Modulation of fungal toxin production by natural extracts

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PUBLICATIONS and COMMUNICATIONS

1.1 Articles

-**Deciphering the Anti-Aflatoxinogenic Properties of Eugenol Using a Large-Scale q-PCR Approach**
Isaura Caceres, Rhoda El Khoury, Ángel Medina, Yannick Lippi, Claire Naylies, Ali Atoui, André El Khoury, Isabelle P. Oswald, Jean-Denis Bailly, Olivier Puel.
Published in Toxins. 8 (Basel), 123. DOI: 10.3390/toxins8050123.

-**The anti-aflatoxigenic molecule Piperine modulates aflatoxin's pathway and oxidative stress response in *Aspergillus flavus***
Isaura Caceres, Rhoda El Khoury, Sylviane Bailly, Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly.
Submitted Article in Molecular Microbiology Journal.

-**Identification of an anti-aflatoxigenic aqueous extract from *Micromeria graeca* and elucidation of its molecular mechanism in *Aspergillus flavus***
Rhoda El Khoury, Isaura Caceres, Olivier Puel, Sylviane Bailly, Ali Atoui, Isabelle P. Oswald, André El Khoury, Jean-Denis Bailly.
Submitted Article in Food Chemistry Journal.

-**Inhibition of Aflatoxin B1 biosynthesis by aqueous extracts of Mexican plants: *Mimosa tenuiflora* and *Larrea tridentata***
Isaura Caceres, Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly.
Manuscript in preparation.

1.2 Lectures

English presentations:

-Hyssop, *Hyssopus officinalis*, a natural plant extract limiting aflatoxin production by *Aspergillus flavus*
Rhoda El Khoury, Isaura Caceres, Ali Atoui, Olivier Puel, Isabelle P. Oswald, Jean-Denis Bailly, André El Khoury.
21th Annual LAAS Conference, Beyrouth, Liban (15-17 April, 2015).

-The anti-aflatoxigenic effect of Eugenol and Mexican plant extracts using a q-PCR molecular tool
Isaura Caceres, Rhoda El Khoury, Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly.
Doctoral Students' Day of the SEVAB Doctoral School, INSA, Toulouse, France (18th March, 2016).

-Use of a large-scale q-PCR approach to understand the anti-aflatoxigenic effect of Eugenol.
Isaura Caceres, Rhoda El Khoury, Claire Naylies, Yannick Lippi, Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly.
38th Mycotoxin Workshop -Federal Institute for Risk Assessment Berlin, Germany (2-4 May, 2016).

-Use of a large-scale qPCR approach to understand the anti-aflatoxigenic effect of Eugenol.
Rhoda El Khoury, Isaura Caceres, Ángel Medina, Yannick Lippi, Claire Naylies, Ali Atoui, André El Khoury, Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly.
ICFM Workshop, Freising, Germany. (13-15 June, 2016).

French presentations :

-Inhibition de la synthèse de l'AFB1 par l'Eugénol : Caractérisation du Mécanisme d'Action.
Isaura Caceres, Rhoda El Khoury, Claire Naylies, Yannick Lippi, Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly.
6ème Journées Mycotoxines - Campus INP-ENSAT, Toulouse, France (15-16 Mars, 2016).

-Des remèdes naturels pour barrer la route aux toxines des moisissures
Isaura Caceres, Amaranta Carvajal, Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly.
Tête à tête avec des jeunes chercheurs, Museum of Toulouse (3th April, 2016).

Spanish presentations:

-Efecto anti-aflatoxinogénico de plantas nativas Mexicanas contra *Aspergillus flavus*
Isaura Caceres, Isabelle P. Oswald, Olivier Puel, Norma Almaraz, Jean-Denis Bailly
Innovation Match MX- Expo-Guadalajara, Jalisco, México (6-8 April, 2016).

1.3 Posters

-Elucidation of the molecular mechanism of action induced by the natural compound Piperine on Aflatoxin B1 production.
Isaura Caceres, Rhoda El Khoury, Claire Naylies, Yannick Lippi, Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly.
-ICFM 2016 Workshop-Kardinal Döpfner Haus, Freising, Germany (13-15 June, 2016).

-Identification of a new aqueous *Micromeria graeca* extract as an anti-aflatoxinogenic agent and elucidation of its molecular mechanism in *Aspergillus flavus.*
Rhoda El Khoury, Isaura Caceres, Olivier Puel, Sylviane Bailly, Yannick Lippi, Ali Atoui, Isabelle P. Oswald, André El Khoury, Jean-Denis Bailly.
6ème journée des Mycotoxines, Toulouse, France (March 2016).

1.4 Awards

- Best oral presentation (2nd place) during the Doctoral Students' Day of SEVAB.
- Invescap scholarship during the "Innovation Match MX" event.
- Winner logo design for representing the Doctoral School SEVAB.

02

INFORMATION LISTS

2.1 Figure List

2.2 Table List

2.3 Annexes list

2.4 Abbreviations list

A. flavus	*Aspergillus flavus*
A. nidulans	*Aspergillus nidulans*
A. parasiticus	*Aspergillus parasiticus*
AFs	Aflatoxins
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AVF	Averufin
AVN	Averantin
A_w	Activity of water
bp	Base pair
bZIP	Basic Leucine Zipper
CAST	Council for Agricultural Science and Technology
CAT	Catalases
CCR	Carbon Catabolic Repression
CPA	Cyclopiazonic Acid
CRE	CAMP-Response Element
DNA	Deoxyribonucleic Acid
Da	Dalton
DBD	DNA-binding domain
DHDMST	Dihydrodemethyl-sterigmatocystin
DHOMST	Dihydro-*O*-methylsterigmatocystin
DHST	Dihydrosterigmatocystin
DMST	Demethylsterigmatocystin
DON	Deoxynivalenol
EO	Essential Oils
EPA	Enviromental Protection Agency
EST	Expressed Sequence Tags
FAO	Food and Agriculture Organisation
FAS	Fatty acid synthase
FDA	Food and Drug Administration
FUM	Fumonisins
GAP	Good Agricultural Practices
GMP	Good Manufacturing Practices
GPCR	G-Protein-Coupled Receptor
GRAS	Generally Regarded as Safe
HAVN	5'-Hydroxy-Averantin
HCC	Hepatocellular Carcinoma
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer

KA	Kojic Acid
kDa	kiloDalton
MEA	Malt Extract Agar
MFS	Major Facilitator Superfamily
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NATs	Natural Antisense Transcripts
NOR	Norsolorinic acid
OAVN	5'-Oxoaverantin
OMST	*O*-methylsterigmatocystin
OTA	Ochratoxin
PKS	Polyketide synthase
ppb	Particules per billion
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
SOD	Superoxide Dismutase
ST	Sterigmatocystin
T-2	T-2 Toxin
TF	Transcriptional factors
VAL	Versiconal
VERA	Versicolorin A
VERB	Versicolorin B
VHA	Versiconal Hemiacetal acetate
WHO	World Health Organisation
ZEN	Zearalenone

03

INTRODUCTION

3.1 Background

During the last 900 million years, microorganisms belonging to the fungi kingdom have successfully evolved making of them, one of the largest groups within the actual world's biodiversity. Although their impact may go unnoticed, they have critical repercussions in our daily live. In general, fungi are intrinsically involved in environmental transformation as the principal decomposers of organic material as well as they have an important symbiotic relationship with other species such as prokaryotes, plants, animals and humans (Galagan *et al.,* 2005).

In the last decades, the study of these microorganisms in mycology field has radically evolved since the development of DNA molecular techniques. These technologies carried out important advances in fungal knowledge and generated new arguments to better understand fungal adaptive mechanisms. Moreover, they also opened a new vision of biology study, taking in consideration the uncovered information of fungi's world that is waiting to be deciphered.

As an example, in the 90's, the estimated number of fungal species was of the order of 1.5 million (Hawksworth, 1997) while this number rapidly raised to 5.1 million whit the use of computer-aid sequence comparison (Blackwell, 2011).

In toxicology field, and regarding to molds study, this number represents an alarming rate due to the potential raise of toxigenic strains which is today, one of the most current problems of food contamination. In fact, several species of molds that contaminate food can produce toxic secondary metabolites compromising food safety and by consequence, human and animal's health.

Taking advantage on DNA technologies and regarding the improvement of food safety, we estimate that the molecular study of these fungal contaminants can strategically contribute to understand the mechanisms underlying toxin production. This knowledge might be used to develop new strategies to target and control fungal toxicity.

Based on this statement, we focused our research on the *Aspergillus* genus and more specifically to the *Flavi* section. Within this group, *Aspergillus flavus* is classified as one of the principal crop contaminants and moreover, as one of the main producers of the carcinogenic toxin Aflatoxin B1. In the present study, several aspects of this species will be presented with a deeper study on its genetic machinery to produce Aflatoxin B1, as well as natural strategies aiming the inhibition of its production.

3.2 Importance of molds/mycotoxins occurrence in food safety

Molds are double-sided microorganisms that have led to a wide gamma of industrial benefits and parallely represent one of the principal problems in food contamination.

Within their useful utilization in industry, some of them are essential elements for biotechnological production of enzymes, pigments and pharmaceuticals (De Jongh and Nielsen, 2007; Blanc *et al.,* 1995). In food industrial processes, several of them also play an important role. As an example, species of the *Aspergillus* genus such as *A. sojae, A. tamarii* and *A. oryzae* are currently used in oriental food to make products like soy sauce or sake beverage. They are also used to produce enzymes such as pectin, esterases and lipases among others (Campbell-Platt and Cook, 1989; Pariza and Johnson, 2001). Reinforcing this idea, species belonging to the *Penicillium* genus such as *P. roqueforti* and *P. camemberti* are key factors in the manufacture of mold-ripened cheeses while *P. nalgiovensis* is also used for dry sausages preparation (Scott, 1981).

Today, it is clear that the utilization of molds in food industry can represent a helpful tool as long as the nature of the strains allow their manipulation without representing a danger for human or animal's health.

Indeed, while some fungal species are useful for industrial processes other ones, known as toxigenic strains, can produce toxic secondary metabolites called mycotoxins.

Several of these fungal strains are susceptible to colonize crops and feed products contaminating the matrix with their toxin and compromising food safety.

Even if the final target of fungal contamination involves health's issues, this problem starts at earlier agricultural steps. In fact, losses of food commodities contaminated with mycotoxins represent above the 25% of spoiled food in the world (FAO, 2003). Although the economic costs are impossible to be exactly estimated, the Food and Drug Administration (FDA) has evaluated that, for the United States, these losses can exceed the $900 million per year (CAST report, 2003).

In addition to this, mycotoxin contamination is an affair that goes beyond economical issues since to date, their regulation in some countries is still until under consideration. Unfortunately, for some developing countries, allowed levels of mycotoxins remain

higher than others or in the most preoccupying situations, they are not controlled by stipulated regulations.

Based on the above facts, it is understood that the study of fungal toxic metabolites is an important issue since it represents not only a sanitary and economic problem but also, an agricultural challenge for the 21st century.

Considering the fact that human population is estimated to increase to 9.1 billion people in 2050, ensuring safe food supply is a main concern that must be taken in consideration. Under this perspective, research has the engagement to develop effective strategies to control fungal contamination and more important, mycotoxin production.

3.2.1 Mycotoxin Definition

The term "mycotoxin" is reserved to toxic chemical compounds produced by several fungal species that may colonize crops and contaminate them with toxins either in the field or after harvest (Turner *et al.,* 2009).

Mycotoxins are produced by filamentous microfungi that cause diseases in vertebrates when ingested, absorbed through skin or inhaled (Frisvad *et al.,* 2007). Contrary to primary metabolites, mycotoxin production is not essential for fungal growth processes and thus, these compounds are considered as secondary metabolites (Drew and Demain, 1977).

During years, the production of mycotoxins was believed to occurs only at the idiophase fungal stage (Dutton, 1988). Nevertheless, recent discoveries cast doubt of when and why these compounds are produced by fungi. As recent propositions, they are highly supposed to serve as fungal defense skills giving a competitive advantage to the producer. It is also believed that they form part of the reproductive fungal processes or serve as interspecies competition (Thippeswamy *et al.,* 2014; Vaishnav and Demain, 2011; Magan and Aldred, 2007).

Mycotoxins are low weight compounds (<1000 Daltons) that are mainly produced by the fungal genus *Aspergillus*, *Penicillum, Fusarium, Alternaria* and *Claviceps.*

To date, more than 400 mycotoxins have been discovered but according to the exisiting knowledge, it is usually admitted that about 30 of them are of great importance for human and animal's health. This consideration is based on their toxicity and/or prevalence in foods and feeds (Iram *et al.,* 2016; AFSSA report, 2009).

Mycotoxins are very diverse in terms of chemical structure and their toxicity has to be considered as a separate special issue for each of them. Indeed, toxicity of mycotoxins not only depends on their chemical nature but also on the metabolic pathway that they can induce after ingestion by human or animals. In fact, after their ingestion, these compounds can also be transformed *in vivo* into new toxic metabolites due to the metabolisation process (Benkerroum, 2016).

3.2.2 Bookmark of mycotoxin's effects in human's health

Undoubtedly, the presence of opportunistic molds dates to millions years ago. Back in the history, there are early references of fungal contaminants signs and thus, effects of mycotoxins in human existence. It might be "the noxious pustule in the ear of grain" that was described on Assyrian cuneiform tablets or the "grasses that cause abortion of pregnant womans" described in the sacred Parsees books. It is believed that this observation came from the contaminated rye, which led to civilizations such as Greek and Roman to stop its consumption (Lapinskas, 2007). It was not until the first sounded mycotoxicosis case in humans, called at the time "St. Anthony's fire" and caused by ergot alkaloids, that we have a documented register of mycotoxin effects in humans. This phenomenon was in fact, the result of the reintroduction of rye in Europe which caused outbreaks of ergotism and attired the attention of middle-age artists who capture in their paints, persons suffering of this disease (Figure 1). Since then, an important list of human accidents caused by several mycotoxins has been identified and registered.

Figure 1: Ergotism Art Illustration; **A:** "Victims of ergotism" by Peter Bruegel and **B:**"Saint Anthony Tormented by Demons" by Matthias Grünewald.

For instance, in 1891 "yellow rice disease" was identified in Japan and was demonstrated to be caused by citrinin consumption, a mycotoxin produced by some *Penicillium* species. Another outbreak was also reported in the ancient USSR between 1930 and 1940 with a disease called aleukia that was due to the trichothecenes of *Fusarium* species (Peraica *et al.,* 1999).
Latter, in the 90's decades, another mycotoxicosis case occurred due to the contamination of food with fumonisins that affected Indian people (Bhat *et al.,* 1997).

Concerning the incidents caused by the *Aspergillus* species, during the 70's, two outbreaks of contaminated maize with Aflatoxins were reported in India. The first one resulted in the intoxication of 994 persons where 97 of them died and the second one involved 400 people where more than 100 persons did not survived (Krishnamachari, 1975; Peraica *et al.,* 1999).
More recently, in 2004, another case involving Aflatoxins occurred in Kenya resulting in the intoxication of 317 persons among which 125 deceased. It has to be noted that Kenya is, unfortunately, one of the most impacted countries due to mycotoxins and that several cases of intoxication have been already registered (Lewis *et al.,* 2005; Accinelli *et al.*2014; Perrone *et al.,* 2014).

Within the last years in Europe and during the period of February to March 2013, countries as Serbia, Croatia and Romania also reported milk's contamination with AFM1, which is an hepatic metabolite from Aflatoxin B1 (De Saeger *et al.,* 2016).

As demonstrated, mycotoxin occurrence and their intoxication cases have been reported during the last decades causing irreparable human loses. This reinforce the importance of surveying mycotoxin's occurrence in food and feed commodities in order to prevent the mycotoxin exposure at long-term as well as fatal incidents.

3.2.3 Mycotoxin occurrence in Food

According to this, several research organisms survey the ocurrence of the major mycotoxins in the world.

In fact, these types of contaminants can be present in a wide gamma of products such as fruits, vegetables, milk, grains, coffee, etc. Nevertheless, within the products designated to human consumption, one of the most contaminated matrixes concern cereals and especially maize and wheat which represent the stapple food for many countries.

Within the mycotoxins' classification, 7 major groups are regularly under surveillance. These groups are listed in Table 1 compiling their corresponding producing species, the most frequently contaminated products and their deleterious effects.

Table 1: Major mycotoxins and corresponding producing species, most frequent source and effects (AFSSA, 2009; CAST, 2003; Krska and Crews, 2008; Bbosa, 2013).

Mycotoxin	Type	Genus	Mainly Producers	Contaminated products	Effects
Aflatoxins	B1, B2, G1, G2, M1	*Aspergillus*	*Aspergillus flavus*, *A. parasiticus* *A. nomius* for AFB1 and *Aspergillii* from the *Flavi* section from Aflatoxin in general	Maize, wheat, sorghum, rice, soybean, pearl millet, sunflower,tree nuts, almonds, pistachio, coconut, milk cotton, dried fruits, spices, peppers	Hepatoxic Carcinonogenic Immunotoxic Teratogenic
Trichothecenes	Deoxynivalenol	*Fusarium*	*F. graminearum, F. culmorum, F. sporotrichoides, F. langsethiae, F. tricinictum, F. poae, F. solani, F. equiseti*	Cereals: wheat, maize, rice and sorghum	Immunotoxic Digestive problems Haematopoietic
	T-2 Toxin and HT-2		*F. tricinctum, F. langsthiae, F. sporotrichioides, F. poae F. equiseti*	Cereals: wheat, maize, rice, soy, beans and barley	Genotoxic Immunotoxic Reprotoxic Neurotoxic
Fumonisins	B1, B2, B3	*Fusarium*	*F. verticillioides, F. proliferatum*	Cereals: maize, rice and sorghum	Carcinogenic Neurotoxic
Ochratoxin	A	*Penicillium*	*P. verrucosum*	Cereals, Cacao, coffee, wine, grape juice and spices	Nephrotoxic Immunotoxic Teratogenic
		Aspergillus	*A. ochraceus, A. carbonarius*		
Zearalenone	F-2 Toxin	*Fusarium*	*F. graminearum, F. culmorum, F. crookwellense*	Cereals: maize, sorghum, soy, wheat, rice and oat	Reprotoxic Immunotoxic
Citrinin		*Penicillum*	*Penicilium citrinum*	Stored grains	Nephrotoxic
Patulin		*Penicillum*	*P. expansum*	Apples and pears as well as derivate juices	Neurotoxic Genotoxic Cytotoxic
		Aspergillus	*A. clavatus*		
		Byssochlamys	*B. nivea*		
Ergot Alkaloids		*Claviceps*	*C. purpurea, C. paspali, C. Africana, C. fusiformis*	Rye, wheat and triticale	Neurotoxic Digestive problems Vasoconstriction

In addition to this, it is thus understood that the detection and quantification of these major mycotoxins has to be updated within the years in order to measure their occurrence in food commodities.

For that, several surveys have been performed and Figure 2 shows the results of one of the latest studies published in 2015.

