cell and leukocyte count, reduction in plasma glucose and stomach toxicity for animals. There are few studies on the ht-2 toxin, its deacetylated form, which has alleged health impacts. Unfortunately, too little is known on t-2 & ht-2 impacts on human health (Li *et al.*, 2011).

Citrinin is not carcinogenic for humans (Group 3 (IARC, 2014)) despite an identified *in vitro* genotoxicity (Knasmuller *et al.*, 2004). It is teratogenic and nephrotoxic for animals (Reddy *et al.*, 1982; Flajs & Peraica, 2009) but not enough data are available to identify its impacts on human health.

1.1.3.c Co-contamination

In addition to the studies linking a single mycotoxin exposure to its toxicity impacts, other research have been done *in vivo* on co-contamination, the effects of double or multiple mycotoxins exposure, on animal health. Those impacts can be organised in 4 categories: synergic, additive, less than additive or antagonistic effects categories (Figure 1).

Figure 1 - Synergistic, additive, less than additive and antagonistic interactions as described by Grenier & Oswald, (2011). Effects of toxin A, toxin B and both toxins addition versus the control.
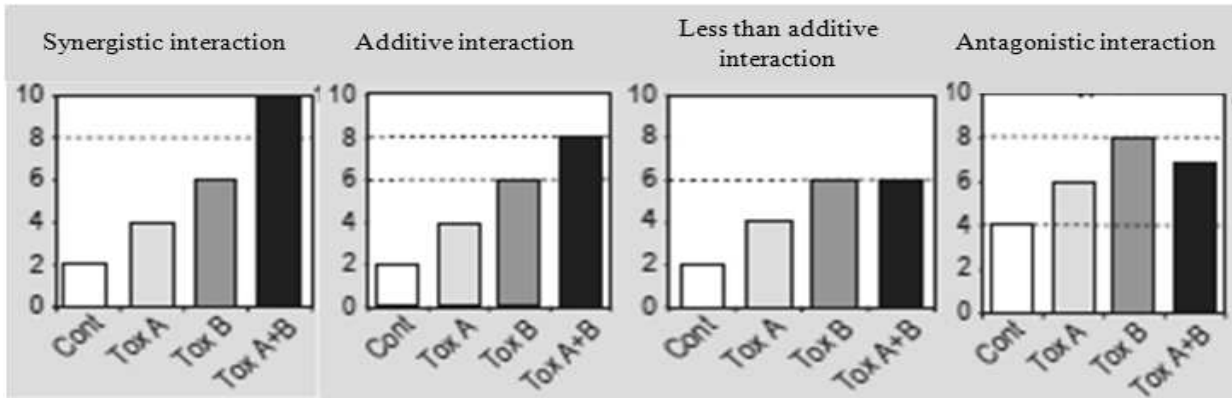
Most studies have focused on Aflatoxin B1 (AFB1) and FB1 co-contamination. Grenier & Oswald (2011) made a summary of all research to date and found the four different categories of effects in a wide range of animals:

In almost all cases of co-contamination, the synergistic and/or additive effects were the reduction of the entire body weight (bw) gain. In some cases, the antagonistic effects were the reduction of organs, including liver and kidneys. For example, the aspartate amino-transferase is an enzyme monitored in blood. Its higher concentration is a sign of organs malfunction including liver, kidneys, brain and heart (Ozer *et al.*, 2008). This enzyme amounts were measured in broilers after a 33 days exposure to different AFB1/FB1 mg.kg^{-1} of feed alone or in combination. At 0.05/50, synergistic effects were shown; at 0.05/200, additive and at 0.2/50 or 0.2/200, antagonistic impacts. This example reveals the complexity of mycotoxins co-contamination. Despite measuring the impact of 2 toxins on the same animal species, results differ depending on the ratios (Tessari *et al.*, 2010).

Another example is AFB1 and OTA co-contamination. Synergistic effects were less predominant than in AFB1/FB1 co-contamination. Nonetheless, there were many additive and less than additive effects of AFB1 and OTA co-contamination on bw reduction and embryos mortality/egg production (Grenier & Oswald, 2011). This example highlights the need to study double and multiple contamination of mycotoxins to identify the most synergistic risks on animal and human health.

1.1.4. Food chain contamination and consumer exposure

Each mycotoxin is produced by 1 or several fungal genera. Depending on environmental conditions, each fungal species produces a wide range of different mycotoxins (Garcia *et al.*, 2009). For instance, the single genus *Aspergillus* produces mycotoxins such as aflatoxins, ochratoxins, patulin and fumonisins (Frisvad *et al.*, 2007). Table 4 represents the main fungal genera with their related mycotoxins and the main commodities they can contaminate:

Table 4 - Mycotoxins, their related commodities and examples of associated producing fungi (engormix, 2014).

Mycotoxin	Commodity	Associated fungi
Aflatoxin	Peanuts, pistachios and other nuts, corn, cottonseed, cereals	*Aspergillus flavus, A. parasiticus*
Fumonisins	Corn, other cereals	*Fusarium verticillioides, F. proliferatum*
Ochratoxin	Vegetables, cereals, coffee beans	*Aspergillus westerdijkiae, Penicillium verrucosum*
Patulin	Apples, grapes, other fruits	*Penicillium expansum, Aspergillus giganteus*
Trichothecenes	Wheat, corn	*Fusarium tricinctum, F. poae and other Fusaria*

Mouldy commodities are not directly linked to a high mycotoxin content. On the contrary, macroscopically clean commodities can be highly contaminated with mycotoxins. This makes it difficult to sort commodities according to the level of mycotoxins contamination. The absence of mycotoxins in mouldy commodities can be due to a non-mycotoxigenic fungal colonization or to environmental conditions not propitious to mycotoxins production. Those environmental parameters are listed in figure 2.

To manage mycotoxins, it is crucial to first pay attention to the fields (parts **1.** and **2.** of figure 2). Fields crops (**1.**) are naturally contaminated by a variety of mycotoxigenic fungi, depending on weather conditions, farmer practices, fungal competition and other parameters (listed in **1.**). For example, the French maize is usually contaminated at field with *Fusarium* species including *F. graminearum* and section *liseola* ("Les Fiches Accidents," 2014). Yet, in Italy, with its warmer climates, maize is usually contaminated by other fungi such as *Aspergillus* sp. (Giorni *et al.*, 2007). These fungal contaminations have an impact on mycotoxins occurrence not only at field but also during storage (parts **3.**, **4.**, **5.** and **6.** (before and after process)) (Magan & Aldred, 2007).

RISK FACTORS AFFECTING MYCOTOXIN OCCURRENCE IN POULTRY PRODUCTION

1. Field crops
Field mycotoxin contamination

- Biological factors
  - Susceptible crops
  - Compatible toxigenic fungi
  - Environmental factors
- Temperature
- Moisture
- Physical damage
- Mold contamination
- Insect/pest control
- Crop rotation, tillage

Goal: Follow sustainable agriculture practices to minimize mold growth and mycotoxin production.

2. Harvesting
Field mycotoxin contamination

- Crop maturity
- Temperature
- Moisture
- Physical damage
- Cross-contamination

Goal: Harvest the crop at the correct stage and with optimum moisture content.

3. Drying/silo storage
Storage mycotoxin contamination

- Temperature
- Moisture
- Mold control
- Storage duration

Goal: Maintain uniformly clean and dry grain and use of mold inhibitors is recommended.

4. Feedmills
Storage mycotoxin contamination

- Temperature
- Moisture
- Season
- Storage duration
- Mold control
- Inventory rotation (First in - first out)
- Hygiene
- Pest control

Goal: Watch moisture levels of finished feed to avoid contamination post-production.

5. Local farm
Storage mycotoxin contamination

- Temperature
- Moisture
- Season
- Hygiene
- Pest control
- Damp grains, feed bridging across the bin
- Inventory rotation (First in - first out)

Goal: Protect feed from being wet and avoid rodent exposure.

6. Feed processing

- Contamination of deposits in dead ends, mixer and conveyers
- Pest control
- Mold control
- Hygiene
- Post-pelleting temperature and humidity control

Goal: Maintain cleanliness of feed mill equipments and storage bins.

7. Farm environment

- Feed residue in feed troughs
- Straw and wet bedding
- Season and moisture
- Speed of feed consumption

Goal: Avoid caking of feed in feeders and pitch out any moldy feed.

8. Mycotoxin symptoms on the farm

- DON: intestinal damage, poor gut immunity, increased mortality
- T-2 toxin: oral ulcers, increased disease susceptibility, reduced anti-oxidant status
- Zearalenone: poor reproduction, chick developmental abnormalities
- Fumonisins: reduced weight gain and antibody titers
- Aflatoxins: liver damage, poor hatchability, increased carcass condemnations
- Ochratoxins: kidney damage, gout, wet litter, increased shell stains and meat spots

Goal: Observe symptoms carefully to introduce preventive and therapeutic measures. Use of mycotoxin adsorbents in feed is recommended.

Enlarged liver, a symptom of AFLATOXINS

Alltech FEI World Equestrian Games 2010™

Known Mycotoxins

Alltech

Figure 2 - Risk factors affecting mycotoxin occurrence in feed, taking poultry as a case study (knownmycotoxins.com, 2014)

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Managing storage conditions is the second crucial step (parts **3.**,**4.**, **5.** and **6.**). Drying duration, water activity (a_w), temperature, CO_2 monitoring and other parameters listed in **3.**,**4.**, **5.** and **6.** are key factors to limit the production of mycotoxins during storage (Chulze, 2010).

The processing steps (parts **4.** (milling process) and **6.**) can reduce or increase the amount of mycotoxins. For example, during starch extraction, only 8.7% of the initial AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) (AFT) content is in the starch. Most of the initial AFT content (36.9%) is in feed by-products (e.g. **6.**) (including fiber, germ and gluten) and can impact animals health (**8.**) (Aly, 2002).

The main feedstuffs at risk of contamination are maize, groundnuts, copra, palm nuts and oilseed cakes. A recent international survey (2012 and 2013) of 4,200 samples of feedstuffs analysed the presence of AFT, ZEA, D.O.N., fumonisins and OTA (Table 5). More than 50% of samples were found positive for D.O.N. and fumonisins. 25 to 30% of the samples were found positive for AFT with an average concentration of 33.5 µg.kg^{-1} (2012 and 2013). The main contaminant was D.O.N.. 59-64% of samples were found positive with an average concentration of 770-1,088 µg.kg^{-1} depending on the year (2012-2013) (Nährer & Kovalsky, 2014).

Table 5 - Overview of Biomin's worldwide survey (2012 and 2013) (Nährer & Kovalsky, 2014)

Global results	Afla 2012	Afla 2013	ZEN 2012	ZEN 2013	DON 2012	DON 2013	FUM 2012	FUM 2013	OTA 2012	OTA 2013
Number of tests	2,636	2,839	3,320	3,470	3,712	3,931	2,570	2,699	2,230	2,459
Percent positive (%)	25	30	46	37	64	59	56	55	31	23
Average of positives (µg/kg)	34	33	251	133	1,088	770	1,350	1,421	5	10
Maximum (µg/kg)	6,323	1,563	9,854	5,324	30,200	29,267	42,120	26,828	170	595
Commodity tested	Ground-nut cake	Maize	Corn Gluten Meal	Maize	Maize	Barley	Maize	Dried Distiller's Grains	Maize	Finished Feed
Source country	Myanmar	China	China	China	USA	China	Malaysia	US	India	Spain

Depending on the geographic region, prevalence of mycotoxins differs (Figure 3). For instance, in Central Europe, the most predominant mycotoxin is D.O.N. with 66% of samples positive, followed by fumonisins 36% and AFT 29%. Notwithstanding, in Southern Europe, results have found a higher occurrence of mycotoxins with fumonisins at 71%, AFT at 55% and D.O.N. at 50%.
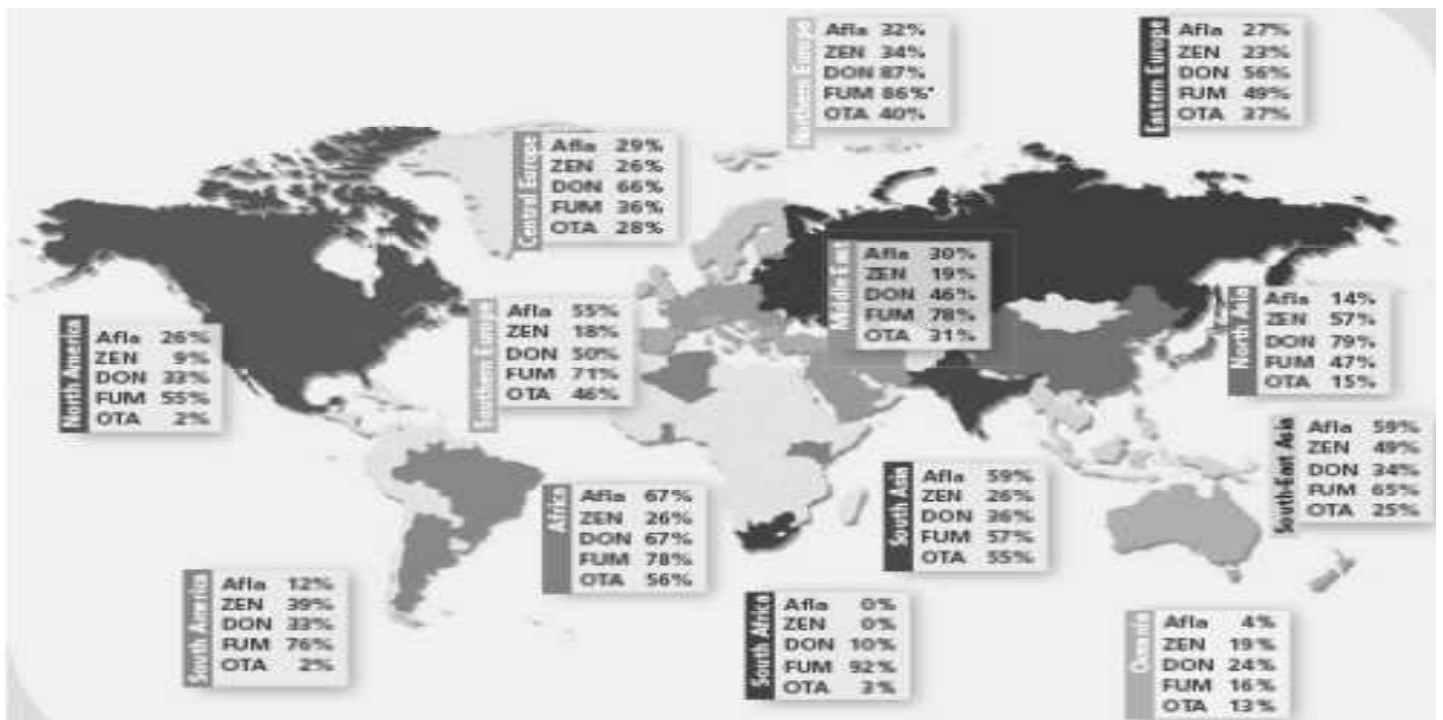
Figure 3 - Prevalence of mycotoxins in different regions according to the percentage of positive samples (Nährer & Kovalsky, 2014)

The level of mycotoxins exposure in crops is not a direct indicator of human exposure. It is very difficult to obtain reliable and accurate data due to censorship and to the heterogeneity of mycotoxins content in commodities. The results of recent studies in China, Korea, Malaysia and South Africa showed maximum levels of AFT daily intake ranging from 2.69 to 133 ng.kg^{-1} of bw. European countries are usually less exposed (< 1 ng.kg^{-1} bw) with the exception of Greece. For instance, 2 French studies estimated children daily exposure between 1 and 10 pg.kg^{-1} bw. This can be explained by the European regulatory environment (Marin *et al.*, 2013).

1.1.5. Regulations and socio-economic impacts

Confronted by the variety of contaminated commodities and toxicological data, the European Food Safety Agency (EFSA) listed all the sources of possible consumer exposure and proposed recommendations of maximum levels. The EU then issued regulations to limit consumer exposure based on those recommendations. Table 6 gives an overview of the various maximum limits applied for foodstuffs in Europe (1881/2006 modified on 6th March 2014, European Union). The limits for AFT will be developed in chapter 1.2.3. The regulation sets concentration limits for D.O.N. from 1,250 µg.kg^{-1} in unprocessed cereals and milling fractions of maize (particles size higher than 500 µm) to 200 µg.kg^{-1} for baby food (infants and young children).

The EU has the lowest limits for mycotoxins contamination in the world. Although regulations aim to protect EU citizens health, their impact on international trade can be drastic. Wu (2008) studied the economic impacts of AFB1 limits on peanuts trade. In the EU, food processing industries experienced greater occurrences of supply shortage and a reduced space to set their own prices and substitute goods. In the countries supplying peanuts to the EU; trading routes changed as soon as the EU laws were enforced. Peanuts started to be traded between countries with identical or similar regulations on AFB1. Nations with poor or poorly enforced standards in terms of AFB1 contamination began to trade peanuts between themselves at a cheaper price (Wu, 2008). In the short term, EU regulations on AFT have reduced the ability of low-income nations to export certain foodstuffs to the EU market and to gain revenues from this trade.

Table 6 - Maximum levels authorised for mycotoxins in foodstuffs. (1881/2006 modified on 6th March 2014) (European Union, 2006). AFT= total among of AFT; M1 = aflatoxin M1.

Mycotoxins	Foodstuffs	maximum levels (μg.kg^{-1})		
		B1	AFT	M1
aflatoxins	Dietary foods for special medical purposes	0.1	-	0.025
	Infant milk and follow-on milk	-	-	0.025
	Raw milk, heat-treated milk and milk for the manufacture of milk-	-	-	0.05
	cereals & food for babies	0.1	-	-
	Groundnuts, nuts, dried fruit and cereals and derivated ingredients	2	4	-
	Tree nuts, dried fruit and cereals sorted/treated before human consumption, spices (*Capsicum spp, Piper spp, Pyristica fragnans, Zingiber officinale, Curcuma longa*)	5	10	-
	Dried figs	6	10	-
	Almonds, pistachios and apricot kernels, intended for direct human	8	10	-
	Hazelnuts, Brazil nuts,Groundnuts sorted/treated before human	8	15	-
	Almonds, pistachios and apricot kernels sorted/treated before human consumption	12	15	-
ochratoxin A	Dietary foods for special medical purposes, baby foods	0.5		
	Wine, grape juice and wine based products	2		
	All products derived from unprocessed cereals	3		
	Unprocessed cereals, roasted coffee	5		
	Wheat gluten not sold directly to the consumer	8		
	Dried vine fruit, soluble coffee	10		
	Spices	15		
	Liquorice root, ingredient for herbal infusion	20		
	Liquorice extract, in particular beverages & confectionary	80		
patulin	Apple juice, solid apple products for infants & young children	10		
	Solid apple products	25		
	Fruit juices & spirit drinks	50		
Deoxynivalenol	baby foods for infants and young children	200		
	Cereals, pasta, Milling fractions of maize with particle size > 500 micron	750		
	Unprocessed cereals, Milling fractions of maize with particle size $\leq$ 500 micron	1,250		
	Unprocessed durum wheat, oats and maize	1,750		
zearalenon	cereals based foods for infants and young children	20		
	Bread	50		
	Cereals intended for direct human consumption	75		
	Unprocessed cereals other than maize	100		
	Milling fractions of maize >500 micron / $\leq$ 500 micron	200 / 300		
	Unprocessed maize (exception of wet milling)	350		
	Refined maize oil	400		
fumonisins (B1 + B2)	Baby foods for infants and young children	200		
	Breakfast cereals and snacks	800		
	Maize intended for direct human consumption	1000		
	Milling fractions of maize >500 micron / $\leq$ 500 micron	1,400 / 2,000		
	Unprocessed maize (exception of wet milling)	4,000		
t-2 + ht-2	Unprocessed cereals and cereal products	/		
citrinin	Food supplements based on rice fermented with red yeast *Monascus purpureus*	2,000		

1.1.6. "Emerging" mycotoxins

Currently, more than 300 mycotoxins have been identified. However, only 14 have been regulated due to their high exposure risk and health threats for EU consumers. Among the remaining mycotoxins, some are in the EU's priority lists. They are shown in table 7:

EU Mycotoxins monitoring		EU Mycotoxins follow-up
alternariol	ergometrine	
alternariol monomethyl ether	ergotamine	sterigmatocystin
	ergosine	nivalenol
tenuazonic acid	ergocristine	enniatins
tentoxin	ergocryptine	beauvericin
altenuene	ergocornine	diacetoxyscirpenol
phomopsins	related -inines.	moniliformin

Table 7 - Mycotoxins under surveillance (monitoring and follow-up) by the EU

(European Union, 2012; Verstraete, 2013).

The first and second columns list mycotoxins whose presence in feed and food the EU recommends monitoring to evaluate consumer exposure.

The third column list mycotoxins whose contamination and toxicity risks have to be evaluated in the forthcoming years.

The "emerging" status of other mycotoxins is also directly linked to the availability of reliable techniques to analyse those mycotoxins and to develop reliable exposure data. The development of measurement techniques including LC-MS has revealed possible threats due to masked, hidden, bound or glycosylated mycotoxins, including D.O.N. metabolites with a high toxicity potential (Verstraete, 2013).

1.2. Aflatoxins

1.2.1 Presentation

There are currently 41,100 scientific publications among which more than 10,000 focus on aflatoxin. Figure 4 represents the number of publications per year on aflatoxin since the first characterisation in 1962. Nowadays, more than 300 publications are published every year on this subject.
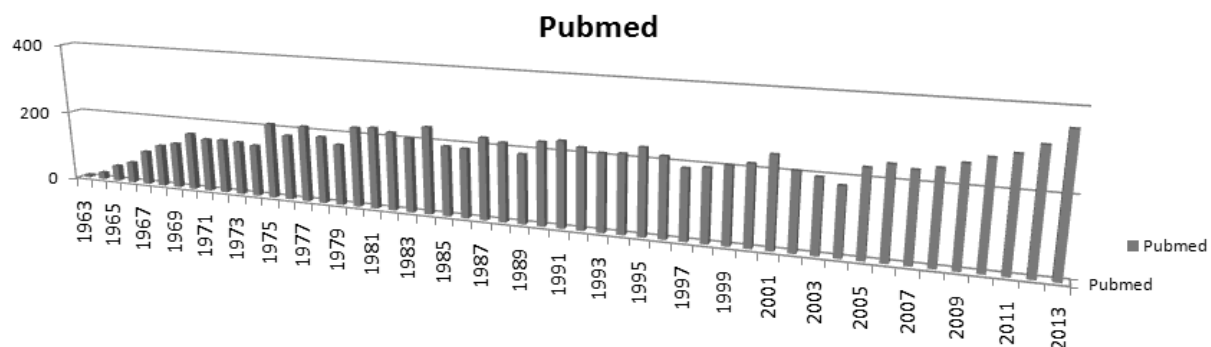
Figure 4 - Number of publications per year on "Aflatoxin" between 1963 and 2013 (Pubmed).

In 1962, the Turkey X disease led to the discovery of AFT. Nesbitt *et al.*, (1962) identified 2 main types based on their fluorescence: the "B" aflatoxins (AFB) with a violet-blue fluorescence (445 nm) and the "G" aflatoxins (AFG) with a green fluorescence (455 nm).
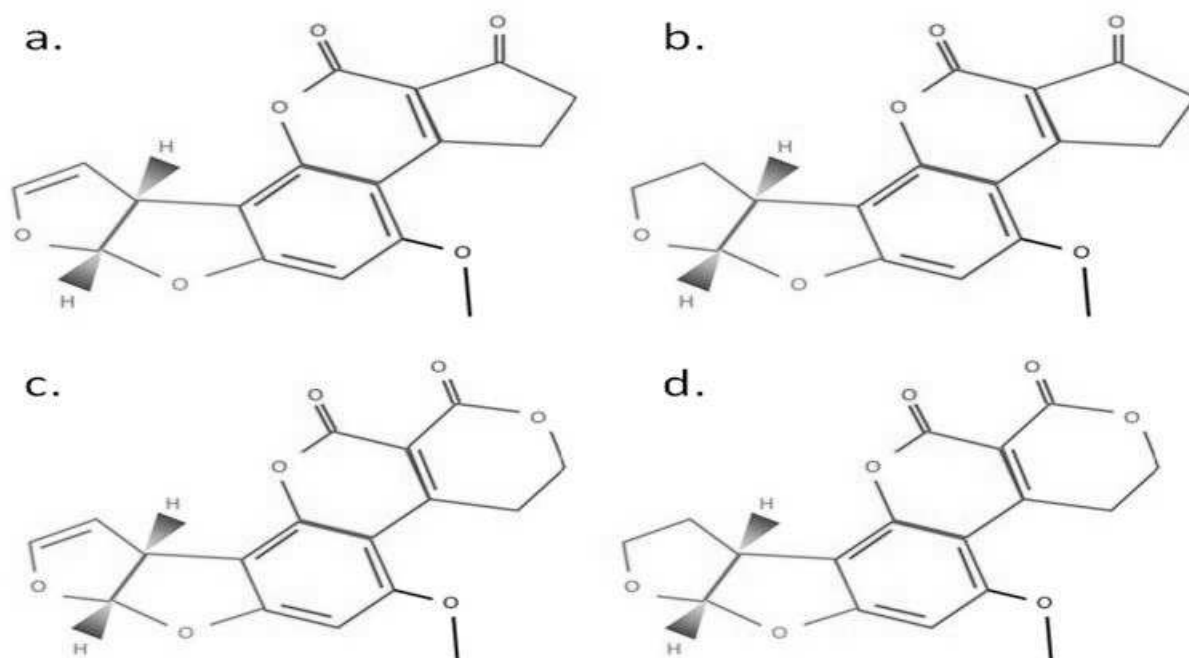
Figure 5 - 2D representation of AFB1 (a.), AFB2 (b.), AFG1 (c.) and AFG2 (d.) structures

The AFB are made of AFB1 and AFB2. The chemical structure of AFB1 (figure 5 a.), is based on a coumarin group (in red) attached to a bisfuran ring (in green) and a pentanon

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group (in blue). AFB1 molecular weight is 312 g.mol^{-1}. Unlike AFB1, the AFB2 structure (figure 5 b.) does not have a double bond in the bisfuran ring.

The AFG chemical structure is close to the B's, with the same coumarin and bisfuran ring. The difference is that AFG have a furan group (in purple) where AFB aflatoxins have a pentan group. The distinction between AFG1 and AFG2 is the same as between AFB1 and AFB2 (Figure 5 c. and d.).

Shortly after the discovery of AFB and AFG, Allcroft & Carnaghan (1963) fed cows with naturally AFB1 contaminated groundnuts meal (daily intake of 2 to 10.8 mg). The cows' milk was given to ducklings which developed liver lesions. An investigation of the milk revealed the presence of aflatoxin M1 (AFM1) (Figure 6). The name AFM1 comes from cows' milk. It is a result of AFB1 hydroxylation by the cow's metabolism (Allcroft & Carnaghan, 1963).
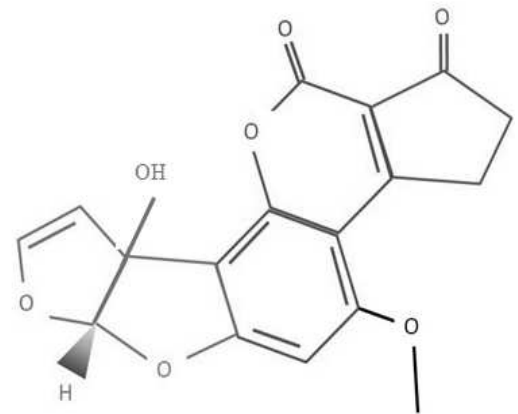
entation of AFM1 structure

Evolutionary studies estimate that AFT have been produced for more than 400 million years (Alkhayyat & Yu, 2014). However, until now, questions remain on the incentives for fungi to produce AFT. The hypotheses of Cary & Ehrlich (2006) are that AFT could:

(i)   be a defense response by fungi to stress;

(ii)   provide protection from UV damage;

(iii)   be by-products of primary metabolism;

(iv)   be virulence factors;

(v)   increase asexual spore production;

(vi)   provide protection from predators for reproductive structures such as conidia and sclerotia.

1.2.2. Toxicity

The toxicity of AFT has already been broadly studied (Peers & Linsell, 1977; Williams *et al.*, 2004; IARC, 2012). Since 2012, those AFT are considered as carcinogen for humans (except for AFM1) (Group 1 (IARC, 2014)). The main target organ is the liver (Peers & Linsell, 1977) but other targets remain. Exposure happens through ingestion, inhalation or intradermal contact. The median lethal dose (LD50) for AFB1 ranges from 0.3 mg.kg^{-1} bw for rabbits to 18 mg.kg^{-1} bw for rats (Nutrition, 2014).

1.2.2.a Impacts on human health

Human ingestion of AFB1 can cause many different symptoms. Those symptoms are mainly due to its 8-9 epoxide form. This form, as well as other AFB1 metabolites, are represented in figure 7:
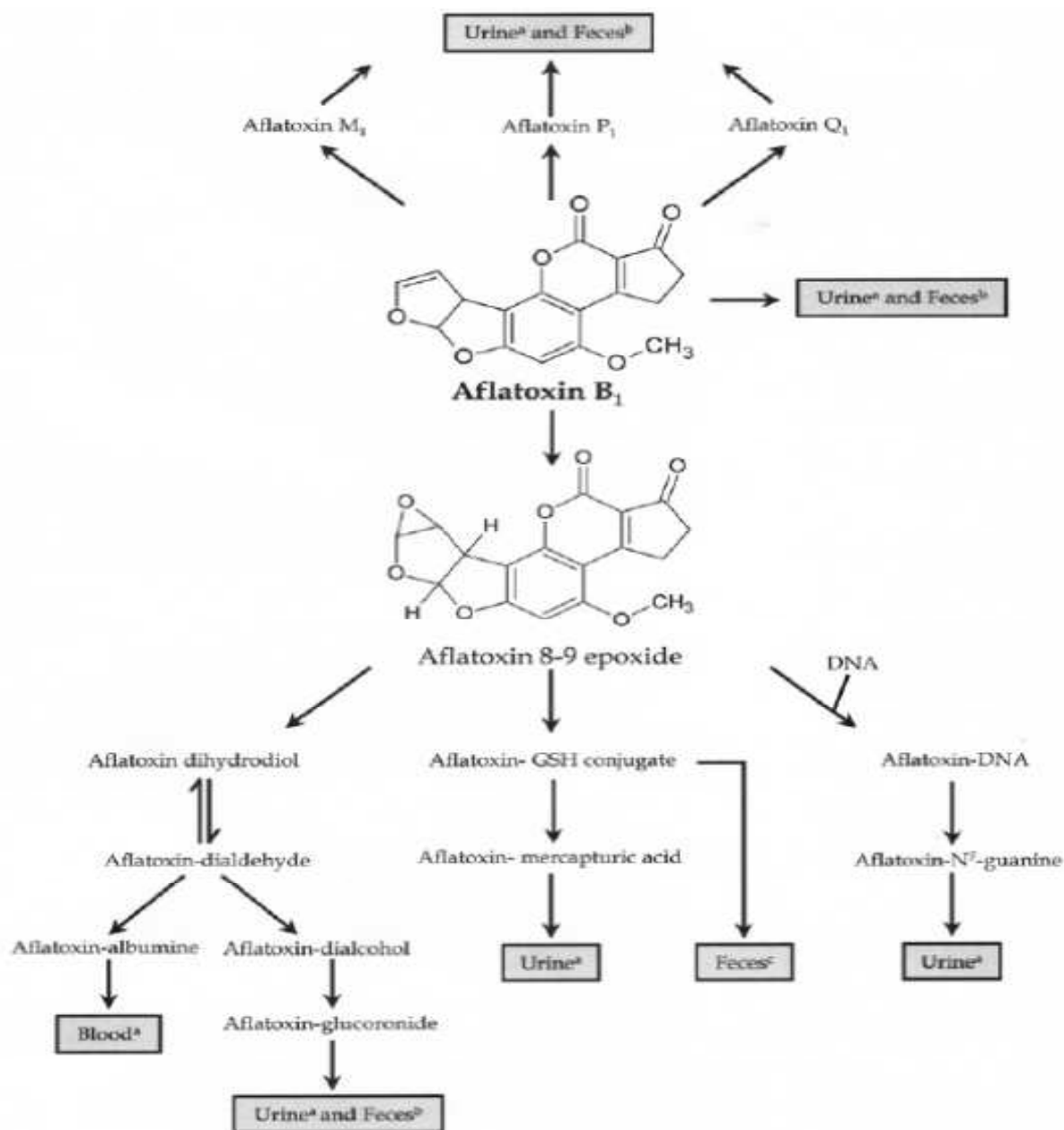
Figure 7 - Pathways of AFB1 biotransformation and excretion in humans (Vincenzi _et al._, 2011). The grey boxes highlight the fluids of excretion. AFQ1: 3α-hydroxylation of AFB1. AFP1: O-demethylation of AFB1.

a. Experimental and human evidence of metabolites excretion; b. Scarce or no evidence available; c. Only experimental evidence available.

Aflatoxin 8-9 epoxide is the result of AFB1 conversion by cytochrome P450 family into its carcinogenic form. This form can bind DNA or be hydrolysed and converted into

aflatoxin dihydriol to become aflatoxin albumine adducts in blood (AF-alb). The latter is a frequently used biomarker. Symptoms in humans are linked to AFT metabolites. The exposure can be acute (high levels (mg daily intake) of AFT resulting in immediate symptoms) or chronicle (low to moderate levels (µg daily intake) exposed regularly), and can impact various organs such as liver and kidneys.

Acute exposure:

The most occurring symptoms are linked to chronicle AFT exposure. However, in cases of exceptionally high AFT contents, clinical symptoms such as hepatitis, bile duct proliferation, edema, anorexia, malaise, reduced kidney function and lethargy can appear (Williams *et al.*, 2004; Lizárraga-Paulín *et al.*, 2011). Shortly after identifying AFT, a link between AFB1 food exposure and human hepatitis was found. The first case of endemic "aflatoxicosis" revealed that 397 persons were daily exposed to 2-6 mg of AFB1 during a month. Among these, 106 died due to a complete liver or/and kidneys failure (Krishnamachari *et al.*, 1975). Since then, despite improvements in food safety, endemic aflatoxicosis still occur in many African and Indian countries. The last case was reported in April 2004 with a maize concentration of up to 46.4 mg.kg^{-1}. This led to 317 cases of aflatoxicosis, among which 125 led to deaths (CDC, 2014).

Chronicle exposure:

HepatoCellular Carcinoma (HCC):

HCC is a common form of cancer: around 500,000 new cases are diagnosed every year around the world (El-Serag, 2011). Many cohort studies have been conducted in China to gain a precise knowledge of the incidence of AFT on HCC occurrence. Blood and/or urine samples were taken from more than 43,000 persons (aflatoxins and AFT biomarkers were quantified). Exposure to AFT led to a 2.4 to 5.5 fold increase of HCC occurrence (Ross *et al.*, 1992; Qian *et al.*, 1994; Wang *et al.*, 1996). Those data led to the classification of AFT as carcinogenic for humans. Further investigations revealed that in 36% of HCC cases, AFT exposure was correlated with a G to T transversion at codon 249 of the *TP53* tumour-suppressor gene (Stern *et al.*, 2001).

Furthermore, there is an epidemic link between HCC cases and the Hepatitis B Virus (HBV). A cohort study evaluated the impact of HBV and AFB1 exposure on HCC incidence (Wu *et al.*, 2009). As shown in table 8, the relative risk of HCC incidence in HBV-positive patients rises from 1 to 7. In case of high exposure to AFB1 ($\geq$ 59.8 fmol.mg^{-1} of AF-alb) this

risk reaches 10.4. AFB1 exposure and HBV have a synergistic effect on the development of HCC.

Table 8 - Cohort studies of AFB1 exposure, HBV and HCC (IARC, 2012). HBsAG positive = HBV-positive patients. HBsAG negative = HBV negative patients.

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases	Relative risk (95% CI)*
Wu et al. (2009) Taiwan, China	Update of the Wang et al (1996) cohort followed to June 2004	Aflatoxin urinary and albumin biomarkers and HBV status.	HCC (155)	*AFB1-albumin adducts*		
				HBsAG negative:		
				AFB1 <59.8	44	1.0
				AFB1 ≥59.8	31	1.6 (0.9-3.0)
				HBsAG positive:		
				AFB1 <59.8	111	7.0 (4.4-11.1)
				AFB1 ≥59.8	44	10.4(5.7-18.8)

Impacts on the immune system:

Chronicle AFT exposure can also impact the immune system. There is currently few data available on those impacts on humans. Nonetheless, Jiang *et al*., (2005) studied the AF-alb concentration of 64 Ghanaians. The AF-alb concentration ranged from 0.33 to 2.27 pmol.mg^{-1} albumin. They showed that the number of leukocytes was the same, independently of AF-alb content. However, T/B lymphocytes and activity markers of leukocytes were shown to be significantly lower in case of high AF-alb. This data suggests a reduction of cellular immunity in case of AFB1 exposure (Jiang *et al.*, 2005).

The Human Immunodeficiency Virus (HIV) leads to a progressive failure of the immune system. Jolly *et al*., (2011) studied 314 Ghanaians (including 155 HIV-positive). They first demonstrated that HIV-positive Ghanaians had a rate of AF-alb significantly higher than the seronegatives. Moreover, statistical analysis revealed a significant correlation between the quantity of AF-alb and the HIV viral load in HIV-positive persons (Jolly *et al.*, 2011).

Child growth retardation:

Another impact of chronicle exposure to AFT is child growth retardation. Gong *et al.*, (2004) monitored the height and AF-alb concentration of 200 children between 16 and 37 months old in Benin. A reverse correlation was established between the amount of AF-alb concentration and children height. For example, over an 8-month period, the children highly exposed to AFT grew 1.7 cm lesser than children less exposed. These data led to the conclusion that AFT exposure has an impact on infant growth (Gong *et al.*, 2004).

<u>Infertility and birth incomes:</u>

Only 1 publication deals with the links between human infertility and AFT chronicle exposure. Ibeh *et al.,* (1994) showed that, among 100 men, AFB1 is present in the semen of 40% of infertile and 8% of fertile men (in the samples found positive, the average AFB1 concentration was 1.660 μg.ml^{-1} and 1.041 μg.ml^{-1} respectively). Feeding rats during 14 days at 8.5 mg.g^{-1} of AFB1 led to similar semen abnormalities (Ibeh *et al.*, 1994).

Shuaib *et al.,* (2010) reviewed birth outcomes in correlation with aflatoxin exposure. They highlighted that aflatoxin exposure is associated with reduced birth weight and increased occurrence of still born and jaundice.

1.2.2.b Impacts on animal health

Many studies have been done on animals to evaluate the toxicity of AFT. Hereby, we will restrict our focus to impacts solely identified on animals. Among the symptoms not found in humans are pulmonary disease and tracheal exudates in horses and mucosa accumulation, pulmonary edema, capillarity fragility and icterus injuries in swine (Table 9).

AFT exposure has different impacts on animals depending on the species studied, the inter-individual response and the dose ingested. For instance, monogastric animals are more vulnerable to AFB1 exposure than ruminants (polygastric). Monogastric animals develop symptoms with feed contaminated above 50 μg.kg^{-1} while cattle symptoms occur above 1.5-2.23 mg.kg^{-1} (Eaton, 1994). Table 9 proposed by Lizzarraga-Paulin *et al.,* (2011) summarizes the major effects detected in different animals species:

Table 9 - Major diseases caused by aflatoxicosis in some animal species (Lizárraga-Paulín *et al.*, 2011)

Species	Disease	Symptoms	References
Horses	When eating: Liver damage, centrilobular hepatic necrosis phagocytosed haemosiderin in Kupffer cells, bile-duct hyperplasia, congestion of renal vessels and adrenal cortex. When inhaling: Chronic obstructive pulmonary disease (COPD), yellow-brown liver with centrilobular necrosis, icterus hemorrhage, tracheal exudates and brown urine.	When eating: Anorexia, icterus, rapid weight loss and dead. When inhaling: Chronic cough, nasal discharge, expiratory dyspnoea reduced exercise tolerance, inappetence, depression, fever, tremor, ataxia, cough and dead.	Greene & Oehme, 1976; Meerdink, 2002; Basalan et al., 2004; Caloni & Cortinovis, 2010.
Chickens	Immunosuppression, liver and kidney damage, periportal fatty infiltrations, increase in connective tissue, hemorrhages, susceptibility to opportunistic infectious agents and poor response to vaccination programs.	Low productivity, low growth, low weight, low stance, but no evident clinical symptoms and death.	Newberne & Butler, 1969; Arafa et al. 1981; Chen et al., 1984; Oguz & Kutoglu, 2000; Okiki et al. 2010.
Swine	Immunosuppression, expression of opportunistic infections, liver swollen, liver congestion, hydrothorax, edematous gall bladder, petechiated and ecchymotic mucosa, extensive centrilobular liver necrosis, haemorrhage, hepatic centrilobular cellular infiltration, hepatocyte swelling, bile stasis, hepatocyte vacuolation and bile ductule hyperplasia.	Low growth rate, gastrointestinal problems, anoroexia, ill thrift and dead.	Newberne and & Butler, 1969; Ketterer et al, 1982; Luzi et al, 2002; Gimeno, 2004.
Cattle	Fibrosis with biliary proliferation in livers, venocclusive disease increase in connective tissue, degeneration of centrilobular hepatic cells, proliferation of connective tissue, generalized liver damage and immunosupression.	Icterus, rapid weight loss and dead.	Newberne and & Butler, 1969 Vaid et al. 1981) (Gimeno 2004.
Other Animals	Pulmonary edema, generalized liver damage, coagulopathy, capillary fragility, hemorrhage, prolonged clotting times, urine pigmentation, icterus and hepatic injury.	Depression, anorexia, weight loss, bleeding, decline in feed consumption and production, gastrointestinal damage and death.	Newberne & Butler, 1969; Ostrowski-Meissner, 1983; Richard et al, 1986; Cova et al. 1990; Mckenzie et al, 1998; Klein et al, 2002; Bintvihok 2001.