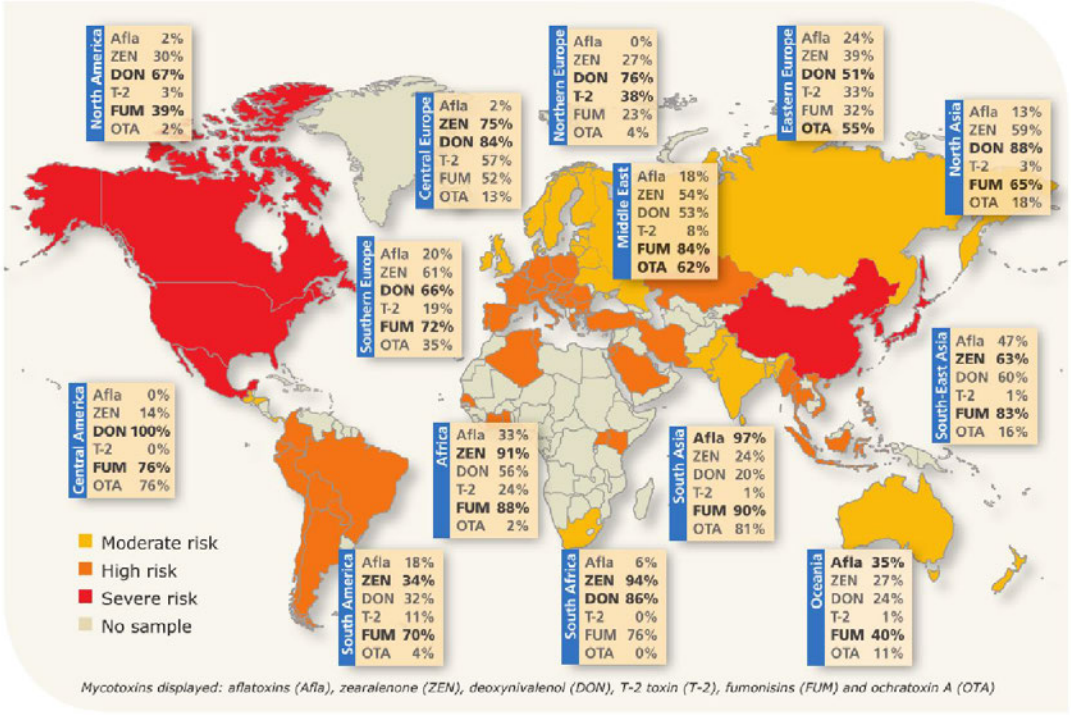


Figure 2: Global distribution of the principal mycotoxins reported by Biomin, (2015).

Afla, Aflatoxins; ZEN, Zearalenone; DON, Deoxynivalenol; T-2, T-2 Toxin, FUM, Fumonisins and OTA, Ochratoxins.
Low risk indicates that average levels of single mycotoxin presence of a given zone do not exceed minimum risk thresholds for livestock. The average level does not preclude specific, severe instances of mycotoxin contamination in farm or fields locally, nor does it account for the negative impacts of multiple mycotoxins presence. Moderate risk indicates the presence of one to two major mycotoxins at levels known to cause harm in animals. High risk indicates the presence of three to four major mycotoxins at levels known to cause harm in animals. Severe risk indicates the presence of five or more major mycotoxins at levels known to cause harm in animals.

Between the years 2004 and 2011, another important worldwide study was performed by Streit *et al.,* (2013) which determined the presence of Aflatoxins (AF), Zearalenone (ZEN), Deoxynivalenol (DON), Fumonisins (FUM) and Ochratoxins (OTA) by analyzing 17,316 feed samples.

Results showed that among the positive samples, 72% of them were contaminated with at least one mycotoxin and the remaining 38% were found to be multi-contaminated with several mycotoxins.

During the 8 years of the study, researchers advised that in general, a stable percentage of occurrences were observed for all mycotoxins with the exception of Aflatoxins. In fact, a raised in Aflatoxin's detection was observed between the years 2005 to 2009 in some tropical countries.

This fact constitutes an alarming signal since within the major mycotoxins, Aflatoxins are one of the most dangerous groups due to their effect on human's and animal's health (WHO, 1998).

In fact, Aflatoxins are also known to be potent mutagenic, teratogenic, hepatotoxic, immunosuppressive as well as they inhibit several metabolic systems (IARC Monograph, 1993; Minto and Townsend, 1997).

Within the Aflatoxins family, Aflatoxin B1 (AFB1) is classified in Group 1 by the International Agency for Research on Cancer (IARC) since demonstrated as "Carcinogenic" for humans and animals.

In consequence, to date Aflatoxins and specially AFB1, are subject of many researches since they are considered as a major health problem. According to this, a especial attention will given to this group in the next sections.

3.3 Aflatoxins: A major public health issue

3.3.1 Discovery

Aflatoxins were discovered above 50 years ago due to a famous acute animal poisoning initially named "Turkey X disease" that occurred in England (Blound, 1961). This case led to the intoxication and death of above 100,000 turkey poults that ingested Brazilian groundnuts cake (*Arachis hypogaea*). Although turkey's death was mainly attributed to Aflatoxins presence, further studies suggested that the clinical observations in animals also included the effect of cyclopiazonic acid, another mycotoxin potentially produced by the same fungal species (Cole, 1986; Bradburn *et al.,* 1994). This hypothesis was strengthen with the isolation from contaminated feed of an *Aspergillus flavus* strain that

effectively produces both toxic secondary metabolites (Amare and Keller, 2014). Since then, these toxins were subsequently named Aflatoxins: A (for *Aspergillus*) fla (for *flavus*) toxins.

Turkey X disease was certainly the most sounded case of intoxication in poultry but within the same time-frame, other cases of animal poisoning were also reported (Asplin and Carnaghan, 1961).

In fact, the term mycotoxin was stablished just after the "Turkey X disease" case marking the start of the "mycotoxin gold rush era" between the years 1960 and 1975 (Maggon *et al.,* 1977).

The mycotoxin term included a number of previously known fungal toxins (*e.g.,* ergot alkaloids), other compounds that were originally considered as antibiotics (*e.g.,* patulin) and a number of new toxic secondary metabolites (Bennett, 2003). Additionally, in the 60's decade, one of the first experiments with aflatoxin-contaminated meals was performed in ducks and rapidly reported as cause of liver-tumors (Carnaghan, 1965). This report led to an important number of experiments that were performed in order to elucidate the effects of Aflatoxins in vertebrates.

3.3.2 Aflatoxins chemical properties

In general, Aflatoxins are difuranocoumarin derivatives formed by two furans and a coumarin ring. They are very stable in long-term storage and heat-resistant molecules with denaturation temperatures overpassing the 200°C which make their elimination a real challenge for food industry (*e.g.* denaturation temperatures for Aflatoxin B1: 268-269°C) (Hayes and Forsythe, 1998; I.A.R.C. Monograph, 2002). They are highly liposoluble compounds and thus, soluble in moderately polar solvent such as chloroform, methanol and dimethyl sulfoxide while they are only slightly soluble in water (10–20 mg/L) (Jalili, 2015).

To date, even if nearly of 20 aflatoxins have been described (*e.g*., P1, Q1 ,B2a, G2a, D1, B3), only 4 of them are produced by fungi and correspond to: Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1) and Aflatoxin G2 (AFG2) (Ashiq *et al.,* 2014). The abbreviations of these major Aflatoxins are described by the AF prefix indicating their Aflatoxin's nature, the numbers 1 and 2 indicating major and minor compounds

and B or G due to their natural blue or green fluorescence under ultraviolet light (Dalvi, 1986).

In terms of structure, B-types Aflatoxins have a cyclopentenone ring that is replaced by a lactone in G-series (Figure 3).

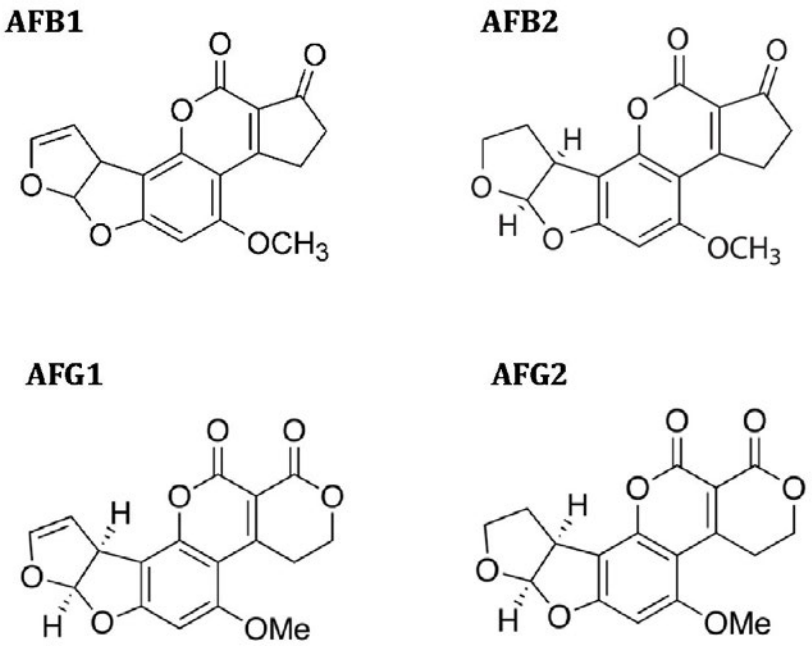


Figure 3: Structures of the 4 mould-produced aflatoxins

3.3.3 The genus *Aspergillus*

Aflatoxins B1, B2, G1 and G2 are produced by a group of filamentous fungi classified in the *Aspergillus* genus and mainly produced by several species belonging to the *Flavi* section.

The *Aspergillus* name was firstly established in 1729 by the priest and biologist Pier Micheli to design molds with conidial heads and stalks which literally reminded to an aspergillum: a liturgical device used to sprinkle holy water (Varga and Samson, 2008).

The opportunistic molds that produce Aflatoxins can grow on minimal media containing only nitrate and sucrose as only source of substrate, but they can also grow on complex media such as cereals, oil crops, beans and peas among others (Frisvad *et al.,* 2007).

The classification of *Aspergillus* species is taxonomically organized into sections and subgenera according to their morphology and teleomorph relationships.

In general, fungi classification and sub-divisions has been subject of debate within the last 200 years and they can vary depending on authors. In order to classify the Aflatoxin producers, we now present a simplified classification of the *Aspergillus* species based on the above works: Hawksworth, Sutton and Ainsworth, (1983); Griffin, (1994); Geiser *et al.,* (2008); Galagan *et al.,* (2005) and O'Brien *et al.,* (2005) and represented in Figure 4.

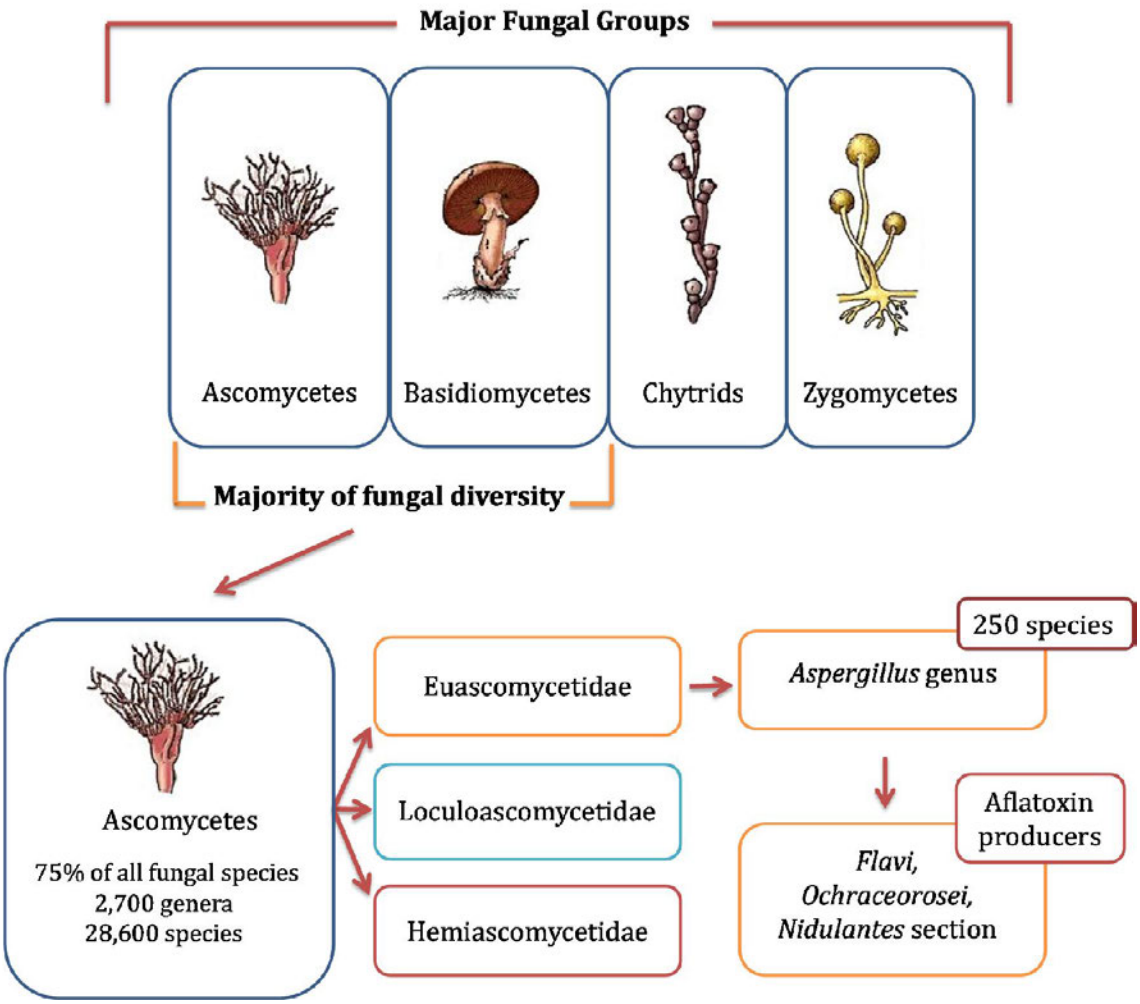


Figure 4: Taxonomic classification of the Aflatoxin producers.

3.3.4 The Aflatoxin B1 producers

Within the *Aspergillus* genus, at least 20 species belonging mostly to the *Flavi*, *Nidulantes* and *Ochraceorosei* sections are capable to produce different types of Aflatoxins and they are listed in Table 2 (Varga *et al.,* 2009; Soares *et al.,* 2012).

Table 2: Aflatoxin producers belonging to the *Flavi, Nidulantes* and *Ochraceorosei* sections. Adapted from Varga, (2009; 2011) and Soares *et al.,* (2012).

	AFB1	AFB2	AFG1	AFG2
Aspergillus* section *Flavi				
A. arachidicola				
A. bombycis				
A. flavus				
A. minisclerotigenes				
A. nomius				
A. novoparsiticus				
A. parasiticus				
A. parvisclerotigenus				
A. pseudocaelatus				
A. pseudonomius				
A. pseudotamarii				
A. togoensis				
A. transmontanensis				
A. mottae				
A. sergii				
Aspergillus* section *Ochraceorosei				
A. ochraceoroseus				
A. rambellii				
Aspergillus* section *Nidulantes				
A. astellatus				
A. olivicola				
A. venezeulensis				

In general, the ideal temperature for Aflatoxins production is of 29-30°C and their production significantly decrease at temperatures below of 25°C as well as it is inhibited at 37 °C or above. In terms of fungal development, water activity (A_w) below of 0.85 decrease fungal growth and sporulation while those factors are completely inhibited at A_w values of 0.75 (Bhatnagar *et al.,* 2006).

In crops, Aflatoxin B1 production is mainly produced by a co-occurrence of *Aspergillus flavus (A. flavus)* and *Aspergillus parasiticus (A. parasiticus)* strains (Payne and Brown, 1998; Baranyi *et al.,* 2015). In order to better show the characteristics of both species, their aspects are shown in Figures 5 and 6.

In terms of morphology, the colony appearance of *Aspergillus flavus* is floccose in the center with white mycelium (Figure 5). Macro and microscopic characteristics include species with conidial heads in shades of yellow-green with biserate columnar or radiates, globose vesicle, rough conidiophores with globose conidia and reddish-brown to black spherical sclerotia (Smith *et al.,* 2008b).

For species that produce sclerotia, a classification depending on their size is divided in: L strains (average sclerotia >400 µm) or S strains (average sclerotia <400 µm) (Balter, 2006; Perrone *et al.,* 2014).

Otherwise, colonies of *Aspergillus parasiticus strain* (Figure 6) are dark green and more floccose that those of *Aspergillus flavus* in MEA medium. Their conidiophores are colorless, variable in size (300-700 µm) and their wall is smooth rough in the distal part. The vesicles are globular (20-35 µm) and phialides are mostly uniseriate (>90%). Conidies are very echinulate (3.5-5.5 pm) (Samson *et al.,* 2010).

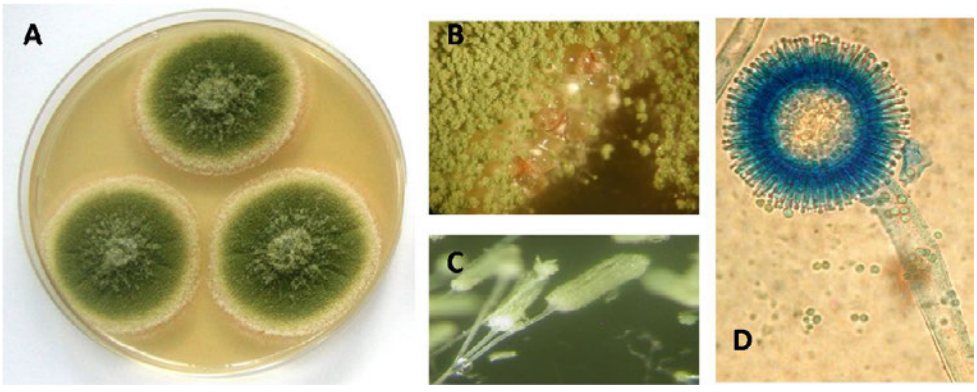


Figure 5: Macroscopic and microscopic aspect of a classical *Aspergillus flavus* strain cultured in Malt Extract Agar (MEA). **A**: Macroscopic aspect; **B**: Binocular perspective of basal mycelium with sclerotias; **C**: Aerial mycelium heads; **D**: Conidiophore with biseriate sub-globose vesicle (*Photos S. Bailly*).

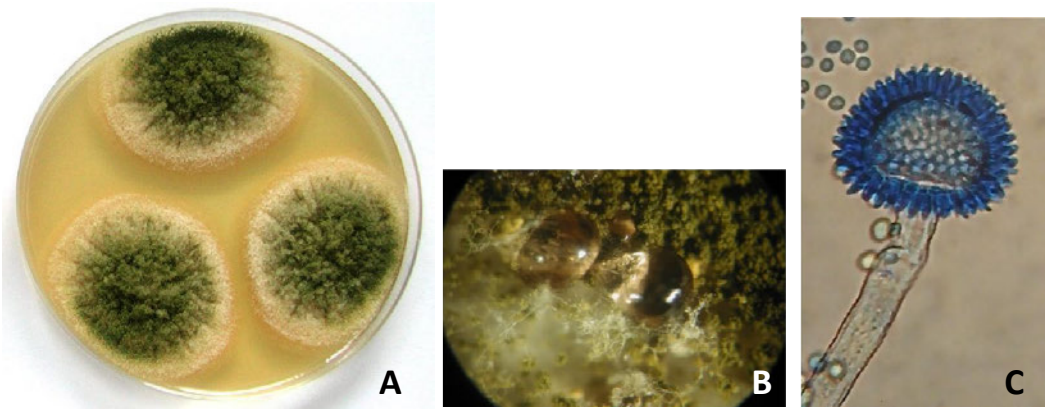


Figure 6: Macroscopic and microscopic aspect of *Aspergillus parasiticus* strain, cultured on Malt Extract Agar (MEA). **A**: macroscopic aspect; **B**: Binocular perspective of basal mycelium with sclerotias; **C:** Microscopic image of a classic conidiophore with uniseriate globose vesicle (*Photos S. Bailly*).

The development of the latter species is only mildly influenced by nutritional composition of substrates and they are potentially able to grow in many foods and feeds. Temperature and water activity of the substrates represent the principal parameters influencing the development and toxigenesis of *Aspergillii* of the *Flavi* section.

Such physiological requirements explain why Aflatoxins are the major contaminants in tropical and subtropical regions where mean climatic conditions are often very favorable for both, fungal development and toxigenesis.

In addition to this, developing countries that present these climate conditions have a weak or miss in mycotoxins regulations leading to permissible limits of Aflatoxins consumption (Benkerroum, 2016).

In addition to this, and as it will be described in the next section, climate conditions are now changing which represent an important problem regarding mycotoxin's contamination.

3.3.5 Effect of climate change in Aflatoxin's occurrence

In the decades to come, one of the factors that will likely have a great effect on mycotoxin contamination is global climate change. Indeed, most of the aflatoxigenic fungal species can be easily developed in tropical and sub-tropical regions exposing human populations to related health hazard.

It is the case in many developing countries belonging to Latin America, Africa and Asia where above 500 million of people are chronically exposed to exceeded normative levels of aflatoxins (Moy and Miller, 2016).

Due to climate change, this problematic could be extended to other regions considered as free until now, as is the case of Europe (Baranyi *et al.,* 2015).

Taking in consideration the expected raise of +2°C hypothesized for the European region as well as changes in CO_2 levels and higher levels of drought in the next years, preventive strategies have to be adapted to cope aflatoxin's contamination (Botana and Sainz, 2015; Tollefson, 2015).

Indeed, with this panoramic it is considered that among all mycotoxins, the most likely to increase in the near future will be the Aflatoxins (Wu and Mitchell, 2016).

As a demostration of this fact and mentioned in the 3.2.3 section, Aflatoxins' concentrations rose in several countries between the years 2005 and 2009. This increment was principally observed in tropical areas but it was also related to more frequent climatic aberrations in other regions (Streit *et al.,* 2013). For instance, in the North of Italy, a recent outbreak of Aflatoxin B1 contamination in maize was already registered (Perrone *et al.,* 2014).

In order to prevent this contamination, several precautions have been already taken and a recent study using computer-modeling simulation to predict AFB1's contamination in European maize and wheat crops was performed. In the latter study, an estimation for the next 100 years considering two climate scenarios of +2°C and +5°C was taken in consideration.

Results showed that in both conditions, the most concerned areas that will be highly exposed to Aflatoxin contamination are: Eastern Europe, Balkan Peninsula and the Mediterranean regions (Battilani *et al.,* 2016).

Taking in consideration AFB1's occurrence in temperate countries, as well as its potential to increase in other ones, this mycotoxin deserves a special attention as one of the most preoccupying issues.

In order to better control its incidence it is thus necessary to understand how this fungal metabolite is synthesized and which fungal mechanisms are involved in this process.

From now, fungal genetic mechanisms involved in AFB1 production as well as their interaction with the corresponding enzymatic cascade leading to toxin production will be presented.

3.3.6 Aflatoxin B1 toxicity

-*Aflatoxins regulations*

Because of AFB1's toxicity, more than 100 countries regulate the levels of Aflatoxins in the food and feed supplies (Van Egmond *et al.,* 2007). In general, levels for food intended for human vary from 2-30 µg of Aflatoxin/kg of food depending on matrix, region and country. For instance, the limit imposed by the US Food and Drug Administration (FDA) stipulate a 20 ppb limit (parts per billion in µg/kg equivalent) for total Aflatoxin amount on food or feed substrate and this amount is a common limit for many commodities in most of the countries (Yu, 2012; Zain, 2011).

Otherwise, the European Commission is stricter regarding to Aflatoxin's control according to the Directive 200/32/EC (Mai, 2002) and Commission (EC) No. 1881/2006 (December, 2006).

In this regulation and regarding AFB1, it is stipulated a maximal concentration between 2-5 ppb for cereals; 2, 5 or 8 ppb (depending on transformation processes) for groundnuts and dried fruits; 5 ppb for spices and 0.1 ppb for food commodities and cereals intended to children.

- *Aflatoxin toxicity*

Aflatoxin toxicity depends on the amount of toxin ingested and on the duration of the exposure. In addition to this, gender, age, tolerance and health conditions of animals/humans are also important factors that have to be taken in consideration.

In fact, Aflatoxin B1 toxicity can vary depending on animal susceptibility; while sheep, rats and dogs are highly sensible, monkey, chicken, mice and humans are more resistant (Bbosa *et al.,* 2013).

In humans, AFB1 can lead to numerous diseases depending on doses and exposure time. Taking in consideration these parameters, two major types of toxicity can be distinguished:

1) Acute
2) Chronic which includes: Teratogenic, Carcinogenic and Mutagenic effects.

1. - *Acute toxicity or Aflatoxicosis*:

Acute toxic accidents in which high levels of AFB1 are ingested cause intoxication symptoms such as jaundice, vomiting, abdominal pain and liver failure with documented fatality rates as high as 40% (Centers for Disease Control and Prevention, 2004).
For instance, in the episode of Aflatoxicosis occurred in India in the 70's where at least 100 persons died, the consumption of Aflatoxins in a single day was calculated to be among 2-6 mg. Since then, the lethal dose for adult humans was stipulated between 10-20 mg (Bennett, 2003).
Nowadays, aflatoxicosis is rarely observed because of the sanitary regulations and preventive methods to avoid food contamination. Nevertheless, these kinds of intoxication accidents still occur and some of them are related to the fact that even if food commodities are contaminated, they represent the only source of available food which raises the risk of intoxication resulting in human death.

2. - *Chronic Toxicity:*

This type of toxicity appears in case of prolonged exposure to low or moderate amounts of AFB1. In humans, such exposure can be revealed using biological markers such as blood, urine and milk samples in which the presence of Aflatoxin metabolites (AFM1), AFB1-albumin or other derivate products can be detected (Williams *et al.,* 2004). A chronic exposure to AFB1 can lead to the suppression of humoral and cell immunity, malabsorption of nutrients that in consequence lead to nutritional deficiencies such as malnutrition, stunted growth, kwashiorkor and marasmus diseases in children, infertility in men and interference with various endocrine glands responsible for the synthesis of various hormones (Stack and Carlson, 2003; Bbosa *et al.,* 2013).

-*Teratogenic effect:*

AFB1 can transgress the placenta barrier and thus, contaminate the fetus *in utero* associated with congenital malformations. Strong evidence indicates that transplacental transfer of AFB1 in humans may also increase the risk of childhood cancer (Wangikar *et al.,* 2007).

-*Mutagenic and Carcinogenic effects*:

Aflatoxin B1 is among the existing mycotoxins, the most potent pre-carcinogenic molecule. Once this toxin is inside the human body, the molecule is transformed *in vivo* and can be highly associated with an increase incidence in Hepatocellular Carcinoma (HCC). In addition to this, HCC occurrence is highly associated with the Hepatitis B virus since both are synergists in humans. Patients that are seropositive for Hepatitis B and that are highly exposed to Aflatoxin B1 are 10-fold more susceptible to develop HCC (Smela *et al.,* 2001).

The toxic *in vivo* process is triggered by consumption of contaminated products.
After entering to the body, the AFB1 is absorbed across the intestine membranes, reaches blood circulation and arrives to the liver system.
Once in the liver, AFB1 is metabolized by the human cytochrome P-450 system (principally by CYP3A4 and CYP1A2) at the 8,9-vinyl bond to produce an unstable intermediate compound identified as AFB1-exo-8,9-epoxide (Wu *et al.,* 2013).
The transformation of AFB1 into a exo-8,9-epoxide occurs by an structural change that is highlighted with orange cadres in Figure 7 (Wan and Hsieh, 1980).

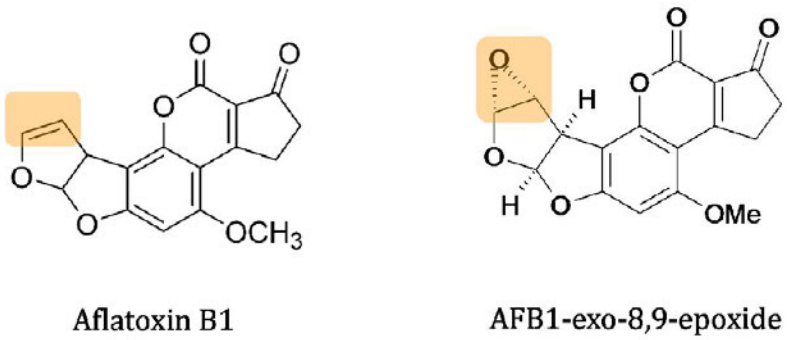


Figure 7: Aflatoxin B1 structure and its transformation to exo-8,9-epoxide

Once the AFB1-exo-8,9-epoxide is formed, it can be covalently bounded to different cellular components:

- to proteins in order to form AFB1-albumin and other protein adducts (Bbosa *et al.,* 2013; Bedard and Massey, 2006; Wu and Santella, 2012).

- to DNA in order to form AFB1-guanine adducts:
The formation of guanine adducts can lead to HCC in humans. In fact, these compounds can bind to DNA specifically to the sequence of the *p53* gene encoding for a tumor-suppressor factor (Hsu *et al.,* 1991). The DNA binding occurs in the codon 249 where an induction of G→T mutations occurs and that is the principal marker in patients that developed HCC due to AFB1 (Bressac *et al.,* 1991). The graphical process is described in Figure 8.

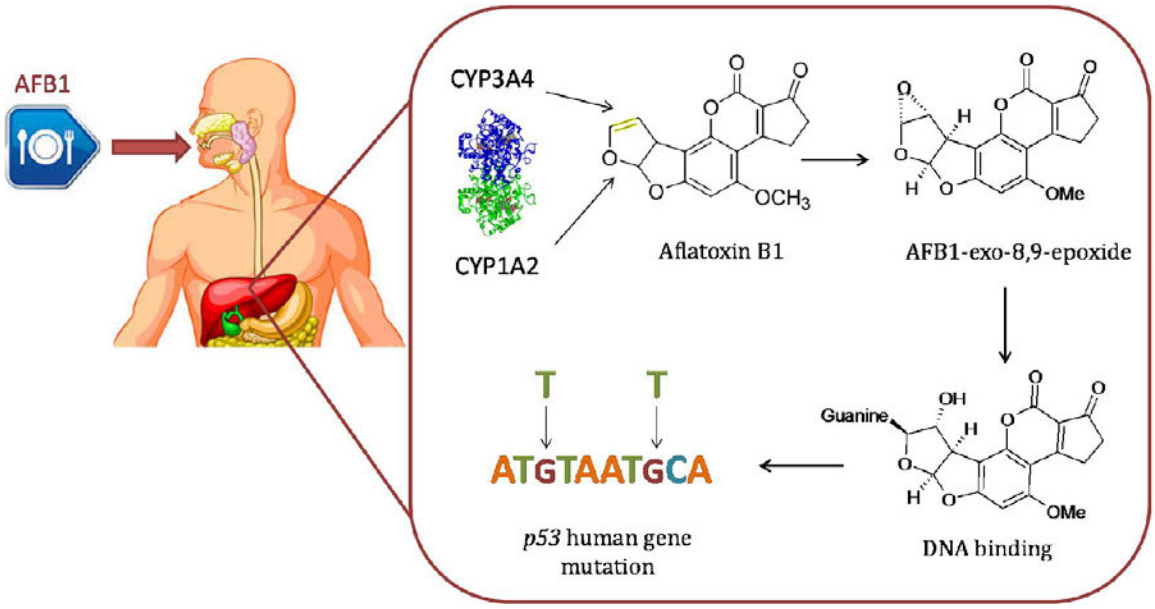


Figure 8: Biotransformation of AFB1 molecule *in vivo.*

In addition to this, AFB1 can also be metabolized into a hydroxylated metabolite known as AFM1. The M denomination comes from the fact that this compound can be excreted in mammals' fluids such as Milk but it can also be detected in urine, bile and human feces. As a member of Aflatoxins' group, AFM1 presents similarities with the other Aflatoxins such as a high heat resistance, making of this molecule resistant to pasteurization processes and other food treatments (Vidal *et al.,* 2013).

To conclude, AFB1 toxicity depends of intake conditions where the amounts and exposure time will determine the impact of damage in human or animals' health.

3.4 Aflatoxin B1 Biosynthetic Pathway

3.4.1 Description of the Aflatoxin B1 Gene Cluster

Genetic information of fungal secondary metabolites is usually (but not always) contained in clusters that can span in average more than 10,000 bases (Brakhage, 2012). A cluster is a group of genes (2 or more) that are in charge to synthesize one product, and for the present purpose, secondary metabolites such as AFB1.

In *Aspergillus* genus, the DNA information is contained in a total of 8 chromosomes (Robinow and Caten, 1969). Within this organization, the enzymatic Aflatoxins' pathway is genetically took in charge by the 54th gene cluster localized at 80 kb away from telomere of the chromosome 3 (Georgianna and Payne, 2009). This cluster of 75 kB includes 30 genes and its activation is principally regulated by two principal genes named *aflR* and *aflS* (Figure 9) (Yu, 2012; Chang, 2003).

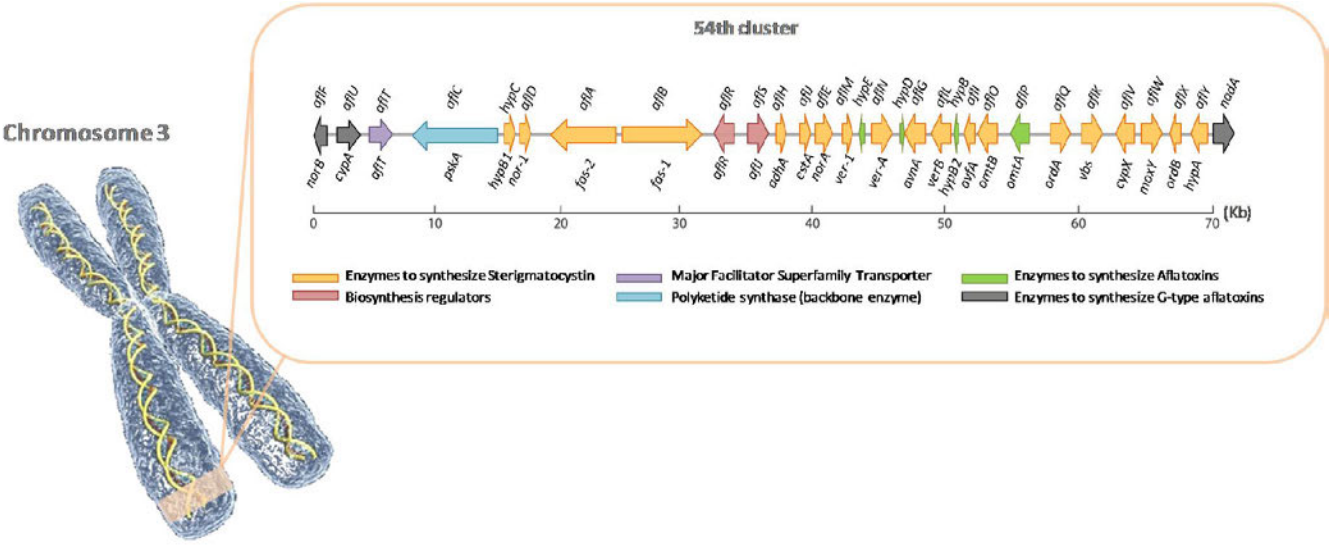


Figure 9: Aflatoxin gene cluster localization and organization adapted from (Amaike and Keller, 2011; Chang *et al.,* 2004; Ehrlich, 2009).

The Aflatoxin gene cluster has been widely studied in several *Aspergillus* strains such as *Aspergillus flavus* and *Aspergillus parasiticus* since these fungal strains are the mainly producers of AFB1 (Jiujiang *et al.,* 1995; Yu *et al.,* 2004a). Nevertheless, this cluster is also highly studied in the sterigmatocystin producer strain *A. nidulans,* due to the fact

that this species shares 25 homologous genes of the AFB1 cluster and also to the fact that sterigmatocystin is the latter metabolite precursor in the AFB1's biosynthetic pathway (Ehrlich, 2009).
In fact, the homology of the clustered genes between *A. parasiticus* and *A. flavus* is about 90–99%, while between the *A. parasiticus* and *A. nidulans* is of 55–75% (Yu *et al.,* 2000b). In terms of involved genes, there exists a difference between biosynthesis of Aflatoxins B-type from the G-type. This difference consists due to the fact that three within the AFB1 genes participate only for G-type aflatoxins' formation and they are namely *aflU, aflF* and *nadA* (Ehrlich *et al.,* 2004b; Ehrlich *et al.,* 2008). The first one corresponds to a cytochrome P450 monooxygenase, the second one to an aryl alcohol dehydrogenase and the third one is a reductase. Due to the fact that a special attention will be only given to the AFB1 production, these three genes will be not discussed.

Since the performance of DNA technologies, the elucidation of the role that each gene has in AFB1 production has been mostly performed by the use of molecular techniques such as Gene cloning, Chromosome walking and Expressed Sequence Tags (EST).
The development of those techniques allowed an important number of experiments that elucidated the predicted roles of genes belonging to the AFB1 cluster and that participate in the enzymatic cascade that is responsible for toxin synthesis.

3.4.2 Enzymatic Cascade Pathway of Aflatoxin B1

AFB1 is produced by a polyketide pathway proposed by Birch in 1976 and nowadays, at least 27 enzymatic reactions have been demonstrated to be involved in this process.
It is considered that, among the natural products, Aflatoxin's biosynthesis is one of the longer and complex process due to the quantity of oxidative rearrangements (Minto and Townsend, 1997). Within this pathway there are three critical oxygen elements that were characterized by Dutton (1988):

i) Monooxygenases: in charge to incorporate one oxygen atom to another atom being reduced by a Nicotinamide Adenine Dinucleotide Phosphate (NADPH).
ii) Dioxygenases: often involved in ring-cleavage reactions.
iii) Baeyer-Villiger reactions: in charge to insert oxygen atoms between two carbons.

Within the same enzymatic pathway, the cytochromes P450 also play an important role. These enzymes are absolutely required to make the backbone of ring structures by attaching functional groups (methyl, acetyl, etc.) and thus, inducing oxidative reactions leading to secondary metabolites biosynthesis (Nelson, 2011). It is important to note that the aflatoxin gene cluster contains the highest number of cytochromes P450 (at least seven) among the pathways that are in charge of produced secondary metabolites (Roze *et al.,* 2015).

Figure 10 shows a synthesized schema of the pathways leading to the four aflatoxins produced by fungi (AFB1; AFB2; AFG1 and AFG2) where some of the intermediate metabolites leading to final toxin productions are presented.