1.2.3. Aflatoxins exposure

One of the best ways to reduce the health impacts mentioned here above is to identify the risks of AFT exposure in food and feed.

Tajkarimi *et al.,* (2011) has ranked the risks of AFT exposure according to commodities:

(i)   high risk: maize (cereal), peanut, pistachio, brazil nut (nut), cottonseed, copra (oilseed) and coconut meat;

(ii)   moderate risk: wheat, oat, millet, barley, rice sorghum (cereal), cassava (root), soybean, bean, pulse (bean);

(iii)   low risk: cocoa bean, linseed, melon seed, sunflower seed.

In terms of high risk commodities, Ezekiel *et al.,* (2013) studied the AFB1 content of a Nigerian peanut-based meal called 'kulikuli'. The highest AFB1 content detected was 2,824 $\mu g.kg^{-1}$ in the Lagos state, Nigeria. Another study focused on Iranian pistachios (2009-2011) and their highest AFT content was 390.49 $\mu g.kg^{-1}$ (Dini *et al.*, 2013). As for oilseed, a 2010-2011 survey identified the highest concentration at 14.4 $\mu g.kg^{-1}$ AFT in cottonseeds (Feizy *et al.*, 2012).

Faced with those risks, the EU set maximum authorised levels of AFT in various products to reduce consumers exposure (See table 6 in chapter 1.1.5.). Regulations do not only apply to AFT but also specifically to AFB1 and AFM1 in milk and milk products. The maximum levels for AFB1 range from 12 $\mu g.kg^{-1}$ in almonds, pistachios and apricot kernels (before being sorted for human consumption) to 0.1 $\mu g.kg^{-1}$ for baby food and dietary food for medical purposes. For feed (Annex 1), the maximum levels of AFB1 range from 20 $\mu g.kg^{-1}$ for feed materials and for cattle, sheep, goats, pigs and poultry to 5 $\mu g.kg^{-1}$ for dairy cattle, calves, sheep, lambs, goats, kids, piglets and young poultry animals.

Levels of enforcement of those EU regulations are high and notifications of non-conformity are included in the Rapid Alert System for Food and Feed (RASFF). In 2013, there were 340 notifications for AFB1 and AFT. Table 10 represents the number of notifications and the level of contamination. Those data give an idea of AFT occurrence in food and feed. For example, the highest concentration of AFB1 and AFT (28,000 $\mu g.kg^{-1}$ and 31,100 $\mu g.kg^{-1}$ respectively) was found in shelled peanuts from China. Nuts and associated products represent the highest number of notifications in 2013, followed by fruits and vegetables. 41 out of the 44 notifications for fruits and vegetables related to Turkish dried figs. Notifications have also been placed on herbs and spices, especially spices coming from

India and Indonesia. A great proportion of notifications on feed materials (21/35) and cereals and bakery products (4/10) are associated with maize (RASFF, 2014).

Products	Number of notifications	AFB1 $\mu g.kg^{-1}$	AFT $\mu g.kg^{-1}$
nuts, nut product and seed	213	2.6-28000	4.1-31100
fruits and vegetables	44	3.2-14400	3.2-29000
feed materials	35	21.6-230	26.7-230
herbs and spice	32	7.3-97	7.9-115
cereals and bakery products	10	3.9-86.1	6.2-49.3
compounds feeds	1	5.91	10.03
ice and desserts	1	5.72	6.72
other food product /mixed	1	0.38	/
pet food	1	376	/
prepared dishes and snacks	1	28	33.4
soups, broths, sauces and condiments	1	5.5	/

Maize is the cereal with the highest risk of AFT accumulation. As maize is the most consumed commodity in many countries, its contamination is of greatest concern. In 2011, the Food and Agriculture Organisation estimated the daily intake of maize at more than 50 g.day^{-1} in 61 countries ("FAOSTAT," 2014).

The levels of maize contamination by AFT differ widely (from none to 46.4 mg.kg^{-1} in Kenyan maize, 2004). There are numerous and regular surveys worldwide to monitor AFT occurrence. In 2011, Indians analysed 639 samples of maize pre and post-harvest. Among these, 22.97% (pre-harvest) and 53.93% (post-harvest) had AFB1 levels between 0.4 and 149.32 µg.kg^{-1} (Karthikeyan *et al.*, 2013). Another example is a Cameroonian survey on poultry maize. The analysis of 77 samples revealed that 9.1% were positive with AFT levels between 2 and 42 µg.kg^{-1} (Kana *et al.*, 2013). To avoid acute exposure, many countries set maximum levels of AFT in maize for food, ranging from 40 (China, Nigeria) to 4 µg.kg^{-1} (EU). Unfortunately, these regulations are often difficult to enforce.

Those different AFT regulations for maize are represented in Figure 8:

Aflatoxin regulations for maize
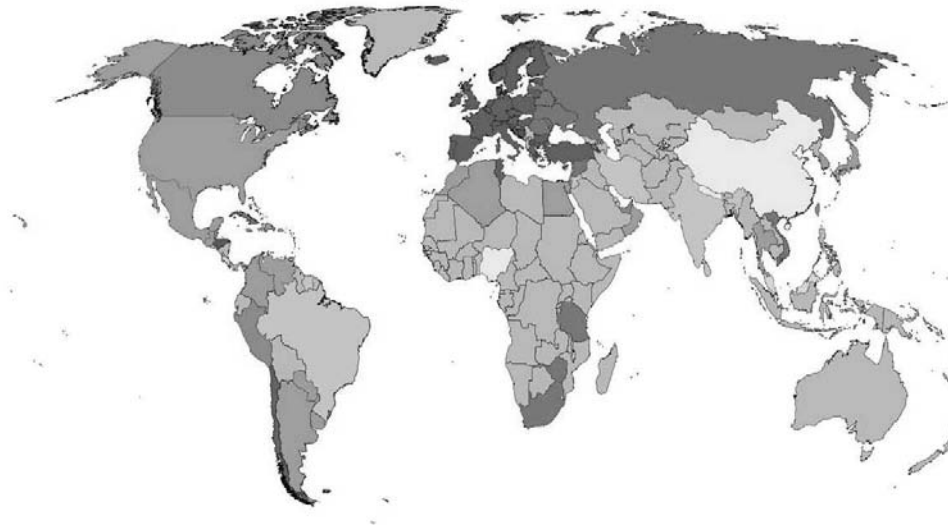
Figure 8 - Maximum levels of AFT authorised in maize per country (Wu & Guclu, 2012).

No regulation,

40 µg.kg^{-1}(e.g. China)

4 µg.kg^{-1} (e.g. EU).

Red represents the most stringent regulations

In Europe, 2 recent surveys on AFT occurrence have been done. A Serbian study revealed that among the 137 maize samples analyzed in 2012, 68.5% had between 1.01 and 86.1 µg.kg^{-1} of AFT content (Kos *et al.*, 2013). In Croatia, 633 maize samples were analyzed in 3 different regions in 2013. 38.1% of them were contaminated with AFB1 levels from 1.1 to 2,072 µg.kg^{-1}, the most contaminated region being Eastern Croatia (Pleadin *et al.*, 2014).

Although maize is not the main source of RASFF notifications, in France, it is the crop with the highest AFT risk. In 2012, the French maize production was 15.61 million tones and in 2011, the French maize exportation represented 1.9 billion € ("FAOSTAT," 2014). In the last 10 years, there has been no RASFF notifications on maize in France. However, Italy has received more than 15 notifications (2004-2014) due to AFT in maize, with 4 in the last 3 years. Moreover, recent meteorological models (figure 9) highlight potential AFT risks in France in the years to come. Indeed, the business-as-usual scenario (figure 9 a.) predicts negligible to low risks in South West France for the 2001-2100 period. Nonetheless, in case of climate change leading to a global temperature increase of 3°C (figure 9 b.), AFB1 risks could be medium in South West France. In the case of a 5°C increase (figure 9 c.), AFB1 risks could be high in South West France and medium to high in other French regions. Those data suggest that AFT could become an emerging issue in France in the coming years.

a.   b.   c.

High AFB1 risk

Medium AFB1 risk

Low AFB1 risk

Negligeable AFB1 risk
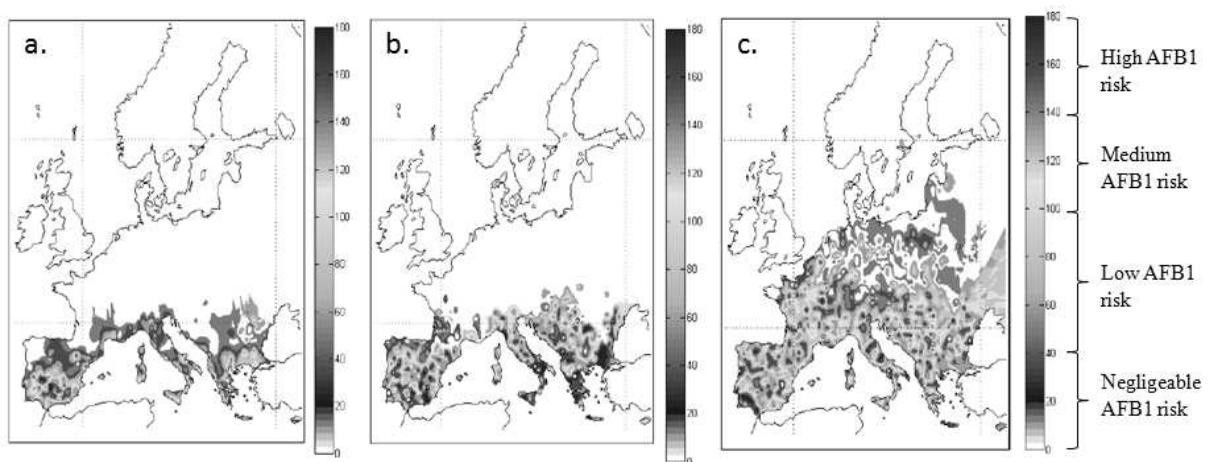
...narios for the 2000-2100
...°C scenario. (Battilani *et*

1.2.4. The fungi producing aflatoxins

One of the best ways to manage the risks of AFT exposure in food and feed is to identify the fungi producing aflatoxins.

AFT are mainly produced by the *Aspergillus* genus (Table 11). This genus was first classified by Micheli in 1729 (Wilson *et al.*, 2002). It is characterised by its ability to spread easily and its high concentration in the air. Moreover, thanks to its ubiquity, it can grow as soon as enough water and nutrients are available in its environment (Bennett, 2010). Furthermore, it is a *Deuteromycota,* meaning it has mainly an asexual mode of reproduction. Nowadays, the *Aspergillus* genus comprises 260 or 837 species depending on classification criteria (Geiser *et al.*, 2007; Index Fungorum *et al.*, 2014). They are divided in 22 different sections including *Flavi, Ochraceorosei* and *Nidulantes* (Table 11) (Varga & Samson, 2008).

Table 11 - Summary of species producing aflatoxins.

Genus	Section	Species	Country of origins	Isolated from	Aflatoxins B1, B2	Aflatoxins G1, G2	Reference
Aspergillus	Flavi	A. arachidicola	Argentina	peanuts	+	+	Pildain et al., 2008
		A. bombycis	Japan	silkworm	+	+	Peterson et al., 2001
		A. flavus	UK	feed	+	-	Nesbitt et al., 1962
		A. minisclerotigenes	Argentina	peanuts	+	+	Pildain et al., 2008
		A. mottae	Portugal	maize	+	+	Soares et al., 2012
		A. nomius	USA	insects and wheat	+	+	Kurtzman et al., 1987
		A. novoparasiticus	Brazil	medical environment	+	+	Goncalves et al., 2012
		A. parasiticus	USA	nuts	+	+	Codner et al., 1963
		A. parvisclerotigenus	Nigeria	nuts	+	+	Frisvad et al., 2005
		A. pseudocaelatus	Argentina	nuts	+	+	Varga et al., 2011
		A. pseudonomius	USA	insects and soil	+	-	Varga et al., 2011
		A. pseudotamarii	Japan	tea field soil	+	-	Ito et al., 2001
		A. sergii	Portugal	almonds	+	+	Soares et al., 2012
		A. transmontanensis	Portugal	almonds	+	+	Soares et al., 2012
	Ochraceorosei	A. ochraceoroseus	Ivory Coast	soil	+	-	Frisvad et al., 2005
		A. rambellii	Ivory Coast	soil	+	-	Frisvad et al., 2005
	Nidulantes	E. venezuelensis	Venezuela	red mangrove sponge	+	-	Frisvad & Samson, 2004
		A. togoensis	Central Africa	moldy fruits	+	-	Rank et al., 2011
		E. olivicola	Italy	olive	+	-	Zalar et al., 2008
		E. astellata	Galapagos	holly	+	-	Frisvad & Samson, 2004
Fusarium		F. kyushuense	Japan	diseased wheat	+	+	Schmidt-Heydt et al., 2009

A.: *Aspergillus*, E.: *Emericella (sexual form of some Aspergillus)*, F.: *Fusarium*

The predominant AFT producer is *Aspergillus flavus*, which only biosynthesises AFB. The second predominant producers are *A. parasiticus* and *A. nomius* which produce AFB and additionally AFG (Dorner *et al.*, 1984; Kurtzman *et al.*, 1987). An exhaustive list of the current scientific literature on AFT producers is drawn in table 11. *Flavi*, *Ochraceorosei* and *Nidulantes* are the 3 sections of *Aspergillus* producing aflatoxins. AFG production by species belonging to section *Ochraceorosei* and *Nidulantes* are not currently described.

There is however an exception to those rules. Schmidt-Heydt *et al.*, (2009) demonstrated that *Fusarium* genus can produce AFT (Schmidt-Heydt *et al.*, 2009).

Hereafter, we will focus on *A. flavus* as the most occurring contaminant and *A. parasiticus* as a model of AFT production.

1.2.4.a *Aspergillus flavus*

A. flavus is currently the main AFB1 producer in many commodities, including maize (Klich, 2002; Giorni *et al.*, 2007; Reddy *et al.*, 2011; Ezekiel *et al.*, 2013; Mauro *et al.*, 2013; Adjovi *et al.*, 2014). The colour of *A. flavus* is white with usually yellow-green to green colours due to conidiogenesis. Figure 10 represents a mature conidiophore (cd) composed of an hyphae (h) (≤1 mm long) connected to the cd. It is composed of a core element: a vesicule (v), surrounded by phialides (p) which produce smooth conidies (c). It is often biseriated with a metula (a sterile branch upon which p develop) situated between v and p.
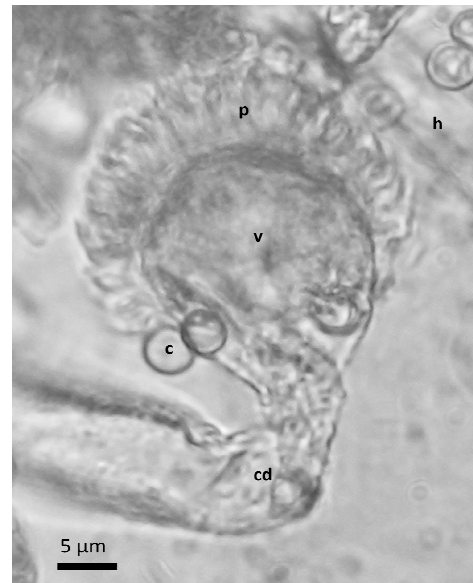
lus flavus **by microscopy.**
ɪodiophores; h= hyphae;
vesicule. (Personal data)

To understand the occurrence of *A. flavus* at field, figure 11 represents *A. flavus* pathogenic and saprophytic stages. The saprophytic stage on plant residues is usually characterized by the presence of various size sclerotia. The latter has hyphae which regroup themselves and create a melanin-based surface (black colour). They germ and sporulate on the plant or in the soil. The resulting conidies are transported mainly through wind and insects before starting their pathogenic stage on the crop. With its high capacity of germination, *A. flavus* can easily use again insects and wind to realize secondary inoculum on surrounding crops (Abbas *et al.*, 2009). Although *A. flavus* main mode of reproduction is by asexual conidial sporulation, it is capable of *Petromyces* sexual reproduction (Horn *et al.*, 2009).

There are 2 different types of strains among *A. flavus*, depending on their sclerotia size and their ability to produce AFB. The Large (L) strains have large sclerotia (300-700 µm) and produce no or little AFB. The Small (S) strains have smaller sclerotia (150-250 µm) and produce larger amounts of AFB (Cotty, 1989).
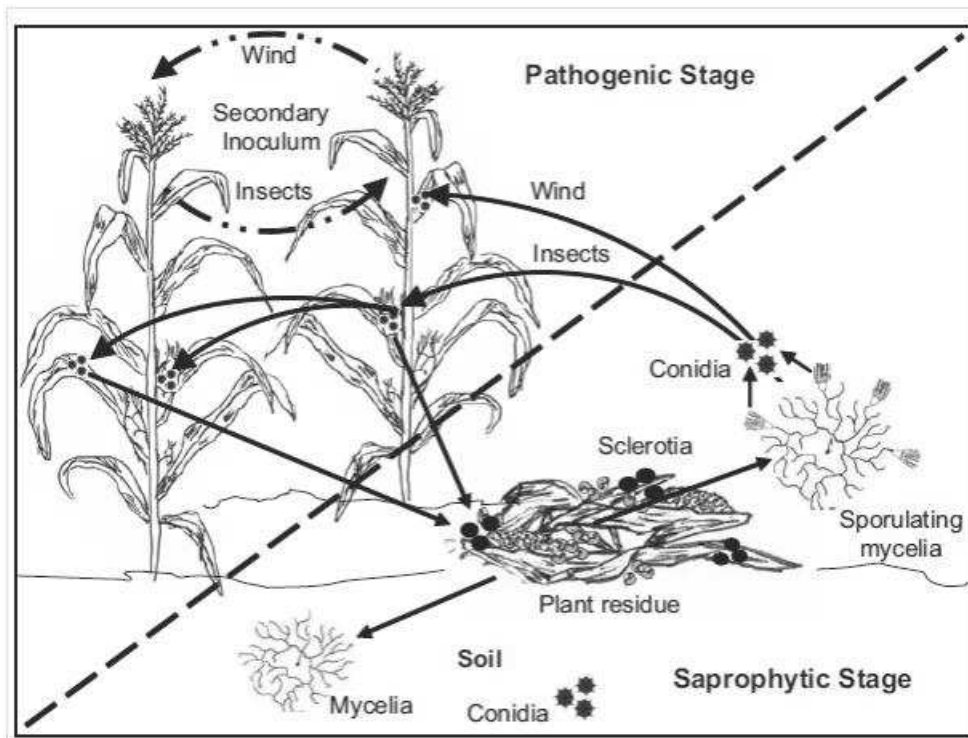
1.2.4.b *Aspergillus parasiticus*

A. parasiticus was known as the second producer of AFT in food (Giorni *et al.*, 2007). However, the recent re-sectorization of *Aspergillus* species led to the reconsideration of occurrence studies based only on micro or macromorphological recognition of *A. parasiticus* (Leslie & Logrieco, 2014). *A. parasiticus* is a model fungus for AFT production. This fungus has a life cycle similar to *A. flavus* as shown in figure 11. Similar structures of h, c, cd and p can be recognized (Figure 12), There are however differences between *A. flavus* and *A. parasiticus*. The latter:

spergillus parasiticus by c: conidies, cd: h: hyphae, p: phialides Personal data)

(i) has a darker green conidiation colour;

(ii) has rough conidies;

(iii) is > 90% mostly uniseriate (no metula);

(iv) both fungi also have different DNA sequences e.g.: ITS, β-tubulin and calmodulin sequences.

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Other criteria such as extrolites profiles can differentiate them. For instance, their aflatoxin gene cluster, enabling *A. parasiticus* to produce AFG, is different.

Studying this gene cluster is key to understand the production of AFT.

1.2.5. The biosynthesis pathway of aflatoxins

Before looking into the genetic parameters affecting the production of AFT, it is essential to recall that genetic regulation factors together with environmental factors influence the production of AFT. Unlike this document which deals with 1 factor (genetic regulation) before another (environment), interactions between those 2 parameters are much more interwoven.

Shortly after the discovery of the Turkey X disease, the biosynthesis study of AFT began with the characterisation of UV irradiated mutants (Lee *et al.*, 1971).

Nowadays, we know that the entire cluster is a sequenced 75 kb cluster located in the subtelomeric region of chromosome 3 (Ehrlich *et al.*, 2005b). Both *A. flavus* and *A. parasiticus* have this cluster in the same gene order. There is a slight difference between the 2 though. *A. flavus* has a deletion in the cluster from 0.8 (L strains) to 1.5 kb (S strains) depending on the isolate. This deletion covers the 5' ends of *aflF* and *aflU*, and their entire 279 bp intergenic region. This is the reason why *A. flavus* does not produce AFG. In the cluster, DNA studies revealed that *A. flavus* /*A. parasiticus* share a 96% homology (Ehrlich *et al.*, 2005b).

We know that there are putatively 30 genes in this cluster, thanks to identification studies mostly done on *A. parasiticus* (Yu, 2012). Figure 13 represents the *A. parasiticus* cluster, the predicted genes and their associated enzymes in the biosynthesis pathway. Aflatoxin biosynthesis uses 1 AcetylCoA and 9 MalonylCoA as first substrates. Hereafter, we will describe *A. parasiticus* genes, their encoding proteins and the known precursors involved in AFT production.

Below is a description of each step of the biosynthesis (numbered in figure 13).
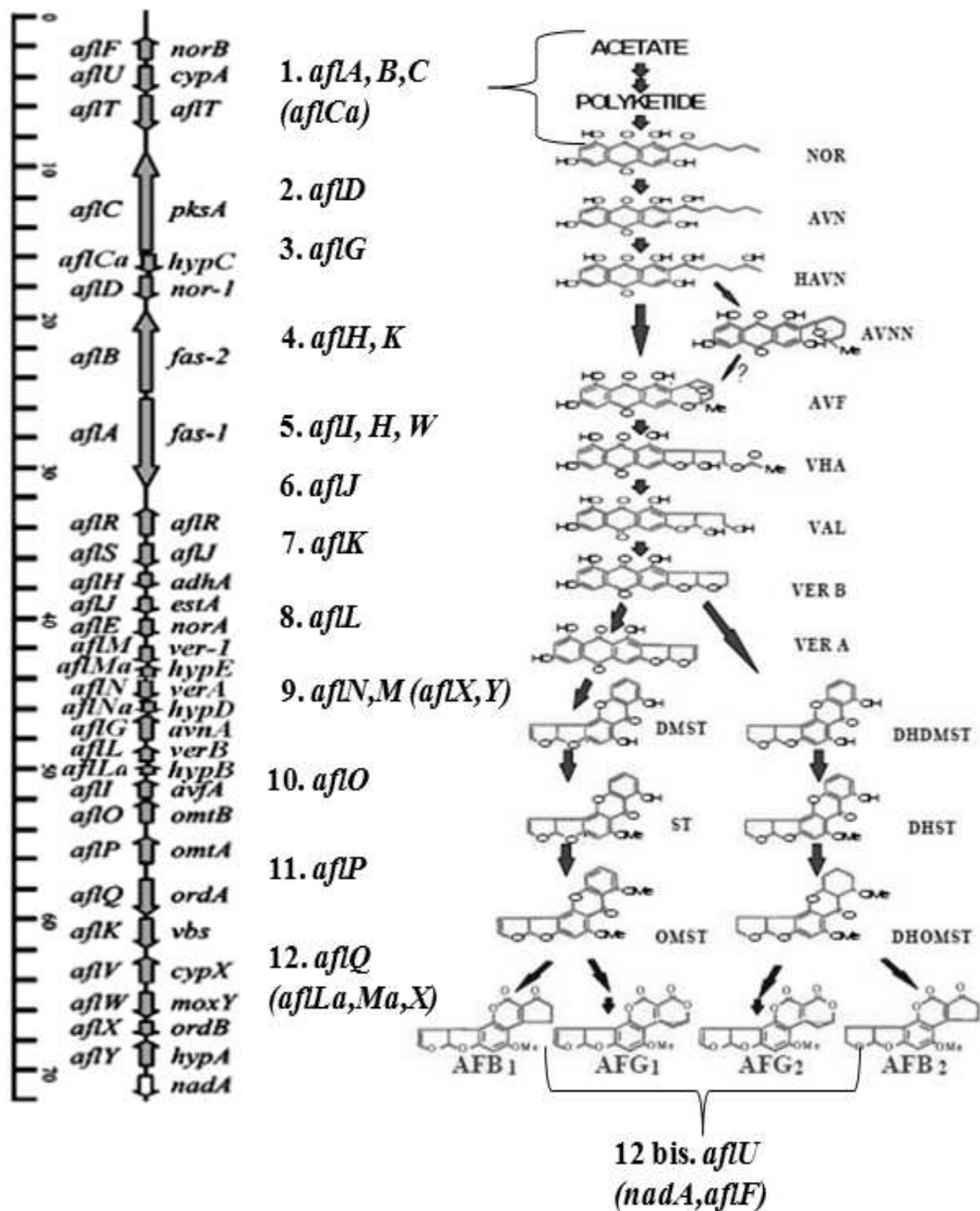
Figure 13 - The genes cluster of the biosynthesis pathway of aflatoxin in *A. parasiticus* **according to Yu (2012); Georginna & Payne (2009). New gene names are labelled on the left and old gene names are labelled on the right of the cluster. Number 1. to 12. and their associated genes (predicted genes in brackets) represent the steps described in chapter 1.2.5.**

NOR= norsolorinic acid; AVN= averantin; HAVN= 5'-hydroxy-averantin; AVNN= averufanin; AVF= averufin; VHA= versiconal hemiacetal acetate; VAL= versiconal; VERB= versicolorin B; VERA= versicolorin A; DMST= demethylsterigmatocystin; DHDMST= dihydrodemethylsterigmatocystin; ST= sterigmatocysin; DHST= dihydrosterigmatocysin; OMST= O-methylsterigmatocystin and DHOMST= dihydro-O-methylsterigmatocystin.

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The first step of the biosynthesis pathway is the synthesis of Norsolorinic acid (NOR). As with the other steps described below, we will describe the gene first and then focus on the functions of the encoded proteins responsible for AFT production.

1.) The synthesis of NOR

genes: *aflA* (*fas-2*) & *aflB* (*fas-1*) & *aflC* (*pksA*) and putative gene: *aflCa* (*hypC*)

proteins and functions: AflA (Fatty acid synthase α) & AflB (Fatty acid synthase β) & AflC (Polyketide synthase). These 3 proteins constitute a complex, called NorS, of 1.4x10^6 Da (partially purified in *A. parasiticus*). As represented in figure 14, the first role of NorS is the synthesis of a hexanoyl primer thanks to the addition

Figure 14 - Formation of norsolorinic acid: the first stable metabolite of aflatoxin biosynthesis (Ehrlich, 2009). Norsolorinic Acid Anthrone (NAA) = Noranthrone. In red, the predicted role of AlfCa.

of 2 MalonylCoA units. This primer is then transferred to the acyl carrier or β-ketoacyl synthase domain of AflC (Watanabe & Townsend, 2002) and is converted into Noranthrone (NAA) by the iterative addition of 7 other MalonylCoA units. However, this intermediate is not stable in time and can be converted spontaneously into NOR or by the putative NAA oxidase: AflCa (Figure 14) (Ehrlich, 2009). NOR is the first stable metabolite of the aflatoxin biosynthesis. *A. parasiticus* mutated strains of *aflD* (*nor-1*) show accumulation of a red-orange pigment: NOR (Lee *et al.*, 1971).

2.) The conversion of NOR into Averantin (AVN)

gene: *aflD* (*nor-1*)

protein and function: AflD is a ketoreductase (Trail *et al.*, 1994) involved in the reduction of the NOR 1'-keto group into the 1'-hydroxyl group of AVN (Figure 15). Although the function is confirmed, mutated strains of *aflD* do not completely stop AVN conversion. The other mechanisms leading to this reduction are not understood yet.
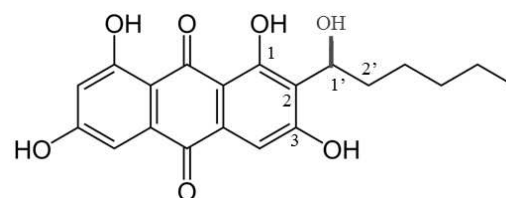
Figure 15 - Averantin (AVN). The red part represents AflD action.

3.) The conversion of AVN into 5'-Hydroxyaverantin (HAVN)

gene: *aflG* (*avnA, ord-1*)

protein and function: AflG is a cytochrome P450 monooxygenase involved in the hydroxylation of the AVN 5'-keto group into the 5'-hydroxyl group of the HAVN (Figure 16) (Yabe *et al.*, 1991).
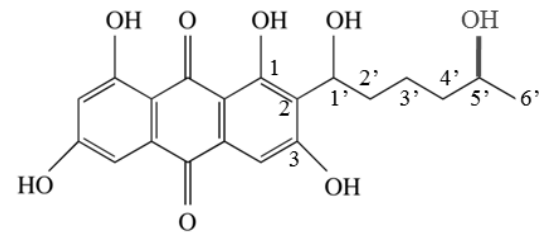
Iydroxyaverantin (HAVN). The red
AflG action.

4.) The conversion of HAVN into Averufin (AVF)

genes: *aflH* (*adhA*), *aflK* (*vbs*)

proteins and functions: This HAVN dehydrogenase is involved in the dehydrogenation (NAD dependent) of the 5'-hydroxyl group of HAVN to the 5'-oxide group of the 5'-oxoaverantin (OAVN) (Figure 17) (Sakuno *et al.*, 2003). *AflH* deletion mutants did not completely lose the possibility to produce OAVN, suggesting that other potential mechanisms might be involved. AflK is a OAVN cyclase and is involved in the dehydratation of the 5'-oxide of OAVN leading to the formation of the (2'-5') AVF (Figure 18) (Sakuno *et al.*, 2005).

oaverantin (OAVN). The red part
ction.
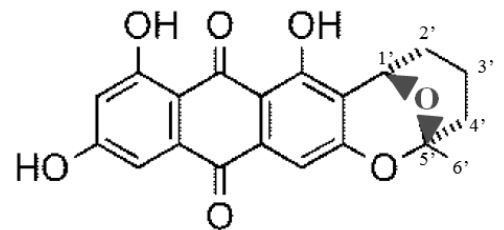
ifin (AVF). The red part represents

5.) The conversion of AVF into Versiconal Hemiacetal Acetate (VHA)

genes: *aflV* (*cypX*), *aflI* (*avfA*), *aflW* (*moxY*)

proteins and putative functions: AflV is a cytochrome P450 oxidoreductase (Wen *et al.*, 2005). As proposed in figure 19, a loss of an hydride group from the 2'-C of AVF by AflV is the initiation step. The predicted metabolite is then hydrated, and AflI supposedly acts as an oxidoreductase (Figure 19) (Yu *et al.*, 2000).
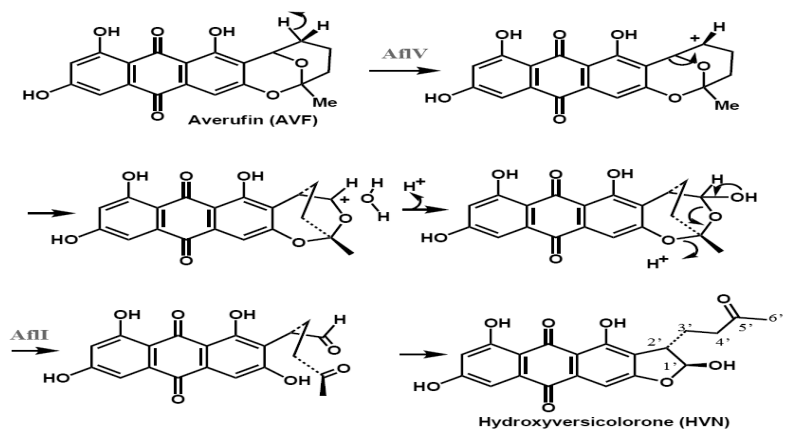
of AVF into Hydroversicolorone
(2009).

AflW monooxygenase is responsible for the insertion of an oxygen atom between the 4' and the 5' keton groups of HAVN and leads to the formation of VHA (Figure 20) (Wen *et al.*, 2005).
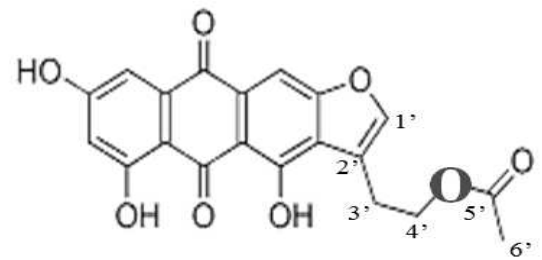
nal Hemiacetal Acetate (VHA).
ents AflW action.

6.) The conversion of VHA into Versiconal (VAL)

gene: *aflJ (estA)*

protein and function: AflJ is an esterase which catalyses the removal of an acetate at the extremity of VHA to convert it into VAL (Figure 21) (Chang *et al.*, 2004).
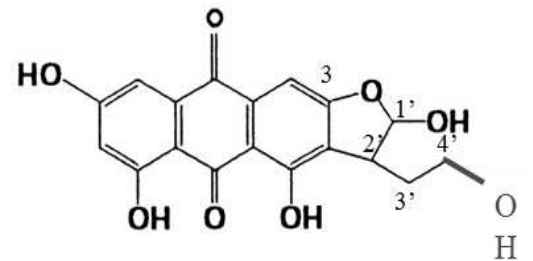
rsiconal (VAL). The red part
tion.

7.) The conversion of VAL into Versicolorin B (VERB)

gene: *aflK (vbs)*

protein and function: AflK is a cyclase already involved in **4.)** (Figure 22). Nonetheless, AflK was first identified in the cyclodehydratation of VAL to VERB (Lin & Anderson, 1992). It is a key step of aflatoxin biosynthesis because it is responsible for the closure of the bisfuran ring. It is also the last common precursor of the AFB1-AFG1 and AFB2-AFG2 pathways.
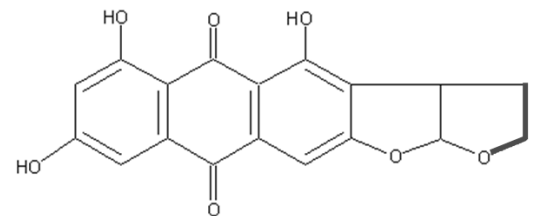
sicolorin B (VERB). The red part
action.

8.) The conversion of VERB into Versicolorin A (VERA) - AFB1-AFG1 pathway only.

gene: *aflL (verB)*

protein and function: AflL is a cytochrome P450 monooxygenase which converts the tetrahydrobisfuran ring into a dihydrobisfuran ring (Kelkar *et al.*, 1997) (Figure 23).

AFB1- AFG1 pathway   AFB2-AFG2 pathway

No metabolite
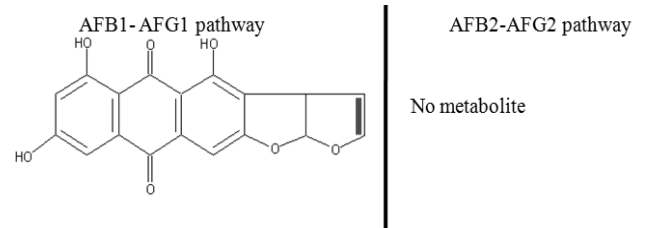
n A (VERA). The red part represents

9.) The conversion of VERA into Demethylsterigmatocystin (DMST) and VERB into Dihydrodemethylsterigmatocystin (DHDMST)

genes: *aflN (verA)* & *aflM(ver-1)* putative genes: *aflY (hypA)* & *aflX (ordB)*

proteins and functions: AflN is a cytochrome P450 monooxygenase (Keller *et al.*, 1994) and AflM a deoxygenase (Skory *et al.*, 1992). AflY and AflX are predicted to be a monooxigenase (Ehrlich *et al.*, 2005a) and an oxidoreductase, respectively. Both putative enzymatic functions are represented in figure 24 (Cary &

the conversion of VERA into ɡested intermediates shown in

Ehrlich, 2006; Ehrlich, 2009). These enzymatic steps lead to the production of DMST in **AFB1-AFG1 pathway**.

In the case of **AFB2-AFG2 pathway**, the same enzymatic steps are proposed with VERB substrate instead of VERA leading to the formation of DHDMST. The difference between DMST and DHDMST is the same as the difference between VERA and VERB. It is due to the double bond in the bisfuran ring.

10.) The conversion of DMST into Sterigmatocystin (ST) and DHDMST into Dihydrosterigmatocystin (DHST)

gene: *aflO (omtB, dmtA)*

protein and function: AflO is an O-methyltransferase. It catalyzes the transfer between the methyl group of S-adenosylmethionine and the hydroxyls of DMST and DHDMST. This leads to the production of ST and DHST, depending on the pathway (Motomura *et al.*, 1999) (Figure 25).
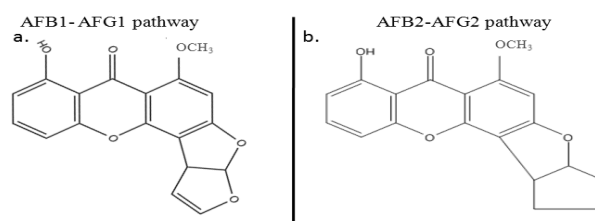
ure: a. Sterigmatocystin (ST); b. T). The red parts represent AflO

11.) The conversion of ST into O-methylsterigmatocystin (OMST) and DHST into dihydro-O-methylsterigmatocystin (DHOMST)

gene: *aflP (omtA)*

protein and function: AflP is the second O-methyltransferase of the pathway. It is specific
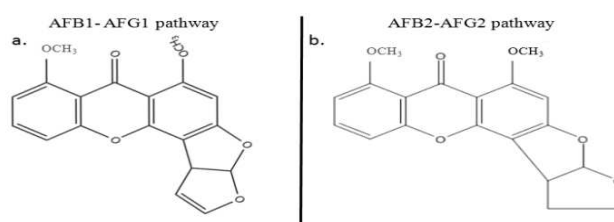
ucture: a. O-methylsterigmatocystin ːhylsterigmatocystin (DHOMST). The ion.

to ST and DHST substrates (Yu *et al.*, 1993). It allows the production of OMST and DHOMST (Figure 26). Indeed, a lack of *aflP* orthologue *A. nidulans* prevents the production of AFT (Yu, 2012).