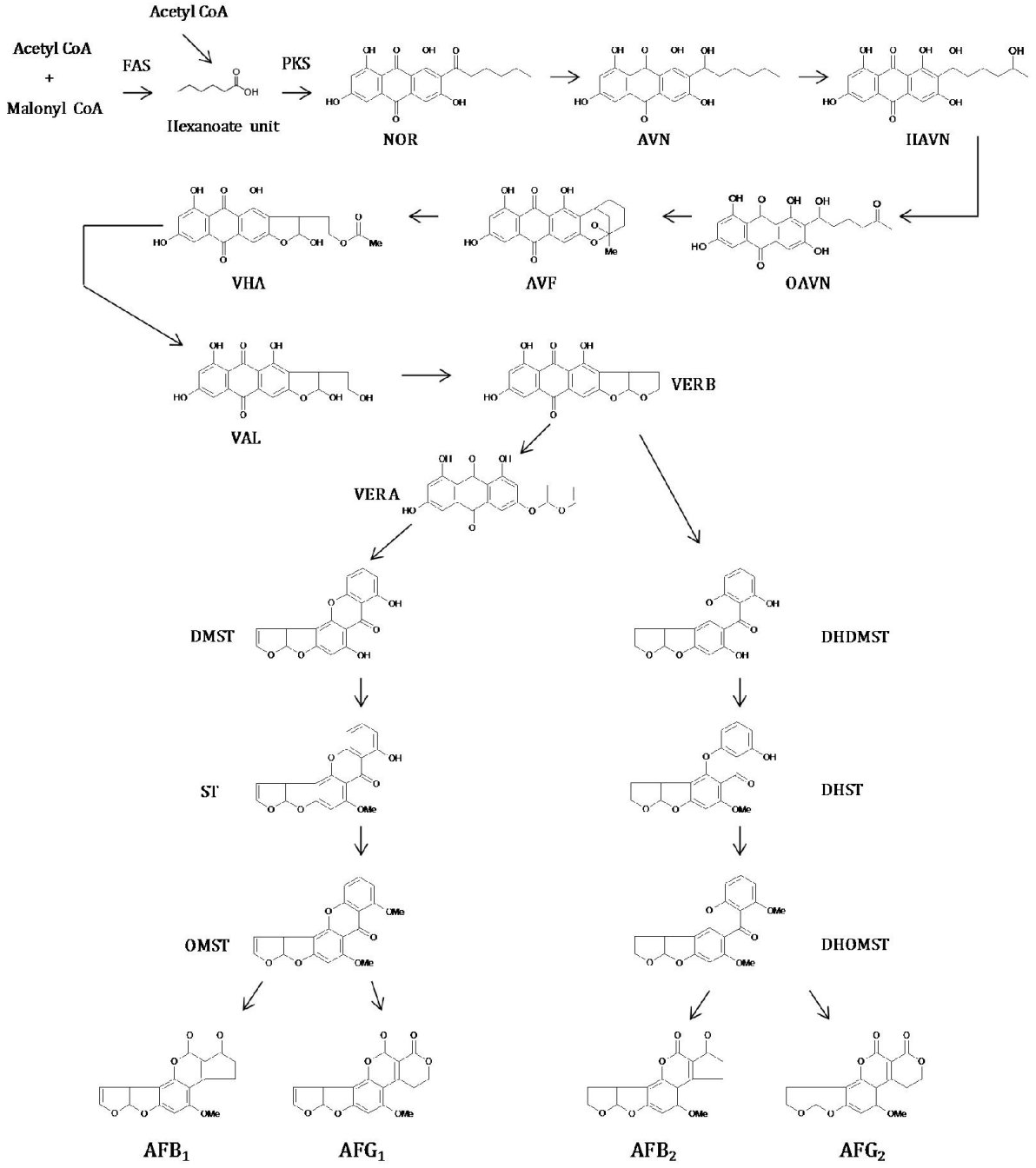


Figure 10: Synthesized schema of Aflatoxins' Biosynthesis

Abbreviations: FAS, Fatty acid synthase; PKS, Polyketide synthase; NOR, Norsolorinic acid; AVN, Averantin; HAVN, 5'-hydroxy-averantin; OAVN, 5'-Oxoaverantin; AVF, averufin; VHA, Versiconal Hemiacetal acetate; VAL, Versiconal; VERB, Versicolorin B; VERA, Versicolorin A, DMST, Demethylsterigmatocystin; DHDMST, Dihydrodemethyl-sterigmatocystin; ST, Sterigmatocystin; DHST, Dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, Dihydro-O-methylsterigmatocystin; AFB1, Aflatoxin B1; AFG1, Aflatoxin G1; AFB2, Aflatoxin B2; AFG2, Aflatoxin G2.

Since only the AFB1 pathway will be described, the different steps involved in its biosynthesis as well as the corresponding genes will be further described in detail.
In order to follow the continuity of the different steps of the pathway, figure 11 shows some of the stables metabolites that lead in chronological order to the AFB1 production.

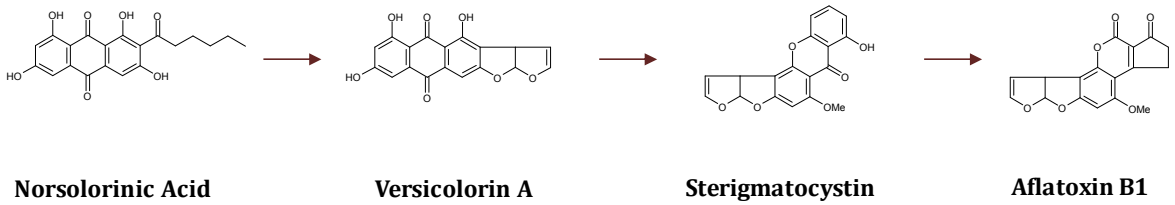


Figure 11: Stable metabolites of Aflatoxin B1 production.

Concerning the genetic information that will be presented, it has to be noted that the names of the genes coding for the AFB1 production have changed with the time and in order to identify them properly, latest names are firstly described while ancient names are in brackets. In addition to this, genes are indicated in italic letters while their corresponding synthesized proteins are not.

3.4.2.1 Conversion of Acetate into Norsolorinic Acid

Aflatoxins are polyketide-derivate that initially require the formation of hexanoate units (from acetyl-CoA and malonyl-CoA) to start an enzymatic cascade reaction (Roze *et al.,* 2013).
First steps in the pathway lead to the transformation from the starter unit hexanoate into the first stable metabolite: Norsolorinic acid (NOR). These reactions are principally encoded by four genes. The first two encoding for fatty acids synthases (*aflA*, *aflB*), the third one corresponding to a polyketide synthase (*aflC)* and the last one (*hypC*) coding for an anthrone oxidase (Ehrlich, 2009; Payne and Brown, 1998; Yu *et al.,* 2004b).

The latter genes will be further described in detail:

- ***aflA*** (*fas-2*) and ***aflB*** (*fas-1*) are one of the largest genes in the aflatoxin pathway and were formerly named "*fas"* since they code for fatty acid synthases. Their corresponding synthesized proteins are α and β sub-units that are principally in charge for the transformation of the hexanoate unit into a polyketide structure (Yu *et al.,* 2004; Roze *et al.,* 2013). These genes were characterized by Watanabe and Townsend (2002) demonstrating that their corresponding enzymes are absolutely necessary to synthesize Norsolorinic Acid (NOR).

- ***aflC*** (*pksA*) is a gene coding for the synthesis of polyketide skeletons. In general, secondary metabolites that are acetate-derivatives (as it is the case of Aflatoxin B1) present a chain elongation that is in charge of these polyketases (*e.g.* from 2ManonylCoA to 7 ManonylCoA).
In the fungal strain *A. parasiticus*, this enzyme has conserved regions with other fungal PKSs (*e.g. A. nidulans*) including β-ketoacyl synthase, acyltransferase, acyl carrier-protein and thioesterase domains (Chang *et al.,* 1995a). Disruption of *aflC* in *A. parasiticus,* resulted in the accumulation of Norsolorinic Acid with absence of AFB1 production and others aflatoxin intermediates compounds (Feng and Leonard, 1995).
This observation led to know that this polyketide synthase, as well as both genes coding for fatty acids, are key elements in the first steps of the AFB1 production.
Interestingly, it seems that *aflC* is an evolved gene of fatty acid synthases generated by gene duplication (Bennett and Christensen, 1983). Additionally to this, this enzyme is also involved in further transformations of the polyketide structure into Norsoloric Acid Anthrone (NAA) (Roze *et al.,* 2013).

- ***hypC*** (*hypB1*) is a gene located in the intergenic region between *aflC* and *aflD* and it has numerous homologous within the *Aspergillus* strains. This 17-kDa enzyme was demonstrated to be involved in the catalytic conversion of NAA into NOR (Ehrlich *et al.,* 2010a). Based on EST technique, *hypC* is expressed only in Aflatoxin conductive conditions. Disruption of the *hypC* gene in *A. parasiticus* resulted in an accumulation of Norsolorinic Acid and in lower levels of Aflatoxins (Ehrlich, 2009; Yu, 2012).

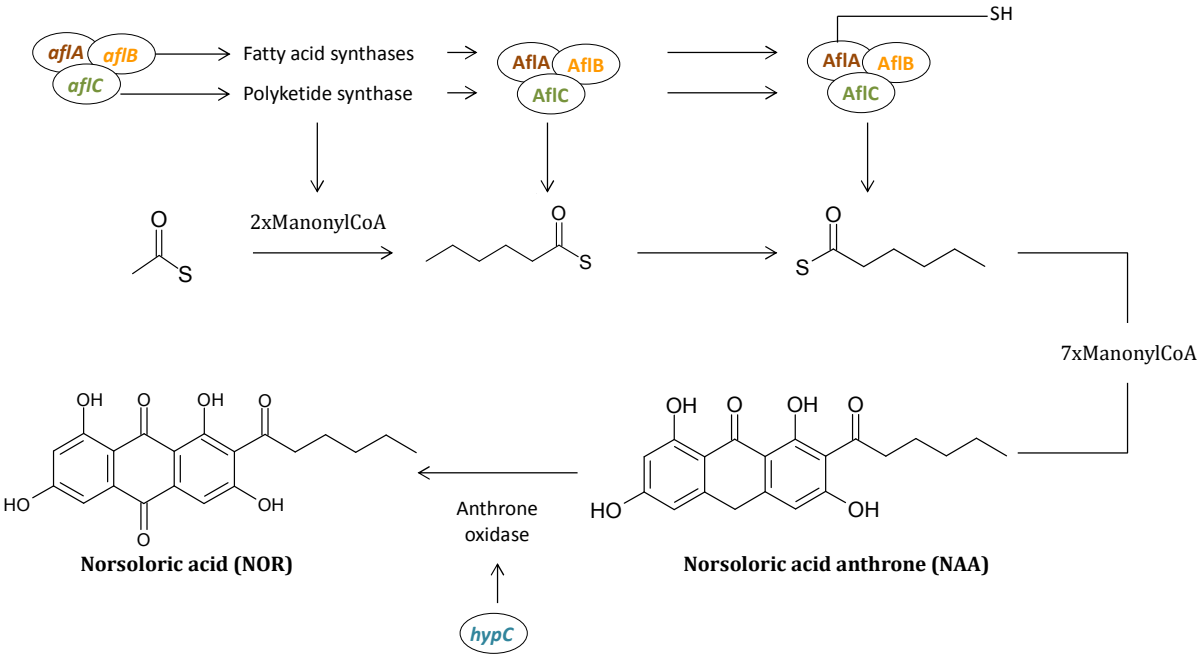


Figure 12: First steps of the Aflatoxin enzymatic cascade coded by *aflA*, *aflB*, *aflC* and *hypC* adapted from Ehrlich (2009).

3.4.2.2 Conversion of Norsolorinic Acid into Averantin

In this step of the pathway, Norsolorinic acid is further transformed into Averantin (AVN) and this transformation is governed by the *aflD* gene (Dutton, 1988). For years, the implication of two other genes (*aflE* and *aflF*) was associated in this step. Nevertheless, new evidence of their implication in other steps within the AFB1 biosynthesis changed this idea and these genes will be described latter.

- ***aflD*** (*nor-1*) is a norsolorinic acid ketoreductase needed for the conversion of the 1'-keto group in NOR to the 1'-hydroxyl group of AVN (Zhou and Linz, 1999). It was firstly cloned by Papa (1982) in an *A. flavus* strain and to date, it is known that null *nor-1* mutants still synthesize lower amounts of aflatoxins but accumulate higher amounts of NOR (Yabe *et al.,* 1991).

Several factors can modulate *aflD* expression. Indeed, its transcription can be stimulated in the presence of glucose and recent studies also hypothesized that a new protein (p32) could be intrinsically involved in the Nor-1 promoter region and contribute to its

regulation (Roze *et al.,* 2004). Localization of the Nor-1 protein using fluorescent probes, demonstrated that this protein mainly occurs in the cytoplasm and vacuoles (Hong and Linz, 2009).

3.4.2.3 Conversion of Averantin into Averufin

In the 90's, Yabe *et al.*(1991) identified in an *A. parasiticus* strain, two additional enzymatic steps involved in the conversion of Averantin into Averufin (AVF). It firstly involved the intervention of a monooxygenase to convert AVN into 5'hydroxyaverantin (HAVN) and secondly, of a dehydrogenase to transforms HAVN into Averufin.
Even if the transformation between HAVN into AVF was firstly proposed to be in one step, Sakuno *et al.,* (2003) discovered that whitin this process there exists an intermediate compound identified as 5'-oxoaverantin (OAVN). With this study not only the transformation of OAVN into AVF was discovered, but also a "novel enzyme" in charge of this process was reported.
In parallel, the genes in charge of the above enzymatic processes were identified and nowadays are known as: *aflG*, *aflH* and *aflK*.

- ***aflG*** (*avnA*) encodes a cytochrome P-450 monooxygenase that catalyzes the hydroxylation of the polyketide anthraquinone averantin, into 5'hydroxyaverantin (Yu *et al.,* 1997; Cary *et al.,* 2000b; Yu *et al.,* 2004a). This enzyme belongs to a superfamily of seven P-450 monooxygenases intervening in the AF pathway (Roze *et al.,* 2015).

- ***aflH*** (*adhA*) previously called *adhA* since it codes for an alcohol dehydrogenase, is needed for the conversion of HAVN to OAVN. Disruption of this gene resulted in a strain that accumulates HAVN thus, explaining it essential presence to further steps in the aflatoxin pathway (Chang *et al.,* 2000d). Even if almost all genes belonging to AF cluster are homologous within the *Aspergillus* species that produce AFB1, this is the exception since the *aflH* gene in *A. flavus* and the *adhA* gene in *A. parasiticus,* do not share significant homology at either DNA or amino acid levels (Yu, 2012).

- ***aflK*** (*vbs*) which is mainly associated to the Versiconal to Versicolorin B conversion (further steps in the biosynthetic pathway) was identified as the "novel enzyme"

required to transform OAVN into AVF that was reported by Sakuno *et al.,* (2003). This research group not only elucidated the presence of an intermediate between HAVN and AVF but also, two years later, identified this enzyme as the cyclase coded by the *vbs* gene (*aflK*). Until now, this report is the first to demonstrate that the same enzyme can catalyze two different reactions within the AFB1 pathway. They hypothesized that this strange phenomenon could be due to an evolution of the AFB1 gene cluster that previously had 2 copies for the *aflK* gene (Sakuno *et al.,* 2005).

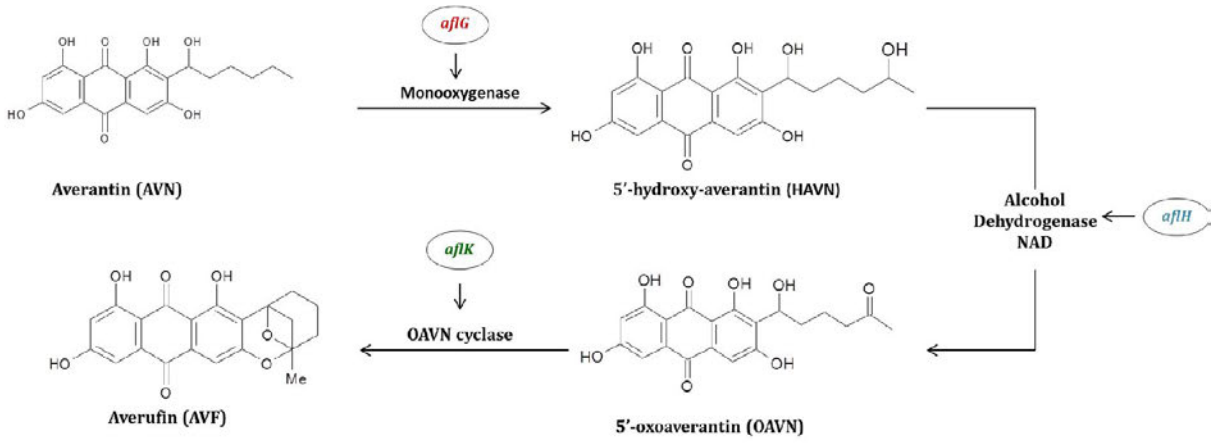


Figure 13: Conversion of Averantin into Averufin coded by *aflG*, *aflH*, and *aflK* and adapted from Yabe *et al.,* 1991 and Sakuno *et al.,* (2005).

3.4.2.4 Conversion from Averufin into Versiconal Hemiacetal Acetate

Transition of Averufin into Versiconal hemiacetal acetate (VHA) is due to the intervention of three genes: *aflI*, *aflV* and *aflW*. The first two ones, being P450 cytochromes, are likely involved in the ring-closure step of hydroxyversicolorone (HVN) while *aflW* coding for a monooxygenase transforms HVN to VHA (Wen *et al.,* 2005; Yu, 2012).

- ***aflI*** (*avfA*) is a gene encoding for an enzyme that is involved in the transformation of AVF into versiconal hemiacetal acetate and that is present in both, *A. parasiticus* and *A. flavus* (Yu *et al.,* 2000b). Deletion of the *aflI* gene led to accumulation of Averufin (AVF) and it is mainly supposed that this enzyme catalyzes the ring-closure step during

formation of hydroxyversicolorone (HVN). Nevertheless, the precise role for AflI in the oxidation of AVF has never been exactly determined (Ehrlich, 2009).

- ***aflV*** *(cypX)* and *aflW* (*moxY*) are two genes that have been mostly characterized by Wen *et al.,* (2005) and Yu *et al.,* (2004a). *aflV* (monooxygenase) has been confirmed to catalyze the reaction from AVF to HVN while *aflW* was also confirmed to catalyzed the transformation from HVN to VHA by a Baeyer-Villiger reaction. In *A. parasiticus* both genes were separately deleted resulting in a lack of AFB1 production.