12.) The conversion of OMST into AFB1 and DHOMST into AFB2

gene: *aflQ (ordA)* and putative genes: *aflLa (hypB), aflX (ordB), aflMa (hypE),*

proteins and functions: AflQ is a cytochrome P450 monooxygenase (Prieto & Woloshuk, 1997) involved in the conversion of OMST into AFB1. Ehrlich (2009) proposed a more detailed metabolism pathway which is represented in figure 27. In this predicted scheme, AflQ could be involved in C-11 hydroxylation and AflLa could introduce an oxygen into the keto-tautomer of 11-hydroxyOMST. Those reactions could lead to a 370 Da metabolite. AflMa is suspected to be involved in the demethylation of the A-ring and may act conjointly with a cytochrome P450 monooxygenase (figure 27) as the last step prior to AFT production in both aflatoxins pathways.
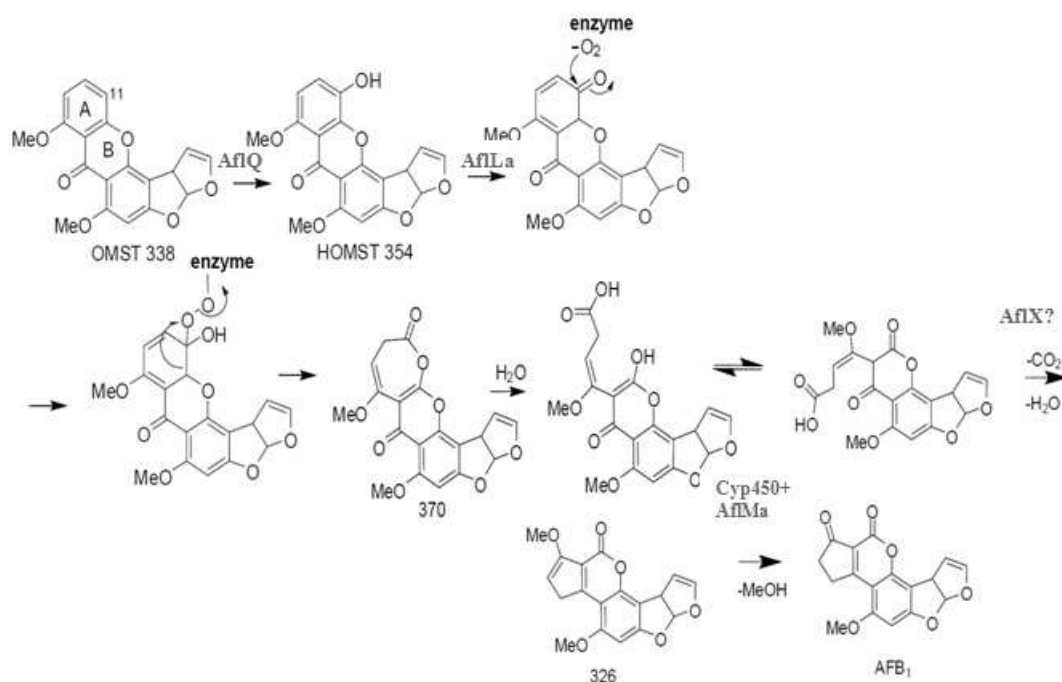
Figure 27 - Speculated last steps from OMST to AFB1 production according to Ehlrich (2009). HOMST= 11-HydroxyOMST. The numbers under the molecules are the atomic mass in Dalton.

12. bis) The conversion of OMST into AFG1 and DHOMST into AFG2

 gene: *aflU(cypA)* putatives genes: *nadA ,aflF(norB)*

The 370 Da metabolite proposed in figure 27 is the probable substrate for AflU oxidations to produce AFG. NadA and AflF could be good candidates to support AflU activity in AFG production as their function has not been determined yet (Ehrlich *et al.*, 2004).

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1.2.6 The genetic regulation of aflatoxins production

The steps, described above and leading to the production of AFT, are modulated by certain transcription factors. The aflatoxin biosynthesis pathway is regulated by specific (AflR, AflS) as well as general transcription regulators.

1.2.6.a AflR, a specific transcription factor

aflR is the ninth gene of the aflatoxin biosynthesis cluster. It encodes a Cys_6Zn_2 transcription factor needed for AFT production (Payne *et al.*, 1993). The composition of the AflR transcription factor is represented in figure 28 (blue part).

e consensus domains for , AflR,...). NLD= Nuclear
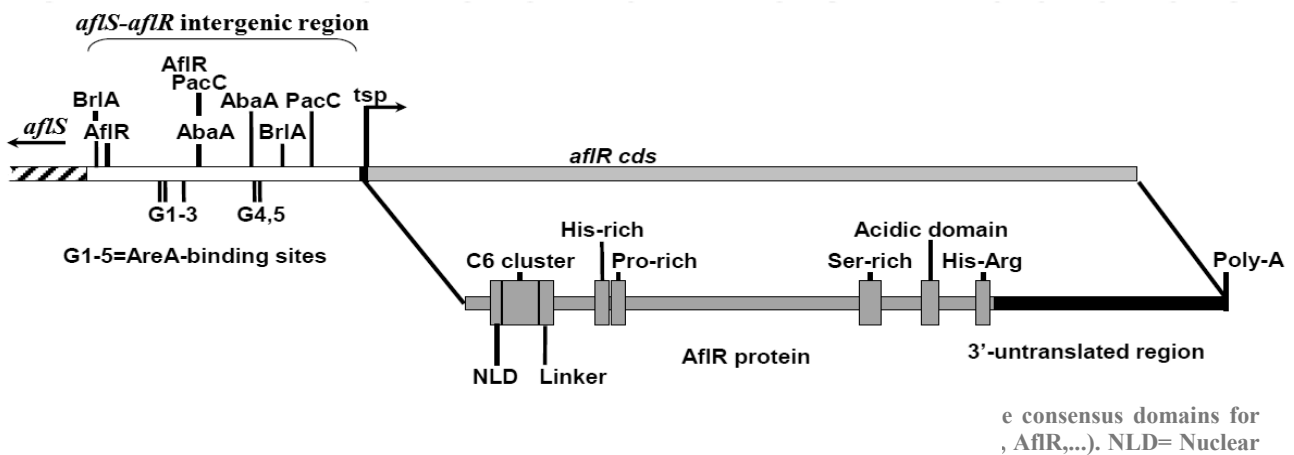
Among the different regions, the AflR N-terminal region (C6 Cluster in figure 28) is the DNA-binding domain, including: the Nuclear Localization Domain (NLD) that ensures AflR transfer from cytoplasm to nucleus (Ehrlich *et al.*, 1998) and the linker region that is possibly involved in DNA-binding specificity.

The specific DNA sequence is composed of 11 bp: 5'-TCGSWNNSCGR-3' (with S: Guanine (G) or Cytosine (C); W: Adenine (A) or Thymine (T) and R: A or G) with the strongest binding site being 5'-WCGSNNNSCGA-3'. These AflR binding sites are usually localized at 200 bp (mainly in the promoter region) prior to the aflatoxin genes translation start point (tsp) except for *aflT* and *avfA* (Ehrlich, 2009). There is upstream of the *aflR* gene transcription start point (TSP) (white part in figure 28), a partial AflR binding site, suggesting an autoregulation. In the same intergenic region, other binding sites from various DNA-binding proteins suggest that many regulation systems may impact *aflR* expression.

Price *et al.*, (2006) studied 40% of the *A. parasiticus* transcriptome in a wild type strain and its Δ*aflR* mutant which lacks the capacity to produce AFT. The microarray results are presented in figure 29. They revealed that most of the aflatoxin gene in the cluster were down regulated in the mutant (except for *aflF, I ,Ma, N* and *Na*) (Price *et al.*, 2006).
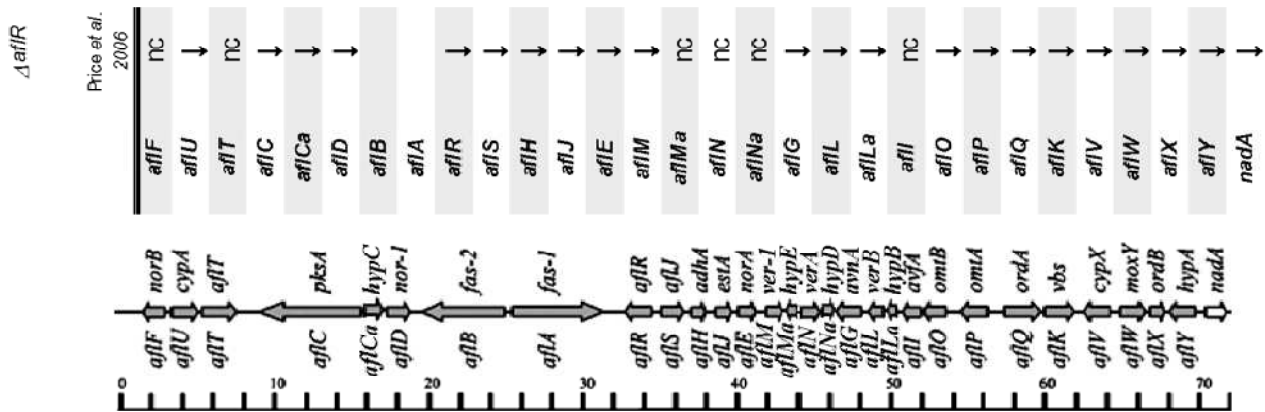
Figure 29 - Scheme representing the impacts of Δ*aflR* mutant on the aflatoxin gene cluster proposed by Georgianna & Payne (2009). An arrow represents a down regulation. nc= low level changes.

1.2.6.b AflS, a putative transcription factor

aflS is the tenth gene of the aflatoxin biosynthesis cluster and share the same intergenic region with *aflR* (white in figure 28). Knockout mutants revealed that *aflS* is required for AFT production but AflS function remains to be characterised. Its three main potential roles are:

AflS is a potential coactivator of AflR (Chang, 2003), although deletion of *aflS* did not have any impact on *aflR* transcripts. AflS specifically interacts with the His-Arg (figure 28) of AflR (Chang, 2003).

A second role is impact on early genes of the aflatoxin biosynthesis (Meyers *et al.*, 1998). Δ*aflS* mutants repressed *aflC, aflD, aflM* and *aflP* transcription by 5 to 20 fold but had no impact on *aflR* expression (Meyers *et al.*, 1998). However, another study rejected AflS impact on *aflM* and *aflP* expression (Du *et al.*, 2007). The effect of AflS on the expression of early genes could be linked to its coactivating functions.

The last role is AflS potential interaction with LaeA (Ehrlich, 2009). Ehrlich *et al.*, (2009) hypothesised that LaeA could need AflS to target specific gene cluster.

AflS is sensitive to incubation temperature. At 30°C, expression of *aflS* and *aflR* were higher of 5 and 24 fold compared to 37°C. For instance, this temperature sensitivity could regulates AFT production (Yu *et al.*, 2011).

1.2.6.c General transcription regulators

There are 7 well known general transcription regulators which regulate the aflatoxin biosynthesis pathway. Each pathway is relevant to this study as it explains the expression or inhibition of certain aflatoxin genes.

The production of fungal secondary metabolites is regulated by a complex system of proteins (Alkhayyat & Yu, 2014). Figure 30 represents 3 of the well known pathways regulating aflatoxin biosynthesis.
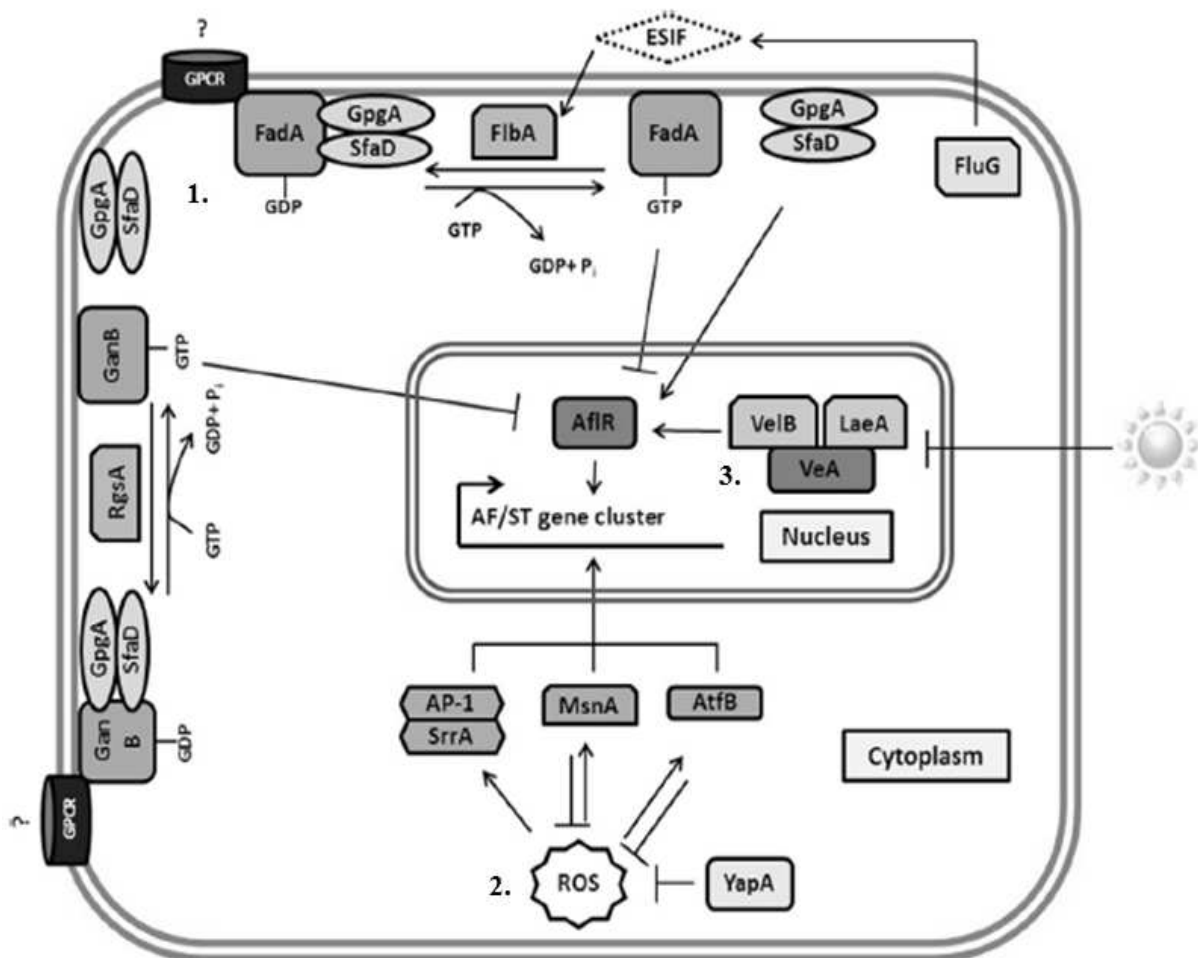
Figure 30 - Various upstream elements influencing the Aflatoxin/Sterigmatocystin (AF/ST) gene cluster. Representation of the different signaling elements, including: the heterotrimeric G protein signaling elements (FadA, GanB, GpgA and SfaD), the light velvet complex (VelB/VeA/LaeA) and the redox status (YapA, MsnA, AtfB, Ap-1 and SrrA) (Alkhayyat & Yu, 2014).

One general transcription regulator, the heterotrimeric G protein pathways (G proteins), is represented by Number **1.** in figure 30.

In eukaryotes, those pathways are associated with the cellular plasmid membranes and are transduction signals necessary to ensure the appropriate physiological status of the cell in

response to external signals. Those G proteins are composed of 3 subunits (α, β and γ) which lose their activity when regrouped in a trimeric form (Figure 30). The activation is due to a GTP-bound to the Gα subunit. Concerning AFT production, 2 Gα subunits: GanB and FadA, were shown to inhibit ST/AF production in case of GTP-binding via inhibition of *aflR* gene expression (Figure 30) (Hicks *et al.*, 1997; Han *et al.*, 2004). However, the Gβγ subunits (SfaD and GpgA) were shown to activate ST biosynthesis, suggesting differential roles on ST production depending on the G protein subunits studied (Seo & Yu, 2006).

A second transcription regulator is the response to the Reactive Oxygen Species (ROS).

Number **2.** in figure 30 represents a proposed model of action of this response. A disruption of *yapA* gene leads to an accumulation of aflatoxin biosynthesis suggesting YapA could inhibit ROS accumulation (Reverberi *et al.*, 2007). In case of ROS accumulation, 4 DNA-binding transcription factors (MsnA, AtfB and AP-1/SrrA complex) were shown to bind specific DNA regions (1 for each of them) and induce aflatoxin biosynthesis by promoting aflatoxin genes (Hong *et al.*, 2013).

A third transcription regulator is the light-sensitive *velvet* complex (VeA, VelB and LaeA), as represented in figure 30 (in a generic manner) and in figure 31 (in a more specific way).

Light leads to a low *veA* expression level while VeA remains in the cytoplasm. However, in the dark, *veA* expression is higher and VeA is transported by the importer α carrier (KapA) into the nucleus (Stinnett *et al.*, 2007). For LaeA to have an inhibition impact on HepA, it must be fixed on the VeA/VelB complex. HepA is a structural adapter putatively involved in the assembly of macromolecular complexes in the chromatin (Wang *et al.*, 2000; Bayram *et al.*, 2008). This HepA inhibition

Figure 31 - Proposed model for the *velvet* complex (Alkhayyat & Yu 2014).

represses the conversion of heterochromatin into euchromatin at the *aflR* locus (Reyes-Dominguez *et al.*, 2010).

A fourth general transcription regulator, the PpoABC proteins, is represented in figure 32.

The *ppoABC* genes encode 3 different putative fatty acid oxygenases responsible for fungal oxylipins productions (Tsitsigiannis *et al.*, 2005). Those proteins, VeA, hydroxylated linoleic (psiα) and oleic acids (psiβ), are known to be involved in the shift between sexual and asexual development (Tsitsigiannis *et al.*, 2004; Bayram *et al.*, 2008). A *ppoAC* double deletion led to no ST production while a single deletion of *ppoB* led to an increased accumulation of ST. Recent studies suggest that the various Ppo oxygenases could result in oxylipins accumulation outside the fungal cell and could trigger G proteins mechanisms (Tsitsigiannis & Keller, 2007)
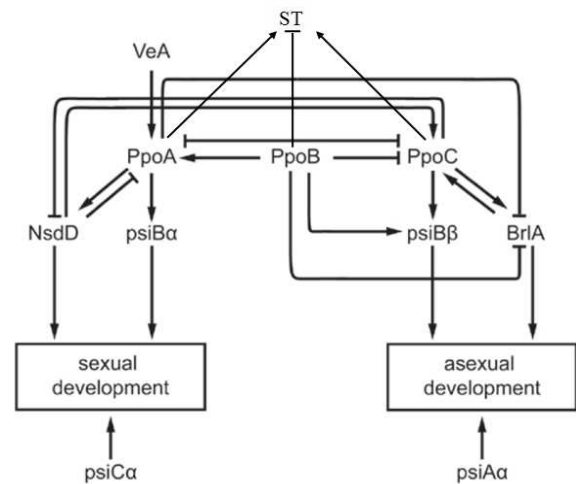
Figure 32 - Ppo impacts on the production of ST. psi = hydroxylated linoleic (α) and oleic (β) with the hydroxy groups represented by a letter; psiA (5'-8'-dihydroxy-), psiB (8'-hydroxy-) and psiC (lactone ring at the 5' of psiA) modified from Krijgsheld *et al.*, (2013).

3 other examples of global transcription regulators include responses to extracellular stimuli. Figure 33 summarises the different pathways. Each of them is briefly described hereafter.

CreA is a zinc finger transcription factor involved in the activation of metabolic pathways in response to a carbon source (Figure 33) (Dowzer & Kelly, 1991). Aflatoxin biosynthesis is enhanced by high concentrations of glucose (≥ 1 M) (Wiseman & Buchanan, 1987). Further characterisation is needed to understand the involved pathways.

AreA is also a zinc finger transcription factor. It regulates the nitrogen metabolism (Wilson & Arst, 1998). An AreA-binding site is located in the *aflS-aflR* intergenic region (Figure 28) and could activate aflatoxin biosynthesis.
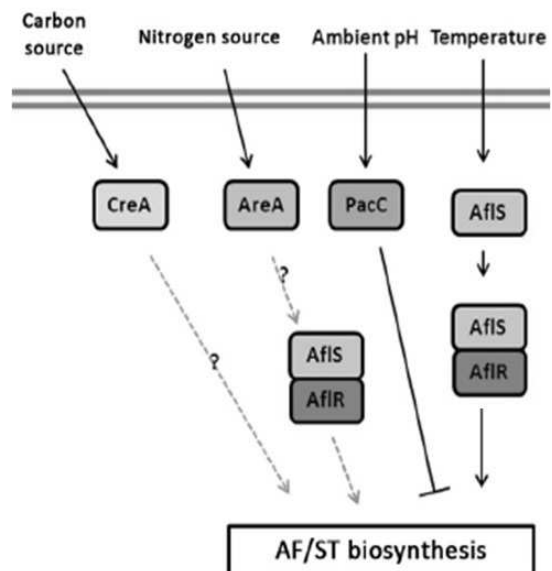
Figure 33 - Other environmental stimuli impacting AF/ST biosynthesis according to Alkhayyat & Yu (2014). Dashes represent unproven connections.

PacC is a zinc finger transcription factor (Figure 33). It negatively regulates the ST production of *A. nidulans* in alkalin conditions (Keller *et al.*, 1997). Its inhibition depends on the pH and can be cancelled in acid conditions.

In addition to all the regulation systems described above, other mechanisms can impact AFT production such as production localisation and excretion system.

1.2.6.d Aflatoxin excretion

In the last decade, many discoveries have been made about the aflatoxin excretion system. Roze *et al.*, (2011) developed a 2-level model represented in figure 34:
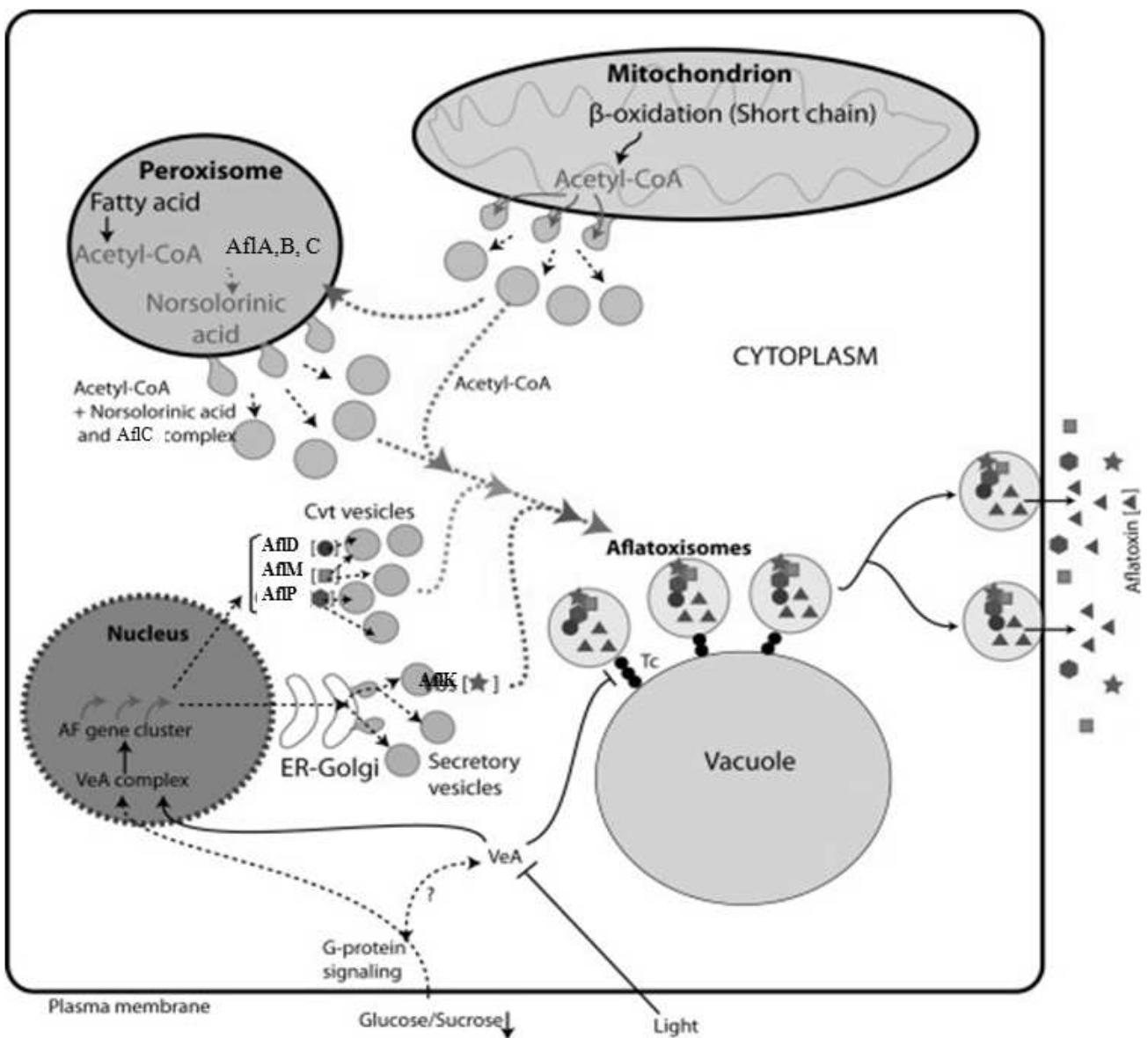
thesized pathways are

(i) Darkness triggers VeA activity which up-regulates gene transcription via the activation of transcription factors. Inside Peroxisomes, Acetyl-CoA from Mitochondria is converted into NOR due to AflA, B and C activity. NOR is then transferred into the aflatoxin specific peroxisomal vesicules: aflatoxisomes.

(ii) AflD, M and P are synthesised in free ribosome in the cytoplasm and then transported in aflatoxisomes. In aflatoxisomes, ST is converted into AFB1. VeA inhibits the activity of the C Vps tethering complex (Tc) (responsible for vesicules regroupment in vacuole), resulting in the accumulation of aflatoxisomes.

The 2 levels lead to first the accumulation of NOR and second the conversion of NOR into aflatoxin by the enzymes (AflD, M, P and K). VeA represses the fusion of aflatoxisomes and the vacuole. This repression leads to AFT accumulation. Aflatoxisomes are then exported outside the cell by exocytosis (Chanda *et al.*, 2009).

In conclusion, we resumed that the biosynthesis of AFT is due to 12 different major steps involving various proteins. Furthermore, this biosynthesis is regulated by specific transcription factors, unspecific transcriptional regulators and an excretion system.

1.2.7. Abiotic parameters: impact on aflatoxins production

Having observed genetic parameters, attention now needs to be paid to environmental factors, which, in conjunction with genetic parameters, influence the production of aflatoxins.

There are 2 types of environmental parameters influencing the production of aflatoxins: abiotic or biotic. We will describe below the main abiotic parameters: water activity (a_w) and temperature, gas composition, medium composition, pH, light and chemical compounds addition.

1.2.7.a. A_w and Temperature (°C)

Many publications have focused on the interaction between a_w and temperature on *A. parasiticus* and *A. flavus* growth and AFT production. This interaction is regarded as the principal controlling factor (Abdel-Hadi *et al.*, 2012).

The a_w is a physical measurement which represents the amount of freely available water in a substrate. As a mean of measurement, a_w of 1 is pure water. Micro-organisms can use this freely available water to grow and to achieve enzymatic reactions. With a moisture sorption isotherm, a link can be made between a_w and the total moisture content of a specific

substrate (relation between water added (moisture content) and the a_w measured at equilibrium).

Garcia *et al.,* (2011) studied the growth of *A. parasiticus* on malt extract agar medium at different temperatures (10, 15, 20, 25, 30, 37 and 42°C) and various a_w (0.80, 0.85, 0.87, 0.89, 0.91 and 0.93). Radial fungal growth was observed daily for 90 days. The estimated growth optimum conditions were a temperature of 31.1°C and an a_w of 0.94. They predicted a lag phase of 7 days at 22-37°C at 0.87 of a_w and of 30 days regardless of the temperature at 0.80 of a_w. However, in this study, the authors do not measure AFT production.

Abdel-Hadi *et al.,* (2012) studied a_w and temperature impacts on *A. flavus* NRRL3357 growth and AFB1 production. *A. flavus* was incubated for 10 days on a YES medium (20 g yeast extract, 150 g sucrose, 1 g $MgSO_4.7H_2O$). The growth optimum (Figure 35 A.) was temperatures of 30-35°C at 0.99 a_w (black bars). Sub-optimal growth occurred at 25-37°C at 0.95 (dark gray bars). At the driest conditions tested (0.85), growth only occurred at 30-37°C (white bars).

AFB1 production revealed a different pattern (Figure 35 B.). The production optimum occurred at 25-30°C at 0.99. Sub-optimal AFB1 production occurred at 0.95 with a gradual increase of AFB1 production from 20 to 35°C (from 0.3 to 1.7 mg.kg^{-1}). No AFB1 was produced at 0.85 and at 25-30°C for 0.90. 40°C was the least conducive tested temperature for AFB1 production.
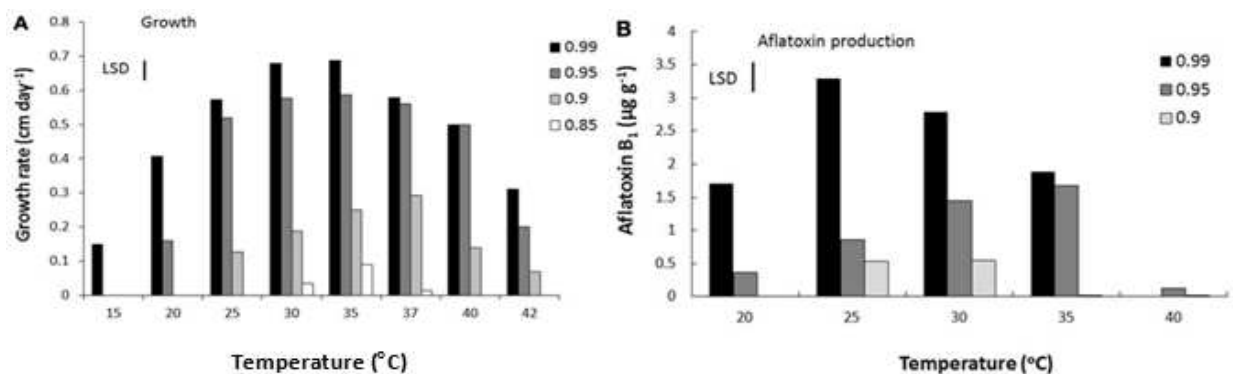
0 days culture of *A. flavus*

Another study tested 2 *A. flavus* isolates (BAFC4274 (A) and BAFC4275 (B)) at various a_w (0.83, 0.86, 0.90, 0.94, 0.96 and 0.98) and temperatures (10, 15, 25, 30, 35 and 40°C) for solely AFB1 production at 4 incubation times (7, 14, 21 and 28 days). Figure 36 represents the results after 21 days of incubation for the 2 strains. Both showed similar

profiles. AFB1 is produced at 15-35°C and at all the a_w tested. The optimum AFB1 production for both strains was 30°C at 0.96 a_w (Astoreca *et al.*, 2014).
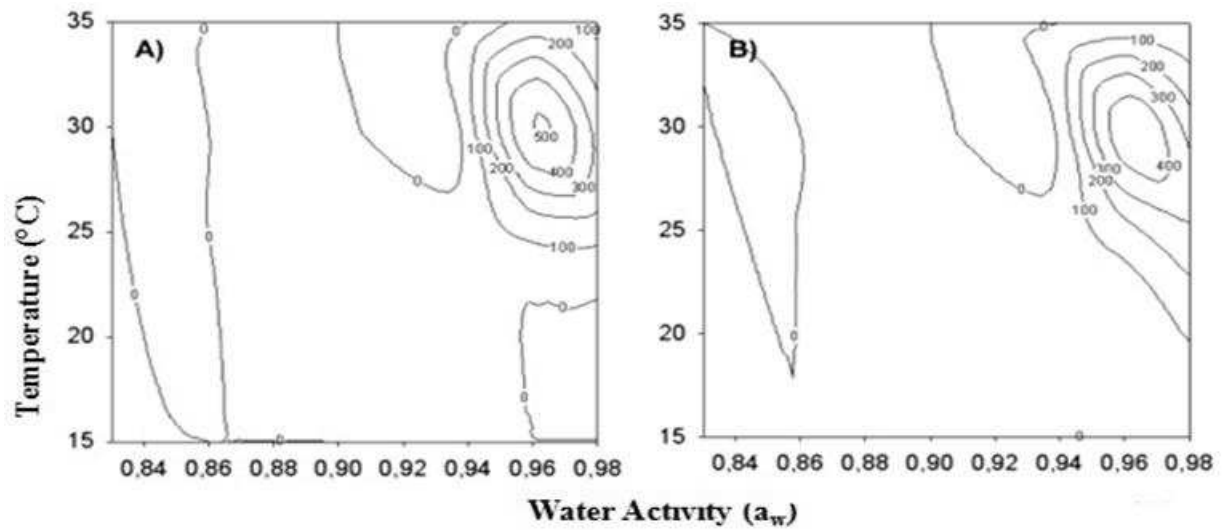
Figure 36 - 2- dimensional contour maps of AFB1 production profiles for A) BAFC4272 and B) BAFC4275 isolates on CYA medium (21 days after inoculation). The numbers on the contour lines refer to the mean AFB1 concentration (μg.g^{-1}) (Astoreca *et al.*, 2014).

Those 3 examples are the most recent of many studies which have led to the accumulation of a significant amount of data on how a_w and temperature influence *A. flavus* growth and AFB production. They all agreed that the optimum conditions for AFT production were different that those for fungal growth.

Those data, obtained *in vitro*, are valuable as a first step to reduce the incidence of AFT in the field and in storage. At the field level, some predictions can be obtained based on known data on air relative humidity and temperature. For instance, Battilani & Logrieco (2014) have proposed a world map of AFT risks (figure 37). Those results show that the main countries at risks are mostly located in tropical and sub-tropical regions where high temperature and low a_w occur.
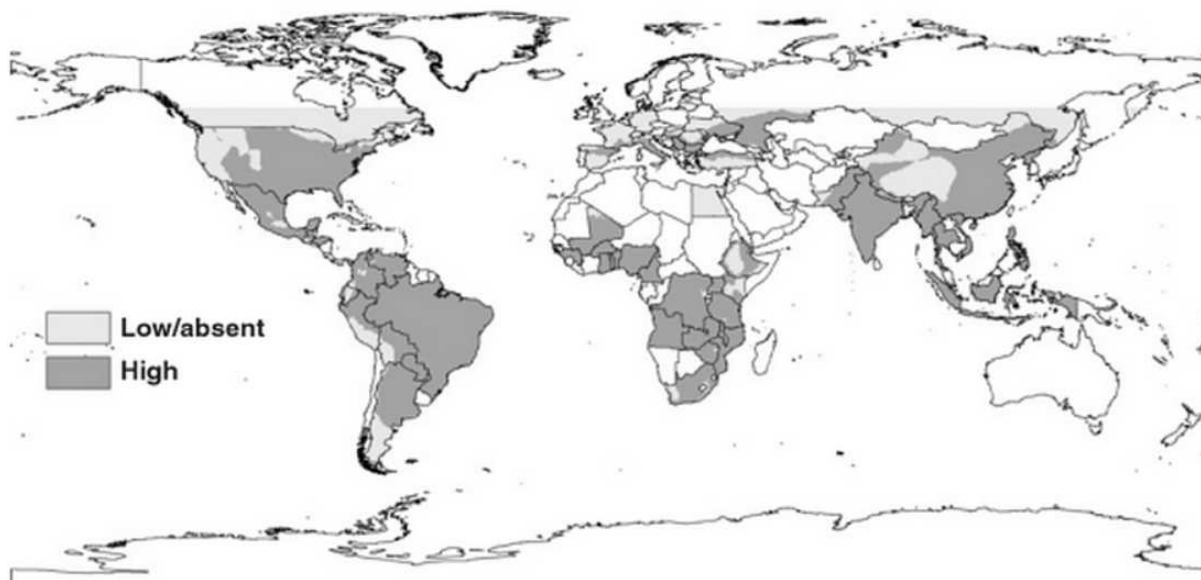
Figure 37 - Global risks of AFB1 contamination in maize. The prediction is based on the aridity index during heading and ear ripening (Battilani & Logrieco, 2014).

In addition to temperature and a_w, gaz composition is also an abiotic parameter influencing the production of aflatoxins.

1.2.7.b. gaz composition

Currently, the atmospheric CO_2 concentration is around 400 mg.dm^{-3} in the air (Foucart, 2014). Climate change experts predict this figure could double or triple in the next 10 to 25 years (Medina *et al.*, 2014). In terms of confined environments, CO_2 concentration can rise. In storage bins for instance, an increase of CO_2 concentration is an indicator of grain spoilage. In maize storage bins, a CO_2 concentration higher than 500 mg.dm^{-3} is correlated with mold spoilage and insect activity (Maier *et al.*, 2010). Although high CO_2 concentration does not seemingly impact *Aspergillus* fungal growth (Garcia *et al.*, 2011; Medina *et al.*, 2014), it enhances AFB1 production (Giorni *et al.*, 2008).

Recently, Medina *et al,.* (2014) reviewed a_w x temperature x CO_2 impacts on AFB1 production. They studied the impact of high CO_2 concentration (650 and 1000 mg.dm^{-3}) on AFB1 production (34 and 37°C, a_w of 0.92, 0.95 and 0.97). At 34°C and with a high CO_2 concentration, an a_w of 0.92 led to the most important increase in AFB1 production. At 37°C, at all a_w tested and with a high CO_2 concentration, the increase in AFB1 production ranged from 15.1 to 79.2 fold compared to control.

Those data confirm the impact of CO_2 concentration on AFT production. Thus, a_w x temperature x CO_2 content can be monitored in maize grain storage to minimize AFT production (Maier *et al.*, 2010).

In complement to CO_2 content, a low O_2 concentration can enhance the shelf life of food. At 25°C on CYA and PDA media, a O_2 concentration under 0.5% and 20% CO_2 concentrations completely inhibited the production of *A. flavus* AFB (Taniwaki *et al.*, 2009).

In addition to temperature, water availability and gas composition, medium composition are also an abiotic parameter influencing the production of aflatoxins.

1.2.7.c. Medium composition

Ahmad *et al.,* (2013) studied the impacts of different combined concentrations in the medium of sorbitol (A), fructose (B), ammonium sulfate (C), KH_2PO_4 (D) and $MgSO_4$ (E) on AFB1 production by *A. flavus*. After 15 days of incubation at 28°C, the highest levels of AFB1 production were obtained at 5, 5, 0.5 0.36 and 0 g.l^{-1} of A, B, C, D and E, respectively. This multifactorial testing led to the elaboration of a predicting model. The latter showed a positive correlation between AFB1 production and A, B and C concentrations and a negative correlation with D and E concentrations.

Those data help to understand the impact of different media on fungal growth and AFT production. However, extrapolations from those predictions to the food matrix are difficult (Garcia *et al.*, 2011). To obtain data close to the maize matrix, maize-based media have been developed. For example, Astoreca *et al,.* (2014) tested AFB1 production by 2 different strains on 2 different media: Czapek Yeast Agar (CYA) and Corn Extract agar (CEM). Both aflatoxigenic strains produced less AFB1 on CEM medium in comparison with CYA. At its climax point, the production of AFB1 was reduced 868 fold on CEM (Astoreca *et al.*, 2014).

To further understand the specificity of maize-based media on AFT production. Giorni *et al.*, (2011) tested maize at different ripening stage in the medium composition. The different maize tested had little impact on *A. flavus* growth and AFB1 production (Giorni *et al.*, 2011).

A recent study compared *A. flavus* growth and AFB production on maize-based medium and on maize grain at 0.99 and 0.90 a_w during 30 days. It obtained a similar fungal growth on maize grain and on agar medium. In both experiments, there was a correlation between fungal growth and AFB production. On maize grain, this correlation was even higher. On agar medium, the delay before AFB production was 1 day under both a_w values whereas, on maize, it was 4 to 8 days at 0.90 and 2 days at 0.99 (Garcia *et al.*, 2013).

In conclusion, we showed that the medium composition impacts fungal growth and AFT production. Results vary between *in vitro* media and maize substrates. Nevertheless, maize-based medium appear to be the closest substitutes to maize even if AFT production occurred earlier on the maize-based medium.

In addition to temperature and a_w, gas composition and to media composition, substrates, pH is also an abiotic parameter influencing the production of AFT.

1.2.7.d. pH

Keller *et al.,* (1997) studied the impacts of acid (4 to 6) neutral (7) and alkalin (8) pH on AFB1 and ST production. A completed ammonium medium was realised in various pH conditions and *A. parasiticus* was incubated up to 36 hours. The results showed that AFB1 production increases in acidified media with 1,062 ng.ml^{-1} of AFB1 produced at pH 4 versus 19 ng.ml^{-1} at pH 6. The incubation on an alkalin medium (pH 8) revealed a production of 22 ng.ml^{-1} of AFB1. This production is close to pH 6 but inferior to the control medium (pH 2.7) (394 ng.ml^{-1}). The mechanisms involved in the impact of pH are well known and detailed in chapter 1.2.5.

In addition to temperature and a_w, gas composition, media composition and to pH, light is also an abiotic parameter influencing the production of AFT.

1.2.7.e. Light

Joffe & Lisker (1969) were the first to study the effects of light on AFB production. They revealed that on Czapek's medium, the production of AFB increases in dark conditions. One of the most representative examples was at pH 6, 24°C, where AFB production was 5 fold higher in dark versus light conditions (Joffe & Lisker, 1969). This light responsiveness seems to be correlated with the glucose amount in the medium (0.3 to 3% tested). Atoui *et al.,* (2010) incubated *A. nidulans* 5 days on a glucose minimum medium. At a 1% glucose concentration, light inhibited ST production, compared to dark conditions. On the contrary, a 2% glucose concentration triggered ST production under light (Atoui *et al.*, 2010). The mechanisms involved in the impact of light are also described in chapter 1.2.5..