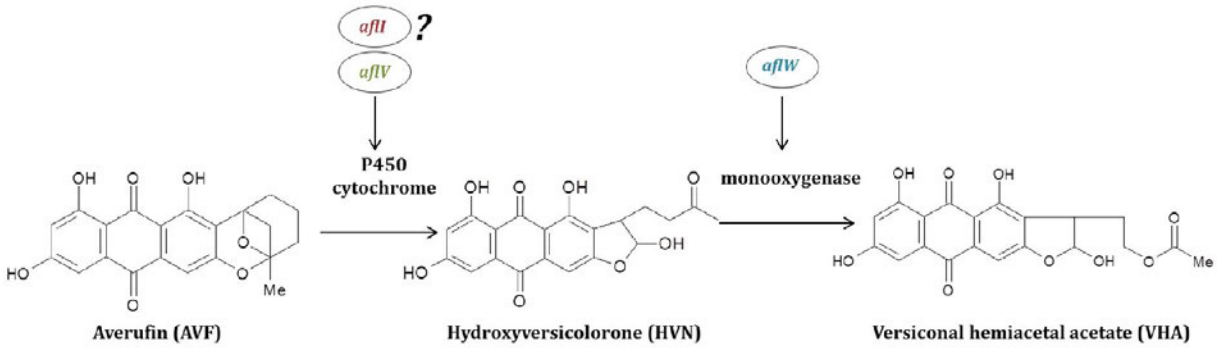


Figure 14: Conversion of Averufin into Versiconal Hemiacetal Acetate adapted from Ehlrich, (2009) and Wen *et al.,* (2005).

3.4.2.5 Conversion from Versiconal Hemiacetal Acetate into Versicolorin B

Transformation from VHA into Versiconal (VAL) is in charge of the gene *aflJ* and then, *aflK* intervenes to transform this latter into Versicolorin B (VERB) (Yu *et al.,* 2004b).

- ***aflJ*** *(estA)* encodes for an esterase that was firstly purified in *A. parasiticus.* Its deletion results in the accumulation of two compounds: VHA and Versicolorin A (Hsieh *et al.,* 1989; Chang *et al.,* 2004a). Several esterase activities are associated with the conversion of VHA into other intermediates, but only *aflI* belongs to the aflatoxin gene cluster. Its involvement in the conversion of VHA to VAL was finally characterized by Yabe *et al.,*

(2003) and Chang *et al.,* (2004a) in *A. parasiticus,* allowing the elucidation of the chemical reactions involved in this step.

With this characterization, not only this part of the AFB1 pathway was elucidated, but this research group also reported the reversible transformation of VHA and VAL into Versiconol Acetate (VOAc) and Versiconol (VOH) respectively and demonstrated that *aflJ* catalyzes both reactions during AFB1 biosynthesis.

- ***aflK*** (*vbs*) is a gene encoding for a cyclase intervening during the transformation of VAL into VERB but also, as already mentioned, for previous steps in the pathway to transform OAVN into AVF.

Concerning its intervention in this part of the pathway, this enzyme represents a critical step since it is in charge of the closure of the bisfuran ring of aflatoxin that is required to bind to DNA and that give to Aflatoxins their mutagenic effect (Yu *et al.,* 2004b).

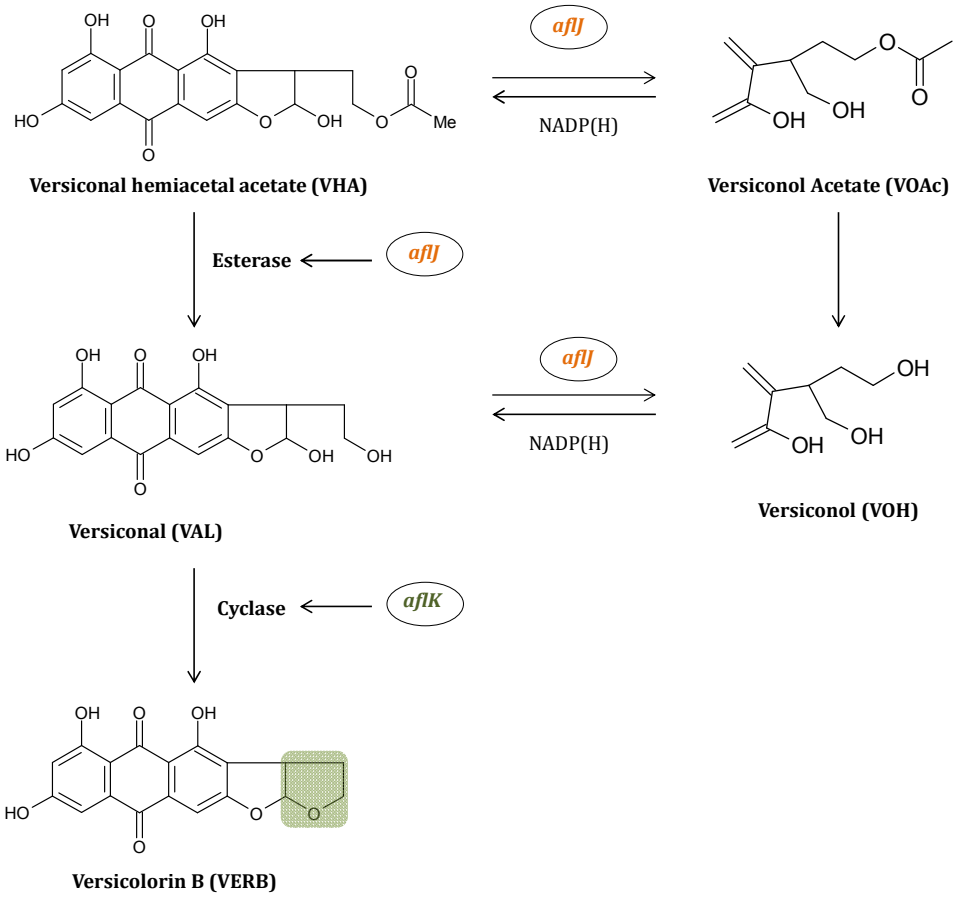


Figure 15: Conversion of Versiconal Hemiacetal Acetate into Versicolorin B adapted from Yabe *et al.,* (2003), Chang *et al.,* (2004a) and Yu *et al.,* (2004b). Green cadre represents the closure of the bisfuran-ring present in aflatoxin structures.

3.4.2.6 Conversion from Versicolorin B into Demethylsterigmatocystin

The transition from VERB into Versicolorin A (VERA) is coded by *aflL* while transformation of VERA into Demethylsterigmatocystin (DMST) is coded by 4 other genes: *aflM*, *aflN, aflY* and *aflX* (Cary *et al.,* 2006).

- ***aflL*** (*verB*) codes for a cytochrome P450 monooxygenase/desaturase which converts VERB into VERA. The main characteristic of this enzyme is that it represents the first and principal branch leading to the formation to B or G-types aflatoxins (Figure 10) (Yabe *et al.,* 1991). The *aflL* gene is present in both, *A. parasiticus* and *A. flavus* gene clusters (Yu, 2012).

- ***aflM*** (*ver-1*) coding for an enzyme during the middle steps of the aflatoxin biosynthetic pathway, was predicted to encode a ketoreductase involved in the conversion of VERA into DMST. Its involvement in aflatoxin pathway was firstly identified and cloned in the *A. parasiticus* genome (Liang *et al.,* 1996, Skory *et al.,* 1992; Trail *et al.,* 1995; Mahanti *et al.,* 1996) and its chemical characterization was widely described by Henry and Townsend (2005). Its homologous gene in *A. nidulans* was also identified as *stcU* by Keller *et al.,* (1994). Recent studies demonstrated that this gene contains CAMP-Response Element sites (CRE) that are also present in genes coding for fungal oxidative stress response (Hong *et al.,* 2013).

- ***aflN*** (*verA*) codes for a cytochrome P450-type monooxygenase and although its exact function is yet to be determined, it seems to work in parallel with *aflM* for the transformation of VERA. Interestingly, even if they seem to work together, no significant sequence homology between both at either DNA or amino acid levels was identified (Yu, *et al.,* 2004b). In 2005, Ehrlich *et al.,* suggested that this protein could be involved in the passage of VERA into an hypothetical intermediate and in 2006, Cary *et al.,* confirmed the same observation.
This gene was also identified as essential for sterigmatocystin (ST) production in *A. nidulans* since its disruption resulted in the inability to convert VERA into ST (Keller *et al.,* 1994).

- ***aflY*** (*hypA*) is one of the AFB1 genes that has been recently described. Its involvement in aflatoxin enzymatic pathway was characterized in *A. parasiticus* and it seems most likely to intervene between two hypothetical intermediates structures among the transformation of VERA into DMST. In addition, this transformation seems to be catalyzed by a Baeyer-Villiger reaction (Cary *et al.,* 2006; Ehrlich *et al.,* 2005).
Disrupted strains for this gene accumulated VERA, being the principal reason to include this gene within the VERB-DMST group. To date, no *aflY* gene homolog has been already identified in the *A. nidulans* gene cluster (Yu *et al.,* 2004b).

- ***aflX*** (*ordB*) was characterized by Cary *et al.,* (2006). It encodes for an oxido-reductase most likely involved in a ring-opening rearrangement of the epoxide produced by the VERA. Nevertheless, further studies demonstrated that this gene catalyzes the oxidative decarboxylation and ring-closure of the Baeyer-Villiger intermediate that results from AflY-catalyzed oxidation. The disruption of *aflX* in *Aspergillus flavus* resulted in accumulation of VERA and reduced amounts of aflatoxin (Ehrlich, 2009).

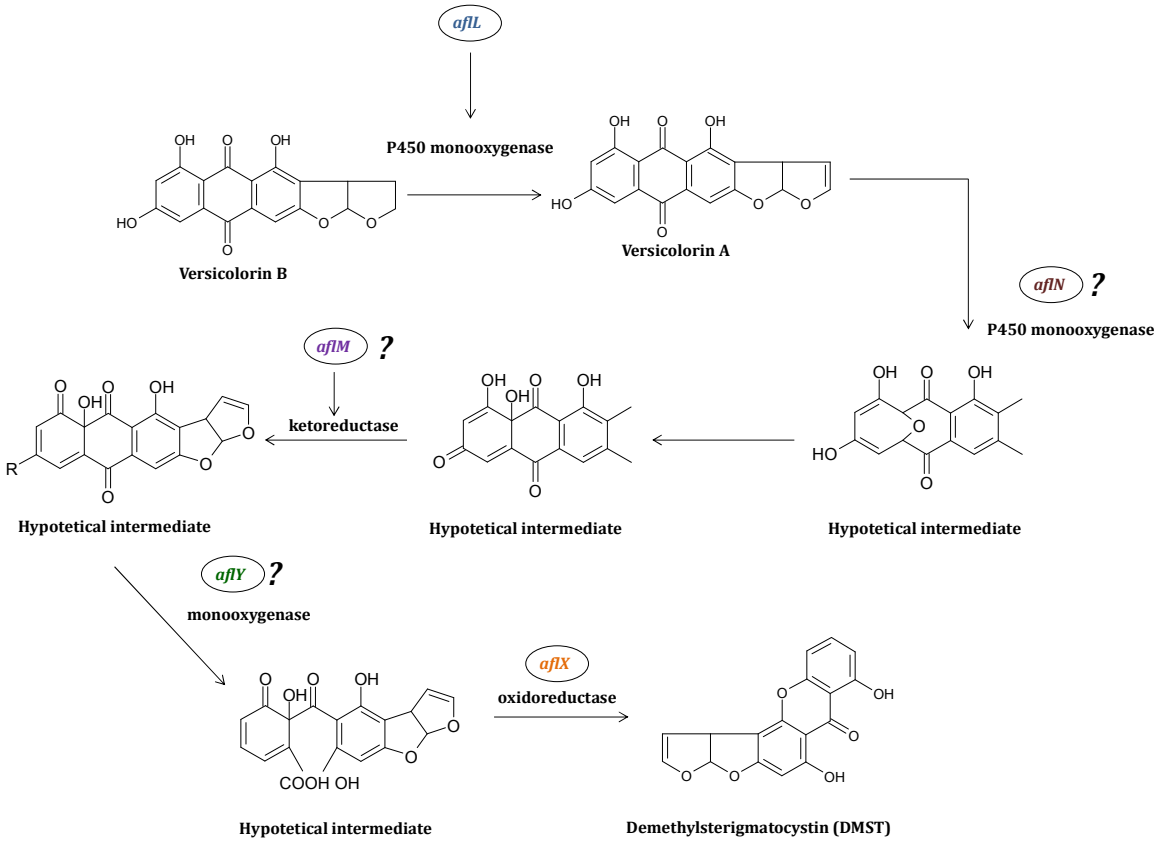


Figure 16: Schematic representation of the enzymatic transformation from Versicolorin B into Demethylsterigmatocystin including the corresponding hypothetical structures and genes involved. This schema is a compilation from information reported by Ehrlich, (2009), Henry and Townsend (2005), Cary *et al.,* (2006) and Ehrlich *et al.,* (2005).

3.4.2.7 Conversion from Demethylsterigmatocystin into *O*-methylsterigmatocystin

Final steps of the enzymatic AFB1 pathway corresponding from DMST into *O*-methylsterigmatocystin (OMST) are principally governed by two genes: *aflO* and *aflP.* Sterigmatocystin is, as previously mentioned, the late stable intermediate of AFB1 but it is also the final synthesized metabolite of *Aspergillus nidulans.*

- ***aflO*** (*omtB*) encoding for an *O*-methyltransferase, is involved in the conversion of DMST to Sterigmatocystin. This gene has been described in *A. parasiticus*, *A. flavus* and *A. nidulans* (*stcP*). It was also reported that in *A. parasiticus,* this gene is only expressed in

aflatoxin-conducive conditions (Yu *et al.*, 2000). The expression of *aflO* was analyzed in *A. flavus* and *A. parasiticus* strains, demonstrating a relationship between higher mRNA levels and increased AFB1 amounts (Jamali *et al.,* 2013; Scherm *et al.,* 2005)

- ***aflP*** (*omtA*) is the principal gene in charge of transforming ST into OMST. It corresponds to one of the enzymes identified in *A. parasiticus* as *O*-methyltransferases (Yabe *et al.,* 1989). This gene was initially named *omt-1*, then *omtA* and finally renamed *aflP* (Yu *et al.,* 1993). The corresponding homologue of *aflP* was also identified few years later in *A. flavus* (Yu *et al.,* 1995). *aflP* is also only expressed in aflatoxin permissive conditions (Scherm *et al.,* 2005; Yu *et al.,* 2000b) and it was hypothesized that *aflP* could play different roles than in the AF's enzymatic cascade and it is also suspected to be involved in fungal conidiation (Lee *et al.,* 2002a).

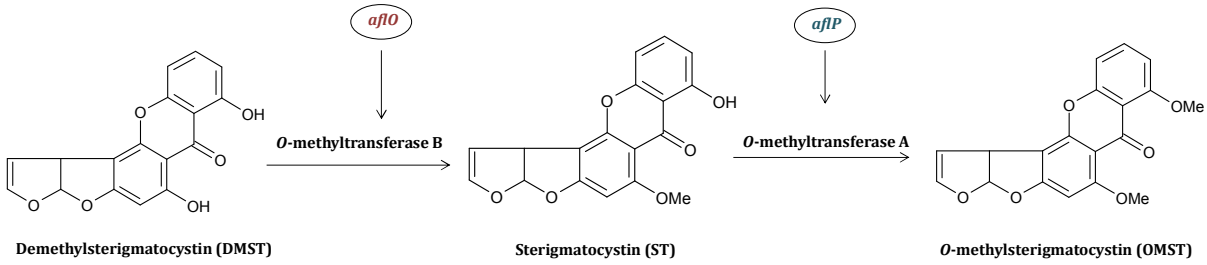


Figure 17: Conversion of Demethylsterigmatocystin into *O*-methylsterigmatocystin by the genes *aflO* and *aflP*.

3.4.2.8 Conversion from *O*-methylsterigmatocystin into AFB1

Finally, conversion of OMST into AFB1 is in charge of the genes: *aflQ*, *hypB*, *aflE* and *hypE*. Within this final transformation, the exact intervention of *aflQ* and *hypB* has been identified while for the other genes only some partial information is known.

- ***aflQ*** (*ordA*) is a gene adjacent to the *aflP* gene in the AFB1 cluster and codes for a P-450 monooxygenase. It was identified by chromosome walking and cloned in *A. parasiticus* demostrating that this gene is involved in the conversion of OMST into AFB1 by an oxidation of the A-ring of OMST (Yu *et al.,* 1998; Ehrlich *et al.,* 2008). This reaction led to an AFB1 precursor named 11-hydroxyOMST (HOMST) (Ehrlich, 2009; Zeng *et al.,* 2011). It was recently suggested that *aflQ* expression can be used to identify the aflatoxigenic *A.*

flavus strains since this gene (in experiments with contaminated pistachios) is not expressed when the amounts of AFB1 are lower than 30 ng/mg (Jamali *et al.,* 2013). The corresponding protein of OrdA can be also called CYP64 (Roze *et al.,* 2015).

- ***hypB*** (*hypB2*): is a gene coding for an oxidase involved in the second step of the transformation from 11' Hydroxy-*O*-methylsterigmatocystin (HOMST) into a 370 Da 7-ring lactone (later precursor of AFB1). The transformation of this latter lactone into another unknown intermediate compound could be done by hydrolytic enzymes encoded by genes that do not form part of the AF cluster (Ehrlich, 2009). Analysis of *hypB* sequenced by EST demonstrated that this gene is a homolog of the *hypC* gene (Yu and Ehrlich, 2011). Disruption of the *hypB* gene resulted in decreased amounts of Aflatoxins (Ehrlich *et al.,* 2009).

- ***aflE*** (*norA*) is a homologous gene of *aflD* in the aflatoxin cluster encoding for a short chain aryl alcohol dehydrogenase (Yu, 2012). First characterizations of *aflE* in *A. parasiticus* suggested that this gene, along with *aflF,* was involved in the transformation from NOR into AVN. At the time, this idea seemed to explain that the strains with an *aflD* deletion still produce low amounts of aflatoxins since it was believed that AflE and AflF could complement Nor-1's function (Cary *et al.,* 1996). However, further studies performed by Ehrlich *et al.,* (2009) in *A. flavus,* demonstrated that *aflE* is instead mainly involved in the two final steps of the AFB1 formation (even if the exact position has not been defined). Experiments performed with null *aflE* strains not only demonstrated its involvement in later steps of the AFB1 pathway, but also allowed the discovery of a minor toxin produced in *A. flavus.* In fact, *aflE* deletion resulted in reduced amounts of AFB1 but in increased quantities of a new identified metabolite: deoxyAFB1.

- ***hypE*** (*aflLa*) was identified in *Aspergillus flavus* by the EST technique. Disruption of this gene led to the accumulation of deoxyAFB1 (Holmes, 2008). Since the HypE protein conserves a catalytic ether domain, it is believed that it works together with another enzyme in the enzymatic pathway. This idea comes from the fact that in bacteria, proteins with such domain are known to work in parallel with cytochrome P450 oxidases. Since AflV and AflQ are P-450 cytochromes they were proposed to interact with HypE (Ehrlich, 2009).

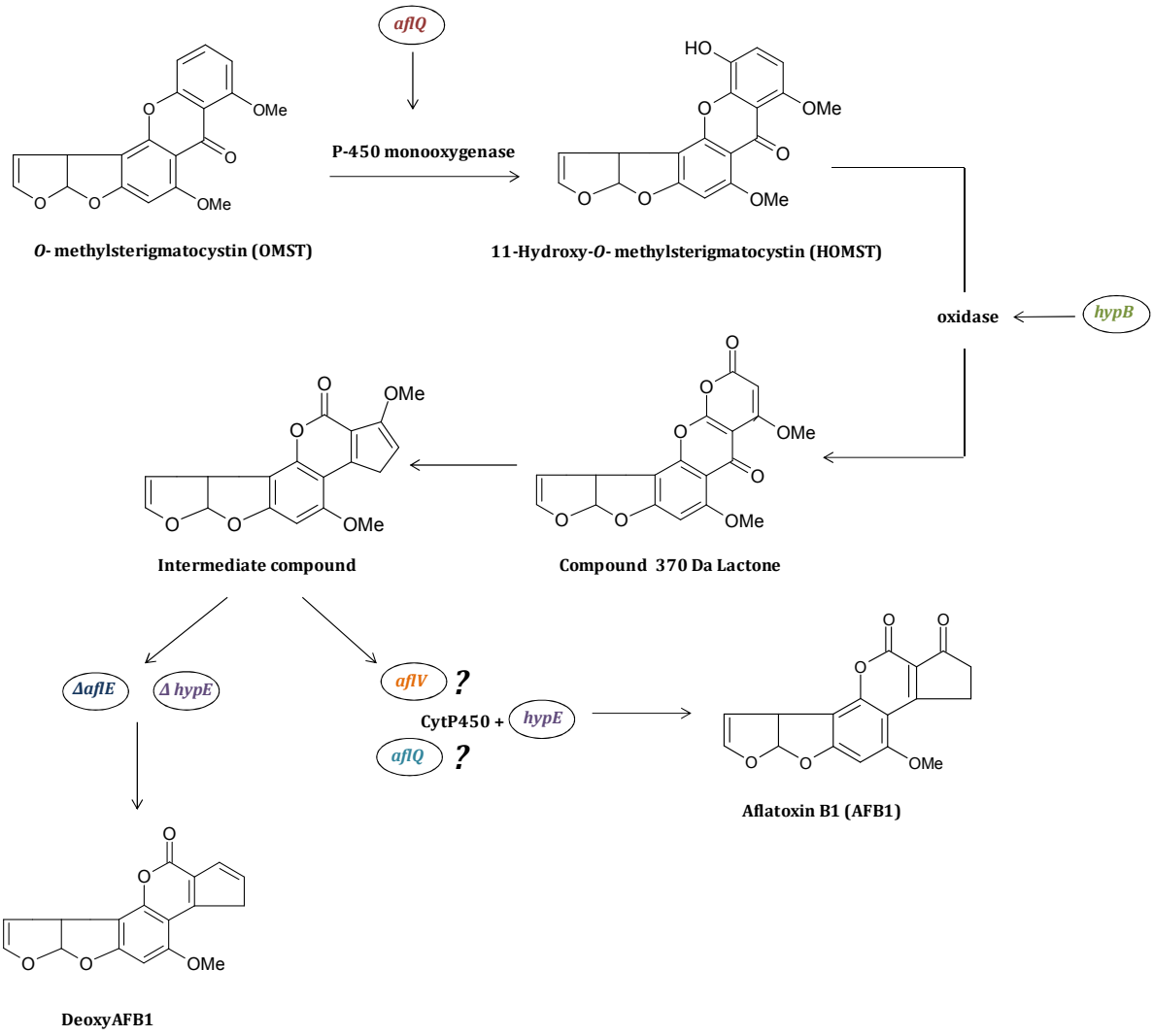


Figure 18: Last steps of AFB1 formation adapted from (Ehrlich *et al.,* 2008; Ehrlich, 2009 and Zeng *et al.,* 2011).

As demonstrated, AFB1 production is a long and complex cascade that involves, most of the times, oxygenated steps to its formation. Table 3 summarizes this process by presenting the identified genes involved, its ancient and recent name and the corresponding enzymes function that participates in each of the AFB1 steps transformations.

Gene	Ancient name	Enzyme	Involved step in AF enzymatic cascade
aflA	*fas-2*	fatty acid synthase α	manolyl-coA --> Norsoloric acid anthrone
aflB	*fas-1*	fatty acid synthase β	manolyl-coA --> Norsoloric acid anthrone
aflC	*pksA*	polyketide synthase	manolyl-coA --> Norsoloric acid anthrone
hypC	*hypB1*	anthrone oxidase	Norsoloric acid anthrone --> Norsolorinic Acid
aflD	*nor-1*	ketoreductase	Norsolorinic Acid --> Averantin
aflG	*avnA*	cytochrome P-450 monooxygenase	Averantin --> 5'hydroxy-averantin
aflH	*adhA*	alcohol dehydrogenase	5'hydroxy-averantin --> 5'oxoaverantin
aflK	*vbs*	cyclase	5'oxoaverantin --> Averufin
aflI	*avfA*	cytochrome P-450 monooxygenase	Averufin--> Hydroversicolorone ??
aflV	*cypX*	cytochrome P-450 monooxygenase	Averufin--> Hydroversicolorone
aflW	*moxY*	monooxygenase	Hydroversicolorone --> Versiconal Hemiacetal Acetate
aflJ	*estA*	Esterase enzyme	Versiconal Hemiacetal Acetate --> Versiconal Versiconal Hemiacetal Acetate --> Versiconol Acetate Versiconol --> Versiconal
aflK	*vbs*	cyclase	Versiconal --> Versicolorin B
aflL	*verB*	cytochrome P-450 monooxygenase	Versicolorin B --> Versicolorin A
aflM	*ver-1*	ketoreductase enzyme	Versicolorin A --> Demethylsterigmatocystin (unnassigned specific localisation)
aflN	*verA*	cytochrome P-450 monooxygenase	Versicolorin A --> Demethylsterigmatocystin (unnassigned specific localisation)
aflY	*hypA*	monooxygenase	Versicolorin A --> Demethylsterigmatocystin (unnassigned specific localisation)
aflX	*ordB*	oxidoreductase	Latter intermediate of Versicolorin A --> Demethylsterigmatocystin
aflO	*omtB*	*O* -methyltransferase B	Demethylsterigmatocystin --> Sterigmatocystin
aflP	*omtA*	*O* -methyltransferase A	Sterigmatocystin --> *O* -methylsterigmatocystin
aflQ	*ordA*	cytochrome P-450 monooxygenase	*O* -methylsterigmatocystin --> 11'Hydroxy-*O* -methylsterigmatocystin
hypB	*hypB2*	oxidase	11'Hydroxy-O-methylsterigmatocystin --> Compound 370 Da Lactone
aflE	*norA*	aryl alcohol dehydrogenase	Final steps of AFB1 formation (unnassigned specific localisation)
hypE	*aflLa*	oxidase	Intermediate compound--> Aflatoxin B1

Table 3: Recapitulative steps and involved genes of the AFB1 enzymatic cascade biosynthesis.

3.4.2.9 Other genes that are presumably not involved in aflatoxin enzymatic cascade but that are present in AFB1 cluster

Within the AFB1 gene cluster there exist other 2 genes for which their role is still unknown.

- ***aflT*** which is a gene coding for a fungal transporter belonging to the major facilitator superfamily (MFS). This gene was widely characterized by Chang *et al.,* (2004b) who demonstrated that even if this gene resides in the AFB1 gene cluster, its role is not linked to aflatoxin biosynthesis since its deletion in *A. parasiticus* does not affect final amounts of aflatoxin compared to control. These results were in agreement with the fact that *aflT* is neither regulated by principal activator of the AFB1 pathway AflR nor by its co-activator AflS.

Results also suggested that *aflT* could be instead regulated by another external regulatory factor, Fad-A, belonging to the G-protein signaling pathway. In the same study, experiments using the yeast *Saccharomyces cerevisiae* led authors to suggest that *aflT* could not be implicated in aflatoxin's transportation. Nevertheless, Chanda *et al.,* (2010) suggested that AflT could resides in the aflatoxisomes which are vesicles implicated in exocytose of aflatoxins. Taken together the exact role of *aflT* is today not clear and has to be elucidated.

- ***hypD*** (*aflNa*) codes for a 129 Da integral membrane binding protein but its role also remains unclear. Ehrlich (2009) characterized this protein in *A. parasiticus* by EST demonstrating that it conserves a DUF6 domain, which is common in several fungi and thus, it probably has an important role in fungi.
They also deleted this gene resulting in increased sporulated cultures with reduced levels of AFB1 production. Taking into consideration that no official transporter for aflatoxin was identified, HypD has been suggested as to be an AF efflux pump.

As shown in this section, AFB1 is the result of a coordinated enzymatic cascade where at least 20 enzymatic steps coded by genes belonging to the AFB1 gene cluster are involved. Whether or not they participate in AFB1 production, most of the genes

belonging to this pathway are regulated by two major internal regulators of the gene cluster *aflR* and *aflS* that will be further described.

3.4.3 Regulation of Aflatoxin B1 synthesis

Principal Regulators of AFB1 cluster

- *The AflR transcription factor*

In *A. flavus*, *A. parasiticus* and *A. nidulans,* the corresponding aflatoxin and sterigmatocystin biosynthetic pathways are mainly regulated by the *aflR* gene (Jiujiang *et al.,* 1995; Price *et al.,* 2006; Yu and Keller, 2005).
In *A. flavus* species, the AflR protein binds to at least 17 of the residing genes in the AF gene cluster which results in the activation of the enzymatic cascade leading to the different AFs production (Figure 19B) (Ehrlich, 2009).
AflR is also classified as a zinc cluster $Zn(II)_2Cys_6$ transcription factor of the Gal4-type family (Shimizu *et al.,* 2003).
This kind of transcription factors have a specific structure that is attributed only to fungi kingdom. They are capable to bind to DNA by using a DNA-binding domain (DBD) which is one of the most important elements in transcriptional and translational processes. In fact, this regulation process occurs in the nucleus; once there, the zinc finger proteins of AflR bind to DNA and it is believed that this binding occurs as a homodimer manner as it is shown in Figure 19 (A) (MacPherson *et al.,* 2006; Woloshuk *et al.,* 1994).

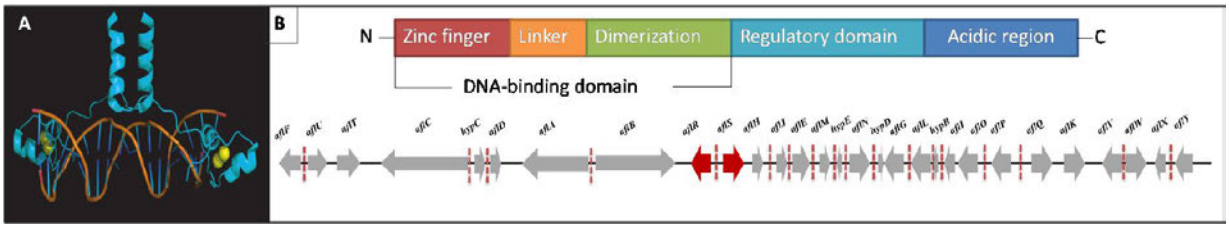


Figure 19: Representation of the AflR binding to DNA, its functional domains and regulated genes belonging to the aflatoxin cluster. **A)** Structure of a DNA-Binding Domain (DBD) $Zn(II)_2Cys_6$ regulator of the Gal4p family binding as homodimer. The yellow spheres represent both atoms of zinc. **B)** At the top, functional domains of the zinc cluster proteins. DBD domain is divided into three regions that correspond to DNA specific binding (Zinc finger); protein-DNA binding (Linker) and protein-protein interaction (Dimerization). In the bottom, aflatoxin gene cluster; the dotted red lines represent the binding sites of AflR in the above pathway. This figure is an adaptation from the original works reported by Ehrlich, (2009) and MacPherson *et al.,* (2006).

In particular, AflR was demonstrated to preferentially recognize the palindromic motif 5'-TCGN$_5$CGA-3'. Nevertheless, it also binds to others sequences such as 5'-TTAGGCCTAA-3' as well as in a lesser extend to the 5'-TCGCAGCCCGG-3' motif.
The AflR binding motifs were found to be located relatively close from the translation starting site from –80 to –600 bp with the majority of them at –100 to –200 bp (Ehrlich *et al.,* 1999b; Payne and Brown, 1998; Yu and Ehrlich, 2011).

Interestingly, AflR conserves within its own promoter the palindrome motif 5'-TTAGGCCTAA-3' suggesting that AflR, apart from being the principal modulator of the AF gene cluster, it could also be auto-regulated (Price *et al.,* 2006; Chang *et al.,* 1995b).
In addition to this, *aflR* can act either as positive or negative regulator within the AF gene cluster. For instance, in *A. flavus*, an over-expression of *aflR* can up-regulate AF genes resulting in a 50-times increased production of aflatoxin (Flaherty and Payne, 1997). Similarly, its deletion has a negative effect on aflatoxin pathway genes (Cary *et al.,* 2000a).
Finally, it has been demonstrated that even if *aflR* is the principal activator of the AFB1 gene cluster, it can interacts with another gene named *aflS* which attends as an enhancer element of this regulatory process (Chang, 2003).

- *The AflS transcription enhancer*

aflS (previously named *aflJ*) was firstly characterized by Meyers *et al.,* (1998) and was demonstrated to be necessary for aflatoxin regulation. In *A. parasiticus*, this gene encodes a 438-amino acid protein and no homology to the existent enzymatic or regulatory domains has been yet found (Yu, 2012). The *aflS* gene is adjacent to *aflR* in the AFB1 biosynthetic cluster and it was demonstrated that *aflS* is also regulated by *aflR* Ehrlich, (2009).

Both genes share a 737-bp intergenic region from their translational starting sites. Nevertheless, it was demonstrated that *aflS* interacts with *aflR* but not with the biosynthetic enzymes demonstrating the co-activate function of *aflS* (Chang *et al.,* 2003; Du *et al.,* 2007).

Disruption of *aflS* in *A. parasiticus* resulted in mutants that lost the ability to synthesize AF intermediates accompanied with a 5- to 20-fold reduction of some AFB1 genes expression such as *aflC*, *aflD*, *aflM* and *aflP* (Chang *et al.,* 2003).

Otherwise in *A. flavus*, its over-expression resulted in higher levels of AFB1 production with a 4-to 5-fold increased levels of *aflC* and *aflD* (Du *et al.,* 2007).

Even if to date it is unclear how *aflS* increases the transcription levels of the genes involved in AFB1 pathway, it has been demonstrated that a dimer between both corresponding proteins is formed in order to activate the AF gene cluster. This phenomenon was proposed by Du *et al.,* (2007) indicating that this dimer-complex could recognize specific sites in the promoter regions of *aflC* and *aflD* (genes coding for early steps in the AFB1 enzymatic pathway) increasing their transcription and thus, AFB1 production.

Additional information on this protein-dimer formation was recently proposed. Taking in consideration that when both genes (*aflR* and *aflS*) are normally expressed, a protein complex made of 4 AflS for 1 AflR is formed allowing the correct binding process to the promoter regions of the aflatoxin pathway genes (Du *et al.,* 2007; Kong *et al.,* 2014).The graphical illustration of these complex is shown in Figure 20.

Even if a great advance in the characterization of *aflS* has been made, to date, the exact mechanism of action by which this gene modulates transcription of AFB1 pathway is still under investigation (Yu, 2012).

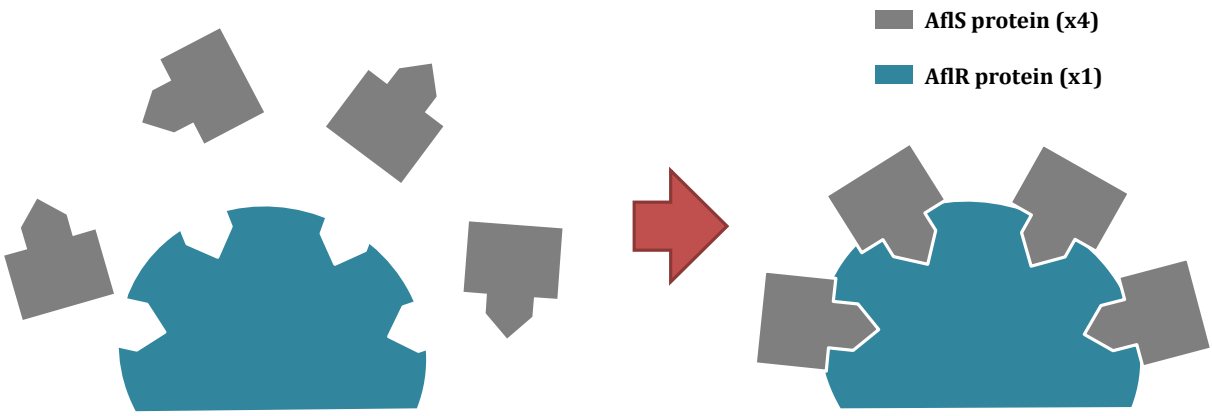


Figure 20: Hypothetical AflR-AflS protein-complex simulating a relation of 4 proteins of AflS for 1 protein of AflR.

In general, it is clear that the activation of the aflatoxin gene cluster is mainly governed by *aflR* and *aflS*. Nevertheless, AFB1's production also depends of other complex mechanisms that involve response to environmental stimuli in which a great number of genetic elements are involved. These factors are known as external regulatory factors and in order to have a general idea of this external system, some of them will be described in the next section.

3.4.4 External Regulatory Factors

External regulatory factors can be classified in this work as the genes that do not belong to the AFB1 gene cluster but that can have a direct or indirect relation with the cluster and thus with AFB1 production.

There exists a lot of information demonstrating that AFB1 production can be modulated by different external regulatory factors. For instance, the source of carbon, the light exposure or the different responses to oxidative stress are some of the external factors that can influence AFB1 production.

To date, the interaction between the genes coding for external regulators and the genes belonging to AFB1 cluster is not completely understood. In addition to this, to date a recapitulation of all these gene connections has not been already reported.

Based on bibliographic data, one of the principal aims of the present work was to link together those different external regulatory factors with the AFB1 gene cluster and by consequence, with their possible relation with toxin production.

For this purpose, a gene map linking all these interactions was constructed and is represented on Figure 21.

It has to be noted that the constructed gene map, containing more than 80 genes, is a hypothetical illustration that was constructed using both, confirmed interactions with AFB1 gene cluster but also with other genes that could be indirectly related to that cluster.