Lastly, chemical compounds addition are also an abiotic parameter influencing the production of AFT.

1.2.7.f. Chemical compounds addition

Numerous studies have tested the impact of essential oils, fungal/bacterial extracts and chemical components (from plants, bacteria, fungi or chemically produced) on the production of AFT. Holmes *et al.,* (2008) and Razzaghi-Abyaneh *et al.,* (2010) reviewed part of them. The chemical components listed above come from various different families of compounds including alkaloids, coumarins, flavonoids, oxylipins, etc. Most of the studies have been undertaken on synthetic media. A smaller proportion of studies have investigated field/storage conditions. Only a few are at the stage of potential commercial development. Hereafter are examples of chemical compounds and their impacts on AFT production.

In maize storage, aflatoxigenic fungi are naturally present on the grain or introduced by the borers. The 2(3)-*tert*-Butyl-4-Hydroxyanisole *(*BHA) has recently been studied for its capacity to act as insecticide and AFB repressor. At a 20 mM concentration, this anti-oxidant inhibited fungal growth and AFB production on maize grain (Nesci *et al.*, 2008). At the same concentration, BHA was also an insecticide against 2 insects (*Stiphilus zeamais* and *Tribolium confusum*) commonly found in maize. BHA application could be part of an integrated system for commercial storage facilities (Nesci, 2012).

Among the numerous examples of chemical components, we will only focus on the ones produced by *Streptomyces* species.

Among these, Dioctatin A, Aflastatin A and Blasticidins A were shown to have inhibition effects on AFT production by *A. parasiticus* (Sakuda *et al.*, 1996; Ono *et al.*, 1997; Yoshinari *et al.*, 2007). Various concentrations of Dioctatin A were added to Potato dextrose broth and *A. parasiticus* was incubated during 4 days at 28°C. Results showed a maximum AFB1 inhibition of 97% at a 50 µM concentration of Dioctatin A in the medium. The fungal weight was not impacted even if conidiation was altered by the treatment. Moreover, the expression of *aflC, aflM, aflP* and *aflR* was repressed (Yoshinari *et al.*, 2007).

Another chemical component, Aflastatin A, was added to both liquid and solid media at 0.5 µg.ml^{-1}. It completely inhibited aflatoxin production in both media by *A. parasiticus*. Fungal growth was only impacted on the agar plate (37% reduction) (Ono *et al.*, 1997). Further investigations revealed a reduction in *aflC* and *aflR* expressions (Kondo *et al.*, 2001).

With regards to the last example, the production of AFT was reduced 166 fold by Blasticidin A and mycelial dry weight was reduced 2 fold when 1.0 µM was added into *A. parasiticus* liquid culture. Moreover, there was no expression of *aflC* and *aflM* and there was a reduction in the expression of *aflP* and *aflR* (Sakuda *et al.*, 2000).

Those chemical components offer a viable option to reduce AFT production in the field and storage. Nonetheless, current agricultural practices tend to reduce the use of pesticides. A negative public opinion could be an issue where those treatments marketed. Moreover, adding a single compound can lead to resistance from the fungus as well as environmental issues. This explains actual scientific moves towards the development of biocontrols.

1.2.8. Biotic parameters: impact on aflatoxins production

The second type of environmental parameters influencing the production of AFT is biotic parameters. Prevention of AFT accumulation is presented hereafter, AFT biotransformation will be developed in chapter 1.2.9..

We will describe below the main biotic parameters: maize susceptibility, fungal interactions (intra and inter-species interactions) and bacterial interactions.

1.2.8.a. Maize susceptibility

Maize is more susceptible to *A. flavus* in case of drought, nutriment deprivation, insect attacks and fungal attacks. Good agricultural practices are defined and available for farmers (resumed in Chapter 1.1.4). Hereafter we will briefly describe direct and indirect susceptibility management.

Direct susceptibility management is done through gene selection. Maize lines available on the market required many agronomic traits. They are genetically identified thanks to both Quantitative Trait Loci (QTL) (DNA sections linked with a quantitative trait) and gene identification (Warburton & Williams, 2014). Many QTL were identified in maize with regards to *A. flavus* and aflatoxin accumulation. However, few genes have been identified yet. For example, the gene AW424439 was identified thanks to QTL analysis and was predicted to be involved in systemic response to fungal infection (Mylroie *et al.*, 2013). Unfortunately the success of those techniques is currently limited (Abbas *et al.*, 2009).

Indirect susceptibility management is done through the Bt maize. It is a genetically modified maize made to resist to certain maize borers. In 2006, different lines of maize were harvested in USA. AFB1 occurrence in cobs from Bt-maize lines was 6.2 fold less than non-Bt lines (Abbas *et al.*, 2009). This is due to the reduction of *A. flavus* inoculum in the cobs (no borers entry) and other unknown mechanisms (Accinelli *et al.*, 2014).

In conclusion, we demonstrated that fungal interactions can strongly impact AFT production. Furthermore, intra-species and inter-species interactions are important biotic parameters. All in all, AFT production is triggered by many micro-organisms in conjunction with abiotic parameters.

1.2.8.b. Fungal interactions:

Numerous fungal genera are present in the maize ecosystem. For example, Pereira *et al.,* (2009) studied maize seedling ecosystem and identified 5 different fungal genera (*Aspergillus, Penicillium, Trichoderma, Monilella* and *Fusarium*) (Pereira *et al.*, 2009). This complex ecosystem can also be enriched by the addition of biocontrol agents. Both the ecosystem and biocontrol agents can impact *A. flavus* growth and AFB production.

We will thus describe below intra-species and inter-species interactions. Examples of fungi in the maize ecosystem and biocontrol agents will be given.

Intra-species interactions:

In the maize ecosystem, both aflatoxigenic and non-aflatoxigenic *A. flavus* are present. For the past 20 years, Dorner and colleagues have developed a methodology based on these non-aflatoxigenic strains (Dorner *et al.*, 1998; Dorner, 2004, 2009; Dorner & Lamb, 2006).

Local *A. flavus* or *A. parasiticus* strains are harvested. They are then selected thanks to a multi-step process:

(i) harmlessness verification: they are genetically analysed to confirm their inability to produce AFT and Cyclopiazonic Acid (CPA);

(ii) Simple Sequence Repeats (SSR) analysis: the strain which are the furthest from aflatoxigenic strains are selected;

(iii) maize grain competition: their capacity to get the upper hand on aflatoxigenic strains is validated *in vitro*;

(iv) non vegetative compatibility: their capacity not to form variable heterokaryon is verified. The remaining strains become adequate biocontrol.

For example, those biocontrols were tested at peanuts field. A survey on treated peanuts revealed an AFB1 reduction of 85.2% (78.9 to 11.7 µg.kg^{-1}after storage). Therefore, it was branded afla-guard$^{®}$ and was first commercialized for peanut and maize fields in the USA (Mehl & Cotty, 2010). Recently, based on the same methodology, afla-safe$^{®}$ was also commercialised in Africa (Atehnkeng *et al.*, 2008; Ogunbayo *et al.*, 2013).

However, those biocontrols have limitations:

The first limitation is *A. flavus* capacity to sexually recombine. Indeed, in dark and nutrient-deprived conditions, *A. flavus* sexually recombines (Horn *et al.*, 2009). This recombination between atoxigenic and toxigenic strains causes phenotypes with the capacity to produce AFB (Olarte *et al.*, 2012).

The second limitation is *A. flavus* capacity to produce toxic metabolites. Recently, 56 putative metabolites clusters were alleged in *A. flavus* (Ehrlich & Mack, 2014). Thus, *A. flavus* capacity to produce toxigenic metabolites is probably underestimated in the supposed non-toxigenic strains.

Inter-species interactions:

In the maize ecosystem, among the numerous fungal species, *A. flavus* isolates can usually be detected (Giorni *et al.*, 2007; Pereira *et al.*, 2009). Those former co-existing species can affect *A. flavus metabolism.* In France*, F. graminearum and F. verticillioides* are natural contaminants in maize fields (Picot *et al.*, 2012). As phytopathogens, they are often associated with huge grain loss. They can impact on one another secondary metabolism. For example, *F. graminearum* impacts *F. verticillioides (F. moniliforme)* growth and FB1 production. On maize grain, 3 a_w (0.98, 0.95 and 0.93) and 2 temperatures (15, 25°C) were tested. *F. verticillioides* growth was reduced by *F. graminearum* under all the tested conditions. In addition, the production of FB1 was inhibited at 15°C and promoted at 25°C (for 0.95 and 0.98) (Marín *et al.*, 2001).

The example given above illustrates how fungal interaction impacts on one another secondary metabolism. Thus, those fungi could impact *A. flavus* growth and AFB production. *F. verticillioides* and *A. flavus* interaction was studied to provide some preliminary answers. On maize grain, *F. verticillioides* primarily overlapped *A. flavus.* However, no data were collected on AFB production (Marin *et al.*, 1998).

Besides the natural ecosystem, fungal biocontrol have been shown to impact AFB1 production. Lyophilised filtrat of *T. versicolor* CF 117 was added to contaminated (*A. parasiticus*) maize seeds. After 20 days at 30°C, AFB1 production in the seeds was reduced by 97%. In addition, expressions of *aflE* and *aflR* were delayed and reduced. The active compounds were identified. They were in the exopolysaccharide fraction of the extract and were linked to some proteins (Zjalic *et al.*, 2006). Based on those results, Trametano® is a promising tool in maize storage conditions with a long-lasting impact of up to 6 months (Scarpari *et al.*, 2014).

Another biocontrol candidate is lyophilised filtrat of *Lentinula edodes* CF 42. The latter was added to potato dextrose broth. After 7 days at 30°C, AFB1 production was divided by 375 fold. In addition, expressions of *aflE* and *aflR* were delayed and reduced. β-glucans were predicted to be the active compounds (Reverberi *et al.*, 2005). However, before they can be placed on the market, further studies are needed.

A last example of fungal biocontrol is *Pichia anomala* WRL076. The capacity of this yeast to inhibit NOR and AFB1 accumulation (by *A. parasiticus*) was tested. After 10 days at 28°C, no NOR was detected and AFB1 production in potato dextrose agar was reduced 80 fold (Hua *et al.*, 1999). The volatile compound 2-phenylethanol was identified as the active compound. Depending on the incubation time, it reduced *A. flavus* expression of *aflO, aflQ* and *aflK* up to 10,000 fold (Hua *et al.*, 2014). Based on those results, this yeast is currently tested in California for tree nuts (Hua *et al.*, 2014).

In addition to fungal interactions, bacterial interactions is also a biotic parameter influencing the production of aflatoxins.

1.2.8.c. Bacterial interactions:

Numerous bacterial genera are also present in the maize ecosystem. For example, Pereira *et al.*, (2009) studied maize seedling ecosystem and identified 5 different bacterial groups (Gram + spore-forming rods, cocci, irregular rods and rods; Gram - rods). This complex ecosystem can also be enriched by the addition of biocontrol agents. Both could impact *A. flavus* growth and/or AFB production.

Bacterial molecules are well studied as potential chemicals against AFT production (Ono *et al.*, 1997; Sakuda *et al.*, 2000; Yoshinari *et al.*, 2007). However, bacterial filtrats or bacteria are less studied. Only few were tested for potential fields or storage application. Examples are given below.

Lactobacillus sp. are rod-shaped Gram-positive bacterium naturally found in the soil. The capacity of *L. platarum* K35 to inhibit AFB1 accumulation (by *A. parasiticus* and *A. flavus*) was tested. After 48h at 37°C, for both fungi, growth and AFB1 production were completely inhibited. Multiple potential active compounds were identified. However, impact *in vivo* has not been studied (Sangmanee & Hongpattarakere, 2014). Other *Lactobacillus sp.* showed similar pattern and are reviewed in Dalié *et al.,* (2009). Many bacteria also have antifungal interest (Aouiche *et al.*, 2012; Muzammil, 2012; Badji *et al.*, 2013). However, we will focus hereafter on examples of bacteria mainly inhibiting AFT production.

Pseudomonas sp. and *Bacillus* sp. are rod-shape bacterium Gram-negative and Gram-positive, respectively. They are naturally found in the non-rhizosphere of maize soil. Their capacity to inhibit AFB1 accumulation by 8 strains (*A. flavus* and *A. parasiticus*) was assessed. After 11 days at 25°C, AFB1 production in malt extract medium (at 0.982 and 0.955 a_w) was effectively inhibited by some strains: *Bacillus subtilis* RCB 6, 55 and 90 and *Pseudomonas solanacearum* RCB 110 (Nesci *et al.*, 2005). Unfortunately, no additional data are available on those strains.

Stenotrophomonas sp. are rod-shaped Gram-negative bacterium naturally found in the soil. The capacity of *Stenotrophomonas rhizophila 27* to inhibit AFB1 accumulation (by *A. parasiticus* and *A. flavus*) was tested. After 3 days at 27°C, AFB1 production in potato dexrtrose broth was reduced to non-detected depending on the bacterial concentration without affecting fungal growth. The cyclo (L-Ala-L-Pro) and cyclo (L-Val-L-Pro) diketopiperazines were identified as the main active compounds. For instance, co-cultures tests reduced *A. parasiticus* expression of *aflC*, *aflO* and *aflR* by 5, 6 and 2 fold, respectively. After 3 weeks, first results in traditional storage conditions in Thailand showed an AFT reduction of up to 3 fold. However, before they can be placed on the market, further studies are needed (Jermnak *et al.*, 2013).

Achromobacter sp. are straight-rods Gram-negative bacterium. *A. xylosoxidans* was isolated from human ear and is a bacteria potentially promoting plant growth. Its capacity to inhibit NOR accumulation by *A. parasiticus* was tested. After 3 to 7 days at 28°C, NOR was not detected. Cyclo (L-Leu–L-Pro) was the active compound. This is because *A. parasiticus* expression of *hexB*, *aflO* and *aflR* seemed to be reduced when Cyclo (L-Leu–L-Pro) was added in the medium (3.5 mg.ml^{-1}) (Yan *et al.*, 2004). Unfortunately, no additional data are available on this strain.

The last example is *Streptococcus lactis.* It is a cocci-shaped Gram-positif bacterium found in milk. Its capacity to inhibit AFB1 accumulation (by *A. flavus*) was tested. After 5 days at 28°C, AFB1 accumulation was reduced by 15 fold. The active compound was not identified. Moreover, *S. lactis* reduced pure-AFB1 and pure-AFG1 concentration. After 2 days at 28°C, no AFB1 or AFG1 were detected (initial concentrations were 18 µg.ml^{-1} each). (Coallier-Ascah & Idziak, 1985). The mechanisms which led to the reduction of pure-AFB1 are further described in chapter 1.2.9.

In addition to fungal interactions and bacterial interactions, maize susceptibility is also a biotic parameter influencing the production of AFT.

1.2.8.d. *Streptomyces*: good biocontrol candidates

Introduction of *Streptomyces:*

Streptomyces are members of the Actinobacteria class. The later were first characterized in the 1830s. Nowadays, they are defined as gram-positive bacteria, mainly aerobic, chimio-heterotrophe and ubiquitous. Their fungi-like life cycle includes: growth as mycelium of branching hyphal filaments, and reproduction by sending up aerial branches that turn into chains of spores. They are identified by molecular biology thanks to their high DNA-content of G and C (> 55%) and their identified similarity by rRNA 16S gene-sequencing and by DNA-DNA hybridisation (e.g.: chapter 3.1) (Whitman *et al.*, 2012).

Among these, the genus of *Streptomyces* sp. is the most predominent (Labeda *et al.*, 2011). It is an historical source of secondary metabolites applied as antibiotics for medical and agricultural use. It has also been identified as source of aflatoxin inhibitors (e.g.: chapter 1.2.7.f). This genus can be differentiated from other Actinobacteria by its mycelium morphology. The latter is regrouped in 3 types: the *Rectus Flexibilis* (RF), the *Retinaculum Apertum* (RA) and S= *Spira* (Figure 38).
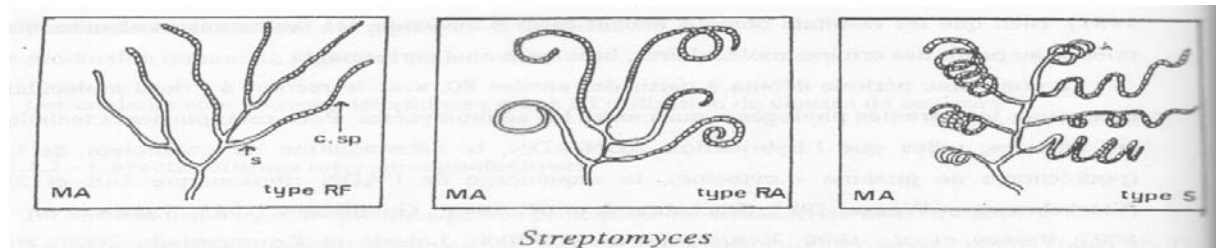
Figure 38 - Micromorphology of *Streptomyces* sp. (Bergey, 1989). MA= Aerial Mycelium, RF= *Rectus Flexibilis*, RA= *Retinaculum Apertum*, S= *Spira*.

In addition to morphological identification, *Streptomyces* sp. are also identified thanks to chemical characterisation. Indeed, their cell wall includes the LL isomer of the diaminopimelic acid and glycine (group IC), their cell menbrane includes phosphatidylethanolamine (group PII) and they have a G and C content between 68 and 78% (Garrity *et al.*, 2004; Meklat, 2012).

Potential biocontrol characteristics:

As a potential biocontrol, *Streptomyces* sp. has many interesting characteristics. The most interesting ones are succinctly presented below:

(i) *Streptomyces* are ubiquitous bacteria. Indeed, they are found in various environments as in the soil of maize field. A study conducted during 8 years at maize field, identified actinobacteria as redundant community with 0.4 nmol.g^{-1} of dry soil (quantification of actinobacteria-specific lipid (10Me18:0) by phospholipids fatty acid analysis) (Dong *et al.*, 2014). As an endogenous bacterium of the maize field community, biocontrol based on actinobacteria (including *Sptreptomyces*) are likely to survive in this environment.

(ii) Since their discovery, *Streptomyces* have been a source of metabolites production. Those metabolites are sources of antibiotics useful for the pharmaceutical and agricultural industries. Among these, some were identified as aflatoxins inhibitors and are presented in chapter 1.2.7.f. Those inhibitors of aflatoxins production are but few examples of *Streptomyces* metabolites that could possibly lead to the inhibition of aflatoxins production.

(iii) *Listeria, Salmonella*, etc. are bacterial species known to be threats for human health. *Streptomyces*, in contrast, are harmless bacteria. Only few reports identified *Streptomyces* as harmful to human health and they were mainly associated with immuno-deficiency (Carey *et al.*, 2001; Moss *et al.*, 2003; Riviere *et al.*, 2012). The main threat linked to those bacteria enhancement in our diet could be linked with the toxicity of their produced metabolites. To minimize this impact, *Streptomyces* will be chosen having mutual antagonism on contact rather than inhibition of *Aspergillus* at distance as fungicides are known to have little impact on aflatoxins production (Abbas *et al.*, 2009).

(iv) In France, on the maize crop, no treatment is sprayed after the 6 leaves stage due to limitation in agricultural machinery. This problem negates the application of the biocontrol at the end of maize maturation (aflatoxin production time (Abbas *et al.*, 2009)). Thus, a potential solution is the development of biocontrol able to survive in the crop until the targeted period. Actinobacteria are known to have endophytic abilities. Moreover, a recent study showed that they can be endophyte and survive in the maize crop (Costa *et al.*, 2013). A part of the Aflafree project will focus on verifying the endophyte abilites of the most interesting strains selected after *in vitro* direct interaction.

1.2.9. The reduction of aflatoxins

Having described the impact of genetic and environmental parameters on the production of AFT, attention now needs to be paid on mechanisms which reduce AFT.

AFT are stable molecules (268-269°C) which resist chemical and physical treatments. This makes decontamination of contaminated food and feedstuffs difficult without altering the initial nutritional values of the infected commodities. However, different decontamination techniques are available to reduce AFT content.

There are 3 types of decontamination techniques. They will be presented in order of importance: physical and chemical methods; adsorbents and biological mechanisms (binding and degradation).

1.2.9. Physical and chemical methods:

During food processing, many physical methods can impact AFT content. Hereafter, we will only focus on the main examples.

Sorting is a preliminary method to reduce AFT content. Various sorting criteria are based on the grain (damaged, small or discolored) or the fungal infection (UV and IR-based). UV-based sorting is frequently used, even though IR-based methods showed more interesting results. Indeed, the initial average contamination of AFT (53 µg.kg^{-1}) was reduced by 81% thanks to a IR-based (750 and 1,200 nm filters) sorting (Pearson *et al.*, 2004).

Alkaline cooking is another step in food processing. Maize is often consumed as tortillas. Water boiling or microwave heating is a key process in tortillas production. Both decreased AFT content up to 84% (Torres *et al.*, 2001; Pérez-Flores *et al.*, 2011). Lesser AFB1 reduction was achieved in baked muffins with only a 13% removal (Stoloff & Trucksess, 1981).

During food processing, many chemical methods can impact AFT content, including ammoniation and ozone. Ammoniation hydrolyses the lactone ring of aflatoxin (up to 90% removal) and leads to less toxic compounds (e. g. aflatoxin D1). Ozone react with the furan ring of aflatoxins (up to 95% removal) (Grenier *et al.*, 2014).

More physical and chemical methods are reviewed in Grenier *et al.*, (2014). In addition to physical and chemical methods, adsorbents are also used as decontamination technique to reduce aflatoxins.

1.2.9.b Adsorbents

Adsorbents are mineral or organic based compounds. Numerous binders have been evaluated in *in vivo* studies. Among these are activated charcoal, silicate binders and other minerals adsorbents. Jard *et al.,* (2011) summarised the different binders available.

Bentonite is the most representative example as it is the latest adsorbent authorised for commercialisation in the EU. Adsorbents need to meet several criteria to be placed on the market such as high bound efficacy, stability in animal's digestive tract (pH resistant) and no adverse effect (on the health and the environment) (Jard *et al.*, 2011; European Union, 2013). At 37°C in 4 μg.ml^{-1} AFB1 concentration, addition of bentonites at 0.02% (w/v) bound more than 90% of AFB1 (pH 5). This binder is added to contaminated feed for ruminants, poultry and pigs at a bentonites concentration of 20 g.kg^{-1}.

1.2.9.c Biological binding and degradation:

Micro-organisms can be able to bind or degradate mycotoxins. Binding and degradation of aflatoxins have been well studied. Some binding molecules and degrading enzymes are identified. These are produced by a wide range of organisms. Those include mushrooms, protozoa, soil-borne bacteria and lactic acid bacteria. Wu *et al.,* (2009) summarised the different biological binders and degradation enzymes currently known. Hereafter, we only focus on examples of bacteria, the domain used in this thesis.

Flavobacterium are rod-shaped Gram-negative bacterium naturally found in the soil. *F. aurantiacum* is the first bacteria studied for AFB1 degradation. After 44 hours in contact with live cells, 74% of AFB1 was removed (Ciegler *et al.*, 1966). After 24 hours at 30°C, AFB1 was removed by 74.5% thanks to the crude protein extract of *F. aurantiacum.* Enzymes were identified as the active compounds of the degradation process (Smiley & Draughon, 2000).

From then on, different bacteria were studied for their potential to remove AFB1. Table 12 summarises them. Some reduction rates of up to 100% were achieved. Teniola *et al.,* (2005) studied cell free extracts of *Rhodococcus erythropolis* DSM 14303 and *Mycobacterium fluoranthenivorans* sp. nov. DSM 44556T for their ability to degrade AFB1. The initial concentration of AFB1 (2.5 mg.l^{-1}) was almost completely degraded in 8 hours. A less efficient bacterium is *Mycobacterium smegmatis.* It took 2 days at 28°C, to remove all the initial concentration of AFB1 (6 mg.l^{-1}) in the medium.

Table 12 - Examples of aflatoxin removal by bacteria.

name	medium	condition	[AFB1] (mg.kg^{-1})	temperature	time	reduction efficiency	actif compound	references
Mycobacterium fluoranthenivorans sp. nov. DSM 44556T	cell extraction buffer	cell-free	2.5	30°C	8h	~100%	enzymes	(Teniola et al., 2005)
Mycobacterium smegmatis	PYB	incubated	6	28°C	48h	~100%	$F_{420}H_2$-dependent reductases	(Taylor et al., 2010)
Nocardia asteroides IFM 8	water bath	bacteria	12	37°C	38h	~100%	?	(Arai et al., 1967)
Rhodococcus erythropolis DSM 14303	cell extraction buffer	cell-free	2.5	30°C	8h	~100%	enzymes	(Teniola et al., 2005)
Rhodococcus strains (32)	LB medium	inoculated in liquid medium	2	28°C	72h	up to 100%	enzymes	(Cserháti et al., 2013)
Bacillus subtilis UTBSP1	Pistachios nuts	bacteria	0.002	30°C	5d	95%	?	(Farzaneh et al., 2012)
Pseudomonas putida (2)	MSG	bacterial pellets	0.2	37°C	24h	90%	?	(Samuel et al., 2014)
Stenotrophomonas maltophilia	NB	AFB1 added in the media	0.1	37°C	72h	82.5%	enzymes	(Guan et al., 2008)
Lactobacillus rhamnosus TISTR 54	PBS	bacterial pellets	15	37°C	1h	79.4%	binding	(Elsanhoty et al., 2013)
Lactobacillus plantarum (PTCC 1058)	corn samples	inoculated on corn	0.24	37°C	4-7d	77%	?	(Khanafari et al., 2007)
Flavobacterium aurantiacum (Nocardia corynebacterioides)	Czapek-Dox medium (solid)	inoculated on Petri dishes	7.5	28°C	44h	74%	enzymes	(Ciegler et al., 1966) (Smiley & Draughon, 2000)
Myxococcus fulvus ANSM068	VY/2 medium	culture supernatant	0.1	30°C	48h	71.9%	Myxobacteria Aflatoxin Degradation Enzyme (MADE)	(Zhao et al., 2011)

PBS: Phosphate Buffered Saline, PYB: Peptone Yeast extract Broth (9 g/l peptone, 4.5 g l^{-1} yeast extract, 23 mM Na$_2$HPO$_4$, 88 mM KH$_2$PO$_4$, 9 mM NaCl, pH 6.0) VY/2 medium: (7 g baker's yeast cake, 1 g CaCl$_2$, 0.5 g MgSO$_4$ and 0.75 mg.l^{-1} nocobalamin, pH 7.4) NB: Nutrient Broth, MSG: Mineral Salt Glucose Medium. (number of strains tested)

These examples highlight how bacteria are interesting AFT reducers. Physical, chemical, adsorbents and biological agents are techniques that the end of a lengthy process of work. AFT management can only be achieved if all the steps in the food processing chain are rightfully managed and monitored. Monitoring of physical parameters and bacterial biocontrol is needed to ensure a safer food process. This explains the objectives of our work.

1.3. Project Aflafree

The previous bibliographic review has highlight the problem of AF contamination in cereals. In Europe, significant AFB1 concentrations in freshly-harvested maize samples are too frequently found (e.g.: summer 2003 in France, regular above regulation samples in northern Italy). The announced global warming suggests an increase of such situations in the near future. Thus we decide to focus on temperature and a_w as the 2 environmental parameters that greatly influence the fungal growth and the mycotoxin production (Figure 39, WP1).

Due to this alarming situation and as an alternative to reduce the chemical inputs, the AFLAFREE project proposes to prove the concept that soil-borne bacteria could be applied as biocontrol against AFT accumulation at maize field. The biocontrol could either interrupt AFT biosynthesis or reduce AFT content. Intermediates of AFT biosynthetic pathway and other side-products will be searched and their residual toxicity will be assessed *in vitro* (Figure 39, WP2).

In addition to *in vitro* testing, the greenhouse tests deal with the optimization of protocol to study *A. flavus*-maize interaction and the biocontrol's impact on this interaction. The best couple bacteria-maize against AFT is studied and validated in greenhouse. Attention is given to conceive the best application of the biocontrol and to validate its safety. The chosen formula will be applied at different physiological maize stages. The efficiency is tested, during ripening and after harvest of maize kernels. To choose the biocontrol, it is necessary to take into account the technical possibilities available at field to treat crop and later grains (Figure 39, WP3).

In complement to the field approach, ECCLOR Europe SAS follows a_w and temperature parameters during the different maize post-harvest stages. Their patented sensors are refined to be used to follow the evolution of a_w and temperature from the maize field to the transformation facilities (Figure 39, WP4).

The results of this project will be delivered in agricultural development and on the maize chain as Good Agricultural Practices accompanied by advices on grain conservation and products process. In the sustainable development spirit, all results will be transferred to the different actors of the maize chain in the form of decision making tools to reduce the sanitary risk, chemical inputs and to be economically feasible. Governance is established based on score-cards including indicators and corrective actions. All this will help to avoid crisis situations (Figure 39, WP5).

This project links 4 partners having different skills (microbiology, molecular biology, plant physiology, electronic, toxicology) to produce a sustainable maize food-chain.

This 42-month long project has been financed by the French National Research Agency and is divided into 5 workpackages (as described previously):
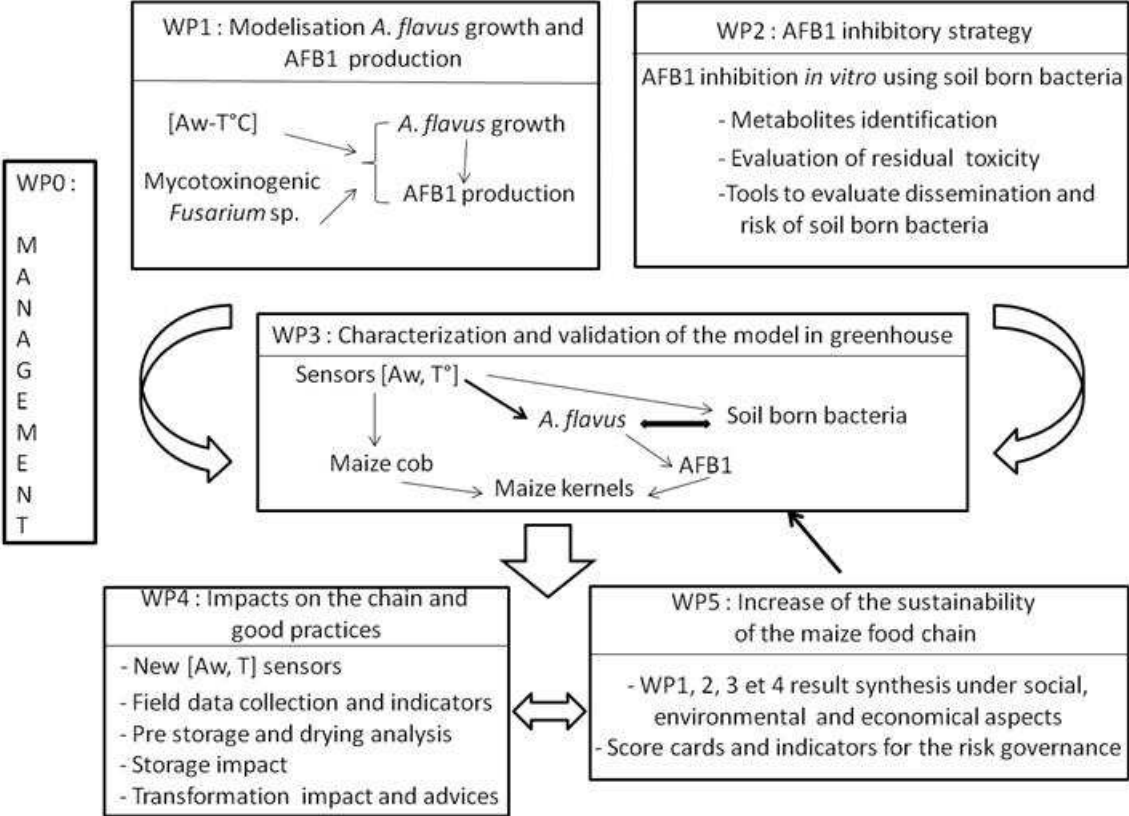
Figure 39 - Organisation of the Project AFLAFREE (2011-2015)

The 3 partners involved are the BioSyM department of the LGC, with Pr. Florence MATHIEU as the project coordinator, ECCLOR Europe SAS and INRA ToxAlim.

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1.3.1. Objectives of the thesis

The objectives of the thesis were:

a) to monitor the entry of *A. flavus* into the French maize ecosystem (e. g. *Fusarium* sp.) and its impact on the management of prestorage to ensure no aflatoxins and D.O.N. risks.

b) to develop a biocontrol (based on actinomycetes) able to reduce (in interaction with *Aspergillus* sp.) AFT contamination at field without impacting the maize microbial ecosystem;

c) to characterise those interactions impacts on the prevention of AFT production (through RT-qPCR) and on the reduction of aflatoxin content (through reduction and adsorption test);

2.Materials and Methods

2.1. Micro-organisms

2.1.1. Fungal strains

In this study, 4 fungal strains were used:

Aspergillus flavus NRRL 62477 (=E73), isolated from Moroccan paprika as described in El Mahgubi *et al.,* (2013).

Aspergillus flavus NCPT180 (=Afc5), isolated from Benin cassava as described in Adjovi *et al.,* (2014).

Aspergillus parasiticus strain NCPT 217, isolated from nuts is a producer of AFT.

The 3 strains were gracefully provided by Dr. Olivier Puel, Toxalim, INRA, Saint Martin du Touch, France.

Fusarium graminearum INRA 155, maize-isolated in south-west of France. This strain was provided by Dr. Christian Barreau, MycSA, INRA Bordeaux, France.

2.1.2. Actinomycete isolates

Isolates were collected and macroscopically identified as actinomycetes by members of Ecole Normale Supérieure (ENS) Kouba, Algeria.

2.2 Media:

2.2.1 Pre-culture media:

Pre-cultures of *A. flavus* and *A. parasiticus* were realized on YEPD medium containing: 5 g.l^{-1} Yeast Extract (Fisher Scientific), 10 g.l^{-1} Casein Peptone (Fisher Scientific), 10 g.l^{-1} α-D-Glucose (Fisher Scientific) and 15 g.l^{-1} Agar (Kalys).

Pre-cultures of *F. graminearum* were realized on PDA medium containing: 20 g.l^{-1} α-D-Glucose, 4 g.l^{-1} Potato Infusion and 15 g.l^{-1} Agar (mixed by Sigma).

Pre-cultures of *Streptomyces* were realized on ISP-2 medium containing: 4 g.l^{-1} α-D-Glucose (Fisher Scientific), 10 g.l^{-1} Malt Extract (Fisher Scientific), 4 g.l^{-1} Yeast Extract (Fisher Scientific) and 20 g.l^{-1} Agar (Kalys) using non-distilled water and adjusted to pH 7.

2.2.2 Growth media:

2.2.2.a *Aspergillus-actinomycetes* interaction

The co-culture between *A. flavus* and the actinomycetes isolates were performed on ISP-2 medium. We selected this medium as it is a standard medium for actinomycetes growth and *A. flavus* grew well and produced high amount (around 1-5 ppm after 7 days) on this medium (preliminary tests).

In case of gene expression study, the Petri dishes were filled with ISP-2 medium (34 ml per Petri dishes). Cellophane sheets (Hutchinson, France) were cut at the Petri dishes shape. The cellophane sheets were exposed 20 minutes for each face to UV lights for sterilization, then, the sheets were displayed on the media thanks to pliers. The sheets were used to recover the fungal biomass without the agar medium.

2.2.2.b a_w study: Maize-based medium

Maize grain was provided by Arterris (harvest 2011, Lespinasse, France). The maize was washed with water and 90° alcohol. 200 $g.l^{-1}$ of maize were added to boiled water. After 30 minutes under agitation, the infusion was cooled down and passed through a Tami (Ø 2 mm). Absorption at 350 nm was validated at 0.6 (±0.05) (after 15 min at 12.000 g). The a_w was measured by HydroPalm Aw1® and measure probe HydroClip AW-DIO (ROTRONIC AG, Basserdorf, Switzerland). The medium had an a_w of 1.

The a_w of the medium was modified thanks to glycerol (Fisher Scientific) addition to obtain the desired values. The experiment was done 3 times. The a_w according to glycerol concentration is represented in figure 40.
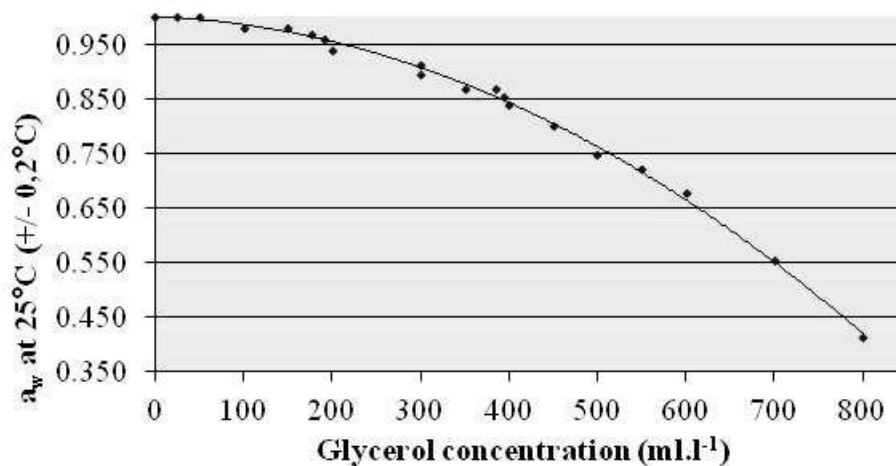
Figure 40- A_w of the maize based infusion depending on glycerol concentration

Nutriments were added to the maize-based infusion (supplemented by glycerol): 5 g.l^{-1} Yeast Extract (Fisher Scientific), 10 g.l^{-1} α-D-Glucose (Fisher Scientific) and 15 g.l^{-1} Agar (Kalys).

To prevent Maillard reaction, the medium was autoclaved separately from the glycerol. Medium was added in sterile condition in the glycerol bottle at a temperature of 60°C (to avoid glycerol high viscosity). The bottle was highly agitated prior to Petri dishes filling.

2.2.2.c AFB1 supplemented media

A 1 mg.ml^{-1} AFB1 solution was prepared in methanol solution. This solution was added to ISP-2 medium after autoclaving to obtain a final concentration of 5 mg.kg^{-1}.

2.2.3 Conservation media:

3 medium were used to conserve strains:

(i)   on Petri dishes for short term conservation (1 week to 6 months);

(ii)   in inclined tubes for intermediate conservation: screw-capped tubes were filled with 12 ml of medium. After autoclaving, the tubes were inclined (around 10°) and were left at room temperature for cooling. The strains were left for growth until sporulation. The inclined tubes were then put at +4°C for conservation (more than a year conservation - transplanting yearly);

(iii)   in cryotubes for long term conservations: strains were grown on Petri dishes. After sporulation, spores were taken with a sterile loop and were put in cryotubes containing a 20% glycerol solution (more than five year conservation - transplanting every 4 years).

Actinomycetes were conserved in different ways to insure reliable isolates conservation. On Petri dishes, actinomycetes were grown on ISP-2 medium and conserved at +4°C. In inclined tubes, on ISP-2 medium and conserved at +4°C and in cryotubes at -20°C.

Fungal strains were also conserved in different ways. On Petri dishes, strains were grown on YEPD and PDA media for *Aspergillus* sp. and *Fusarium* sp., respectively. After sporulation, the Petri dishes were conserved at +4°C. In inclined tubes, on PDA medium and conserved at +4°C and in cryotubes at -20°C and -80°C.