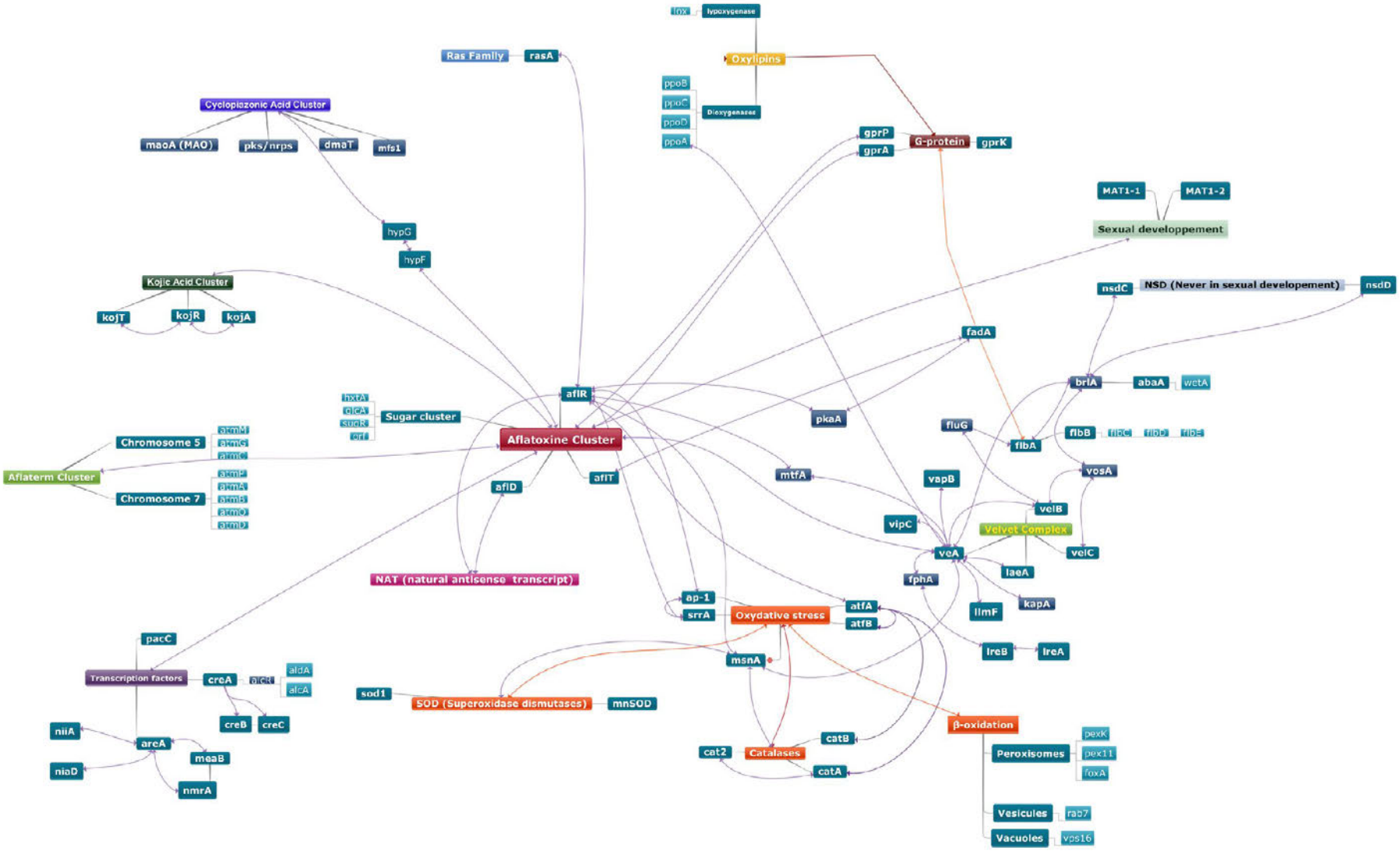


Figure 21: Network of genes intervening directly or indirectly with AFB1 gene cluster and their possible relationships.

For practical explanations, all the above genes were classified in 7 main groups and in different sub-groups corresponding to: cellular signalization, reproductive development, global transcription factors, oxidative stress, natural antisense transcriptions, environmental transcription factors and relation with other secondary metabolites. All these groups and their corresponding sub-groups are represented in Figure 22.

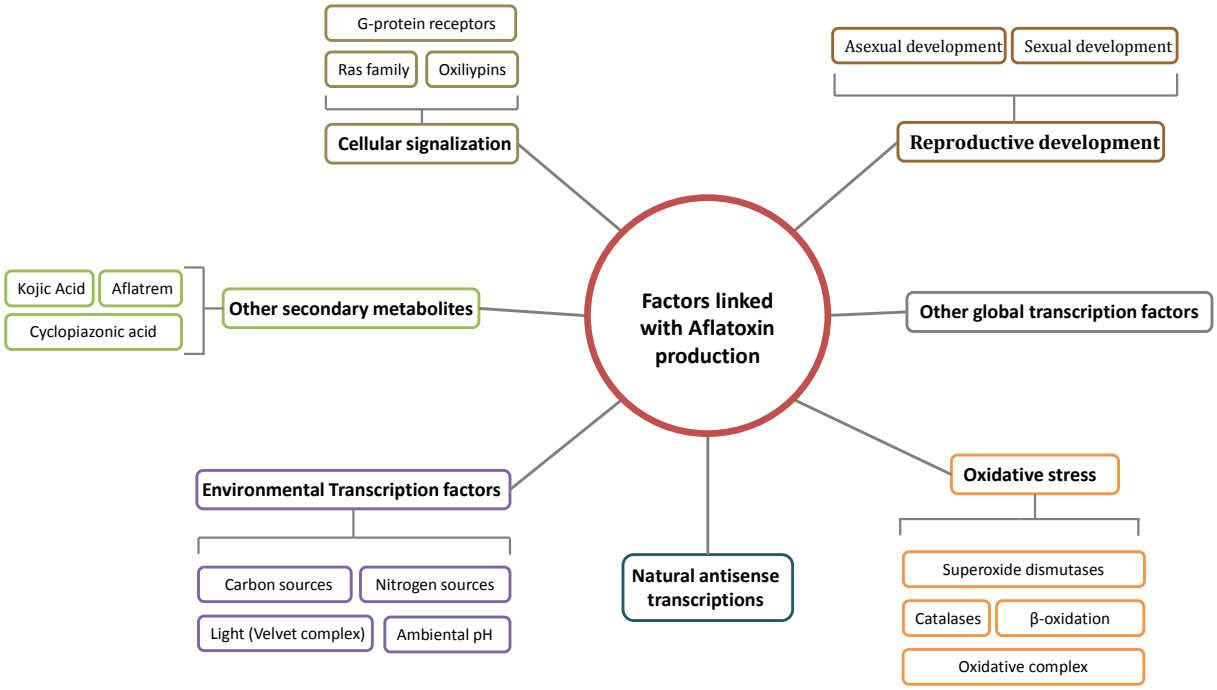


Figure 22: External Regulatory factors that influence AFB1 production grouped as a function of their demonstrated physiological effects

In order to detail the influence that each of these gene groups have over the AFB1 production, recapitulative tables will be further presented. Within these tables, a brief justification of the inclusion of each of the genes in the map gene network will be presented. Since the interactions between genes can be confusing, Figure 21 can help as graphical support.

3.4.4.1 Environmental Transcription factors

During the production of secondary metabolites including AFB1, one of the most influencing factors are the stimuli by environmental factors such as carbon and nitrogen sources as well as pH and light changes (Deepika *et al.,* 2015; Yu and Keller, 2005).

- ***Carbon source utilization***

It has been demonstrated during several decades that the availability and type of carbon source can modulate the amount of secondary metabolites production including AFB1 production. Within the utilization of carbon source, one of the most important elements for these processes resides in the sugar cluster as well as in the CreA-type proteins.

-Sugar cluster

Table 4: Genes involved in sugar utilization

Sugar cluster	
Genes	Function
hxtA *glcA* *sugR* *orf*	Hexose transporter Glucosidase Sugar regulator --
Relation between genes or corresponding proteins: These four genes are involved in sugar utilization and grouped in a 10 kB cluster located next to the AF gene cluster (Bhatnagar *et al.,* 2006).	
Relation with AF production	
- Sugars are preferred carbohydrates for AFB1 production since they are important in generating polyketide starter units (*e.g.* Acetyl-CoA) (Georgianna *et al.,* 2010; Davis, 1968; Maggio-Hall *et al.,* 2005; Nowicki and Foolad, 2012). -Simple sugars (*e.g*. sucrose, glucose, fructose, sorbitol) that are used as principal carbon source have been correlated with higher production of AFs, which is not the case of complex sugars (Bhatnagar *et al.,* 2006; Calvo *et al.,* 2002; Georgianna and Payne, 2009). - Even if glucose is a preferred source for aflatoxin production, this toxin can be also produced using other carbon sources (*e.g.* ribose, xylose, or glycerol) (Woloshuk and Shim, 2013).	

-The presence of glucose is linked with increased levels of *aflR* expression, suggesting that glucose can influence AFB1 production in an indirect manner (Roze *et al.,* 2004).
Impact of sugar elements in a AF/ST producer model
Aspergillus flavus -Within carbon sources, several glucose-derivatives (*e.g.* D-glucal) are non-metabolized by fungi resulting in AFB1 inhibition (Zhang *et al.,* 2014a). ***Aspergillus parasiticus*** -In aflatoxin conductive media, the *hxtA* gene is concurred with the AF genes which could potentially explain the induction of AFB1 synthesis by simple sugars (Yu, 2000a).

-*Carbon Catabolic Repression*

In parallel, when a most favorable source of carbon is detected in the environment, other fungal mechanisms are activated in parallel in order to repress the catabolic activity for the utilization of other carbon sources. This process is known as Carbon Catabolic Repression (CCR) and is a strategic mechanism used by *Aspergilli* to preserve energy (Deepika *et al.,* 2015). It is mainly governed by the transcription factor (TF) CreA as well as genes interacting with this latter.

Table 5*:* Genes involved in carbon catabolic repression

Carbon catabolic repression	
Genes	Function
creA	Cys_2His_2 Zinc finger transcription repressor
creB	Cys_2His_2 Zinc finger ubiquitin processing protease
creC	Cys_2His_2 Zinc finger ubiquitin processing protease
alcR	Regulon specific transcription factor
aldA	Aldehyde dehydrogenase
alcA	Alcohol dehydrogenase
Relation between genes or corresponding proteins: -*creA* represses gene expression of *aclR* which is a positive regulatory factor of genes involved in ethanol pathway: *aldA* and *alcA* (Ruijter and Visser, 1997; Shroff *et al.,* 1996). -CreB-CreC form a protein complex that is essential for CreA function and stability (Ries *et al.,* 2016).	
Relation with AF production	
-CreA is one of the major repressors factors of CCR (Ichinose *et al.,* 2014).	

-It has been suggested that a competitive mode of action occurs between AlcR and CreA for carbon utilization (Brown *et al.,* 2013).

-Gene expression in AFB1 gene cluster is regulated either positively or negatively by CreA (Dowzer and Kelly, 1989; Yu and Keller, 2005).

-Within the AFB1 cluster, several genes have CreA-sites near to their promoter regions (*e.g. aflC*) (Ehrlich *et al.,* 2002; Georgianna and Payne, 2009).

-CreA along with LaeA have been recently demonstrated to intervene in other secondary metabolite production (*Penicillium oxalicum*) (Zhang *et al.,* 2016).

Impact of carbon elements in a AF/ST producer model

Aspergillus nidulans

-CreA is likely to be regulated at post-transcriptional level and its activation or binding to the target DNA can be influenced by protein modification (Mogensena *et al.,* 2006).
-CreA has been shown to block the transcription of genes associated with the utilization of alternative carbon sources when glucose is present (Brown *et al.,* 2013).
-*creA* positively regulates penicillin synthesis (Martin, 2000).
-Deletion of *creA* results in strains that are not able to grow (Shroff *et al.,* 1996).

- *Nitrogen source*

Depending on the *Aspergillus* species, nitrogen sources can affect in a different manner sterigmatocystin and aflatoxin production. This fact involves nitrogen sources as an important factor for secondary metabolite production (Calvo *et al.,* 2002). For this, AreA is a key element since it is in charge of modulates genes coding for the utilization of alternative sources of nitrogen.

Table 6*:* Genes involved in nitrogen utilization

Nitrogen source	
Genes	Function
areA	Zinc finger mediating nitrogen metabolite repression
nmrA	Repressive nitrogen
meaB	Regulatory protein
niiA	Nitrite reductase
niaD	Nitrate reductase

Relation between genes or corresponding proteins:

-*areA* and *meaB* are regulatory genes mediating nitrogen metabolite repression (Kudla *et al.,* 1990; Ruijter and Visser, 1997; Wong *et al.,* 2007; Yin and Keller, 2011).

-AreA regulates nitrate transporters binding at the intergenic regions of *niiA* and *niaD* (Chang *et al.,* 2000c).

-MeaB is a regulatory factor that activates NmrA, which is a repressor of AreA (Amaike *et al.,* 2013).

Relation with AF production

-Nitrogen source is closely linked to AF production since some substrates (*e.g.* asparagine, ammonium salts, glutamate) support aflatoxin production while others do not (*e.g.* sodium, nitrate, tryptophan) (Yu, 2012).

-Area might play a role in regulation of *aflR*/*aflS* due to the GATA elements present in their intergenic region (Chang *et al.,* 2000c).

-*niiA* is one of the genes that does not belong to AFB1 gene cluster but that is regulated by the major regulator, *aflR* (Price *et al.,* 2006).

Impact of nitrogen elements in a AF/ST producer model

Aspergillus flavus

- *aflS* gene expression is modulated by AreA in different sources of nitrate and ammonium (Ehrlich and Cotty, 2002).
- Overexpressing *meaB* strains lost their capacity to produce AFs (Amaike *et al.,* 2013).

Aspergillus parasiticus

-*aflC* and *aflD* genes are expressed in ammonium and peptone media while they are not in nitrate sources (Feng and Leonard, 1998).

-AreA binds to the promoters of *aflR* and *aflS* and affect their expression (Bhatnagar *et al.,* 2006).

Aspergillus nidulans

-Contrary to AFB1 producers, nitrate medium increases the production of sterigmatocystin while ammonium does not (Feng and Leonard, 1998; Bayram and Braus, 2012).

- *pH of medium*

The pH medium is another extracellular condition to which fungal organisms have to respond to survive. The pH also plays an important role in AF/ST production. Nevertheless, its impact on secondary metabolites production depends on each *Aspergillus* species. Within the transcription factors that are involved in pH response, *pacC* plays an essential role.

Table 7*:* Genes involved in pH modulation

pH impact	
Gene	Function
pacC	Cys_2Hys_2 Zinc finger pH regulator
Relation between genes or corresponding proteins: The transcription factor PacC is strongly expressed under alkaline conditions (Tilburn *et al.,* 1995).	
Relation with AF production	
- Acidic conditions have been demonstrated as more favorable than alkaline ones for AF biosynthesis (Keller *et al.,* 1997). - In neutral and alkaline conditions, PacC inhibits acid response genes and induces alkaline ones. ST/AF gene expression could be modulated in response to pH by PacC (Selvig and Alspaugh, 2011; Keller *et al.,* 1997).	
Impact of pH elements in a AF/ST producer model	
Aspergillus flavus - Increased pH in nitrate-base medium results in lowers levels of AF while lower pH (4.0) resulted in 10-fold increased production of AF (Cotty, 1988). ***Aspergillus parasiticus*** - 164 matches to the consensus PacC binding sites have been identified in *aflR* promoter region, but interestingly alkaline conditions where *pacC* is activated do not support AFB1 production (Ehrlich *et al.,* 1999a). - *aflM* was higher expressed in acidic media that in neutral and alkali media. Fungal growth decreases the pH of the medium and increase AF production with time (Keller *et al.,* 1997). - Contrary to this, Buchanan (1975) showed a maximal AF production at pH 7.0 attributing this behavior to composition of the medium rather than pH influence. ***Aspergillus nidulans*** - PacC deleted strains produced 10-fold less ST amounts compared to the control (Keller *et al.,* 1997)	

- *Light*

Luminous stimulus is another important factor for fungi since it has a large effect in fungal adaptation and survival. Light can affect growth as well as it has an impact in several morphological changes. Light occurrence is without a doubt an important key for secondary metabolites biosynthesis and its impact is controlled by the "velvet complex". Within this complex, a special attention was recently given to the major regulator *veA* since it has been demonstrated to govern a great number of genetic elements including, among several others, the photoreceptors elements leading to light-response.

Table 8: Genes involved in light response

Light	
Genes	Function
veA	Global regulator
vapB	Methyl transferase
vipC	Methyltransferase
fphA	Phytochrome-like red-light receptor
kapA	α transport carrier
velB	Velvet-like protein B
laeA	Putative methyltransferase
velC	Velvet-like protein C
lreA	Blue-light sensing protein
lreB	Blue-light sensing protein
llmF	LaeA-like methyltransferase
Relation between genes or corresponding proteins: - Vea-LaeA-VelB forms a trimeric complex called velvet complex. VeA interacts with LaeA in the nucleus and with VelB in the cytoplasm and the nucleus (Bayram *et al.*, 2008). - FphA interacts with VeA in the nucleus (Bayram and Braus, 2012). FphA also interacts with LreB which at the same time interact with LreA (Calvo, 2008). Together FphA, LreA, LreB and VeA form a complex for sensing red and blue light (Purschwitz *et al.,* 2008). - KapA and VeA physically interact in dark conditions. KapA support the entry of the VeA-VelB complex into the nucleus (Sarikaya-Bayram *et al.,* 2015). -VelC as well as VelB form a protein-dimer with vosA (in charge of viability of spores) (Sarikaya-Bayram *et al.,* 2015). -VipC and VapB inhibit the nuclear accumulation of VeA (Sarikaya-Bayram *et al.,* 2014). -LlmF interacts with VeA controlling its subcellular localization (Palmer *et al.,* 2013).	

Relation with AF production
-Light stimulus can modulate secondary metabolite production (Purschwitz *et al.,* 2008). -The trimeric complex (VeA-LaeA-VelB) with others light-receptors proteins, perceive light signal and are essential to coordinate secondary metabolism and development (Bayram and Braus, 2012; Sarikaya-Bayram *et al.,* 2014). - Even if the effect of light and its transmission through *veA* is today not clear, studies in *A. nidulans* demonstrated that, in light conditions, asexual development is enhanced and sexual development as well as ST production are repressed (Fischer, 2008). -Also in dark conditions, nuclear localization of VeA increases its interaction with LaeA (situated exclusively in nucleus) to enhance secondary metabolite production and also with VelB to induce sexual development (Palmer *et al.,* 2013).
Impact of light in AF/ST producer model
Aspergillus flavus -*veA* gene is essential for AFB1's production (Duran *et al.,* 2007; Duran *et al.,* 2009). -In null mutants of *veA* or *laeA*, no *aflR* expression was observed (Amaike and Keller, 2009). -Δ*laeA* reduced *aflR*, *aflD* and *aflS* mRNA expression with no AF production (Chang *et al.,* 2012). -LaeA is a positive regulator of aflatoxin; overexpression of *laeA* results in higher levels of AFB1 (Amaike *et al.,* 2013). -VeA governs 28 out of the 56 secondary metabolites gene clusters (Cary *et al.,* 2015). ***Aspergillus parasiticus*** Deletion of *veA* resulted in loss of aflatoxin intermediates. *veA* is required for *aflR*/*aflS* expression (Calvo *et al.,* 2004). ***Aspergillus nidulans*** - Δ*veA* strains resulted in absence of ST production and *aflR* expression (Kato *et al.,* 2003). - Deletion of the *laeA* gene results in no *aflR* expression (Bok and Keller, 2004). -LreA, LreB and FphA modulate sterigmatocystin and penicillin biosynthesis depending on light and glucose presence (Atoui *et al.,* 2010). -FphA represses sexual development and ST production while LreA and LreB stimulate both processes. Blue light represses ST production and red-light has the opposite effect (Purschwitz *et al.,* 2008). -*velC* deletion reduced sexual fruiting bodies (Park *et al.,* 2014). -LaeA plays both, a role in secondary metabolism and in light control by modifying the levels of VelB and VosA (Bayram and Braus, 2012). -LlmF is a negative factor of sexual development and ST production (Palmer *et al.,* 2013).

3.4.4.2 Natural Antisense transcription

Natural antisense transcripts (NATs) are Ribonucleic Acid (RNA) molecules that are transcribed from the opposite DNA strand and partly overlap, with RNA (Faghihi, 2009). It has been suggested that the expression of the antisense genes may be involved in silencing the cluster via chromatin remodeling (Smith *et al.,* 2008).