2.3 Culture:

2.3.1 Culture for the screening study of good aflatoxin-reducing candidates (results chapter 3.2):

The co-culture screening method is based on the method proposed by Sultan & Magan (2011). *A. flavus* spores are dislodged from the pre-culture with a sterile loop and placed in 10 ml sterile water + 0.05% Tween 20. Spores are counted using a Thoma cell and a 10^6 spores.ml^{-1} concentration solution was prepared. In a Petri dish filled with ISP-2 medium, actinomycetes and *A. flavus* are inoculated on the same day. Inoculation is done with the following instructions: 10 µl of spores suspension from *A. flavus* are spotted at 2 cm from the Petri dish periphery. Actinomycetes streak is inoculated perpendicularly to *A. flavus* - actinomycete axe at 4.5 cm of the *A. flavus* spot. The methodology is represented as figure 1 in Verheecke *et al.,* (2014) are hereabove:
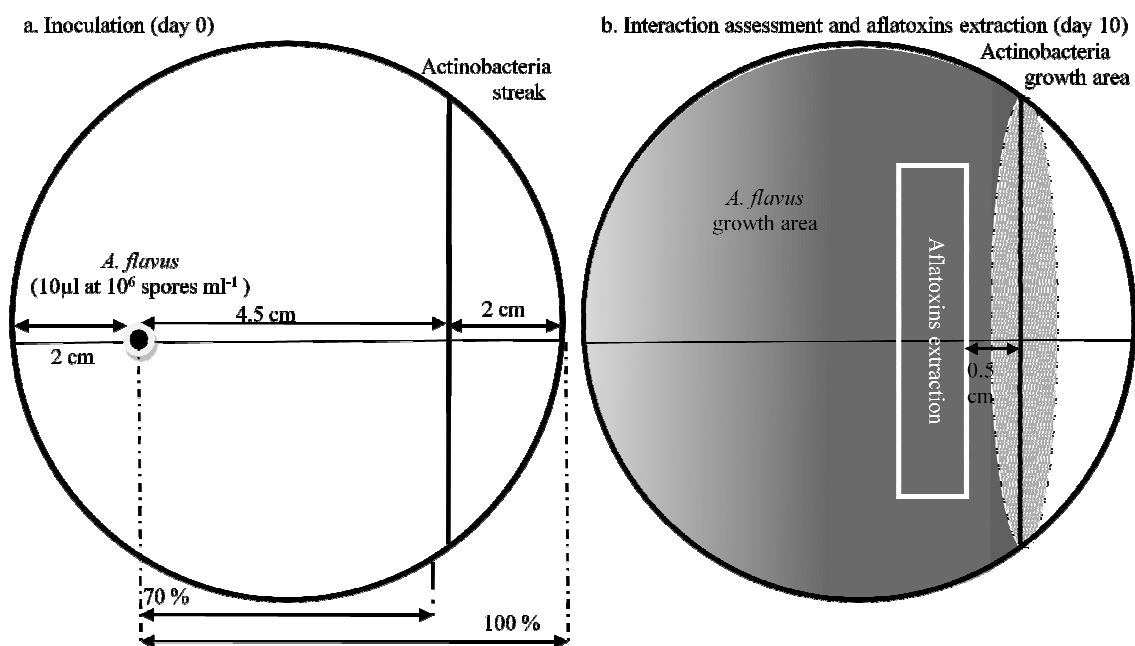
a. Inoculation (day 0) in a Petri dish filled with ISP2 medium, actinomycetes and *A. flavus* are inoculated on the same time. Inoculation is done with the following instructions: 10 µl of spores suspension from *A. flavus* are spotted at 2 cm from the Petri dish periphery. Actinomycete streak is inoculated perpendicularly to *A. flavus* -actinomycete axe at 4.5 cm of the *A. flavus* spot.
b. Interaction assessment and aflatoxins extractions, in case of I_D (2/2). The growth measurements are done and is represented in grey for *A. flavus* and in stripes for the isolate. The aflatoxin extraction area is delimited by a white box.

Figure 41 -Methodology used for interaction assessment and aflatoxins extraction (Verheecke *et al.,* 2014)

The incubation is made during 10 days at 28 °C, growth measurements are done at the end of the incubation time. Experiment is realized twice in triplicate. The interaction between the 2 micro-organisms is observed macroscopically and scored based an Index of Dominance (I_D) (Magan & Lacey, 1984).

2.3.2. Culture for the RT-qPCR study of the impact of selected *Streptomyces* on aflatoxin gene expression (results chapter 3.3):

the co-culture method for RT-qPCR analysis is based on the latter with slight modifications. The methodology is represented is figure 42:
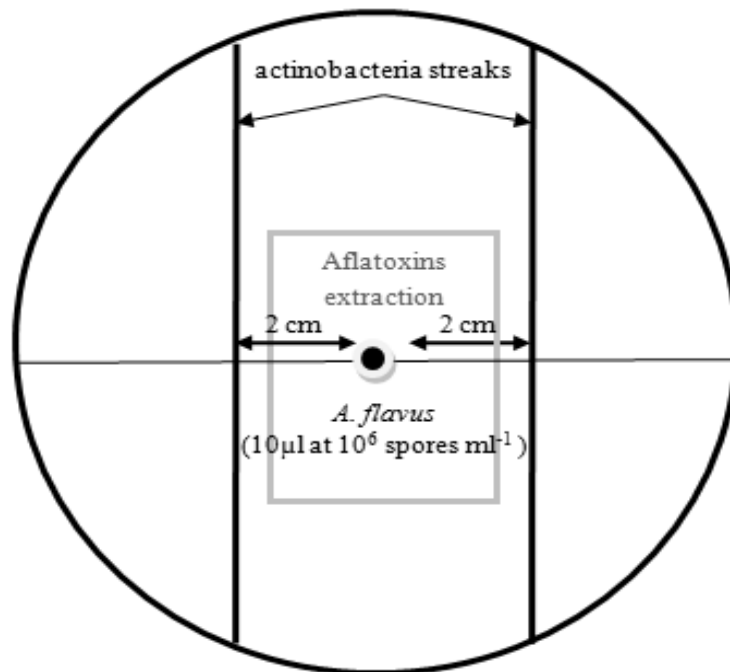
Figure 42 - Methodology used for interaction assessment, RT-qPCR and AFT extractions.

The protocol used is described above:

Day 0: Inoculation in a Petri dish filled with ISP-2 medium, actinomycetes and *A. flavus* are inoculated on the same time. Inoculation is done with the following instructions: 10 µl of spores suspension from *A. flavus* are spotted in the middle of the Petri dishes. Actinomycete streaks are inoculated in parallel with *A. flavus* spot in the center.

90 hours: With a scalpel, the cellophane close to the mycelium growth was cut. At the interaction point, all the eye seen mycelium was taken avoiding bacterial biomass. Fungal biomass was separated from the bacterial one.

Day 7: The fungal biomass (without bacterial biomass) was removed from the cellophane sheet for dry weight (18 hours at 80°C prior to weight measurement) and in the remaining media 3 agar plugs (Ø 9 mm) were taken from the fungal growth area for AFT extraction as shown in a grey box.

2.3.3 Culture for the AFB1-reduction test of 12 chosen actinomycetes (results chapter 3.2):

On AFB1-supplemented medium, actinomycetes were inoculated with a loop to cover completely the Petri dish surface. After a 4 days long incubation period at 28°C, AFB1 was extracted. The actinomycete growth was observed macroscopically in the ISP-2 medium, control medium (methanol addition without AFB1) and in the AFB1-supplemented medium. The experiment was realized twice in triplicate.

2.3.4 Culture for the AFB1 adsorption test for 2 chosen actinomycetes strains (S13 and S06) (results chapter 3.3):

Streptomyces (S13 and S06) spores were dislodged from the pre-culture with a sterile loop and placed in 10 ml sterile water. Spores were counted using a Thoma cell and a 10^6 spores.ml^{-1} concentration solution was prepared. In a glass vial, 990 µl of spore solution and 10 µl of AFB1 (100 µg.ml^{-1}) were added to achieve a global concentration of 1 µg.ml^{-1}. After 1 or 60 minutes at 30°C, the mixture was collected thanks to syringe and needle. It was filtred (PVDF, 13 mm, 0.45 µm, Whatman) and transfer into vial n°1. The filter was rinsed once with sterile water (1 ml) and the rinse water was transferred into vial n°2. Finally, the filter was also rinsed with methanol (Fisher Scientific) and the rinse methanol was transferred into vial n°3. The experiment was done twice in triplicates. A student t-test was realized as a statistical analysis.

2.4 Analytical methods:

2.4.1 Aflatoxins HPLC measurement:

3 agar plugs (Ø 9 mm) are taken: at 5 mm from actinomycete streak (co-culture screening) and randomly on actinomycete growth area (AFB1 reduction test). The 5 mm distance was taken to mesure the direct impact of the *A. flavus* closest to the interaction zone.

The total weight of agar was measured. One milliliter of methanol was added to the plugs, shaken 5 seconds 3 times and incubated 30 min at room temperature. After centrifugation 15 min at 12,470 g, the supernatant was recovered and filtered through 0.45 µm PVDF Whatman filter into a vial and stored at -20°C until analysis.

AFB1 measurement was done by an HPLC Ultimate 3000 (Dionex, FR) coupled with a Coring Cell (Diagnostix Gmbh, GE) for post-column derivatization. The Fluorescence Detector (Ultimate 3000, RS Fluorescence Detector, Dionex) was fixed at an excitation

wavelength λex = 362 nm and emission wavelength λem = 440 nm. 3 differents analytic columns were used. A C18, 5 μm (150 x 4,6 mm) Prontosil ODS1 with a pre-column (10 x 4,3 mm) was first used. To optimize AFT separation two other columns were used, C18 Phenomenex Luna and Kinetex (3 μm, 200 x 4-6 mm). A 10 to 100 μl injection volume was used (depending on the level of AFT quantified) with a Dionex auto-injector. The mobile phase was methanol: acetonitrile (Fisher, UK): water (20 : 20 : 60) with 119 mg.l^{-1} of potassium bromure (acros organics, BE) and 100 μl.l^{-1} of 65% Nitric acid (MERCK, DA) added. The flow rate was 0.8 to 1 ml.min^{-1}. AFT quantification was done with standards (AFB1 produced by *A. flavus*, Sigma-aldrich, France) and the data were treated thanks to Chromeleon software.

The recovery ratio was calculated thanks to the addition of 5 μg.kg^{-1} of AFB1 in the medium. The standard solution and the medium extracted were both analysed in triplicate. A recovery ratio was calculated as 50% ± 5%.

2.4.2 RT-qPCR:

2.4.2.a. Primer design:

Primers were design thanks to Primer-blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with slight parameters modifications:

(i)   annealing temperature T_m: 59°C (T_m variance ≤ 2°C between the primers)

(ii)   80 to 150 pb long amplicon (with an intron if possible)

(iii)   no more than 2 G/C in the last 5 nucleotides (3' end),

To confirm no strong secondary structure (≥-ΔG:4), produced primers were validated by Beacon software (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1).

cDNA structure was checked with the mfold software (http://mfold.rna.albany.edu/?q=mfold). Primers were selected to be outside of the secondary structures of cDNA.

The primers were produced and delivered by IDT (http://eu.idtdna.com/site) and their specificity was validated by qPCR followed by a nucleotide analyzer (experion - 100bp DNA StSens chip (Bio-Rad)).

2.4.2.b. Efficiency determination:

The efficiency of each primer sets was determined as describe: cDNA serial dilutions were made from a randomly chosen sample (100 fg, 1 ng, 10 ng, 100 ng) and were amplified

in duplicate using the same protocol as RT-qPCR. The efficiency was determined by CFX Manager (BioRad) and the efficiencies between 85 and 115% were validated.

2.4.2.c. RT-qPCR:

RNA extraction. The (90 hours aged) fungal biomass was crushed to a fine powder under liquid nitrogen and stored at -80°C until RNA isolation. Approximately 60 mg of mycelia were taken for extraction. Total RNA was isolated using the Aurum Total RNA Kit (BioRad) according to the manufacturer's instructions for eukaryotic and plant cell material with the following modifications: DNase I digestion increased to 1 hour and the elution was done at 70°C for 2 min in elution buffer. Total RNA was eluted into 80 µl and stored at -20°C for short term storage. 1 µl of Total RNA of each sample was loaded into a RNA StSens chip (Bio-Rad) and quantified on nanodrop 2000 (Thermo scientific) according to the manufacturer's instructions. Samples with RQI> 6.5, $A_{260/280}$>2 and $A_{260/230}$>1.3 were taken for further analysis.

Reverse transcription (RT) and qPCR. RT was carried out with the Advantage RT-PCR Kit (Clontech) with Oligo $(dT)_{18}$ primer (eukaryote only) according to the manufacturer's instructions (1 µg total RNA) with 1 modification: reaction incubation at 42°C was increased to 4 hours. RT-qPCR was performed in a CFX96 Touch instrument (Bio-Rad) using SsoAdvanced^{TM} SYBR Green Supermix (Bio-Rad) with the protocol recommended for cDNA by the manufacturer's instructions (annealing temperature: 59°C; concentrations: Primers: 500 nM and cDNA: 100 ng). Each sample was run in duplicate. Following the RT-qPCR, data were analysed using CFX Manager Software (version 3.0, Bio-Rad) for melting curves analysis. The Cq values were analysed thanks to the qbase+ software (biogazelle) (Hellemans *et al.*, 2007). A One-way ANOVA (control versus all strains), paired t-test (control vs each strain) and spearman correlation test were done for statistical analysis.

2.4.2.d. Reference genes validation:

Based on the literature, we studied 7 candidate genes (*act1*, *βtub*, *cox5*, *ef1*, *gpdA*, *hisH4*, *rpl13* and *tbp*) as potentially suitable reference genes (Radonić *et al.*, 2004; Bohle *et al.*, 2007). For the identification of stability and optimal number of reference genes, 8 samples (randomly selected among the different conditions) were tested in triplicate. The gene stability measures V (gene pairwise variation) and M (V of a gene with other genes) were calculated with geNorm software (Vandesompele *et al.*, 2002).

Optimal reference genes were then calculated.

2.4.3 16S sequencing:

For DNA extraction, cultures of actinomycetes were grown for 3 days on ISP-2 medium using non-distilled water (Shirling & Gottlieb, 1966) at pH 7. The protocol for bacterial DNA extraction was taken from Liu *et al.,* (2000). Nucleic acid quantification following DNA extraction was performed using Nanodrop 2000 (Thermo scientific) according to the manufacturer's instructions.

PCR amplification of the 16S region was performed using a C1000 Touch Mycycler (BioRad). The chosen primers were the 27F (5' AGAGTTTGATCCTGGCTCAG 3') and the 1492R (5' GGTTACCTTGTTACGACTT 3'). PCR reactions were performed in 50 µl reactions containing 0.5 µM of each primer, 10 x of reaction buffer containing $MgCl_2$, 10 µM of deoxyribonucleotides (dNTP), and 5 U.μl^{-1} of Taq DNA polymerase (MP Biomedical). Samples were subjected to an initial 1 minute denaturation at 98°C, followed by 30 cycles of 1 minute denaturation at 94°C, 1 minute primer annealing at 57°C, and 2 minutes of extension at 72°C, with a final 10 minutes extension step at 72°C (Zitouni *et al.*, 2005). PCR amplicons were detected by agarose gel electrophoresis and were visualized by UV fluorescence after ethidium bromide staining.

PCR amplicons were sequenced by Beckman Coulter Genomics (Grenoble, France). Sequencing primers were 10-30F (5' GAGTTTGATCCTGGCTCA 3') and 1500R (5' AGAAAGGAGGTGATCCAGCC 3').

The 16S rRNA DNA sequences were compared with the EzTaxon database (http://eztaxon-e.ezbiocloud.net/). Similar 16S rRNA gene sequences were detected and the pairwise similarity was calculated. All the strains 16S sequence and their 3 closest strains were entered into Mega 6 software (Tamura *et al.*, 2013) for analysis. The 16S DNA sequences were aligned against neighboring nucleotide sequences using CLUSTAL W (Larkin *et al.*, 2007).

Phylogenetic tree was constructed by using neighbour-joining (algorithm to compare sequences between each others)(Saitou & Nei, 1987) and Kimura model was used. This latter is based on the assumption that transitional substitutions (A=>G and C=> T) occurred twice less than transversional (G=>C, G=>T, A=>C and A=>T) through time. Bootstrap analysis (Felsenstein, 1985) was performed to evaluate the reliability of the tree topology.

3. Results & Discussion

3.1. Impact of environmental parameters on AFB and D.O.N. production.

Introduction

In this project, our aim was to investigate *A. flavus* impact on the maize microbial ecosystem (*Fusarium* sp.) and its impact on AFB, D.O.N. and fumonisins production. Our objectives included different steps:

(i)   characterisation of *A. flavus* growth and AFB production on a maize-based medium;

(ii)   characterisation on the same medium of *F. graminearum* and *F. verticillioides* growth and their D.O.N. and fumonisins production, respectively;

(iii)   characterisation of the competition between those species 2-by-2 and the 3 together on the same medium;

(iv)   tests *in vitro* on maize grain;

(v)   development on decision making tools available for storage agencies.

As a beginning, we focused on the first 2 steps. Based on the literature, we defined the a_w and temperature conditions where *A. flavus* could grow and produce AFB. However, few data are available on the kinetic of production in controlled environments. These data are crucial for our ultimate step. Indeed, storage agencies needs reliable data to give an order of priority to the dryer. Those decisions will depend on the a_w and temperature of the stored grain and time available prior to mycotoxins risks.

Thus, in the publication hereafter we conduct *in vitro* experiments on maize-based medium to determine the early stages of AFB and D.O.N. production by *A. flavus* and *F. graminearum*, respectively. Different abiotic parameters (time, temperature and a_w) interwoven with sole and mixed inoculation were tested.

Impact of environmental parameters on early Aflatoxins and Deoxynivalenol *in vitro* productions on maize-based medium.

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Running title: T°C, Aw, Time, _A. flavus_ & _F. graminearum_ DON-AFB production

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ABSTRACT (200 words)

BACKGROUND: provides a rationale for the study (understandable to a broad audience) and states the main aim(s).

Aflatoxins and Deoxynivalenol are mycotoxins that can be produced in maize prestorage. Those mycotoxins productions depend on the prestorage conditions (time, temperature and a_w) and natural microbial maize ecosystem (e.g.: presence of *Aspergillus* sp. and *Fusarium* sp.). We studied the impact of these biotic and abiotic parameters on two representative producers: *A. flavus* and *F. graminearum* for aflatoxins (B1+B2) and Deoxynivalenol productions, respectively.

RESULTS: describes the main findings, including important numerical values.

In sole culture, *A. flavus* grew after 1 day and *Fusarium* after 2. Aflatoxin B1 production started at day 2 (36°C, 1) while no Deoxynivalenol was detected after 7 days. Maximum aflatoxin B1 and B2 production occurred after 4 days (28°C, 1). In co-inoculation tests, the growing fungus with the highest growth rate (in separate conditions) seems to overtake the other one.

Keywords: *Aspergillus flavus, Fusarium graminearum,* a_w, temperature, deoxynivalenol, aflatoxins

INTRODUCTION

In France, maize is the second crop production (FranceAgriMer, 2013). Thus, prevention against mycotoxins content above the regulation is crucial. Prestorage is a dangerous step regarding mycotoxins content (Grosjean & Gourdain, 2010). The former consists of temporary storage (1 to 6 days) before the first drying of the long term storage. At this step, high temperatures (30-35°C) and a_w (> 0.80) may occur (personal communication).

At prestorage, fungal growth and mycotoxin production result from a complex interaction of several factors. Those includes abiotic (water activity (a_w), temperature, time, etc.) and biotic parameters (microbial interaction) (Giorni *et al.*, 2008; Formenti *et al.*, 2012; Picot *et al.*, 2012). A monitoring of each parameter involved is essential to understand the overall process and to predict and prevent mycotoxins development during this temporary step. In France, proper prestorage management is based on rapid drying (maximum 48h) and cooling (under 20°C) (Journal officiel, 2011). However, technical constraints can sometimes postpone this drying step.

At field, French maize can be contaminated with *Fusarium sp.* (Picot *et al.*, 2012) and the newcomer *Aspergillus* section *Flavi*. The latter is already present in Italy (Giorni *et al.*, 2007) and starting to occur in France due to climate changes (Battilani *et al.*, 2012). As an effect, prestorage maize can be contaminated with *Fusarium sp.* and *Aspergillus* section *Flavi*, both able to produce mycotoxins.

F. graminearum is one of the most representative of mycotoxigenic *Fusarium sp.* and produces Deoxynivalenol (D.O.N.) (Reid *et al.*, 1999). The latter is colloquially known as the vomiting toxin:"vomitoxin"(Pestka, 2007) and its maximum limits in maize is 750 µg.kg^{-1} for human consumption (European Union, 2006). *A. flavus* is the most representative of aflatoxins B1 and B2 (AFB) producers (Nesbitt *et al.*, 1962). The latter are potent carcinogenic compounds ("IARC Publications list," 2012).

Thus, determination of the safety space-time of maize pre-storage prior to mycotoxins risks is needed. Moreover, the impact of the newcomer *A. flavus* needs to be assessed. Faced with the need for reliable data to prevent D.O.N. and AFB accumulation, maize-based medium have been developed (Garcia *et al.*, 2013). The use of this medium provide preliminary data needed for a future model development dedicated to prestorage.

The aim of this work was to evaluate, *in vitro,* on a maize-based medium, the effect of temperature, a_w, early incubation time and co-inoculation on fungal growth and toxin

production by *A. flavus* (AFB producer) and *F. graminearum* (D.O.N. producer). Both fungi were together and separately incubated on such medium.

Fungal isolates. The fungal strains used were *Aspergillus flavus* NCPT180 and *Fusarium graminearum* INRA 155 (maize-isolated in south-west of France) as they were previously identified as high aflatoxin and D.O.N. producers on maize-based medium, respectively. Both strains were conserved at -20°C in cryotubes in a 20% glycerol solution.

Medium preparation and water activity adjustment.

French dent maize was harvested in 2011. The maize was washed with water and 90° alcohol. 200 g.l^{-1} of maize were added to boiled water. After 30 minutes under agitation, the infusion was cooled down and passed through a Tami (Ø 2 mm). Absorption at 350 nm was validated at 0.6 (±0.05) (after 15 min at 12.000 g). The a_w was measured thanks to HydroPalm Aw1$^®$ and measure probe HydroClip AW-DIO (ROTRONIC AG, Basserdorf, Switzerland). The initial medium had an a_w of 1. The medium a_w was adjusted thanks to the addition of glycerol (Fisher Scientific, France) to obtain the desired a_w values. The sorption isotherm of the media depending on the quantity of glycerol added was evaluated in triplicates. Nutriments were added to the maize-based medium: 5 g.l^{-1}Yeast Extract (Fisher Scientific, France), 10 g.l^{-1} α-D-Glucose (Fisher Scientific, France) and 15 g.l^{-1} Agar (Kalys, France). The medium (pH 5.3) was separately autoclaved from the glycerol. The medium was mixed with the glycerol at 60°C. The a_w of plates was not significantly different after seven days (statistics performed with R (2.15.2), t-test).

Inoculation method. Pre-cultures were realized as previously described (Verheecke *et al.*, 2014). *A. flavus* and/or *F. graminearum* spores are dislodged from the pre-culture with a sterile loop and placed in water. In a Petri dish filled with maize-based medium, *A. flavus* (1.10^6 spores.ml^{-1}) or *F. graminearum* ($2.5.10^5$ spores.ml^{-1}) were centrally inoculated by applying 10 μl of spores suspension. In case of co-inoculation the spore suspension was composed of *A. flavus* (1.10^6 spores.ml^{-1}) and *F. graminearum* (1.10^5 spores.ml^{-1}). All the experiments were done in triplicates.

Incubation and growth assessment. 2 hours prior inoculation, the plates were incubated at the different temperature (12, 20, 28 and 36°C) in incubators (AQUALYTIC FKS 3600 Index 10B, LIEBHERR, Fisher Scientific, France.) supplemented with water containers. During 7 days, three plates per condition (a_w, temperature) were randomly chosen each day of culture. Growth measurements were taken in two directions at right angles to each others. A picture per condition was taken and the mycotoxin extraction was realized.

Mycotoxins extraction and quantification. In the colonized media (sole or co-incubation), 3 agar plugs (Ø 9 mm) were taken randomly at the periphery, in the middle and in the center of the colony. Aflatoxin extraction and quantification were done as previously described (Verheecke *et al.*, 2014) with a slight modification: methanol incubation was extended to 1 hour (limit of detection 0.05 ppb). The same extraction was applied to DON. The HPLC system used for DON analysis was an Ultimate 3000 system (Dionex- Thermo Electron, Fr) with all the RS series modules. A C18 column and its associated pre-column (Prontosil, ODS1 5 µm, 125 x 4 mm) were used at 45°C. Analyses are realized at a flow rate of 1 ml.min^{-1} during a 22 min run divided in 4 steps with different acetonitrile:water (pH 2.6). Step 1, 0 to 10 min the ratio raised from (0:100) to (45:55). Step 2, 10 to 15 min the ratio raised from (45:55) to (90:10). Step 3, during 5 minutes, the ratio remained stable. Step 4, the ratio dropped from (90:10) to (5:95). The quantification is realized by the Chromeleon software, thanks to a standard of DON (Sigma-Aldrich, FR). The limit of quantification is 0.5 mg.kg^{-1}.

RESULTS

***A. flavus* growth.** Fungal growth was highly influenced by the 3 studied parameters: temperature, a_w and time of incubation. 12°C and a_w of 0.75 and 0.80 were the most repressive conditions with no growth after 7 days. At day 1, growth only occurred in 2 conditions: at an a_w of 1, the fungus grew by 0.4 and 0.5 cm at 28°C and 36°C, respectively. At day 2 (a. figure 1), more conditions were permissive to *A. flavus* growth: an a_w of 1 for all the remaining temperature and an a_w of 0.95 for 28 and 36°C. Days 3 and 4 (b. and c. figure 1), both a_w of 1, 0.95 and 0.90 (28°C and 36°C) led to *A. flavus* growth. At day 5 (d. figure 1), a complement growth was observed at 0.85 (36°C). Day 6 and 7 (e. and f. figure 1), only an a_w of 0.85 (20°C, 28°C) remained suppressive for fungal growth. A_w of 0.95 and 36°C were the most permissive conditions: the maximum growth (4.2 cm) was at day 7 (0.95, 36°C).

AFB production by *A. flavus*. AFB were also highly influenced by the 3 studied parameters. 12°C and a_w of 0.75, 0.80 and 0.85 were repressive conditions with no AFB1 production. At 0.90, only between days 6 and 7 AFB1 (not AFB2) occurred, with 0.7 and 11 ppb produced, respectively. At day 2 (Table 1), AFB1 only occurred at 36°C at an a_w of 1. For the following days, the highest AFB detected were at an a_w of 1 for 28°C and an a_w of 0.95 for 36°C. At 20°C, the production of AFB1 started at day 5 and AFB2 at day 6: the maximum occurred at day 7 and an a_w of 1. Overall, the maximum AFB production was achieved at day 4 and an a_w of 1 where 3,070.0 and 41.7 ppb were detected for AFB1 and AFB2, respectively.

***F. graminearum* growth**. Fungal growth was highly influenced by the 3 studied parameters: temperature, a_w and time of incubation. 36°C and a_w of 0.75, 0.80 and 0.85 were the most repressive conditions with no growth after 7 days. At day 1, no growth occurred. At 0.90, growth only occurred (0.3 cm) at day 7 and 28°C. The other 6 incubation days are represented in figure 2 (a. for an a_w of 0.95 and b. for 1). At both a_w and 12°C, growth was delayed by 2 days (day 4) compared to 28 and 36°C. The Petri dishes were completely recovered after 6 days at an a_w of 1 and 28°C (most permissive conditions).

D.O.N. production. No production was detected at 7 days in all the conditions tested.

***A. flavus* and *F. graminearum* co-inoculation.** 0.75, 0.80 and 0.85 were the most repressive conditions with no growth after 7 days. At day 1, growth only occurred in 2 conditions: at an a_w of 1, the fungi grew by 0.5 cm at 28°C and 36°C. The growth at 0.90 started day 3 at 36°C and day 4 at 20 and 28°C. From day 2 to day 5, the fungal growth was higher at 20 and 36°C

than 28°C. Macroscopic study showed that *F. graminearum* was predominant at 20°C, both developed at 28°C and only *A. flavus* at 36°C.

Results concerning metabolites are currently under analysis. Growth and metabolites production at 12°C remains to be investigated.

DISCUSSION

In this work, the impacts of a_w, temperature and time (taken together or alone) on *A. flavus* and *F. graminearum* were monitored on a maize-based medium. The aim was to specifically study those impacts on early days of AFB and D.O.N. production as they would appear in prestorage.

It is known that *A. flavus* is a representative fungus for AFB production. To understand this production many *in vitro* studies have been done on synthetic medium (Garcia *et al.*, 2011; Abdel-Hadi *et al.*, 2012; Astoreca *et al.*, 2014). In this study, we optimized a maize-based medium for AFB production. Giorni *et al.,* (2008) studied AFB1 production by *A. flavus* on Potato Dextrose Agar. For instance, after 7 days (25°C, 0.95) the fungal growth was 4.1 cm and the AFB1 production was 470 ng.g^{-1}. Similar AFB1 results were observed at 28°C in the present study (380 ng.g^{-1}). Nonetheless, in our study we draw attention on the day 4 which revealed to be the optimal incubation time for AFB concentration.

We monitored early days of *F. graminearum* growth and D.O.N. production. Garcia *et al.,* (2012) also monitored *F. graminearum* growth and D.O.N. production on a soybean-based medium at different temperatures (15, 20, 25°C and 30°C). After 7 days, at 25°C, the Petri dishes were saturated by *F. graminearum* growth. It was the only incubation temperature leading to D.O.N. production with 0.11 µg.g^{-1} produced (Garcia *et al.*, 2012). In our study, no D.O.N. production occurred. This difference could be explained by our higher detection limit (0.5 µg.g^{-1}) and differences in medium composition.

Discussion around co-incubation will be added according to the future results.

Our study provided results on biotic and abiotic impacts on AFB and D.O.N. production. Recently, Garcia *et al.,* (2013) developed a model on maize-based medium and maize for AFB production by *A. flavus* (separately incubated). They showed that fungal growth and AFB production were delayed (1 to 2 days) on maize compared to the maize-based medium. Moreover, AFB (up to 120 ng.g^{-1}) was less produced in the maize (10 ng.g^{-1}). In future studies, we may get similar delay when transposed into maize experiments. Such results will help to provide data for risk management of aflatoxins and D.O.N. depending on environmental parameters.

ACKNOWLEDGEMENTS

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Figure Legends:

Figure 1: radial growth of *A. flavus* at day 2 (a.), day 3 (b.), day 4 (c.), day 5 (d.) day 6 (e.) and day 7 (f.) versus temperature and a_w.

Figure 2: radial growth of *F. graminearum* at a_w of 0.95 (a.) and 1 (b.) versus temperature and days of incubation.

Figure 3: radial growth of *A. flavus* and *F. graminearum* (after co-inoculation) at day 2 (a.), day 3 (b.), day 4 (c.), day 5 (d.) day 6 (e.) and day 7 (f.) versus temperature and a_w.

Table 1: Production of AFB1 and AFB2 by *A. flavus* on maize-based medium at different temperatures, a_w and incubation time.

Figure 43 - Figure 1 - radial growth of *A. flavus* at day 2 (a.), day 3 (b.), day 4 (c.), day 5 (d.) day 6 (e.) and day 7 (f.) versus temperature and a_w.
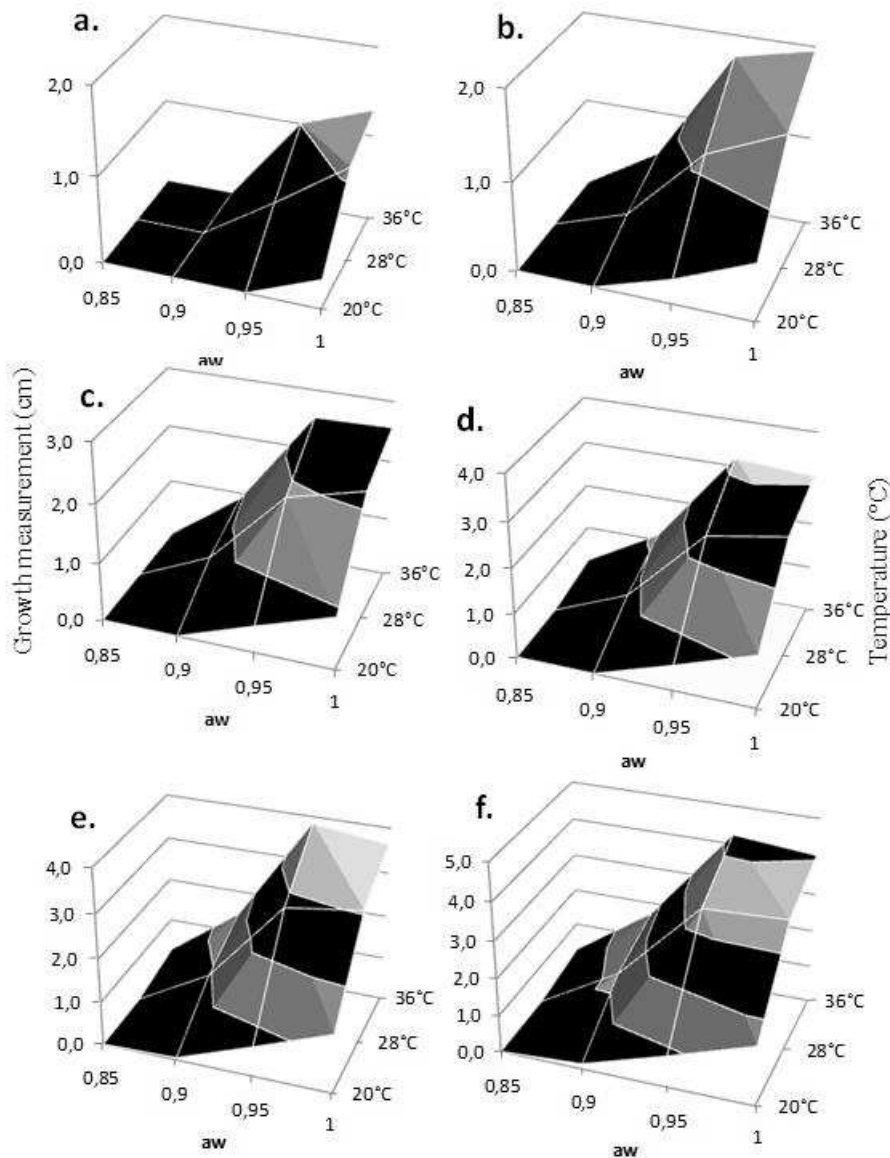
Figure 44 - Figure 2 - radial growth of *F. graminearum* at a_w of 0.95 (a.) and 1 (b.) versus temperature and days of incubation.
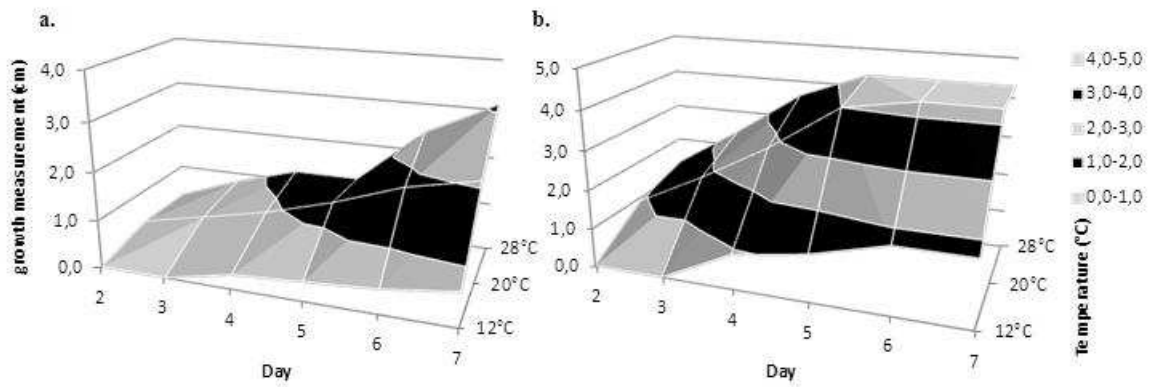
Figure 45 - Figure 3 - radial growth of *A. flavus* and *F. graminearum* (after co-inoculation) at day 2 (a.), day 3 (b.), day 4 (c.), day 5 (d.) day 6 (e.) and day 7 (f.) versus temperature and a_w
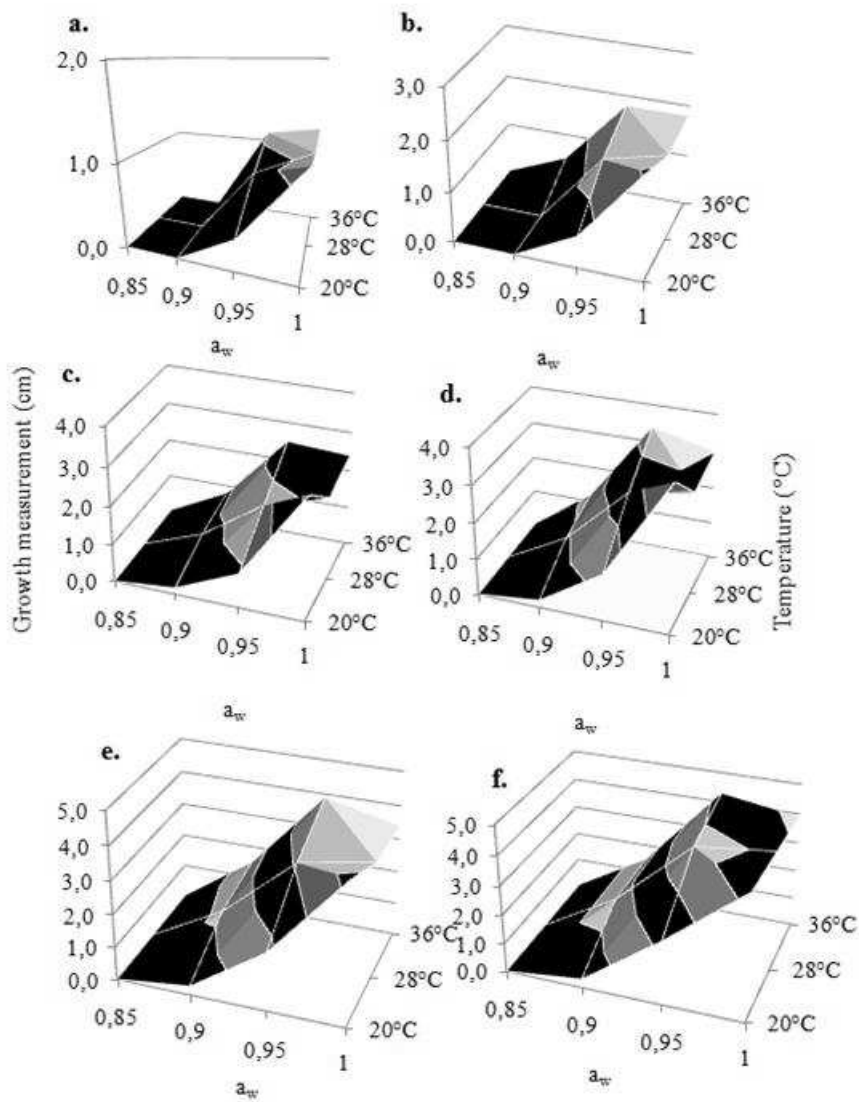
Table 13 -Table 1: Production of AFB1 and AFB2 by *A. flavus* on maize-based medium at different temperatures, a_w and incubation time

		a_w\day	2	3	4	5	6	7
AFB1	20°C	0.95	ND	ND	ND	ND	ND	0.4 ± 0.6
		1	ND	ND	ND	1.4 ± 1.6	13.6 ± 17.7	431.9 ± 59.4
	28°C	0.95	ND	1.1 ± 1.7	7.2 ± 4.0	59.7 ± 54.7	134.6 ± 51.9	380.7 ± 127.7
		1	ND	2286.1 ± 417.3	3070 ± 1068.9	2294.8 ± 583.9	1551.2 ± 195.0	1576.5 ± 397.8
	36°C	0.9	ND	ND	ND	ND	0.7 ± 0.6	11 ± 11.3
		0.95	ND	206.8 ± 93.9	503.4 ± 90.6	977.8 ± 281.8	971.3 ± 125.6	504.2 ± 133.3
		1	3.4 ± 3.2	7.2 ± 2.0	0.6 ± 0.0	2.3 ± 0.7	1.3 ± 0.8	8.7 ± 6.6
AFB2	20°C	0.95	ND	ND	ND	ND	ND	ND
		1	ND	ND	ND	ND	2.1 ± 1.9	14.6 ± 3.3
	28°C	0.95	ND	ND	ND	1.4 ± 1.3	2.9 ± 1.4	12.1 ± 8.0
		1	ND	29.8 ± 7.1	41.7 ± 17.2	32.8 ± 10.5	25.2 ± 5.7	32.5 ± 7.5
	36°C	0.9	ND	ND	ND	ND	ND	ND
		0.95	ND	1.9 ± 1.0	4.4 ± 0.8	7.7 ± 2.4	7.2 ± 6.3	4.7 ± 0.7
		1	ND	ND	0.1 ± 0.2	ND	ND	ND

ND: non detected. Values are represented in ppb $(ng.g^{-1})$

Conclusion

In this work, we wanted to evaluate, *in vitro* on a maize-based medium, the effect of temperature (12, 20, 28 and 36°C), a_w (0.75, 0.80, 0.85, 0.90, 0.95 and 1), early incubation time (day 1 to 7) and co-inoculation on fungal growth and toxin production by *A. flavus* (AFB producer) and *F. graminearum* (D.O.N. producer). We specially focused on early stages as they are the key to the understanding of prestorage risks.

Firstly, *A. flavus* growth was followed. 28°C and 36°C were the most permissive temperature and showed growth after 1 day. For the a_w of 1, 0.95 and 0.90, a reduction of a_w was correlated with a delay of 1 day in fungal growth. At 20°C, growth started at day 2 and a delay was observed with the decrease in a_w (a_w of 1 (day 2), a_w of 0.95 (day 3) and a_w of 0.90 (day 6)). No growth of *A. flavus* was observed at 12°C and at an a_w lower than 0.90 with an exception after 7 days at 36°C.

Secondly, we monitored AFB production by *A. flavus*. At 36°C, only AFB1 was produced after 2 days at an a_w of 1 (3.4 µg.kg^{-1}). Moreover, from day 3 to day 7, AFB1 was produced preferably at an a_w of 0.95 compared to 1. AFB1 production occurred at day 6 for an a_w of 0.90. At 28°C, AFB1 production started at day 3 and was produced preferably at an a_w of 1. At 20°C, AFB1 production started at day 5 for an a_w of 1 and day 7 for an a_w of 0.95.

Thirdly, *F. graminearum* growth and D.O.N. production was followed. Growth only occurred at a_w of 0.95 and 1. Fungal growth started at day 2 and saturated the Petri dishes after 5 days. No D.O.N. was detected after 7 days.

Further characterisation are needed to assess the impact of *A. flavus* co-inoculation on the AFB and D.O.N. risks.

3.2. Study of actinomycetes and Aspergillus flavus interaction

Introduction

In this project, our aim was to identify a bacterial biocontrol able to grow with *A. flavus* and reduce AFT accumulation in maize. Our objectives included different selection criteria:

(i) AFT concentration must be reduced by the biocontrol (ultimate aim *in planta*);

(ii) the maize ecosystem have to be modified as little as possible;

(iii) harmlessness of the biocontrol must be verified;

(iv) the biocontrol have to survive in maize or in maize soil.

As a beginning, we focused on the 2 first criteria. Based on those, we selected potential biocontrol. We decided to focus on actinomycetes.

Those bacteria are soil-borne and can be easily detected on maize (Costa *et al.*, 2013). They are already used as biocontrol agents. For example, in greenhouse maize, *Streptomyces* DAUFPE 11470 and 14632 were tested for their antagonism against *F. moniliforme* (= *F. verticilliodes*). *Streptomyces* showed up to 55% (11470) and 62.5% (14632) reduction of damping-off (Bressan & Figueiredo, 2008). This example, among others, reveals actinomycetes interesting criteria as biocontrols.

Moreover, actinomycetes are also producers of a wide range of secondary metabolites (Neuss *et al.*, 1970; Lamari *et al.*, 2002; Yoshinari *et al.*, 2007). The latter have already been applied in many fields: pharmaceutical, agronomics, etc. As so, choosing actinomycetes increase the chances to get interesting results. Thus, we took actinomycetes available in our own collection (jointly with ENS Kouba) as potential biocontrol agents. Those actinomycetes were chosen as they come from ecosystems where *A. flavus* is a regular contaminant (Algeria).

To choose an efficient biocontrol, it is important to characterise its mode of action. Two hypotheses are suggested: either the biocontrol prevent aflatoxin biosynthesis or/and it reduces pure-AFB1. Firstly, intermediate of aflatoxin biosynthesis may be accumulated. Secondly, side-products of degradation may also be accumulated. Both can impact the harmlessness of the interaction. Thus, in this publication we conducted *in vitro* experiments to segregate AFB-reducing isolates in interaction with *A. flavus*. Moreover, the best candidates were tested for pure-AFB1 reduction as a first characterisation.

ORIGINAL ARTICLE

In vitro interaction of actinomycetes isolates with *Aspergillus flavus*: impact on aflatoxins B1 and B2 production

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Significance and Impact of the Study: Interaction between *Aspergillus flavus* and Actinomycetes isolates was conducted *in vitro*. Actinomycetes isolates having a mutual antagonism in contact with *A. flavus* were chosen for further aflatoxins production study. This is a new approach based to develop biocontrol against aflatoxins accumulation in maize while respecting natural microbial equilibrium.

Abstract

This work aimed to study the interaction between Actinomycetal isolates and *Aspergillus flavus* to promote mutual antagonism in contact. Thirty-seven soilborn *Streptomyces* spp. isolates were chosen as potential candidates. After a 10-day *in vitro* co-incubation period, 27 isolates respond to the criteria, that is, mutual antagonism in contact. Further aflatoxins B1 and B2 analysis revealed that those 27 isolates reduced aflatoxin B1 residual concentration from 38·6 to 4·4%, depending on the isolate. We selected 12 isolates and tested their capacity to reduce AFB1 in pure culture to start identifying the mechanisms involved in its reduction. AFB1 was reduced by eight isolates. The remaining AFB1 concentration varied between 82·2 and 15·6%. These findings led us to suggest that these eight isolates could be used as biocontrol agents against AFB1 and B2 with low risk of impacting the natural microbial equilibrium.

Introduction

Aflatoxins B1 (AFB1) and B2 (AFB2) (AFBs) are secondary metabolites produced by filamentous fungi *Aspergillus flavus*. Aflatoxins are carcinogenic compounds (IARC 2012), and their presence in food is a major food-related health issue. Aflatoxins are commonly found in foodstuffs such as groundnuts, wine, maize and feed products such as wheat (Magan *et al.* 2011). Maize has the highest risk of aflatoxin contamination among cereals in the European Union (Piva *et al.* 2006), which has limited its presence to 4 μg kg^{-1} in maize foodstuff (European Union 2006).

Several studies have analysed aflatoxin contamination in maize. Each step of the cereal food chain can be affected by aflatoxin contamination. Contamination can be controlled at seed, field, production, storage or food processing levels (Abbas *et al.* 2009; Elsanhoty *et al.* 2013). At the field level, this control can be made by jointly observing weather settings [water activity (aw), temperature, etc.] and acting upon agricultural practices (irrigation, fertility, reinforced insects prevention, etc.) (Abbas *et al.* 2009).

In addition, biocontrol agents are able to reduce AFB1 accumulation. Two similar biocontrol agents are already commercialized against AFB1 accumulation: afla-guard® (Circle One Global, Inc., Shellman, GA) and afla-safe®(IITA, Ibandan, Nigeria). These nonaflatoxigenic *A. flavus* strains can prevent aflatoxins occurrence between 70·1 and 99·9% by competing and displacing aflatoxin producers (Atehnkeng *et al.* 2008). This displacing overtakes the maize fungal niche and prevents other mycotoxigenic fungi to colonize maize. This shows that fungus contamination is widely tolerated as long as there is no aflatoxin production (Atehnkeng *et al.* 2008). No other micro-organism is currently available to avoid aflatoxin accumulation in maize.

Other micro-organisms are being tested for their action on AFB1 accumulation or degradation/removal. In terms of preventing accumulation, Sultan and Magan (2011) and Zucchi *et al.* (2008) showed how actinomycetes genera—*Streptomyces* can inhibit AFB1 production *in vitro*. Other bacteria are also being tested as agents for AFB1 degradation or removal: *Nocardia corynebacteroides* (Tejada-Castañeda *et al.* 2008), *Enterococcus faecium* (Topcu *et al.* 2010), *Flavobacterium aurantiacum*, *Mycobacterium fluoranthenivorans* and *Corynebacterium rubrum* have been shown to efficiently detoxify AFB1 (Wu *et al.* 2009). Lactic acid bacteria have been shown to reduce AFB1 concentration by 45% thanks to cell wall surface binding (El-Nezami *et al.* 1998). Another described mechanism is the enzymatic degradation of *Rhodococcus erythropolis* (Alberts *et al.* 2006), *Peniophora*, *Pleurotus ostreatus* and *Trametes versicolor* (Alberts *et al.* 2009). However, there is no usable biocontrol which can both prevent accumulation in the field and remove AFB1.

In this context, our work was planned to screen actinomycetes for their ability to do mutual antagonism at contact with *A. flavus*. The corresponding actinomycetes isolates were investigated for AFBs production reduction. The best performing strains were put in an AFB1-supplemented media and further analysed.

Results and discussion

Actinomycetes were chosen as micro-organisms to be tested due to their tolerance to water stresses, their broad spectrum of metabolite production and survival in most soils and crops (de Araújo *et al.* 2000; Doroshenko *et al.* 2005).

Actinomycetes isolates selection

The 37 strains grew well on ISP-2 medium. They formed nonfragmented and colourless or yellowish brown substrate mycelium. The aerial mycelium was grey, yellowish grey or greyish yellow and produced numerous nonmobile and straight or spiral spore chains carried by sporophores. Diffusible pigments were not observed. This description corresponded to the genus *Streptomyces* (Holt *et al.* 1994).

Screening results

Thirty-seven actinomycete candidates were chosen for screening. After 10 days on ISP-2 medium, three patterns were observed (Table 1). Four of the 37 tested isolates showed an I_D (0/5) that represents an 'actinomycete dominance at distance' pattern and can decrease AFB residual concentration in the medium (rcm). Each of these four

Table 14 - Table 1 - Actinomycetes presenting the same I_D and the impact on AFBs concentration (Pictures in Annex 1)

I_D	Number of strains	AFBs concentration	Number of strains	AFBs rcm in % range
(0/5)	4	Reduction	4	4·4–9·7
		No reduction	0	
(4/0)	6	Reduction	4	8·1–46·1
		No reduction	2	
(2/2)	27	Reduction	27	4·4–38·6
		No reduction	0	

I_D = Applied to *A. flavus*/actinomycete strain (as defined in *Materials and methods*) reduction = AFBs concentration reduction compared with the control no reduction = AFBs concentration equivalent to control ($P < 0.05$).

isolates (S25, S26, S31 and S36) individually inhibits *A. flavus* growth at distance. S25 has the most impact on AFB1 and AFB2 rcm (4·4 and 5·3%).

Among the 37 isolates, six showed an I_D (4/0) that represents a 'dominance of *A. flavus* on actinomycete isolate' pattern (Table 1). For these isolates, macroscopic observations revealed that *A. flavus* completely covered the actinomycete inoculation streak. Regarding AFBs, we observed that S15 had no impact on both AFBs rcm. S7, S12, S24 and S37 presented lower AFBs rcm. The maximum decrease in AFBs content was observed in the presence of S12 or S37 isolates with a rcm of 8·1 and 9·1%, respectively, for AFB1 and of 9·6 and 10·2% for AFB2.

The 27 remaining isolates showed an I_D (2/2) that represents 'mutual antagonism on contact' and promotes both micro-organisms growth. They can reduce AFBs rcm from 38·6 to 4·4% rcm.

We were able to demonstrate different I_D patterns from Sultan and Magan (2011) (Table 1). Indeed, they demonstrated that among the six Egyptian *Streptomyces* tested, five had mutual intermingling with *A. flavus* (I_D 1/1) and one had dominance at a distance (I_D 5/0). However, this can be explained by the previous selection done in our study. Little data are available on *Streptomyces–Aspergillus* micro-organisms interaction, because many studies have focused on *Streptomyces* free cell extracts. Mohamed *et al.* (2013) tested 16 Egyptian rhizosphere *Streptomyces* for their potential antagonism. They revealed that 69% of free cell extracts were not able to reduce fungal growth.

Those studies are showing that a big part of the tested *Streptomyces* are not able to reduce fungal growth. Unlike our study, other researchers have chosen fungal growth inhibition as the first selection criteria for their potential biocontrol agents against mycotoxin production (Sultan and Magan 2011; Haggag and Abdall 2012). Our work focuses on promoting both micro-organisms growth: I_D (2/2).

I_D (2/2)—mutual antagonism on contact

Twenty-seven isolates showed an I_D (2/2) that represents 'mutual antagonism on contact'. The results are presented in Table 2. They are classified from the lowest to the highest impact of specific actinomycete isolates on AFB1 rcm.

We observed that when *A. flavus* is in contact with those actinomycetes streaks, fungal growth is slightly reduced (about 30%). All the 27 isolates reduced AFB rcm compared with the control. S10–S11 (Table 2) showed a slight decrease in AFB1 concentration, particularly with isolate S10 preserving 38·6% rcm. We focused on S3–S35 in Table 2, which have the highest AFB1 rcm reduction.

Co-culture of each isolates from S3 to S17 (as shown in Table 2) had a AFB1 rcm above 10%. In addition, S38–S35 led to an efficient decrease of AFB1 rcm (<10%). In this last group, a co-culture of S38 showed the lowest level of AFB1 reduction. S35 was the most efficient in reducing the amount of AFB1 (rcm of 4·4%).

Focusing on AFB2, we observed that rcm varied between 27·1 and 10·5% for S3–S35 (Table 2). Besides, isolates S23 and S22 were less efficient in AFB2 than AFB1 reduction. If only AFB2 reduction is considered, S35 is again the most efficient candidate (rcm of 5·5%).

Overall, isolates with an AFB1 and AFB2 rcm of <17% (after S11 in Table 2 except S23 and S22) could be interesting candidates for further studies. Considering the decrease of both aflatoxin levels, we concluded that S1, S6, S27 and S35 were effective in co-culture with *A. flavus*. This aflatoxin reduction may be linked to the actinomycete metabolites.

Ono *et al.* (1997) identified a molecule produced by *Streptomyces* sp. MRI142 called aflastatin A which is able to completely inhibit AFB1 production at 0·5 μg ml^{-1} without affecting fungal growth. Thus, our results could be linked with a possible production of aflastatin A by our candidate isolates. Another *Streptomyces* molecule called Dioctatin A was identified by Yoshinari *et al.* (2007). This molecule inhibits *Aspergillus parasiticus* conidiogenesis and AFB1 production. These results are different from our results showing no conidiogenesis macroscopic impact for the 27 isolates tested. Thus, it is unlikely that our results are linked with actinomycetes Dioctatin A.

Focusing on biological control approaches already applied in the field, Nigerian nontoxigenic strains of *A. flavus* were able to reduce to 0·02% AFB1 content *in vitro* on maize kernel (Atehnkeng *et al.* 2008). These results were the first step towards Afla-safe® commercialization. The main criterion in the atoxigenic strains selection was the capacity to outmatch toxigenic strains. Comparatively, we have promoted the isolates that can grow in contact with *A. flavus*. Thus, the actinomycete growth in *A. flavus* presence was monitored.

Actinomycetes growth in *A. flavus* presence

For the 27 isolates in mutual antagonism on contact with *A. flavus*, actinomycete growth was measured to select isolates able to develop when on contact with *A. flavus*. We observed that some of the isolates grew more efficiently than others in *A. flavus* presence. For instance, S10, S30 and S34 had the smallest spread with seven mm. Twenty-one isolates were able to spread between 0·9 and

Table 15 - Table 2 - Effect of different actinomycetes isolates on fungal growth and aflatoxin B1 and aflatoxin B2 concentration. Only the 27 which has shown mutual antagonism on contact with *Aspergillus flavus* (I_D(2/2)) are represented.

Strain	Fungal growth (%)	AFB1 (rcm in%)	AFB2 (rcm in%)	Strain	Fungal growth (%)	AFB1 (rcm in%)	AFB2 (rcm in%)
Control	100·4 ± 1·7[a]	100·5 ± 5·5[a]	100·9 ± 9·4[a]	S8	69·6 ± 4·0[b]	15·1 ± 1·3[c]	16·0 ± 3·7[c]
S10	72·1 ± 4·2[b]	38·6 ± 15·4[b]	33·2 ± 18·1[b,c,d]	S16	67·8 ± 1·4[b]	14·8 ± 2·7[b,c,d]	16·6 ± 2·6[b,c,d]
S18	79·9 ± 22·1[b]	25·8 ± 6·6[b]	20·6 ± 1·4[b]	S4	70·0 ± 4·1[b]	14·6 ± 2·5[b,c,d]	15·8 ± 4·3[c]
S5	87·4 ± 14·8[b]	23·7 ± 8·5[b]	29·7 ± 4·7[b]	S34	71·1 ± 3·1[b]	12·8 ± 2·1[c,d]	15·2 ± 4·7[b,c,d,e]
S20	64·2 ± 1·7[b]	22·5 ± 5·7[b]	22·4 ± 6·4[b,c]	S33	77·0 ± 11·1[b]	11·9 ± 9·8[b,c,d,e]	13·9 ± 10·6[b,c,d,e]
S29	70·7 ± 2·7[b]	20·4 ± 4·0[b,c]	21·1 ± 16·1[b,c,d]	S2	68·7 ± 2·0[b]	11·2 ± 1·9[d]	10·5 ± 3·6[d,e]
S30	65·1 ± 1·3[b]	20·0 ± 8·2[b,c,d]	16·1 ± 6·8[b,c,d,e]	S17	67·7 ± 1·4[b]	10·6 ± 1·4[d]	10·8 ± 2·0[b,c,d,e]
S19	67·4 ± 1·3[b]	20·8 ± 6·8[b,c]	25·8 ± 6·4[b,c,d]	S38	74·7 ± 13·1[b]	8·8 ± 3·8[c,d,e]	13·0 ± 3·5[b,c,d,e]
S14	66·4 ± 0·8[b]	18·2 ± 5·5[b,c,d]	16·7 ± 3·6[b,c,d]	S13	87·1 ± 12·1[b]	7·9 ± 2·2[d,e]	8·3 ± 3·0[b,c,d,e]
S11	68·3 ± 2·8[b]	18·0 ± 1·2[b]	18·6 ± 1·9[b,c]	S27	71·5 ± 11·6[b]	7·0 ± 1·4[e]	8·1 ± 2·0[d,e]
S3	71·4 ± 3·6[b]	16·9 ± 3·0[b,c,d]	17·0 ± 0·9[c]	S28	66·8 ± 0·6[b]	6·9 ± 1·9[d,e]	10·7 ± 2·1[b,c,d,e]
S22	65·9 ± 1·6[b]	16·1 ± 2·0[b,c]	27·1 ± 3·5[b]	S1	72·6 ± 8·1[b]	6·2 ± 0·8[e]	8·7 ± 1·6[d,e]
S21	69·8 ± 2·5[b]	15·8 ± 3·5[b,c,d]	14·2 ± 2·6[b,c,d]	S6	67·9 ± 5·1[b]	5·9 ± 2·1[e]	7·3 ± 2·1[e]
S23	70·9 ± 2·1[b]	15·8 ± 2·3[b,c]	20·4 ± 2·5[b,c]	S35	69·1 ± 3·3[b]	4·4 ± 1·0[e]	5·5 ± 1·3[e]

Data with the same letter are not significantly different ($P < 0.05$).

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1·7 cm. S18 and S6 show the most significant spreading with 1·7 and 1·8 cm, respectively.

Effects of selected actinomycetes isolates on pure AFB1

Thirty-seven isolates were screened for their ability to reduce AFB rcm without having an impact on fungal growth. We revealed that 27 isolates were corresponding to those criterions. Among these, 12 of the most efficient reducers were chosen for further characterization. We decided to test whether they reduce AFB1 concentration in pure culture to start identifying the mechanisms involved in its reduction.

The 12 selected actinomycetes isolates were inoculated in the presence of AFB1 in solid media at a concentration of 5 mg kg^{-1}. Results are shown in Table 3.

Among the 12 chosen actinomycetes, seven showed no macroscopic difference when AFB1 was present in the medium (represented by the minus symbol in Table 3). The remaining five isolates showed phenotypic differences. We observed a lack of white pigmentation for S35 and S38 (linked to sporulation) in the presence of AFB1. S6 and S27 showed a reduction in streak width. S27 had a 1 mm streak width instead of 4 mm in the control, and S34 did not grow in the presence of AFB1.

The AFB1 level in the media was analysed for each of these 12 isolates. Results are shown in Table 3. S13, S17 and S34 had no impact on the AFB1 rcm. S8, S21, S27 and S33 slightly reduced the initial AFB1 concentration (rcm between 82·2 and 69·8%), even if S35 and S38 showed a more significant reduction in the AFB1 concentration (rcm of 29·4 and 38·0%, respectively). Finally, S3, S4 and S6 were extremely efficient in the reduction of AFB1 concentration (rcm: 22·2, 27·3 and 15·6%, respectively). Joining co-culture and pure AFB1 test results, we

can see that S3 reduced AFB1 rcm (16·9%), and this is due to the isolate degradation or adsorption properties. In contrast, S17 reduced AFB1 rcm (10·6%) in co-culture but had no impact in contact with pure AFB1. This could be linked to other mechanisms like aflatoxin biosynthesis inhibition.

In summary, S3, S4, S6 and S35 showed a rcm above 30% and are potential candidates for the reduction of AFB1 concentration. Among these, only S6 and S35 showed a differential phenotype in the presence of AFB1.

For the 12 selected isolates, HPLC chromatograms were investigated. Only three (S3, S4 and S6) revealed a peak emergence in their chromatogram profile. We presumed that this peak is due to aflatoxin degradation or actinomycete metabolites production in response to AFB1 presence. This could be linked to the presence of partially hydrophobic lower molecular weight molecules (shorter retention time) as a result of AFB1 degradation. Taylor et al. (2010) demonstrated that the $F_{420}H_2$ reductase is able to reduce AFB1 α,β-unsaturated ester moiety, which resulted in several low molecules appearance. This reductase commonly found in Actinomycetales genus has not yet been characterized in Streptomyces genus (Purwantini et al. 1997).

Many studies have attempted to control the toxicity of AFB1 disruption by-products. Megalla and Hafez (1982) demonstrated that AFB1 can be converted to less toxic derivates such as aflatoxin B_{2a}. Recently, Samuel et al. (2014) showed that Pseudomonas putida can biotransform AFB1 to less toxic compounds, aflatoxins D. Other studies reported that AFB1 can be degraded by Pseudomonas spp. and other soilborn bacteria. The degradation results revealed a toxicity reduction compared with control sample (Elaasser and El Kassas 2011; Krifaton et al. 2011). These results imply the prospective that our isolates could detoxify the medium. However, they must be investigated for biosafety tests.

Our study has demonstrated that actinomycetes can reduce in vitro A. flavus aflatoxins accumulation without impacting fungal growth. We have also shown that actinomycetes can reduce AFB1 concentration in solid media. Our study is the first step in developing actinomycetes as biocontrol agents against AFB1 on maize grain. Further mechanistic approaches can be done focusing on the impacts of our potential biocontrol candidates on aflatoxins G1 and G2 producer A. parasiticus.

Materials and methods

Fungal strain and actinomycete isolates

The fungal strain used was A. flavus NRRL 62477. Actinomycetes strains were collected from soils of different

Table 16 - Table 3 - Impact of actinomycetes on aflatoxin B1 concentration in the media

Strain	AFB1 impact on actinomycetal growth	AFB1 (rcm in%)
Control	/	$100\cdot0 \pm 2\cdot2^a$
S13	−	$104\cdot9 \pm 17\cdot0^a$
S17	−	$96\cdot6 \pm 17\cdot1^{a,b}$
S34	+	$92\cdot7 \pm 8\cdot5^{a,b}$
S21	−	$82\cdot2 \pm 6\cdot0^b$
S8	−	$78\cdot1 \pm 15\cdot2^b$
S27	+	$76\cdot6 \pm 13\cdot7^b$
S33	−	$69\cdot8 \pm 11\cdot5^b$
S38	+	$38\cdot0 \pm 11\cdot1^c$
S35	+	$29\cdot4 \pm 15\cdot2^c$
S4	−	$27\cdot3 \pm 2\cdot2^d$
S3	−	$22\cdot2 \pm 5\cdot4^d$
S6	+	$15\cdot6 \pm 11\cdot7^d$

Data with the same letter are not significantly different ($P < 0.05$).

locations in Algeria (Adrar, Biskra, Ghardaïa, Hassi R'Mel and Laghouat) by a dilution agar plating method using chitin–vitamin agar medium (Hayakawa and Nonomura 1987) supplemented with cycloheximide (80 mg l^{-1}) and nalidixic acid (15 mg l^{-1}) to suppress the growth of fungi and Gram-negative bacteria, respectively. A first antagonism test was realized against *A. flavus*, and 37 strains showing the less antagonistic characteristics were selected for screening and numbered from 1 to 38. The cultural characteristics of actinomycete strains were observed by naked-eye examination of 14-day-old cultures grown on yeast extract/malt extract agar (ISP-2) medium (Shirling and Gottlieb 1966). Spores and mycelium were examined by light microscopy (Motic; B1 Series). They were conserved at $-20°C$ in cryotubes in a 20% glycerol solution.

Culture media

Precultures of *A. flavus* were inoculated on YEPD medium containing 5 g l^{-1} yeast extract, 10 g l^{-1} casein peptone, 10 g l^{-1} α-D-glucose and 15 g l^{-1} agar. Actinomycete isolates precultures were inoculated on ISP-2 medium (Shirling and Gottlieb 1966) at pH 7. The

micro-organisms were preincubated separately on ISP-2 (actinomycetes) and YEPD (*A. flavus*, when needed), at 28°C for 7 days.

Co-culture screening method

The co-culture screening method is based on the method proposed by Sultan and Magan (2011). *A. flavus* spores were dislodged from the preculture with a sterile loop and placed in 10 ml sterile water +0·05% Tween-20. In a Petri dish filled with ISP-2 medium, actinomycetes and *A. flavus* were inoculated on the same day as described in Fig. 1. The spore suspension from *A. flavus* was spotted, and the actinomycetes were inoculated with a streak. The incubation lasted 10 days at 28°C, and growth measurements were carried out at the end of the incubation period. The experiment was realized twice in triplicate. The interaction between the two micro-organisms was observed macroscopically and scored based on the Index of Dominance (I_D) (Magan and Lacey 1984). The I_D is determined by addition of individual scores based on: mutual intermingling (1/1), mutual antagonism on contact (2/2), mutual antagonism at a distance (3/3), dominance of one species on contact (4/0) or dominance
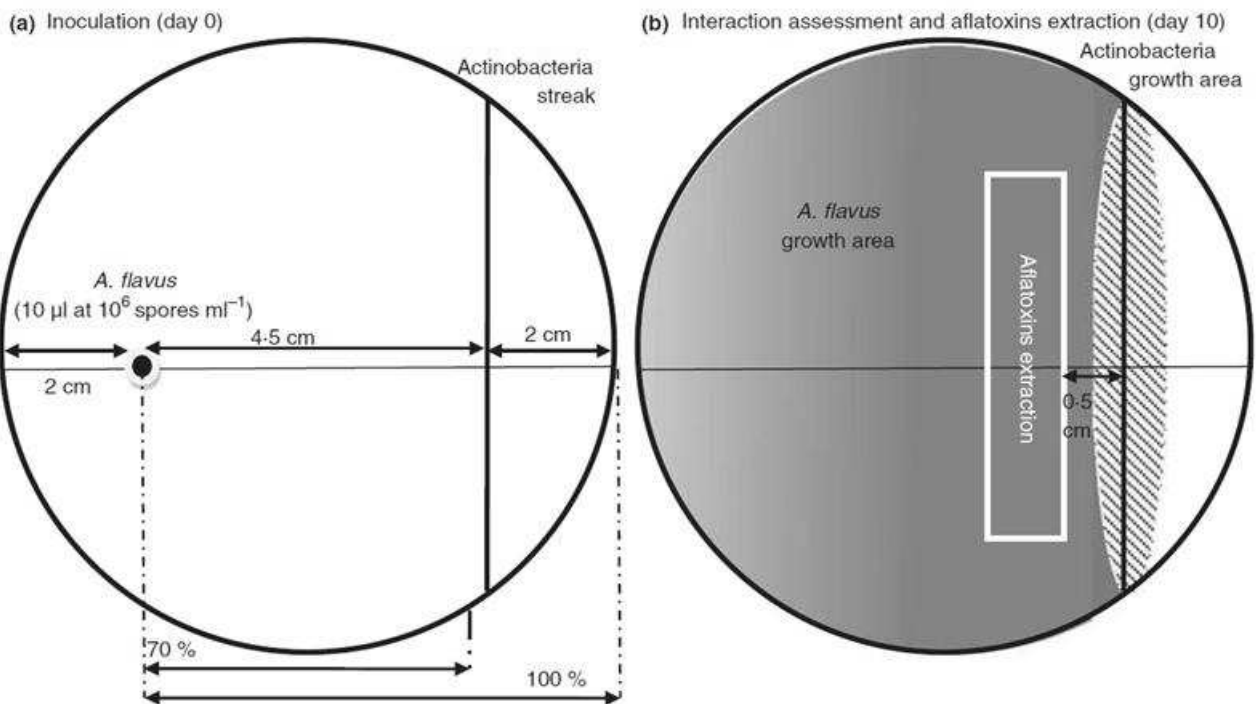
Figure 46 - Figure 1 - a. Inoculation (day 0) in a Petri dish filled with ISP2 medium, actinomycetes and *A. flavus* are inoculated on the same time. Inoculation is done with the following instructions: 10 μl of spores suspension from *A. flavus* are spotted at 2 cm from the Petri dish periphery. Actinomycete streak is inoculated perpendicularly to *A. flavus* -actinomycete axe at 4.5 cm of the *A. flavus* spot.

b. Interaction assessment and aflatoxins extractions, in case of I_D (2/2). The growth measurements are done and is represented in grey for *A. flavus* and in stripes for the isolate. The aflatoxin extraction area is delimited by a white box.

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at a distance (5/0) occurred for each *A. flavus*/actinomycetes interaction.

Solid media AFB1 reduction test

A 1 mg ml^{-1} AFB1 solution was prepared in methanol solution. This solution was added to ISP2 medium after autoclaving to obtain a final concentration of 5 mg kg^{-1}. Actinomycetes were inoculated with a loop to cover completely the Petri dish surface. After a 4 days long incubation period at 28°C, AFB1 was extracted as described below. The actinomycete growth was observed macroscopically in the control media (without AFB1) and in the AFB1-supplemented media. The experiment was realized twice in triplicate.

Aflatoxins extraction

Three agar plugs (Ø 9 mm) were taken both 5 mm away from actinomycete streak for the co-culture screening method and randomly on actinomycete growth area for the solid media AFB1 reduction test.

The total weight was measured. One millilitre of methanol was added to the plugs and shaken for 5 s three times. After 30-min incubation at room temperature, solutions were centrifuged 15 min at 12 470 **g**. The supernatant was taken and filtered through 0·45-μm PVDF Whatman filter into vials and stored at −20°C until analysis. The recovery ratio was 50%.

AFB1 and AFB2 detection and quantification by HPLC

The HPLC system used for aflatoxins analysis was an Ultimate 3000 system (Dionex–Thermo Fisher Scientific, Courtaboeuf, France) with all the RS series modules. A C18 column and its associated precolumn (Phenomenex, Luna 3 μm, 200 × 4·6 mm) was used. The mobile phase and AFB1 derivatization were realized according to the Coring Cell$^®$ instruction (Coring System Diagnostix Gmbh, de). Analyses were realized at a flow rate of 0·8 ml min^{-1} during a 35-min run. The quantification was realized by the Chromeleon software, thanks to standards of AFB1 and AFB2 (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The limit of quantification is 0·5 ppb for each.

Statistical analysis

The statistical analyses were performed with R (2.15.2; Lucent Technologies, Auckland, Australia) for nonparametric events, the package 'nparcomp' was used and the contrast method was Tukey with a confidence level of 95% and a logit transformation.

Conflict of interest

No conflict of Interest declared.

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The 12 best candidates studied for pure-AFB1 reduction had additionally their 16S rRNA gene sequence analysed for identification.

Usually, actinomycetes are identified by a multitude tests:

(i)   Genus identification: isolation on a chitine medium (supplemented with fungicides), morphology (e.g.: mycelium observation), and chemical characterisation (e.g.: amino acid, sugar, lipides), 16S rRNA gene sequencing (e.g.: a pairwise similarity under 95% leads to different genus);

(ii)   Species identification: physiology (e.g.: degrade or tolerate chemical), 16S rRNA gene sequencing (e.g.: for *Streptomyces* pairwise similarity under 97% leads to new species) and DNA-DNA hybridisation (e.g.: for *Streptomyces* an homology under 70% leads to a new species) (Meklat, 2012).

Among these, the 16S rRNA gene sequencing provides two different key information: the species identification and phylogenetic tree design.

In our study, only *Saccharothrix algeriensis* NRRL B-24137 (S34) has already been characterised and identified (Zitouni *et al.*, 2004). The *Streptomyces* genus of the other isolates was previously identified thanks to morphological observations (ENS Kouba). 16S rRNA gene sequencing was done for these strains (except S08).

The first results concern the species identification. It is based on sequence blasting between the new sequence and those available in databases (e.g.: Extaxon). Those results (Table 17) exhibited more than 95% pairwise similarities with *Streptomyces* strains. It confirms the belonging of those isolates into the *Streptomyces* genus.

Among these, the lowest similarity level (95.1%) was for S27 with *S. neopeptinius* KNF 2047. The 16S rRNA gene sequence similarities between S27 and other remaining *Streptomyces* were also below 97%. It is known that a pairwise similarity under 97% with the other *Streptomyces* strains can lead to potential new species. In our results, S27 could possibly be a new species. A complete characterisation of S27 should be subjected to identify a potential new *Streptomyces* species.

The isolates S03 and S04 were highly similar to *S. zaomyceticus* (99.1 and 99.0 %, respectively). Each remaining strain was similar to a distinct *Streptomyces* species: S06 to *S. roseolus* (99.0%), S13 to *S. calvus* (99.2%), S17 to *S. thinghirensis* (99.1%), S21 to *S.*

griseorubens (99.0%). S33, S35 and S38 had the highest similarity rates with: S33 to *S. rochei* (99.6%), S35 to *S. pratensis* (99.9%) and S38 *S. caeruleatus* (99.6%).

All those strains showed 99.0 to 99.9% pairwise similarities with *Streptomyces* strains. Many studies discovered new species, even though high pairwise similarity were detected (Santhanam *et al.*, 2012; Mohammadipanah *et al.*, 2014; Sakiyama *et al.*, 2014). This suggests that further DNA-DNA hybridisation should be done with the strain having the closest sequence to determine the 70% threshold.

Table 17 - Isolates, corresponding strains and their associated pairwise similarity in percent.

| Isolates | 16S Sequencing | | Isolates | 16S Sequencing | |
	Strain	Pairwise Similarity (%)		Strain	Pairwise Similarity (%)
S03	*Streptomyces zaomyceticus*	99.1	S21	*Streptomyces griseorubens*	99.0
S04	*Streptomyces zaomyceticus*	99.0	S27	*Streptomyces neopeptinius*	95.1
S06	*Streptomyces roseolus*	99.5	S33	*Streptomyces rochei*	99.6
S08	no data available		S34	*Saccharotrix algeriensis NRRL B-24137*	
S13	*Streptomyces calvus*	99.2	S35	*Streptomyces pratensis*	99.9
S17	*Streptomyces thinghirensis*	99.1	S38	*Streptomyces caeruleatus*	99.6

Another output of 16S sequencing is the design of phylogenetic trees. This latter is calculated thanks to phylogenetic algorithm and validated by statistical analysis (Meklat, 2012).

This produced tree (Figure 47) showed the close phylogenetic association of strain S03 and S04 with *S. zaomyceticus* and *S. omiyaensis*. S13 and S33 were also closely associated. Similar patterns were observed with S35 and S06, and, S21 and S17. S38 and S27 had the biggest difference of phylogenic association with the other strains. S38 and S27 were the most diverse in base substitution per site. S27 formed an independent phyletic line of its own, and the topology was supported by a high bootstrap value (82%).

Currently, phylogenetic trees are based on 16S rRNA gene sequences. It was made to reflect the evolution pattern between the different species separation. Generally, trees based 16S rRNA sequence find similar results with phenotypic and chemical taxonomy (Meklat *et al.*, 2011, 2013).

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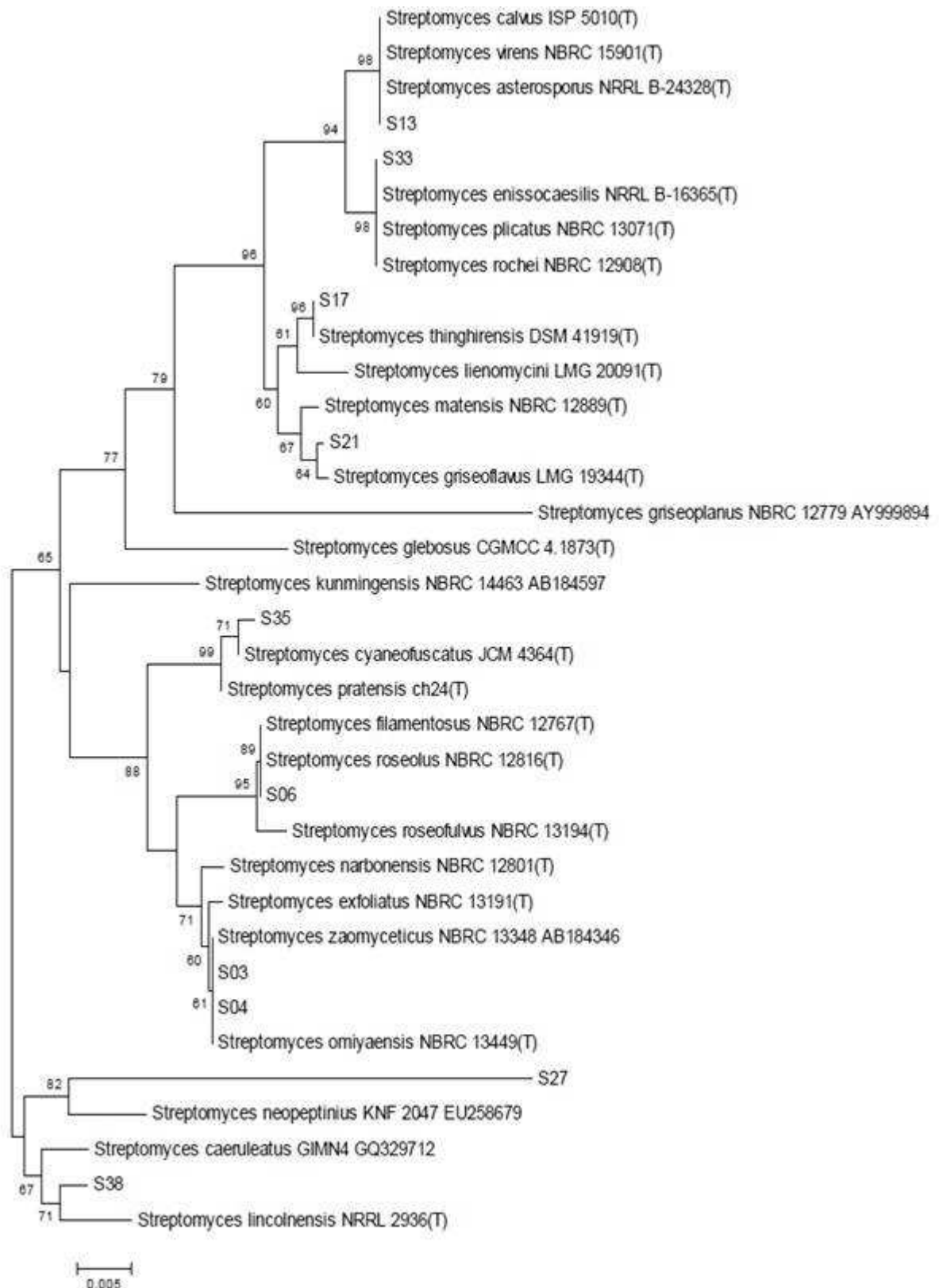
Figure 47 - Phylogenetic tree between our isolates and selected species of *Streptomyces* genus. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 0.22168834 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (only values greater than 50% are given) (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 35 nucleotide sequences. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

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Conclusion

In this chapter, we studied the interaction between actinomycetes isolates and *A. flavus*. Our aim was to study those isolates capacity to reduce AFB production without impacting fungal growth. Among the 37 actinomycetes chosen, after a 10 day co-incubation *in vitro*, only 27 isolates showed mutual antagonism in contact. We evaluated the impact of these isolates on AFB accumulation by *A. flavus*. They reduced AFB1 residual concentration from 38.6% to 4.4%, depending on the isolate.

Among them, 12 were tested for their ability to reduce pure-AFB1 content. After 4 days at 28°C on ISP-2 medium, AFB1 (5 mg.kg^{-1}) was reduced by 8 isolates. The remaining AFB1 concentration varied between 82.2 and 15.6%. 4 isolates reduced AFB1 content under 30% of the control.

These 12 isolates also had their 16S rRNA gene sequence analysed for identification. 1 strain was *Saccharothrix algeriensis* B-24137 and another was not analysed. For the 10 remaining strains, their belonging to *Streptomyces* genus was confirmed. The S27 is potentially a new species whereas for the other strains further DNA-DNA homology tests need to be made.

3.3. Characterisation of the mechanisms involved by RT-qPCR while A. flavus and A. parasiticus interaction and AFB$_1$-adsorption tests

Introduction

A. flavus is currently the main AFB1 producer in many commodities. Thus, it is regularly chosen as a representative of AFB producers (Abbas *et al.*, 2009; Abdel-Hadi *et al.*, 2012; Battilani *et al.*, 2013). Besides, *A. parasiticus* is a representative fungus for AFT production. Thus, we decided also to study the impact of *Streptomyces* on the AFT production by *A. parasiticus*.

We try to elucidate the molecular mechanisms involved in the reduction of AFB1 concentration during *Aspergillus* sp.-*Streptomyces* isolates interaction. To achieve this, we selected 6 *Streptomyces* strains as good candidates. Those 6 strains, showed mutual antagonism in contact with *A. flavus* and reduced AFB concentration under 17%. Moreover, 2 did not reduced (S17, S13), 3 moderately reduced (S27, S35, S38) and 1 highly reduced (S06) pure-AFB1 concentration.

Among the possible mechanisms, prevention of AFT production (repression on aflatoxin genes expression) and/or reduction of AFT content (adsorption or degradation) could be involved.

To study the repression of gene expression, different techniques can be used: microarray, northern blot, Reverse Transcription quantitative PCR (RT-qPCR). RT-qPCR is a powerful technique to obtain quantitative gene expression data. Nevertheless, this sensitive technique can easily give non representative results. Thus, it is critical to ensure adequate RT-qPCR protocol and reference gene normalisation (Bustin *et al.*, 2009).

In the publication hereafter we conduct *in vitro* experiments to study gene expression of aflatoxin genes.

Effect of *Streptomyces* interaction on aflatoxin production by *Aspergillus flavus* and *A. parasiticus*

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SUMMARY

Aspergillus flavus and *A. parasiticus* are producers of aflatoxins, a carcinogenic compound. *Streptomyces* sp. may interact with *Aspergillus* and lead to the *in vitro* reduction of aflatoxin concentration. To shed light on the mechanisms involved, we studied the microbial interaction between *Aspergillus* and six selected *Streptomyces* strains and looked into the quantification of aflatoxins and the expression of 5 genes *aflD*, *aflM*, *aflP*, *aflR* and *aflS* thanks to RT-qPCR.

When *Streptomyces* strains were separately put in contact with *A. flavus* and *A. parasiticus*, the level of aflatoxins production decreased. In terms of gene expression, it was either lower or higher depending on the *Streptomyces* strains and the gene studied. Therefore, we demonstrate that all the six tested strains reduce aflatoxin production and have various impacts on gene expression.

Keywords: *Aspergillus, Streptomyces*, RT-qPCR, gene expression, aflatoxin

INTRODUCTION

Aflatoxins are carcinogenic polyketide-derived furanocoumarins (IARC, 2014) which may contaminate agricultural foodstuffs. *Aspergillus flavus* and *A. parasiticus* are the main producers of aflatoxins. They are found in different crops such as maize, hazelnut, peanut, etc. (Giorni *et al.*, 2007; Passone *et al.*, 2010). To reduce risks associated with aflatoxin ingestion, particularly aflatoxin B1 (AFB1), multiple nations have set maximum authorised levels of aflatoxins in food and feed (Wu & Guclu, 2012).

The 29 aflatoxin pathway genes are regrouped in a 80-kb long cluster, characterized in both *A. flavus* and *A. parasiticus* species (Yu, 2012). In terms of structural genes, early (*aflD*), medium (*aflM*) and late (*aflP*) genes are denominated. *AflD* encodes the reductase enzyme is involved in the conversion of the norsolorinic acid (NOR) into averantin (Papa, 1982). AflM is required for the conversion of versicolorin A (VERA) into demethylsterigmatocystin (DMST) (Skory *et al.*, 1992). *AflP* encodes the methyltransferase which converts Sterigmatocystin (ST) into *O*-methylsterigmatocystin (Bhatnagar *et al.*, 1988). Two regulatory genes are also present, *AflR* encodes a transcription activator which binds a consensus sequence in the promoter regions of aflatoxin genes (Payne *et al.*, 1993) and AflS is a potential co-activator of AflR (Meyers *et al.*, 1998). Schmidt Heydt et al. (2009) showed that the *aflR*:*aflS* ratio can be an indicator of aflatoxin accumulation. This ratio, above 1, promotes aflatoxin accumulation. In addition to *aflR* and *aflS*, the clustered biosynthetic genes are also regulated by aspecific transcriptional regulators.

Yeast, bacteria and fungi can impact the production of aflatoxins (Yin *et al.*, 2008). In *Streptomyces* - *A. flavus* interactions, a significant reduction of *in vitro* AFB1 and aflatoxin B2 (AFB2) (AF) medium concentration (mc) was observed. For instance, *in vitro* interaction between *A. flavus* NRRL 62477 and *Streptomyces* revealed an antagonism upon contact leading to a AF mc at less than 17% of the control (Verheecke *et al.*, 2014). Schroeckh et al. (2009) also revealed that some Actinomycetes strains can specifically induce secondary metabolism of *A. nidulans.*

Previously we have demonstrated that, *Streptomyces* strains inhibit *in vitro* AF accumulation by *A. flavus* (Verheecke *et al.*, 2014). In order to investigate the mechanisms involved, we first studied those six strains in interaction with *A. parasiticus*. As fungal growth wasn't correlated with AF(AFB1, AFB2 and AFG1) reduction, we secondly adopted a RT-qPCR approach to monitor the impacts of *Streptomyces* interactions with *A. flavus* and *A. parasiticus* on AF gene expression.

METHODS

Fungal and *Streptomyces* strains. The fungal strains used were *A. flavus* NRRL 62477 and *A. parasiticus* Afc5. Six actinomycete strains were selected and their 16S rRNA gene was sequenced by the method previously described (Zitouni *et al.*, 2005). The six strains (*Streptomyces roseolus* S06, *S. calvus* S13, *S. thinghirensis* S17, *Streptomyces spp.* S27, *S. griseoplanus* S35, *S. caeruleatus* S38) were kept at -20°C in cryotubes in a 20% glycerol solution.

Interaction method and AF quantification. Pre-cultures and cultures were prepared as previously described (Verheecke *et al.*, 2014) with slight modifications: a sterile 8.5 cm cellophane sheet (Hutchinson, France) was added on ISP-2 prior to inoculum and two streaks (instead of one) of *Streptomyces* culture were inoculated (in parallel) 2 cm away from *A. parasiticus* inoculation. Two sets of plates were inoculated for optimum data analysis, one set for RNA extraction after 90 h and the other after 7 days to determine fungal growth and AF concentration. As for the first set, after 90 hours, the fungal biomass was separated from the bacterial one. With a scalpel and unaided, the cellophane close to the mycelium growth was cut. This enabled to avoid bacterial biomass. The fungal biomass was then removed from the cellophane surface for RNA extraction. As for the second set, after 7 days, the fungal biomass was removed from the cellophane sheet for measurement of dry weight (18 hours at 80°C prior to weight measurement). In the remaining media, three agar plugs (Ø 9 mm) were removed from the fungal growth area for AF extraction (Verheecke *et al.*, 2014). All the experiments were done twice. The same protocol was applied with *A. flavus* in a separate experiment.

RNA extraction and quantification. 60 mg of mycelium were crushed in liquid nitrogen into a fine powder. The powder was then stored at -80°C until RNA isolation. Total RNA was isolated using the Aurum Total RNA Kit (BioRad). The manufacturer instructions for eukaryotic and plant cell materials were followed, except for two modifications: DNase I digestion was extended to one hour and elution was done at 70°C for 2 min in the elution buffer. Total RNA was eluted into 80 µl and stored at -20°C for a short period of time. 1 µl of Total RNA of each sample was loaded into a RNA StSens chip (Bio-Rad) and quantified on nanodrop 2000 (Thermo scientific) according to the manufacturer instructions. Samples with RQI> 7, $A_{260/280}$>2 and $A_{260/230}$>1.3 were selected for further analysis.

RT-qPCR. RT was carried out with the Advantage RT-PCR Kit (Clontech) with Oligo (dT)$_{18}$ primer according to the manufacturer instructions (1 µg total RNA), except for

one modification: incubation at 42°C was extended to four hours. RT-qPCR was performed in a CFX96 Touch instrument (Bio-Rad) using SsoAdvanced[TM] SYBR Green Supermix (Bio-Rad). Primer pairs and associated efficiencies were validated (85-115%) (Table S1) and statistics were made with the qbase+ software.

       Reference genes validation. Seven candidate genes (*act1*, *βtub*, *cox5*, *ef1*, *gpdA*, *rpl13* and *tbp*) were tested as potentially suitable reference genes (Bohle *et al.*, 2007; Radonić *et al.*, 2004). The measures of gene stability V and M were calculated with geNorm software (Vandesompele *et al.*, 2002). This led to the choice of *act1* and *βtub* as optimal reference genes (Figure S1).

RESULTS

Interaction of Streptomyces with *A. parasiticus* and *A. flavus*

After seven days, all the tested *Streptomyces* strains showed a mutual antagonism when in contact with *A. parasiticus*. The fungal residual dry weight (rdw) ranged from 24.7 to 57.2% of the control dry weight (100%) when treated with the six strains (Table 1). The reduction effect increased in the following order: S17 (57.2%) > S38 (44.3%) > S27, S13 (35.2%) > S35 (32.9%) >S06 (24.7%).

Mutual antagonism was also showed in contact with *A. flavus* with the same *Streptomyces* strains, with a lower impact on *A. flavus* growth (Table 2). The rdw ranged from 60.7 to 92.7% of the control dry weight (100%) when treated with the same six strains. The reduction effect increased in the following order: S27 (92.7%) > S13 (81.3%) > S17 (77.7%) > S06 (64.6%) >S38 (62.4%) > S35 (60.7%).

Reduction of AFB1

Among the six studied bacterial strains, all lead to a reduction of AFB1 production by *A. parasiticus* and *A. flavus*. S17 showed a lesser reduction on AFB1 production. It was 13% mc for *A. parasiticus* and 24% for *A. flavus*. S27 and S38 present more significant reduction in the AFB1 concentration: the mc for *A. flavus* and *A. parasiticus* were 4.1% and 8.1% by S27 and 4.5% and 3.1% by S38. S06 and S35 were extremely effective strains. Indeed, no AFB1 was detected in interaction with *A. parasiticus* and mc of 2.3% (S06) and 0.2% (S35) in *A. flavus* interaction. Finally, S13 had varying impact on AFB1 production depending on the strain studied : no AFB1 detected after *A. parasiticus* interaction although 15.6% were detected in *A. flavus* interaction.

Reduction of AFB2 and AFG1

AFG1 production by *A. parasiticus* was monitored in the same conditions. Among the six studied strains, three totally inhibited AFG1 production and three didn't: S17, S27 and S38 with 6.2%, 2.9% and 4.0% respectively (Table 1). AFB2 production by *A. flavus* was monitored. Among the six studied bacterial strains, four totally inhibited AFB2 production and two didn't: S13 and S17 with 9.3% and 5.3% respectively (Table 2). S17 also had a lesser reduction on AFG1 and AFB2 production.

RT-qPCR for aflatoxin gene expression. The study of the gene expression was carried out with *A. flavus* or *A. parasiticus* alone (controls) and in interaction with the 6 *Streptomyces* strains. Five genes (*aflD*, *aflM*, *aflP*, *aflR* and *aflS*) were investigated relatively to two reference genes (*act1* and *βtub*).

Focus on *A. flavus*, the expression of *aflD* and *aflS* was not significantly impacted by the six strains.

S35 repressed the expression of *aflM* (8.4 fold) and *aflR* (1.5 fold). S38 repressed the expression of *aflP* (4.8 fold) and *aflR*.S06 enhanced the expression of *aflR* (2.37 fold).

Regarding *A. parasiticus*, *aflD* expression was not significantly impacted. *AflM* expression was slightly impacted by S13 (7.7 fold), moderately by S35 (33.3 fold) and very highly by S06 (100 fold). S35 and S06 also reduced *aflP* expression 83 and 250 fold, respectively. Regarding *aflS* and *aflR*, S13 significantly reduced *aflS* expression (6.25 fold) and S06 repressed the expression of both *aflS* (10 fold) and *aflR* (14.3 fold).

The ratio *aflR*:*aflS* was monitored in both producing strain. Both positive control were close to 1: 0.8 for *A. parasiticus* and 0.9 for *A. flavus*. It was above 1 for *A. parasiticus* in S06 (1.2), S13 (1.2), S17 (1.4) and S38 (1.5) interactions and for *A. flavus* in S06 (2.9), S17 (1.8) and S35 (1.3) interactions. Otherwise, under 1 ratios were observed.

DISCUSSION

In this work, *Streptomyces spp.* inhibit aflatoxin production by *A. flavus* and *A. parasiticus* in Petri Dishes on ISP-2 medium. It is known that micro-organisms interactions can led to aflatoxin inhibition (by *A. parasiticus* and *A. flavus*) without fungal growth inhibition. *Pichia anomala, Stenotrophomonas sp., Achromobacter sp.* were but three of the many examples (Hua *et al.*, 1999; Jermnak *et al.*, 2013; Yan *et al.*, 2004). In this study, we add *Streptomyces* to this list.

In interaction with the six bacterial strains, significant differences in AF concentration was detected depending on the producing strain. Bluma et al., (2008a, 2008b) studied the impacts of essential oils addition. AF reduction mainly was observed on *A. parasiticus* and *A. flavus* (Bluma *et al.*, 2008a, b). However, in certain conditions, *A. parasiticus* and *A. flavus* overproduced AF compared to the control. In our study, AF production by both producing strains were similarly repressed. Our selected strains clearly have a repressing impact on AF production independently of the strain tested.

This inhibition can be linked to AF biotransformation or production prevention. The latter has already been studied. The concentration of pure-AFB1 was significantly reduced by S06 (15.6% mc), S35 (29.4% mc) and S38 (38.0% mc) in contrast to S27 (76.6%), S17 (96.6%) and S13 (104.9%) (Verheecke *et al.*, 2014). A significant difference in aflatoxin inhibition percentage was observed in the present study, although the same *Streptomyces* strains were used. This potentially suggests additional gene repression involved.

It is known that aflatoxin inhibition can occur through gene repression (Alkhayyat & Yu, 2014; Yu, 2012). The expression of *aflM* was mostly repressed (between 2.2 and 100 fold) in the tested conditions. A disruption of *aflM* homolog in *A. nidulans* (*verA*) led to a reduction of ST production by 200 to 1000 fold (Keller *et al.*, 1994). Just as in this study, repression of *aflM* expression could be linked with the reduction of aflatoxin production.

Other expressions of genes were also repressed. Similar results were shown by the addition of caffeic acid in the media. *AflD* (6.6 fold), *aflM* (7.1 fold), *aflP* (9.1 fold) and *aflS* (1.5 fold) were repressed without affecting fungal growth (by *A. flavus*) (Kim *et al.*, 2008). In our case, the same range of repression was observed in interaction with our *Streptomyces* strains. This suggests that *Streptomyces* were responsible of the gene repression.

The expression of *aflR* was differently impacted. It was enhanced 2.37 fold (by S06 in *A. flavus*) and repressed up to 10 fold (by S06 in *A. parasiticus*). Variation of *aflR* expression was also observed in contact with lyophilized filtrat of *Trametes versicolor* (aflatoxin repressive). In *A. parasiticus*, at 72h, *aflR* expression was enhanced by more than 10 fold (Zjalic *et al.*, 2006). In our study, even though *aflR* expression was enhanced, aflatoxin production was also reduced. This suggests that *Streptomyces* can impact independently from *aflR* the expression of *aflD*, *aflM* and *aflS* (S06).

Depending on fungal and bacterial strains, the ratio *aflR:aflS* was differently impacted. It ranged from 2.9 by S06 in *A. flavus* to 0.5 by S35 in *A. parasiticus* and by S13, S27 and S38 in *A. flavus*. This ratio was first studied under various a_w and temperature. A ratio above one resulted in activation of AFB1 biosynthesis (Schmidt-Heydt *et al.*, 2009). In our study, a ratio above one was not correlated with high AF accumulation and was found in most conditions. Another indicator besides the *aflR:aflS* ratio, could be investigated for AF accumulation in *Streptomyces* interaction.

Streptomyces strains reduce AF accumulation by *A. flavus* and *A. parasiticus*. Those former mainly repressed *aflM* and *aflS* and differently impacted *aflP* and *aflR* expressions. Studies are now in progress to identify the mechanisms involved. Pure-AFB1 reducers and genes repressor *Streptomyces* will be further tested as potential biocontrol agents on maize grain.

ACKNOWLEDGEMENTS

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TABLES:

Table 1: *Streptomyces* strains impact on *A. parasiticus* aflatoxins and gene expression.

Table 2: *Streptomyces* strains impact on *A. flavus* aflatoxins and gene expression.

Table S1: RT-qPCR Primers used for candidate reference genes and genes of interest.

FIGURES:

Figure S1: The gene stability measure M for the seven reference genes candidates.

Table 18 - Table 1 - *Streptomyces* strains impact on *A. parasiticus* aflatoxins and gene expression

		Effect on AFBs		Effect on gene expression					
Strain	fungal growth (%)	Aflatoxin B1 (% mc)	Aflatoxin G1 (% mc)	*aflD*	*aflM*	*aflP*	*aflR*	*aflS*	ratio *aflR:aflS*
Control	103.5 ± 0.9^a	108.3 ± 5.8^a	101.3 ± 10.9^a	1.00	1.00	1.00	1.00	1.00	0.8
S06	24.7 ± 26.4^c	ND^c	ND^c	0.7	0.01*	0*	0.1*	0.07*	1.2
S13	$35.2 \pm 11.6^{b,c}$	ND^c	ND^c	0.67	0.13*	0.08	0.2	0.16*	1.2
S17	57.2 ± 6.6^b	13 ± 3.5^b	$6.2 \pm 0.3^{b,c}$	1.56	2.61	2.28	1.05	0.64	1.4
S27	35.2 ± 17^b	4.1 ± 0.5^b	$2.9 \pm 0.2^{b,c}$	0.84	0.41	0.1	0.39	0.44	0.8
S35	32.9 ± 2.9^c	ND^c	ND^c	0.50	0.03*	0.01*	0.27	0.42	0.5
S38	$44.3 \pm 12^{b,c}$	$4.5 \pm 0.7^{b,c}$	$4.0 \pm 0.3^{b,c}$	0.64	0.28	0.11	0.5	0.44	1.5

Table 1: *Streptomyces* strains impact on *A. parasiticus* aflatoxins and gene expression

data with the same letter are not significantly different (P<0.05)/ * represent a significant difference (P<0.05)

Table 19 - Table - 2 *Streptomyces* strains impact on *A. flavus* aflatoxins and gene expression

		Effect on AFBs		Effect on gene expression					
Strain	fungal growth (%)	Aflatoxin B1 (% mc)	Aflatoxin B2 (% mc)	*aflD*	*aflM*	*aflP*	*aflR*	*aflS*	ratio *aflR:aflS*
Control	100.0 ± 15.4^a	100.0 ± 13.9^a	100.0 ± 17.3^a	1.00	1.00	1.00	1.00	1.00	0.9
S06	64.6 ± 8.6^b	2.3 ± 4.5^c	ND	0.69	0.25	1.57	2.37	0.40	2.9
S13	81.3 ± 16.2^a	15.6 ± 9.2^b	9.3 ± 20.8^b	1.60	0.45	0.41	0.82	0.70	0.5
S17	77.7 ± 11.2^a	24.0 ± 19.8^b	5.3 ± 11.9^b	0.95	0.26	3.03	1.53	0.39	1.8
S27	92.7 ± 18.3^a	8.1 ± 5.1^b	ND	1.42	0.26	0.39	0.88	0.96	0.5
S35	60.7 ± 11.4^b	0.2 ± 0.5^c	ND	0.50	0.12*	1.02	0.63	0.24	1.3
S38	62.4 ± 15.2^b	3.1 ± 5.3^c	ND	1.44	0.14*	0.21*	0.69*	0.62	0.5

Table 2: *Streptomyces* strains impact on *A. flavus* aflatoxins and gene expression.

data with the same letter are not significantly different (P<0.05)/ * represent a significant difference (P<0.05)

Table 20 - Table S1 - RT-qPCR Primers used for candidate reference genes and genes of interest.

Table S1: RT-qPCR Primers used for candidate reference genes and genes of interest.

Primer	Primer Sequence	Protein	Accession no.	Length	Efficiency
act1 F	5' - TTGACAATGGTTCGGGTATGTG - 3'	actin	XM_002378226.1	80	89,7%
act1 R	5' - ACCGACAATGGAGGGGAAGA - 3'				
btub F	5' - TGGTGTTACCGTCAGTCTTC - 3'	β-tubulin	XM_002383269.1	72	91,1%
btub R	5' - ACCATGTTCACAGCCAGTTT - 3'				
cox5 F	5' - CCCTGTTCTACGTCATTCACTTGTT - 3'	cytochrome c oxidase subunit V	XM_002384631.1	99	93,1%
cox5 R	5' - TCTTCTCGGCCTTGGCATACTC - 3'				
ef1F	5' - TCAGGCACGGGGAACTTTG - 3'	translation elongation factor EF-1 (putatif)	XM_002375025.1	123	94,1%
ef1R	5' - CCACTGAACCGAGTCCATCT - 3'				
gapdh F	5' - ACGGCAAGCTCACTGGTATGT - 3'	glyceraldehyde 3-phosphate	XM_002373980.1	120	100,8%
gapdh R	5' - CAGCCTTGATGGTCTTCTTGATGT - 3'				
rpl 13F	5' - TCCGAAATGCAAATGCCCAAA - 3'	60S ribosomal protein L13	XM_002385523.1	88	98,5%
rpl 13R	5' - ACGCAGCTTCATACCATACATC - 3'				
tbp F	5' - TCCCTTCCCTACTCTACATCAC - 3'	Transcription factor TFIID	XM_002374743.1	103	114,0%
tbp R	5' - AGTCAAGTAAGACCTCCAGCAA - 3'				
aflR F	5' - GCAGTCAATGGAACACGGAAA -3'	AflR transcription Factor	XM_002379905.1	136	94,3%
aflR R	5' - CGCCTGAAACGGTGGTAGTG- 3'				
aflS F	5' - CCGTCTATCAACAGCAACACAA - 3'	AflS transcription Factor	XM_002379904.1	72	98,4%
aflS R	5' - GGGGATGGGAATGGGATGGA - 3'				
aflD F	5' - GCTCCCGTCCTACTGTTTC - 3'	AflD Reductase	XM_002379908.1	116	102,6%
aflD R	5' - GCATAGTCGTGCATGTTGGT - 3'				
aflM F	5' - TCGAATTATCCTGACCAGCTCTAAC - 3'	AflM dehydrogenase	XM_002379900.1	109	93,0%
aflM R	5' - TGCCATGCAGCGAACAAA - 3'				
aflP F	5' - TCAGAAAAACGACTGAGCTAGGA - 3'	AflP - O-methyltransferase B	XM_002379891.1	116	94,7%
aflP R	5' - GTGGGAAAGTATTGATTTGGTGAA - 3'				

Figure 48 - Figure S1 - The gene stability measure M for the seven reference genes candidates

Figure S1: The gene stability measure M for the seven reference genes candidates.

Average expression stability of remaining reference targets
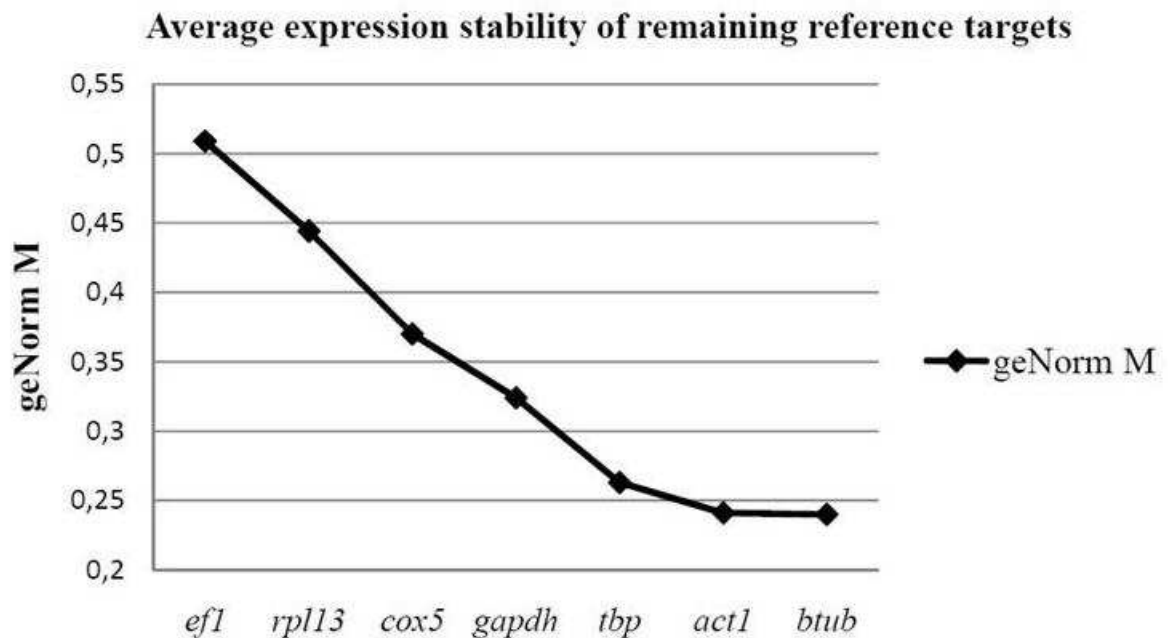
Additional results

To study adsorption mechanisms, additional results were obtained. An AFB1-reducer (S06) and AFB1-non-reducer representative (S13) were chosen. The results are represented in table 21. The AFB1 level was analysed in the supernatant (1) and in the 2 successive washing steps (2 and 3). In control conditions, at both incubation time, 73% of the initial AFB1 was recovered in the supernatant. Then, after a first wash with water and a second with methanol, 14 and 4-6% additional recovery was observed. The total recovery was 90-93%. Similar results were obtained in addition with S06 and S13.

Table 21 - Adsorption test results. Cells were suspended in water in the presence of AFB1 (1 µg) and incubated at 30°C during 1 and 60 minutes. Data are means ± standard deviations in % of the standard. 1= supernatant; 2= washing water and 3= washing methanol.

strain\vial n°	incubation time: 1 minute			Total recovery in %
	1	2	3	
Control	73.5 ± 6.8	14.2 ± 2.0	5.9 ± 3.0	93.6 ± 11.8
S06	81.3 ± 12.7	15.3 ± 1.9	4.4 ± 0.5	101.1 ± 15.2
S13	80.8 ± 9.1	15.7 ± 2.0	4.8 ± 1.2	101.3 ± 12.3
strain\vial n°	incubation time: 60 minutes			Total recovery in %
	1	2	3	
Control	72.7 ± 10.3	13.7 ± 1.0	3.8 ± 0.8	90.2 ± 12.1
S06	71.0 ± 6.9	14.1 ± 1.9	4.2 ± 0.9	89.2 ± 9.7
S13	81.1 ± 8.3	14.3 ± 2.0	3.4 ± 0.5	98.8 ± 11.5

no data were significantly different according to the t-test ($P < 0.05$).

Even though S06 was able to reduce pure-AFB1 (15.6%), those results bring out the absence of binding in the S06's AFB1 reducing process. Indeed, in case of binding, after 1 hour, 79.4% of AFB1 (15 mg.kg^{-1}) was removed from the supernatant by *Lactobacillus rhamnosus* TISTR54 (Elsanhoty *et al.*, 2013). This pattern was not observed with S06. This lack of binding suggests that other mechanisms were involved.

A possible explanation could be linked with the degradation process. In case of higher incubation time (8 hour), Teniola *et al.,* (2005) showed that cell extraction buffer of *M. fluoranthenivorans sp. nov.* DSM 44556T was able to completely degrade AFB1. This degradation was potentially linked to enzymes. Similar enzymes could be investigated.

Conclusion

In this work, the potentials of 6 *Streptomyces* strains to reduce AFT production by *A. flavus* and *A. parasiticus* were evaluated. Confirmation of *Streptomyces* strains capacity to reduce AFB by *A. flavus* was done. Moreover, they were able to reduce AFB1 and AFG1 production by *A. parasiticus* with little impact on fungal growth.

On one hand, expression assays were carried out for *A. flavus* and *A. parasiticus*. The results showed that this interaction significantly impacts gene expression of aflatoxin biosynthesis. In both fungi, expression of *aflM* and *aflS* were mainly repressed. We also revealed that the ratio *aflR:aflS* is not a relevant indicator in *Streptomyces* interaction. We conclude that S06, S35 and S38 were good candidates as biocontrol agents.

On the other hand, we showed that the capacity of S06 to reduce pure-AFB1 is not linked to adsorption mechanisms.

5. Discussion & Perspectives

Discussion and Perspectives

The maize microbial ecosystem is complex. Numerous fungal and bacterial genera are present in this ecosystem. Among these, certain fungi including *Fusarium* sp. can produce mycotoxins. In our study, we chose *F. graminearum* as a representative of those mycotoxigenic fungi in French maize. Our aim was to investigate the impact of *A. flavus* (and its AFB production) entry on *F. graminearum* and its D.O.N. production. We specially focused on the early impacts as our ultimate goal is to prevent AFB and D.O.N. production maize pre-storage. Different abiotic (time, temperature and a_w) and biotic (fungal competition) parameters were tested.

We observed (in sole culture) *Aspergillus* grew after 1 day and *Fusarium* grew after 2. In sole culture, AFB1 production started on the second day (36°C, 1) while no D.O.N. was detected after 7 days. The peak of AFB concentration occurred after 4 days (28°C, 1). In co-inoculation tests, the strain with the highest growth rate (sole culture) overtook the other. Results of AFB and D.O.N. content while co-inoculation remains to be investigated.

Storage facilities such as silos can have different thermal isolation. In case of low isolation, a daily cyclisation of temperature up to 15°C may occur on the silo's side (personal data). Garcia *et al.*, (2012) showed that cycling temperatures (15/20, 15/25 and 25/30) impacted *F. graminearum* D.O.N. production. This latter was either reduced, unchanged or enhanced depending on the cycling temperatures (Garcia *et al.*, 2012). Thus, cycling (e.g. in case of poor thermal isolation) could impact our results on early aflatoxins and D.O.N. production.

In our study, we focused on *F. graminearum* as a producer of D.O.N. and *A. flavus* as an AFB producer. Both fungi produce many other secondary metabolites. *F. graminearum* is a representative of mycotoxigenic fungi in French maize. As a producer of ZEA, nivalenol and 3 or 15-acetylDON (Jennings *et al.*, 2004) it should be investigated accordingly. *A. flavus* is a producer of CPA (Luk *et al.*, 1977). Our chosen strains could produce those secondary metabolites and attention should be given. Furthermore, *F. verticillioides* (FB1 and FB2 producer) is another representative of mycotoxigenic fungi. It would be interesting to inoculate this fungus on the maize-based medium, separetely, and in interaction with the other mycotoxins producers studied in this PhD.

Such experiments along with the impact of CO_2 content on mycotoxins production will provide data to select the critical conditions (a_w, temperature, time, fungi) for maize grain

testing. Obtained data on maize-based medium and on maize grain will provide the details needed to develop modelling tools. Those will be provided to storage agencies to help the prevention of EU-regulated mycotoxins (AFT, D.O.N., ZEA, FB1 and FB2).

Overall, this results will help to understand the impact of *A. flavus* on the French microbial ecosystem of maize. Besides understanding the microbial ecosystem, mycotoxin prevention can be achieved by the use of biocontrol agent.

Our second aim, was to develop a biocontrol (based on actinomycetes) able to reduce (in interaction with *Aspergillus* sp.) AFT contamination at field without impacting the maize microbial ecosystem. The first step in biocontrol development was to distinguish a biocontrol able to inhibit *in vitro* AFB accumulation without impacting the fungal growth. We chose actinomycetes as potential candidates as they are a source of aflatoxin-repressing metabolites.

Interaction between *A. flavus* and actinomycetes isolates was conducted *in vitro*. 27 of 38 actinomycetes isolates showed a mutual antagonism in contact with *A. flavus* and had their impact on AFB was monitored. In current literature, if isolates did not inhibit fungal growth, tests were not realised on their capacity to produce AFB (Sultan & Magan, 2011; Haggag & Abdall, 2012). In our work, we chose to promote both micro-organisms growth. This decision was based on the following facts: we wanted to impact as less as possible the maize microbial ecosystem and to limit the spread of resistant strains.

Our first key finding showed that after a 10 day *in vitro* co-incubation period (28°C), the 27 isolates with mutual antagonism in contact reduced AFB1 residual concentration from 38.6% to 4.4%, depending on the isolate. Similar results were observed in interaction with *Pichia anomala* WRL076: it inhibited AFB1 accumulation (by *A. parasiticus*) on PDA medium by 80 fold (Hua *et al.*, 1999). This range of reduction is similar to our observations.

We conducted our experiments at an a_w of 1 on ISP-2 medium. Interactions can have different impacts at various a_w. The variation of a_w was extensively studied in case of antioxidants or essential oils addition (Passone *et al.*, 2007; Bluma *et al.*, 2008a, 2008b). As *Streptomyces* are xerophilous bacteria, they can develop under a wide range of a_w (down to 0.87) (Stevenson & Hallsworth, 2014). This impact has not been characterized yet in *Aspergillus-Streptomyces* interaction. The former should be tested under various a_w (0.87 to 1) and temperature conditions (20 à 36°C). Those tests could evaluate the efficiencies of those interactions in various climate conditions (found in the field).

Streptomyces strains efficiently reduced AFB production by *A. flavus*. Thus, we looked whether this reduction is similar in interaction with the AFT producer: *A. parasiticus*. 6 of the most AFB1-reducing actinomycetes strains (among the 27 isolates) were chosen for testing.

Our second key finding revealed that in contact with *A. parasiticus*, those strains reduced AFB1 from 13% to non detected levels. As for AFG1, it was reduced from 6.2% to non detected level.

Although the 6 bacterial strains showed significant differences in AFB1 reduction, AFB1 content was clearly reduced independently of the *Aspergillus* sp. strain tested. Bluma *et al.,* (2008a, 2008b) showed that the addition of essential oils can reduce AFB1 production by *A. parasiticus* and *A. flavus*. However, this addition did not always inhibit AFB1 production, compared to the control. On the contrary, our strains were 100% efficient in reducing AFB1 in interaction with *Aspergillus* sp..

We monitored AFB1 and AFG1 content in *Streptomyces-A. parasiticus* interaction. In the literature, most studies provide data on AFB1 alone or total AFT (Zjalic *et al.,* 2006; El-Nagerabi *et al.,* 2012). Notwithstanding, Khosravi *et al.,* (2011) studied the impact of *Cuminum cyminum* essential oil (0.25 mg.ml^{-1}) on *A. parasiticus* growth and AFT production. Both were repressed, with AFB1 and AFG1 being repressed by 94.2% and 98.9%, respectively. In our study, in case of S17, S27 and S38 interaction, the reduction of AFB1 was also slightly lesser than the reduction of AFG1.

To develop larger scale tests, the production of the biocontrol in erlenmeyers and in bioreactors should be developed. Such development will provide enough bacterial biomass to assess its impact on maize (*in vitro*), the maize crop and at field regarding AFT production by *Aspergillus* sp.. Moreover, their impact on other metabolites produced by *Aspergillus* sp. should be studied (e. g.: CPA, aflatrem and kojic acid).

It will also provide a bacterial biomass to assess the biocontrol impact on other mycotoxigenic fungi (e.g. *Fusarium* sp.) and their associated mycotoxins (e.g. D.O.N., ZEA, FB1 and FB2).

Our third key finding revealed that the gene expression of aflatoxin biosynthesis genes in the 2 fungi (*A. flavus* and *A. parasiticus*) were impacted by the same 6 *Streptomyces* strains chosen. The highest levels of repression were found on *aflM* (a structural gene) and *aflS* (a regulatory gene).

We developed a technique to study 5 genes of the aflatoxin biosynthesis. In our study, we monitored gene expression after 90 hours, for optimum gene expression. In *A. parasiticus,* Zjalic *et al.,* (2006) also observed *aflR* expression in contact with lyophilized filtrate of *Trametes versicolor* (aflatoxin repressive). 5 incubation times where monitored (1, 2, 3, 7 and 9 days). The maximum *aflR* expression occurred at 2 and 7 days. In lyophilized filtrate addition, the expression was delayed (about 2 days) compared to the control and the expression values were always under the utmost value of the control (Zjalic *et al.*, 2006).

Our results revealed that S35 and S38 mainly repressed *aflM* and *aflP* expression and at a lesser grade *aflR, aflD* and *aflJ* expression by *Streptomyces-Aspergillus sp*. interaction. This repression can be regulated by a complex combination of transcriptional regulators. Among these, the lack of *laeA* (encoding a methyltransferase) was shown to reduce by 100 fold *aflM* and *aflP* expression and partially reduce *aflR, aflD* and *aflJ* expressions (Chang *et al.*, 2012). Thus, in our case we didn't target *laeA* expression, but *laeA* could have been repressed by S35 and S38.

S38 repressed *aflM*, *aflP* and *aflR* expression while reducing AFB1 production. This could be due to metabolites production. Indeed, *Streptomyces* metabolites have already been studied for their impacts on aflatoxin biosynthesis (Kondo *et al.*, 2001; Yoshinari *et al.*, 2007, 2010). For example, *Streptomyces* sp. SA-2581 produce a metabolite called Dioctatin A., which was shown to repress *aflC*, *aflM*, *aflP* and *aflR* gene expression of *A. parasiticus* while inhibiting AFB1 accumulation (Yoshinari *et al.*, 2007). In our study, S38 repressed *aflM*, *aflP* and *aflR* expression. However, neither conidiogenesis impact nor repression of *aflD* expression were observed. Both achieved with Dioctatin A addition. This suggests another metabolite (e.g. aflastatin A) and/or mechanism involved.

In our study, *aflM* was almost entirely repressed in *A. flavus* and *A. parasiticus* with a reduction of AFB1 by 4.16 fold to non detected levels and 7.7 fold to non detected levels, respectively. *A. nidulans* is a producer of ST. *A. nidulans* disruptant of *verA* (*aflM* homolog) were shown to produce less ST by 200-1000 fold and accumulate VERA (Keller *et al.*, 1994). In *Streptomyces-Aspergillus sp.* interaction, a lack of *aflM* expression could be linked with VERA accumulation.

In *A. flavus*-S06 interaction, no induction of *aflD* or *aflP* and a reduction of AFB1 by 43 fold were detected. Overexpression of *aflR* thanks to constitutive expression induced *aflD* and *aflP* expression and an identical or reduced AFB1 production (compared to wild-type)

(Flaherty & Payne, 1997). This suggests that S06 employed other mechanisms than *aflR* modulation to reduce aflatoxin production.

One of these mechanisms could be linked to PkaA. Calvo *et al.,* (2002) showed that an upregulation of PkaA promoted AflR phosphorylation. This phosphorylation prevents AflR nuclear localisation and inhibits its activation on the aflatoxin structural genes (Calvo *et al.*, 2002). Thus in case of S06 interaction, we would expect a reduction of *pkaA* expression.

However many other approaches should be applied to further understand the impact on gene expression:

(i)   A kinetic of gene expression (e.g. day 1 to 7) should be done in interaction with S06, S35 and S38. Kinetics of expression will provide more data to verify a possible delay in gene expression;

(ii)   Mutated strains of *A. flavus* or *A. parasiticus* can accumulate NOR (lacks the capacity to convert into AVN). *Streptomyces* interaction with those mutated strains should be done to evaluate the proportion of NOR inhibition;

(iii)   It could be interesting to monitor the expression of general transcription regulators (e.g.: *laeA*, *pkaA*) to further understand the mechanisms involved in the reduction of aflatoxin biosynthesis;

(iv)   Techniques such as microarray should be used to monitor the primary metabolism as well as the secondary metabolism involved in aflatoxin repression.

Besides the study of gene expression, identification of a potential metabolite preventing AFT production should be tested. This includes the dosage of metabolites already known as AFT-repressor which could be produced by our biocontrol (e.g.: aflastatin, dioctactin, blasticidin). If none of those are the active compounds, purification of the new active compound will be needed.

In our fourth key findings, these *Streptomyces* strains were tested for their efficiencies to reduce pure-AFB1, apart from reducing gene expression,. Thus, 12 isolates were selected based on their AFB1-prevention rates (under 17% remaining) in *Streptomyces-A. flavus* interaction. They were inoculated in the presence of pure-AFB1 (5 mg kg^{-1}) in solid medium. Among the 12 selected isolates, 8 reduced pure-AFB1 (range between 82.2 and 15.6%). Similar results were observed with *Lactobacillus plantarum* (PTCC 1058) on maize. After 4 to 7 days, 77% of the AFB1 (240 µg.kg^{-1}) was removed. Khanafari *et al.,* (2007) hypothesised

that this reduction could be linked with binding. However, the mechanism remains to be investigated. The 8 remaining strains can reduce pure-AFB1 and this characteristic may depend on binding and/or degradation mechanisms.

Our last key finding, the most efficient strain (S06) was tested for adsorption and showed to be unable to adsorb pure-AFB1. After 1 or 60 minutes at 30°C in contact with pure-AFB1 (1 µg.ml^{-1}), no adsorption was detected. Similar methodology should be applied to identify potential adsorption the other *Streptomyces* strains. The inability of S06 to adsorb pure-AFB1 leads to putative degradation mechanisms. Aside from the $F_{420}H_2$ reductase (Taylor *et al.*, 2010) found in *Actinomycetales*, MADE from *Myxococcus fulvus* NSM068 is also a degradation enzyme. After 48h at 30°C, this enzyme degraded AFB1 (71.89%), AFG1 (68.13%) and AFM1 (63.82%). Characterisation showed an activity in a wide range of pH (4-9) and temperatures (25-50°C) (Zhao *et al.*, 2011). In our study, after 4 days at 28°C, similar reductions of pure-AFB1 were observed. In case of S06, a similar degradation mechanism could be involved.

The AFB1-degradation mechanisms in the biocontrol agent (e.g. S06) should be identified. Special care should be given to S03, S04 and S06 as new peaks on the HPLC chromatogram were identified (Chapter 3.2). The enzyme(s) involved should be purified and characterised. This enzyme identification would be a first for *Streptomyces* genus. The potential metabolites produced must be identified and their harmlessness assessed. This process of degradation should be tested on maize grain. An optimum formulation of this degradation tool should be investigated for maize. This could be sprayed at field (after drought stress), in pre-storage, in storage and in some stages of food processing. In the long run, a potential DNA sequence containing the gene encoding the desired enzyme could be included into genetically modified maize.

A potential application of this thesis could be the use of this kind of biocontrol at field. In order to monitor the dissemination of the biocontrol at field, specific primers should be developed. Thus, in regards to the chosen bacteria, DNA-DNA hybridisation and physiology should be performed to fully identify the biocontrol agent. A whole genome sequencing should be done and the strain should be patented. Moreover, the *Streptomyces*-maize-*Aspergillus* interaction should be studied. Possible endophytism from the biocontrol should be tested as well as possible induction of the plant systemic resistance.

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Annexes

Streptomyces-Aspergillus interactions: Impact on Aflatoxins B accumulation

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ABSTRACT (300 words)

This work aimed to study the interaction between *Streptomyces* bacteria and *Aspergillus flavus* to promote mutual antagonism in contact leading to a reduction of Aflatoxin B1 (AFB1) concentration. Thirty-seven *Streptomyces* isolates were chosen as potential candidates. After a 10 days *in vitro* co-incubation on Petri dishes, 27 of the 37 isolates respond to mutual antagonism in contact criteria. AFB1 and B2 analysis revealed that these isolates led to an AFB1 residual concentration from 38.6% to 4.4% compared to the control, depending on the isolate.

To begin identifying the mechanisms involved, we selected 12 isolates and tested their capacity to reduce AFB1 in pure culture, AFB1 was reduced by eight isolates. The remaining AFB1 concentration (rcm) varied between 82.2 and 15.6%. This phenomenon could be due to degradation or adsorption mechanisms.

To further understand the mechanisms involved, we studied six *Streptomyces* strains (4 strains able to reduce pure AFB1 rcm and 2 not) in interaction with *A. flavus* and we monitored by RT-qPCR, the genes expressions of *aflD*, *aflM*, *aflP*, *aflR* and *aflS*. The tested strains revealed two different patterns. The first one (S06, S35 and S38) showed reduced Aflatoxins concentrations (3.1 to 0.2% residual AFB1) with different impacts on gene expression. Thus, S06 repressed *aflS*, *aflM* and enhanced *aflR* expression, while S35 and S38 generally repressed all the studied genes. The second pattern (S13, S17 and S27) which moderately reduced Aflatoxins concentrations (24.0 to 8.0% residual AFB1). S13 and S27 showed no significant impact on gene expression, while S17 significantly decreased *aflM* and *aflS* expression. These findings led us to suggest that some *Streptomyces* strains have an impact on Aflatoxin gene expression.

This study showed that *Streptomyces* are potential biocontrol candidates as they could prevent production (Aflatoxin gene expression) and decontaminate (AFB1 reduction) Aflatoxin in food and feed.

KEYWORDS: *Aspergillus flavus*, *Streptomyces*, co-culture, degradation, RT-qPCR, gene expression, aflatoxin

Aflatoxins B1 and B2 (AFBs) are secondary metabolites produced by filamentous fungi. Those polyketide-derived furanocoumarins are carcinogenic (IARC 2013) and their contamination in food and feed is a major food-related health issue. Thus, the European Union has limited their presence to four µg kg^{-1} in maize foodstuff (European Union 2006) and China to 40 µg kg^{-1} (Wu & Guclu 2012) to prevent Aflatoxins (AFs) ingestion by the consumer. The most producing fungus, *Aspergillus flavus,* is found in different crops including : maize, hazelnuts, peanuts, etc (Giorni et al. 2007; Passone et al. 2010).

Maize Aflatoxin contamination is already well studied. Abiotic and biotic stimuli can prevent Aflatoxins accumulation. Abiotic stimuli like temperature and water activity (aw) are the most affecting aflatoxin accumulation (Holmquist et al. 1983). Moreover, other abiotic parameters can affect as well, including: pH, carbon sources, chemical compounds, etc. (Keller et al. 1997; Wilkinson et al. 2007; Holmes et al. 2008). At field conditions, where abiotic parameters are difficult to manage, biotic solutions has to be developed.

Techniques based on biocontrol agents are currently available: afla-guard$^®$(USA) and afla-safe$^®$(Africa). Those non aflatoxigenic *A. flavus* overtake the maize fungal niche and prevent other mycotoxigenic fungi colonization. This technique can prevent aflatoxins occurrence between 70.1 to 99.9% (Atehnkeng et al. 2008). Other micro-organisms could be potential aflatoxin inhibitor like *Fusarium spp* and *Streptomyces spp.* They can reduce AFB1 accumulation by *A. parasiticus* on irradiated maize grain and also by *A. flavus in vitro* (Marín et al. 2001; Zucchi et al. 2008).

Biotic stimuli could also act directly on AFs molecules to reduce concentration. Indeed, bacteria were shown to detoxify, bind to their cell wall or enzymatically degrade AFB1 (Wu et al. 2009; Alberts et al. 2009).

In order to understand the mechanisms involved in the prevention of AFs accumulation, we proposed to monitor aflatoxin gene expression. Indeed, the AFs molecular pathway is now well described and the cluster corresponds to a 80-kb DNA sequence containing 25 well characterized genes and five transcripts (Yu 2012). Among these, *aflR* and a*flS* encodes transcription activators with AflS helping AflR to fix a consensus sequence localized in Aflatoxin genes promoters (Payne et al. 1993; Meyers et al. 1998). Concerning structural genes, the most studied are *aflD*, *aflM* and *aflP*, encoding respectively a norsolorinic acid reductase, a versicolorin A deshydrogenase and a sterigmatocystin methyltransferase (Papa 1982; Bhatnagar et al. 1988; Skory et al. 1992). Those 5 genes expressions are affected directly by the primary metabolism thanks to regulators including the

putative methyltransferase LaeA (Yu 2012). Those regulators can be impacted by many stimuli including biotic stimuli.

Biotic interaction can reveal many advantages, thus it is interesting to find field friendly micro-organisms able to inhibit aflatoxin production. To respond to this demand, we investigated Actinomycetes as potential biocontrol agents. Thanks to different techniques (Microbiological, Analytical and Molecular) we investigated *Streptomyces* effect on Aflatoxin accumulation. Those results lead us to identify three strains able to both inhibit Aflatoxin biosynthesis and remove pure AFB1.

Materials And Methods

A. Fungal strain and actinomycete isolates.

The fungal strain used was *A. flavus* NRRL 62477, the Actinomycetes strains were chosen and conserved as described in Verheecke et al. 2014.

B. Interaction method.

The interaction methodology for the screening of Actinomycetes was realized as described in (Verheecke et al. 2014). For the RNA extraction, this methodology was used with slight modifications: (i) a sterile 8.5 cm cellophane sheet (Hutchinson, France) overlaid the media, (ii) two streaks of Actinomycetes were inoculated 2 cm away from *A. flavus* inoculation point, (iii), the fungal biomass, without bacterial biomass, was removed from the cellophane surface after 90 hours of incubation.

C. Aflatoxin extraction and quantification.

Aflatoxin extraction, quantification and statistical analysis were done as previously described (Verheecke et al. 2014).

D. RNA extraction, RT and qPCR.

The fungal biomass was crushed to a fine powder under liquid nitrogen and stored at -80°C . Approximately 60 mg of mycelia were taken for extraction. Total RNA was isolated using the Aurum Total RNA Kit (BioRad) according to the manufacturer's instructions for eukaryotic and plant cell material with the following modifications: DNase I digestion increased to one hour and the elution was done at 70°C for 2 min in elution buffer. RNA quantity and quality was checked by nanodrop (ThermoFisher, France) and experion (BioRad, France) according to manufacturer's instructions.

RT was carried out with the Advantage RT-PCR Kit (Clontech) with Oligo (dT)$_{18}$ primer according to the manufacturer's instructions with one modification: reaction incubation at 42°C was increased to four hours. RT-qPCR was performed in duplicate in a CFX96 Touch instrument (Bio-Rad) using SsoAdvanced^{TM} SYBR Green Supermix (Bio-Rad) with the protocol recommended for cDNA by the manufacturer's instructions. Following the RT-qPCR, data were analyzed using CFX Manager Software (version 3.0, Bio-Rad). The data were statistically analyzed by qbase+ software (biogazelle) with *act1* and *βtub* as reference genes and a One-way ANOVA (control versus all strains), paired t-test (control vs each strain) were done.

E. Solid media AFB1 reduction test

The experiment was done as previously described (Verheecke et al. 2014).

Results

A. Streptomyces-A. flavus *screening results*.

Among the 37 chosen actinomycete candidates only 27 showed mutual antagonism on contact and promote actinomycetal and *A. flavus* growth. However, only 16 strains were also able to reduce both AFBs residual concentration in the media (rcm) to less than 17% compared to the control. As examples, the isolate S17 was able to reduce AFB1 and B2 rcm to 10.6 % and 10.8 % respectively, whereas S35 was able to reduce to 4.4 % and 5.5 %. In order to understand if these strains are able to prevent AFBs accumulation we decided to study the interaction effect on gene expression with a RT-qPCR approach.

B. RT-qPCR Aflatoxin gene expression.

Study of the aflatoxin pathway genes expression was carried out with *A. flavus* alone (control) and also in interaction with six different *Streptomyces* strains. Five genes (*aflD*, *aflM*, *aflP*, *aflR* and *aflS*) were investigated for their relative expression. Table 1 summarize relative gene expression quantities in interaction with *Streptomyces* strains (normalization with the control condition). *aflD* expression was not significantly impacted in all tested conditions. Only S35 and S38 were able to repress significantly gene expression. Both strains repressed a*flM* expression by more than 7 fold. Moreover, S38 was able to significantly repress *aflP* expression by 4.8 fold and *aflR* expression by 1.4 fold. As S35 and S38 are able to repress significantly gene expression, they could prevent aflatoxin accumulation by this strategy. However, we wanted to know if they could also remove pure AFB1.

C. Effects of selected Actinomycetes isolates on pure AFB1

We decided to test if those strains could reduce AFB1 concentration in pure culture to possibly identify decontamination mechanisms. Those strains were inoculated in the presence of AFB1 in solid media at an initial concentration of 5 mg kg^{-1}. As shown in Table 1, S13 and S17 showed no significant impact on the AFB1 rcm. S27 slightly reduced the initial AFB1 concentration to 76.6 % rcm. S35 and S38 showed a more significant reduction of the AFB1 concentration with 29.4 and 38.0% rcm, respectively. Finally, S06 was the most efficient with a rcm of 15.6%.

D. Conclusion

The tested *Streptomyces* strains have been identified as reducing AFBs accumulation in interaction with *A. flavus.* Two different patterns concerning AFBs accumulation impact were shown. S06, S35 and S38 represent the first pattern, with high reduction of AFBs rcm in Petri dishes co-culture and an high capacity to remove pure AFB1 in the media. The second pattern

included S13, S17 and S27 which also reduced AFBs rcm but were less efficient in pure AFB1 removal. Focusing on gene expression, the first pattern revealed a capacity to repress Aflatoxin gene expression with S06 repressing *aflS* (p<0,19) and *aflM* (p<0,19), S35 and S38 repressing *aflM* and *aflR* (p<0,09 et p<0,08, respectively) and S38 repressing *aflP*. While the second pattern showed no significant impact on the studied genes expression.

Discussion

Little data is available on *Streptomyces-Aspergillus* micro-organisms interaction. Many studies have focused on *Streptomyces* metabolites, for example, Mohamed et al., 2013, revealed that 69% of 16 strains free cell extracts were not able to reduce fungal growth (Mohamed *et al.*, 2013). Unlike our study, other researchers have chosen fungal growth inhibition as the main selection criteria for their potential biocontrol agents against mycotoxin production (Sultan & Magan 2011; Haggag & Abdall 2012). Our work focus on promoting both micro-organisms growth and AFB1 concentration reduction.

We obtained these results with 27 of our *Streptomyces* isolates. They have been identified as reducing AFBs accumulation in interaction with *A. flavus.* In 1997, Ono et al. identified *Streptomyces* sp. MRI142 as a producer of aflastatin A. This molecule is able to completely inhibit AFB1 production at 0.5 µg ml^{-1} in the media without impacting the fungal growth (Ono et al. 1997). A possible explanation could be that some of our strains may produce aflastatin A.

Moreover, aflastatin A inhibitory mechanisms were further investigated by RT-qPCR. At a 0,1% (v/v) concentration, aflastatin A inhibited the expression of *aflC, aflM, aflP* and *aflR* in *A. parasiticus* ATCC24690 (Kondo et al. 2001). In our study our strain S38 inhibited *aflM, aflP* and *aflR* expression. These results suggest a possible aflastatin A production by this strain.

Another strain, S35, repressed *aflM* expression by 8.4 fold and AFB1 rcm by 11.4 fold. Our study on S35 and S38 strains impacts revealed lesser expression of *aflM* and *aflP* compared to the control. A possible mechanism involved in S35 and S38 pattern could be linked to LaeA. A gene mutation of *laeA* in *A. flavus* revealed a *aflM* and *aflP* 100 fold less expression (Chang et al. 2012). Thus, a *laeA* repression could be involved in the reduction of aflatoxin production by S35 and S38.

A complementary advantage to our biocontrol candidate could be pure AFB1 removal thanks to adsorption or degradation mechanisms. S06, S35 and S38 were able to highly remove pure AFB1 concentration (15.6, 29.4 and 38% rcm, respectively). This mechanism could be linked to cell wall surface binding like described in lactic acid bacteria (El-Nezami *et al.* 1998). Another possibility is an enzymatic degradation of AFB1. A $F_{420}H_2$ reductase commonly found in *Actinomycetales* genus has been already shown to transform AFB1 into several low

molecules (Taylor et al. 2010). Nevertheless, this reductase has not yet been characterized in *Streptomyces* genus.

The health risks linked to AFB1 degradation by-products has been well studied. These were often found as less toxic than AFB1 like aflatoxin D (Elaasser & El Kassas 2011; Krifaton et al. 2011; Samuel et al. 2014).

Our results showed that S17, S27 and S13 are able to act on Aflatoxin accumulation through mechanisms not yet identified. Concerning S06, S35 and S38, they were able to prevent and remove AFB1 accumulation, suggesting that they are good biocontrol candidates. These 3 strains have to be further investigated in greenhouse to evaluate their ability to maintain their interesting characteristics.

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Table caption :

Table 1: Results concerning Aflatoxins accumulation, gene expression and effect on pure AFB1 by six chosen actinomycetal strains.

Table 1: Results concerning aflatoxins accumulation and gene expression by six chosen *Streptomyces* strains.							
Strain	Effect on AFBs accumulation in co-culture		Effect on gene expression				
	Aflatoxin B1 (% rcm)	Aflatoxin B2 (% rcm)	*aflR*	*aflS*	*aflD*	*aflM*	*aflP*
Control	100.5 ± 5.5^a	100.9 ± 9.4^a	1.00	1.00	1.00	1.00	1.00
S6	2.3 ± 4.5^c	ND	2.37	0.40	0.69	0.25	1.57
S13	15.6 ± 9.2^b	9.3 ± 20.8^b	0.82	0.70	1.60	0.45	0.41
S17	24.0 ± 19.8^b	5.3 ± 11.9^b	1.53	0.39	0.95	0.26	3.03
S27	8.1 ± 5.1^b	ND	0.88	0.96	1.42	0.26	0.39
S35	0.2 ± 0.5^c	ND	0.63	0.24	0.50	0.12*	1.02
S38	3.1 ± 5.3^c	ND	0.69*	0.62	1.44	0.14*	0.21*
data with the same letter are not significantly different ($P<0.05$)/ * represent a significant difference ($P<0.05$). rcm: residual concentration in the media.							

Annex 2 - Maximum levels and Guidance values for mycotoxins in feedstuffs (European Union, 2002, 2006)

Mycotoxins	Feedstuffs	levels (mg.kg⁻¹)
aflatoxin B1	Feed for dairy cattle, calves, sheeps, lambs, goats, kids, pidglets and young poultry animals	0,005
	Complementary and complete feed	0,01
	Feed materials and for cattle, sheep, goats, pigs and poultry (except mentioned above)	0,02
rye ergot	Feed materials and feed containing unground cereals	1 000
deoxynivalenol	Complementary and complete feedingstuffs for pigs	0,9
	Complementary and complete feedingstuffs for calves (<4 months), lambs and kids	2
	Complementary and complete feedingstuffs (with exception of above)	5
	Feed materials: Cereals and cereal products with the exception of maize by-products	8
	Feed materials: Maize by-products	12
zearalenon	complementary and complete feedingstuffs for piglers and gilts	0,1
	Complementary and complete feedingstuffs for sows and fattening pigs	0,25
	complementary and complete feedingstuffs for calves, dairy cattle, sheep and goats	0,5
	Feed materials: Cereals and cereal products with the exception of maize by-products	2
	Feed materials: Maize by-products	3
ochratoxin A	Complementary and complete feedingstuffs for pigs	0,05
	Complementary and complete feedingstuffs for poultry	0,1
	Feed materials: Cereals and cereal products	0,25
fumonisins (B1 + B2)	Feed materials: pigs, horses, rabbits and pet animals	5
	Feed materials: fish	10
	Feed materials: poultry, calves (<4 months), lambs and kids	20
	Feed materials: adult ruminants (>4 months) and mink	50
	Maize and maize products	60
Red: maximum level Black: guidelines values		

218
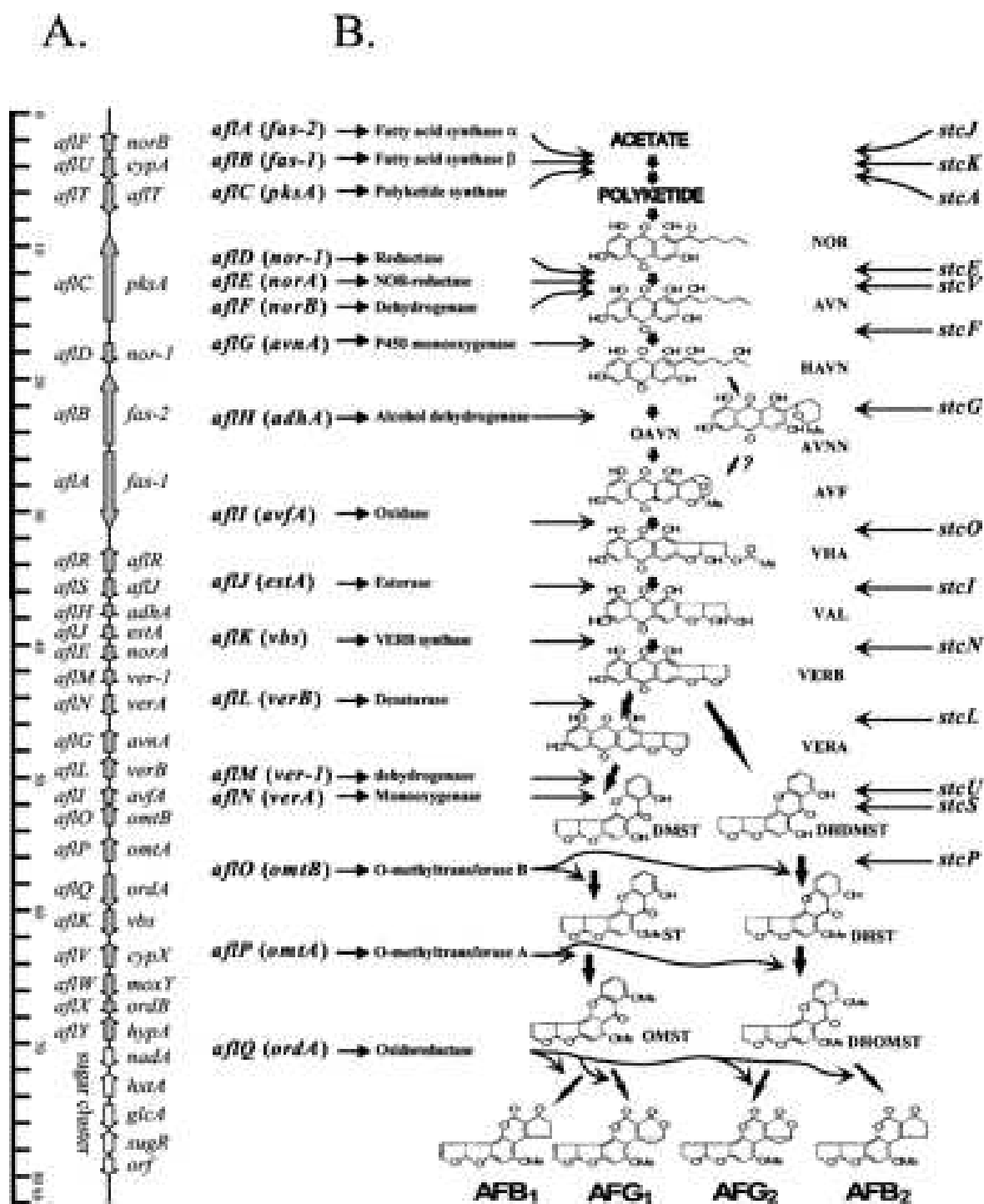
FIG. 1. Clustered genes (A) and the aflatoxin biosynthetic pathway (B). The generally accepted pathway for aflatoxin and ST biosynthesis is presented in panel B. The corresponding genes and their enzymes involved in each bioconversion step are shown in panel A. The vertical line represents the 82-kb 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 direction of gene transcription. The ruler at far left indicates the relative sizes of these genes in kilobases. The ST biosynthetic pathway genes in *A. nidulans* are indicated at the right of panel B. Arrows in panel B 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. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5'-hydroxyaverantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydro-demethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methyl-sterigmatocystin; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2.

Positive control (*A. flavus* only):
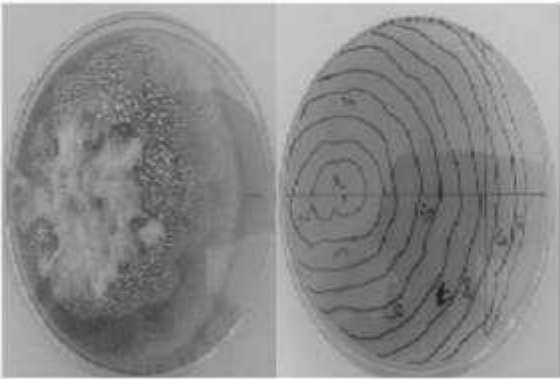
6 strains had dominance *of A. flavus* on contact I$_D$ (4/0):
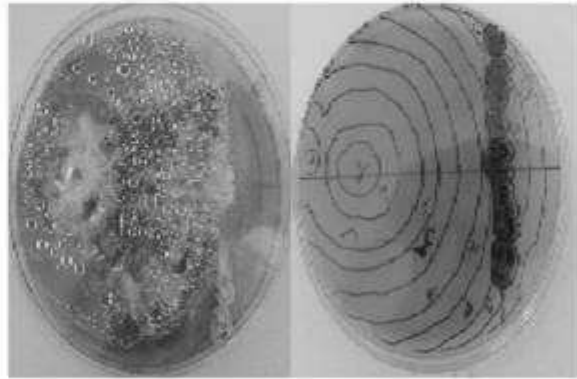
4 strains had dominance at the distance I$_D$ (0/5):
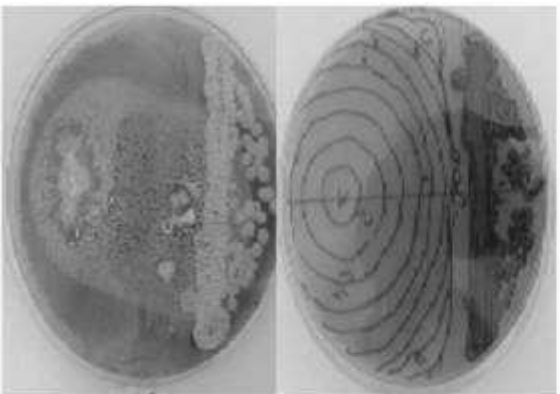
27 strains had mutual antagonismen on contact I$_D$ (2/2):
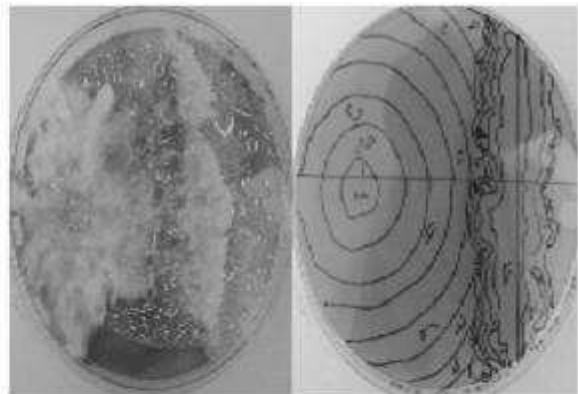
Picture of Petri dishes after 10 days at 28°C (right side up and right side out). I$_D$= Index of dominance applied to *A. flavus*/actinomycetes strains as defined in Verheecke *et al.,* 2014.

Aspergillus flavus
Zea mays   AFB1

Aflatoxins on maize cobs inoculated with Aspergillus flavus in greenhouse

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Summary:

- After no success with inoculation by silk channel, two strains of *Aspergillus flavus* were inoculated separately on maize cobs grown in greenhouse with toothpicks at 3 maturation stages. Dent stage: Pollination + 40 days (P40); P50 days and P60 days.
- *A. flavus* inoculations did not affect the growth of ears (31.4 cm).
- Growth, sporulation and aflatoxin production of *A. flavus* stayed limited to the inoculated kernels under our environmental conditions.
- Earlier stages showed more development of *A. flavus* than later stages.
- There was no difference in development and sporulation between the two strains.
- There were great differences of aflatoxin production between the two strains: 634 µg/kg vs 19 µg/kg at P+40.

Experimental conditions:

Zea mays was grown and pollinated in greenhouse in order to better control contamination environmental conditions during cob maturation: aRH 80% (30 – 100%) ; aT°C 17°C (6 – 34 °C) . A. flavus was grown on PDA at 27 °C. Spores were suspended in water–Tween 80 at 2% at 2x10^7 spores/mL. Two strains of *Aspergillus flavus* were inoculated separately with toothpicks soaked in spores suspension on maize cobs at 3 stages : pollination + 40 days (P40), P50 and P60 days according to a greco-latin experimental design at 3 replications. Cobs were harvested at P77 days at 18 % of kernel moisture.
Grains were grinded under 0.5 mm size. Aflatoxins were extracted on immunoaffinity columns AFLAPREP P07 r–Biopharm and analyzed for aflatoxins by HPLC Ultimate 3000 Dionex, column Luna 3 µm, 200x4.6 mm with coring cell derivatisation.

Results:

A. flavus growth:
- A. flavus did not affect the growth of ears: average length with peduncle of 31.4 cm is NS (Non Significant) according to conditions (p: 0.64) and 4.8 cm diameter.
- A. flavus sporulation was limited to the inoculated kernel areas with an average of 3.85 grains/ear.
- There was no difference in development between the two strains: respectively 4.0 and 3.7 grains for A1 and A2 (NS p: 0.89) for P40 days inoculation.
- At P50 days, the development is about two times lower (HS p: 0.003). Difference between the two strains stayed no significant: A1: 2 grains; A2: 1.7 grains (NS p: 0.77).
- There was no development for inoculations at P60 days.

Synthesis of AFB1 kernel contents
Inoculation Pollination + 40 days
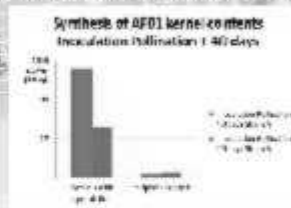
Aflatoxins production:
- Aflatoxin production remained localized on grains with mycelial growth and sporulation.
- Grains immediately around these sporulated grains had only an average of AFB1 of 0.02 µg/kg of dry grains.
- In our experimental conditions a large majority of AFB1 was detected. AFB2 was infrequently detected at a level of about 60 times less. Others aflatoxins were not present.
- On sporulated grains inoculated at P40 days, AFB1 production of A1 strain was 634 µg/kg of dry grains, whereas it was not detected in P50 days inoculation and no symptom and no detection were found for the inoculation at P60 days.
- The 2 strains did not cause the same level of production of AFB1. The A2 strain actually only analyzed on a single repetition produced AFB1 only at 19 µg/kg of dry grains.

Discussion - Conclusion:

Early inoculations allowed better development of *A. flavus* in grain corn cobs in our environmental conditions.
Next experiments will include earlier stages in order to increase symptoms. However, we also keep in mind that in bad prestorage conditions AFB1 is produced on harvest without symptoms.
Next experiments will be carried out at higher RH and T° and will also be inoculated with needles in place of toothpicks to increase grain injury and A. flavus development.
AFB1 was the main aflatoxin produced by our strains, as for natural conditions.
Our 2 strains tested did not cause the same level of production of AFB1. These results need to be confirmed for A2 strain but this fact may be of interest as a working tool to reach our goal to develop a biological control in field against the production of AFB1.

First survey on the trichothecenes-producing *Fusarium culmorum* strains and their impact on infecting barley in Algeria

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INTRODUCTION

Fusarium culmorum (W.G. Smith), the causal agent of *Fusarium* blights, is responsible for devastating disease that lead to extensive yield and quality losses to cereals in humid and semi-humid regions including North Algeria. This fungus produces a wide range of mycotoxins amongst which the deoxynivalenol (DON), a type B trichothecene, is believed to play a determinant role in the pathogenesis toward Triticeae (Wagacha & Muthomi 2007). However, regardless its significant occurrence and impact, little is known about trichothecenes-producing ability of *F. culmorum* infecting barley in Algeria.

Thus, we first evaluate, by PCR assays, the DON-producing ability of several *F. culmorum* strains recovered from the most important cereals cropping areas of Algeria. To determine the variation in pathogenesis among *F. culmorum* strains, a barley seedlings infesting experiment were conduced; then possible relationship between strain pathogenicity and its DON-produced amount were assed after the HPLC-quantification of fungi ability to produce the DON *in vitro*.

PCR SCREENING

Species-specific identification:

Twelve single-spore isolates of *F. culmorum*, noted Fc01 to Fc12, identified in prior on the basis of their conidial morphology according to Leslie and Summerell (2006), were submitted to a PCR-specific identification (Schilling et al. 1996). This molecular identification permitted to confirm the belonging of fungal isolates to *F. culmorum* species.

Trichothecenes-producing ability:

PCR screening of genes related to the trichothecenes biosynthetic pathways were conduced using primers sets designed on gene *Tri5*, *Tri7* and *Tri13* as described by Niessen & Vogel, (1998) and Chandler et al. (2003). This method demonstrated the capacity of the *F. culmorum* isolates to produce trichothecenes among which the DON toxin.

HPLC-QUANTIFICATION OF THE DON PRODUCING CAPACITY

In order to compare isolates through their capacity to produce the DON, fungi were grown in the same standard condition by incubation in liquid Mycotoxin Synthetic (MS) medium for 14 days, in darkness at 25°C (Boutigny et al. 2009). The HPLC analysis was carry out by using an Agilent reverse phase column ZORBAX SB-C18 (Ø 5 µm ; 150 × 4.6 mm) with an UV detection at 218 nm. The final quantification was performed with reference to external calibration curve.

The obtained results (Fig.1) showed significant differences among isolates concerning the DON-produced quantities. The highest DON-productive ability were scored for isolates Fc01 and Fc12 [0.22- 0.23 ng . µl^{-1} MS . mg^{-1} dry biomass].
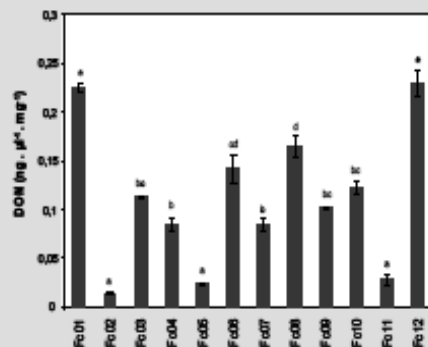

Fig. 1 DON produced in MS medium by *F. culmorum* isolates. Columns with the same letters (as given by analysis of variance, ANOVA) are not significantly different at p = 0.05.

PATHOGENICITY TEST

Isolates pathogenicity were assessed thought the evaluation of their induced disease occurrence, emergence, seedling length and disease severity (according to the scoring system described by Khan et al. (2006)) towards the susceptible barley (*Hordeum distichum* L.) seedlings variety Tichidrette.

The principal component analysis (PCA) from all data recorded on the pathogenicity test (Fig. 2) provided a general view of the disease impact of the twelve *F. culmorum* isolates. Together, the first and the second plot (plot F1 and plot F2) explained 73% of the differences between isolates. PC analysis permitted to assembly (clustering) isolates according their disease impact strength.
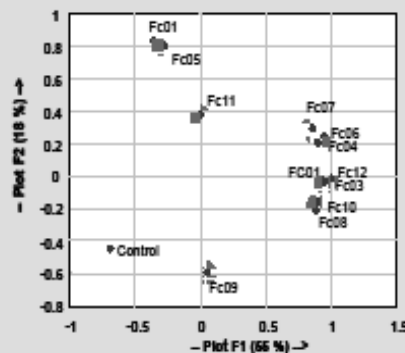

Fig. 2 Principal component plot (F1 vs F2) obtained from data on pathogenicity test of *F. culmorum* isolates. Clusters have been indicated by blue background.
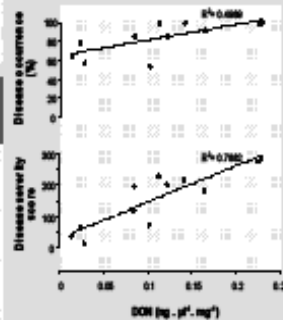

Fig. 3 Relation between the DON concentration produced by *F. culmorum* isolates and both disease occurrence or disease severity.

DON-PRODUCING ABILITY AND PATHOGENICITY RELATIONSHIP

Since the DON is believed to play a determinant role in pathogenicity of *F. culmorum* in different cereal crops, we investigate the relationship of the DON producing capacity and disease impact. Thus, a multiple correspondence analysis (MCA) was carried out based on isolates grouping given by the ANOVA for the DON-producing ability and the isolates clustering given by the PCA for disease impact. This factorial analysis exhibited only 39% of correspondence, which is not sufficient to conclude for a global relationship between the DON-producing ability of isolates and their expressed disease impact. Therefore, correlation between each parameter composing the disease impact (disease occurrence, emergence, plant length and disease severity) and the DON-producing ability of isolates were considered. Indeed, there was significant correlation between the DON producing capacity and increasing in both disease occurrence (r = 0.70, p=0.05) and disease severity (r = 0.88, p=0.05) (Fig. 3).

CONCLUSION

Our survey is the first dealing with the trichothecenes-producing *F. culmorum* strains and their impact on infecting cereals crops in Algeria.

Molecular assay, which were confirmed by HPLC analysis, demonstrated the DON-chemotype nature of isolates sampled from infested barley.

The phytotoxic nature of DON led to hypothesis that its production is an important factor of the pathogenicity of *Fusarium* species were here established through several statistical analysis.

This research highlighted the pathogenic capacity of the *F. culmorum* population sampled from Algerian fields crop and suggested, by this way, to accentuate the surveillance of *Fusarium* diseases, especially those occurring in cereal cultures, which importance is currently under considered by the governmental plant health authority. Thus, a better concern of *F. culmorum*-related diseases and control will increase cereal harvesting yield and grain quality.

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Validation of reference genes for quantitative real-time PCR studies in *Aspergillus parasiticus*

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1. Introduction

-Aflatoxins (AF) are highly toxic and type I carcinogenic (IARC 2012) secondary metabolites of *Aspergillus* spp. that are responsible for the contamination of food and feed crops.

-*Aspergillus parasiticus* and *Aspergillus flavus* are main AF producers, that produce both AFBs and AFGs, or solely AFBs, respectively.

AFLATOXIN B₁ AFLATOXIN B₂ AFLATOXIN G₁ AFLATOXIN G₂
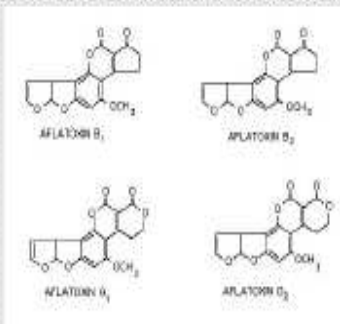
-Studies into the gene expression of the well-known Aflatoxin biosynthetic pathway are important to determining how different stimuli affect Aflatoxin production.

-To study gene expression using RT-qPCR, it is important to have properly validated reference genes for reliable results (Bustin et al., 2009).

2. Candidate reference gene test

-Eight candidate reference genes were selected based on their involvement in different metabolic pathways and previously validated in *A. flavus* (unpublished data).

-Two of the eight target genes, beta-tubulin and glyceraldehyde 3-phosphate, were already sequenced in *A. parasiticus*.

-PCR products of the six remaining genes were sequenced with lengths of sequenced products between 72-143bp

Gene	Protein	Homologue	% similarity
act1	actin	A. flavus - actin	100
btub	β-tubulin	Already sequenced	
cox5	cytochrome c oxidase subunit V	A. flavus - cytochrome c oxidase	98
efl	translation elongation factor EF-1	A. oryzae - RRM0 translation elongation factor	95
Gpd4	glyceraldehyde 3-phosphate dehydrogenase	Already sequenced	
hist1	histone H1 (putatif)	A. oryzae - histone H1	96
RPL13	60S ribosomal protein L13	A. flavus - 60s ribosomal protein L13	97
TBP	Transcription factor TFIID	A. oryzae - TATA box binding protein	85

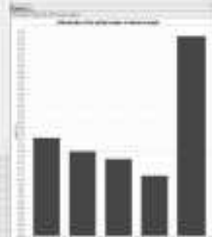*Purple highlight indicates genes not previously sequenced in A. parasiticus

3. Reference gene validation- geNorm

-RT-qPCR results of reference gene expression in *A. parasiticus* under different *Streptomyces*-fungal interactions were analyzed by geNorm for expression stability.
-Rpl13 was excluded from the final analysis following unstable RT-qPCR Results.
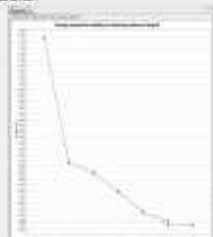
*Fig 1: M - gene expression analysis   *Fig 2: V- optimal # of reference genes

-Fig 1: The lower the M value, the more stable the gene expression: act1, btub, and cox3 showed the lowest M-value, hence the most stable gene expression (Hellemans et al., 2007).
-Fig 2: Based on a GeNorm V < 0.15, 3 reference genes are determined optimal for further RT-qPCR analysis, the addition of more would not have a significant impact on analysis (Hellemans et al., 2007).

4. Conclusions and perspectives

-Here we have identified 8 primer pairs associated to potential reference genes.

-Among these, 6 are newly described in *A. parasiticus*.

-In our assay:
   -7 were possible candidates (1 not stable).
   -GeNorm software validated 3 as reference genes.

-We propose 8 candidates, for use as reference genes in RT-qPCR studies of *A. parasiticus* in different assay conditions.

-Gene expression studies of the Aflatoxin producer *A. parasiticus* can lead to the knowledge needed to inhibit Aflatoxin production in varying conditions.

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