Since their discovery in *Aspergillus flavus,* some of them have been linked to aflatoxin production, even if their role in this process is not clear yet.

Table 9: Natural antisense transcripts

NATs	
Genes	Function
352 cis NATs	Antisense transcripts
Relation with AF production	
-NATs can modulate AF genes and secondary metabolism production. Up-regulation of the cis *aflD* antisense was associated with reduction of AF production (Smith *et al.,* 2008a).	
Impact of NATs elements in a AF/ST producer model	
Aspergillus flavus - Over the 352 cis NATs present in *Aspergillus flavus, aflR* encodes for two antisense transcripts (*aflRas*) (Woloshuk *et al.,* 1994). - NATs might regulate gene expression at post-transcriptional level (Wu *et al.,* 2014).	

3.4.4.3 Reproductive processes

-Sexual development

For years, it was believed that *A. flavus* reproductive process occurs only in asexual manner. Nevertheless, sexual reproduction in *A. flavus* and *A. parasiticus* was recently demonstrated. Both were characterized as heterothallic species containing one of two mating-type genes: *MAT1-1* or *MAT1-2*. Sexual reproduction occurs within conidia or sclerotia when they recombine with the opposite mating-type (Perrone *et al.,* 2014; Bruce *et al.,* 2014; Horn *et al.,* 2009).

Table 10: Genes involved in sexual development

Sexual Development	
Genes	Function
MAT1-1 *MAT1-2*	Sexual mating
Relation between genes or corresponding proteins: Either MAT1-1 or MAT1-2 can be present in *A. flavus* and *A. parasiticus* strains and are in charge of sexual development (Ramirez-Prado *et al.,* 2008).	
Relation with AF production	
-Sexual state of *A. flavus* and *A. parasiticus* are called *Petromyces flavus* and *Petromyces parasiticus* and they share the classification of *Aspergillus* genus of the *Flavi* section. As a novel discovery, not all mycotoxins produced by sexual development have been described. Nevertheless, *P. flavus* can produce B-type AF's as well as Ciclopiazonic Acid (CPA) while *P. parasiticus* produces B and G-type aflatoxins but not CPA (Horn *et al.,* 2009).	
Impact of sexual development in AF/ST producer model	
Aspergillus flavus - Strains of *A. flavus* sexually developed demonstrated that production of AF and CPA is highly heritable. In asexual development, non-aflatoxigenic populations are maintained while in sexual development the aflatoxigenicity increases (Olarte *et al.,* 2012).	

-A*sexual development*

Secondary metabolite production is also coordinated with the general development of the fungus and development is intrinsically linked with conidiation (the asexual reproduction mode).

Table 11: Genes involved in asexual development

Asexual development mode	
Genes	Function
fadA	α subunit of heterotrimeric G-protein
fluG	Developmental regulator
brlA	C_2H_2 zinc finger protein transcriptional activator of conidiophore
abaA	Transcription factor for conidia formation
wetA	Developmental regulatory protein
nsdC	Zinc-finger transcription factor
nsdD	Zinc-finger transcription factor
pkaA	Catalytic subunit of protein kinase A
flbA	RGS protein/ developmental regulator
flbB	bZIP-type transcription factor
flbC	C2H2 conidiation transcription factor, putative
flbD	MYB family conidiophore development
flbE	Developmental regulator
vosA	Spore viability/Developmental regulator

Relation between genes or corresponding proteins:

-*fluG* activates *flbA* and thereby *flbA* represses *fadA* signaling (Calvo *et al.,* 2002; Deepika *et al.,* 2015).
-*fadA* up-regulates *pkaA* (Georgianna and Payne, 2009).
-*flbA* is a regulator of *flbB* which regulates *flbC*, *flbD*, *flbE* (*flb* genes).
-*flb* genes are required with *fluG* for the expression of *brlA* (Payne and Brown, 1998; Yu and Keller, 2005).
-*brlA* is a negative regulator of *abaA* and *abaA* also regulates *brlA*.
-*abaA* is also a repressor of *wetA* (Andrianopoulos and Timberlake, 1994).
-*brlA* is also regulated by *veA* (from the velvet complex) (Kato *et al.,* 2003).
-*vosA* is a repressor of *brlA* (Ni and Yu, 2007). VosA also forms protein-complex with VelB and VelC (velvet proteins) (Bayram and Braus, 2012; Sarikaya-Bayram *et al.,* 2015).
-*nsdC* and *nsdD* are repressors of *brlA* (Cary *et al.,* 2012).

Relation with AF production
- Asexual sporulation and secondary metabolite production has been linked (Calvo *et al.,* 2002; Deepika *et al.,* 2015; Yu and Keller, 2005). - It exists an interconnection between conidiation and AF/ST production principally by genes such as *fadA*, *fluG*, *flb* genes and *brlA* (Payne and Brown, 1998; Yu and Keller, 2005). - FadA governs AF and ST biosynthesis (Calvo *et al.,* 2002; Chang *et al.,* 2004b). *fadA* up-regulates PkaA which down-regulates conidiation and more important, PkaA inhibits AflR activity by phosphorylation (Georgianna and Payne, 2009; Shimizu *et al.,* 2003). *fadA* also regulates *aflT*, the MFS belonging to the AFB1 gene cluster (Chang *et al.,* 2004b). - VosA is necessary for normal spore viability and thus, involved in AFB1 production (Calvo, 2008). - NsdC and NsdD are required for normal AF biosynthesis and conidiophore development (Cary *et al.,* 2012).
Impact of conidiation in model aflatoxin producer
Aspergillus flavus Expression of *aflD*, *aflM*, and *aflP* is strongly reduced in *nsdC* deleted mutants. Loss of NsdC or NsdD results in developmental alterations that impact the ability of AflR to activate expression of the AF biosynthesis genes (Cary *et al.,* 2012). ***Aspergillus parasiticus*** - Mutants defectives in conidiation had reduced AF production (Kale *et al.,* 2003). ***Aspergillus nidulans*** - Mutations in *flbA*, block both ST production and asexual sporulation while its overexpression causes ST accumulation as well as defects in sporulation (Hicks *et al.,* 1997). - *fadA* and *pkaA* mutants resulted in inhibition of conidiation and ST biosynthesis (Shimizu and Keller, 2001). - Loss of *flbA* function resulted in lack of ST and asexual sporulation (Hicks *et al.,* 1997). - FlbA is necessary for ST cluster transcription and biosynthesis (Shwab and Keller, 2008).

3.4.4.4 Oxidative stress

In fungi, changes in environmental conditions can alter the normal intracellular balance between Reactive Oxygen Spices (ROS) production and scavengers. In response of this phenomenon, several transcription factors are involved in order to activate enzymatic defenses that protect cell from excessive levels of ROS and subsequently to possible damages in DNA, proteins and lipids (Montibus *et al.,* 2013). In addition to this, fungal oxidative stress has been also linked with secondary metabolite production and also demonstrated as a pre-requisite for AFB1 production. In fact, it is proposed that in *A. parasiticus* and *A. flavus,* aflatoxin production is part of the fungal oxidative stress response (Roze *et al.,* 2013).

Table 12: Genes involved in oxidative stress response

Oxidative Complex	
Genes	Function
ap-1	bZIP transcription factor
srrA	Transcription factor
atfA	bZIP transcription factor
atfB	bZIP transcription factor
msnA	Transcription factor
Relation between genes or corresponding proteins: -AtfB, SrrA, Ap-1 and MsnA together, constitute a regulatory network involved in oxidative stress response and secondary metabolite production (Roze *et al.,* 2013). - AtfA might interact with AtfB in response to oxidative stress (Amare and Keller, 2014; Hong *et al.,* 2013).	
Relation with AF production	
-The relation between oxidative stress and secondary metabolites production starts to be deeply studied in fungi. Informatic reviews of the involved transcription factors in *Aspergilli* are reviewed by Linz and co-workers (2013) and Montibus *et al.,* (2013). -Aflatoxins and their precursors (*e.g. O*-methylsterigmatocystin, Versicolorin, Norsolorinic Acid) are highly oxigenated molecules thus, subject to redox regulation. It has been demostrated that oxidative stress is a pre-requisite for AFB1 production (Narasaiah *et al.,* 2006). -AFB1 biosynthesis is activated by high levels of oxidative stress-inducing factors (*e.g.* lipid hydroperoxides) whereas it is inhibited by antioxidants (*e.g.* polyphenols) (Grintzalis *et al.,* 2014). -In cell systems, the *ap-1* gene is activated under both, pro-oxidant and antioxidant conditions (Gomez del Arco *et al.,* 1997).	

Impact of oxidative stress elements in AF/ST producer model
Aspergillus flavus *msnA* deletion results in higher amounts of aflatoxins as well as higher levels of kojic acid. *msnA* appears to be necessary to maintain normal oxidative stress state (Chang *et al.,* 2011). ***Aspergillus parasiticus*** - AtfB binds to promoters of seven belonging to aflatoxin gene cluster (Roze *et al.,* 2011). -Ap-1 deletion resulted in increased AF production but also, Ap-1 has binding sites in the promoter region of the *aflR* gene (Reverberi *et al.,* 2008). ***Aspergillus nidulans*** -*atfA* plays an important role to cope oxidative stress (Asano *et al.,* 2007). -Deletion results in a phenotype of hypersensitivity to oxidative stress (Hagiwara *et al.,* 2007; 2008).

-*Superoxide dismutases and Catalases*

Within the response to oxidative stress, superoxide dismutases (SOD), catalases (CAT) and Glutathione Peroxidase (GPX) are in also involved in the fungal mechanism of defense. For instance, SOD act as the first line of fungal defense by converting the radicals into hydrogen peroxide (H_2O_2) and O_2, then the catalases and peroxidases convert H_2O_2 into H_2O and in the case of catalases, into O_2 and H_2O (Weydert and Cullen , 2011).

Table 13: Genes coding for fungal superoxide dismutase and catalases

Superoxide Dismutases and Catalases	
Genes	Function
mnSOD	Manganese Superoxide Dismutase
sod1	Cu, Zn superoxide dismutase
catA	Conidia-specific catalase
catB	Mycelial catalase
hyr1	Glutathione Peroxidase
Relation between genes or corresponding proteins: All genes are involved in cellular defense against oxidative stress (Weydert and Cullen, 2011).	

Relation with AF production
-It has been demonstrated that *mnSOD* and the genes *aflA*, *aflM*, and *aflP* belonging to the aflatoxin cluster are co-regulated (Hong *et al.,* 2013). -In other fungal strains such as *A. ochraceu*s, *ap-1*'s deletion (from the previous table) was also related with the expression of CAT and SOD (Reverberi *et al.,* 2008).
Impact of CAT/SOD in AF/ST producer model
Aspergillus flavus Deletion of *sod* resulted in decreased AF production (He *et al.,* 2007). ***Aspergillus nidulans*** Deletion of *mnSOD* increased both, glutathion reductase and catalase activities while its overproduction lowered the activity of catalase but increased the SOD activity (Leiter *et al.,* 2016).

- β-oxidation

β-oxidation of fatty acids is a fungal process that degrade fatty acids into acetate units. Therefore, since AFB1 biosynthesis is trigger from acetate units, the involved pathways that contribute to the formation of acetate units such as β-oxidation are related with AFB1 production (Maggio-Hall *et al.,* 2004; 2005). In addition to this, β-oxidation of fatty acids occurs in peroxisomes (Reverberi *et al.,* 2012) and several genes can serve as peroxisomes' markers thus, making interesting their study. On the other side, vacuoles and vesicles are also elements that are involved with AFB1 production by forming and then exporting this mycotoxin outside of the cell (Chanda *et al.,* 2009).

Table 14: Genes involved in β-oxidation

β-oxidation	
Genes	Function
pexK	Peroxisome existence
pex11	Peroxisome proliferation
foxA	Fatty acid regulation metabolism by *β* -oxidation
rab7	Vesicle marker
vps16	Vacuole marker
Relation between genes or corresponding proteins: - These genes are mainly used as markers of organelles that are involved in AFB1 production.	

Relation with AF production
-β-oxidation is a fundamental step for aflatoxin biosynthesis (Maggio-Hall *et al.,* 2005). -Peroxisomes are involved in the β-oxidation of fatty acids and probably in the first steps of AFB1 synthesis (Reverberi *et al.,* 2012). -In filamentous fungi, peroxisomes are crucial for the primary metabolism and play a role in the formation of some secondary metabolites (Reverberi *et al.,* 2012). - Vesicles catalyze the final 2 steps in aflatoxin biosynthesis and compartmentalize and export aflatoxin to the cell exterior (Chanda *et al.,* 2009). -Nor-1 (protein involved in the norsolorinic acid biosynthesis and a precursor of AFB1) mainly occurs in the cytoplasm and vacuoles (Hong and Linz, 2009).
Impact of β-oxidation in AF/ST producer model
Aspergillus flavus -Proliferation of peroxisomes enhances AFB1 production (Reverberi *et al.,* 2012). ***Aspergillus parasiticus*** -An increase in vesicle number was positively correlated with aflatoxin accumulation/export (Chanda *et al.,* 2009). ***Aspergillus nidulans*** -Overexpression of *pexK* increased the number of peroxisomes, which was correlated with an increased production of another secondary metabolite, penicillin (Herr and Fischer, 2014). -*pex* mutants are able to grow on acetate but their growth is affected on fatty acids, indicating a requirement for the peroxisomal localization of β-oxidation enzymes (Hynes *et al.,* 2008).

3.4.4.5 Cellular signalization

Cellular signalization in fungi, help to overcome environmental stresses by activating rapid transduction of signal through the cell and thus, allowing the organism to be adapted to its surroundings. Within the members that are involved in this process, G-protein coupled receptors (GPCR) and oxylipins are some of them. It has to be noted, that GPCR are one of the transmembrane receptors that have been recently studied in *Aspergillus flavus* so little is known about their functions in filamentous fungi (Affeldt *et al.,* 2014).

Table 15 Genes involved in cellular signalization

Cellular signalization	
Genes	Function
ppoA	(oxylipin) Dioxygenase
ppoB	(oxylipin) Dioxygenase
ppoC	(oxylipin) Dioxygenase
ppoD	(oxylipin) Dioxygenase
lox	(oxylipin) Lipoxygenase
gprK	GPCR
gprA	GPCR
gprP	GPCR
rasA	GTP-binding protein
Relation between genes or corresponding proteins: -GPCRs are involved in oxylipins response (Affeldt *et al.,* 2014).	
Relation with AF production	
- The proper regulation of G-protein signaling play a central role, among others, in secondary metabolite production (Yu and Keller, 2005). -Recently, the GPCRs were shown to regulate AFB1's synthesis and its precursor, sterigmatocystin (ST) (Affeldt *et al.,* 2014). -In *Aspergillus fumigatus*, *gprK* gene was demonstrated to be necessary for gliotoxin production and oxidative stress response (Jung *et al.,* 2016). -Oxylipins regulate secondary metabolism at a transcriptional level (Tsitsigiannis and Keller, 2006).	

Impact of cellular signalization in AF/ST producer model
Aspergillus flavus -Deletion of *gprK* and *grpA* resulted in higher levels of AF production compared to control strain (Affeldt *et al.,* 2014). -When all four *ppo* genes and the *lox* gene were disrupted at the same time, the mutant strains showed a reduced conidiation and increased their Aflatoxin production in maize and peanut seeds (Amare and Keller, 2014). ***Aspergillus nidulans*** -Δ*ppoA*; Δ*ppoB* and Δ*ppoC* mutants are unable to produce sterigmatocystin (ST) (Tsitsigiannis and Keller, 2006). -RasA has been demonstrated to control *aflR* activity (Shimizu *et al.,* 2003).

3.4.4.6 Relationship with the production of other secondary metabolites

In addition to Aflatoxin production, *A. flavus* can produce other mycotoxins such as Cyclopiazonic acid (CPA), Aflatrem (AFT) and Kojic Acid (KA) (Duran *et al.,* 2007; Shinohara *et al.,* 2011). The implication of these latter with AFB1 production relies in the fact that some of these secondary metabolites are also modulated with variations of AFB1 production.

-*Cyclopiazonic acid*

Also considered as a mycotoxin, this compound is a calcium-dependent ATPase inhibitor, which modifies the Ca++ levels in organisms resulting in an increase of muscle contraction. Ingestion of cyclopiazonic acid has been demonstrated to be toxic for humans and animals (Amare and Keller, 2014; Duran *et al.,* 2007). Specifically, some strains of *A. flavus* are capable to produce this secondary metabolite during *in vitro* and *in vivo* growth (Gilbert *et al.,* 2016). CPA production is principally related to several genes that are clustered near to the AFB1 cluster.

Table 16: Genes involved in Cyclopiazonic acid synthesis

Cyclopiazonic acid	
Genes	Function
dmaT	Dimethylallyl tryptophan synthase
pks/nrps	Hybrid polyketide non-ribosomal peptide synthase
maoA	Flavin adenine dinucleotide oxidoreductase
msf1	Major facilitator superfamily protein (MFS)
hypG	Putative gene
hypF	Putative gene
Relation between genes or corresponding proteins: - CPA production requires four genes (*dmat*, *pks*/*nrps*, *maoA* and *msf1*) localized in a small cluster next to the AFB1 cluster (Chang *et al.,* 2009). -*hypG* and *hypF* are two genes that are localized between AFB1 and CPA clusters but they do not belong to any of the clusters (Georgianna *et al.,* 2010).	

Relation with AF production
- The 55th CPA cluster, is next to the gene cluster (54th) and under conductive conditions it is expected that both metabolites are synthesized (Georgianna *et al.,* 2010). -Disruption of the AF gene cluster and subtelomeric regions resulted in loss of AFB1 but also of CPA (Amare and Keller, 2014).
Impact of cyclopiazonic acid elements in a AF/ST producer model
Aspergillus flavus -Several *A. flavus* strains that lacks of AFB1 cluster are not able to synthesize CPA, suggesting a link between productions of both mycotoxins (Chang *et al.,* 2009). - *dmaT* deletion decrease conidiation and AFB1 biosynthesis (Yang *et al.,* 2016). - *hypG* and *hypF* were demonstrated as non-essential for either CPA or AFB1 production even if they are localized between both clusters (Georgianna *et al.,* 2010). - Inhibition of AFB1 and CPA were observed in parallel using a *Bacillus megaterium* treatment (Kong *et al.,* 2014).

-Aflatrem

Classified as a potent tremorgenic compound (which causes uncontrollable rhythmic movement of one part of the body), this mycotoxin is known to lead to neurological diseases in vertebrates (Gallagher and Wilson, 1978). Aflatrem, different to most mycotoxins produced in one cluster, needs the presence of two clusters for its biosynthesis (Dolezal *et al.,* 2013; Georgianna *et al.,* 2010).

Table 17: Genes involved in Aflatrem synthesis

Aflatrem		
Genes		Function
Cluster 32 7th chromosome	*atmM* *atmG* *atmC*	FAD-dependent monooxygenase GGPP synthase prenyltransferase
Cluster 15 5th chromosome	*atmP* *atmQ*	Putative cytochrome P450 monooxygenase
	atmB *atmA*	Putative polytopic membrane protein
	atmD	Putative aromatic prenyltransferase

Relation between genes or corresponding proteins:
-Genes involved in Aflatrem production resides in two clusters localized in two separate chromosomes (Nicholson *et al.,* 2009). -*atmG*, *atmC* and *atmM* are the principal genes for aflatrem biosynthesis (Dolezal *et al.,* 2013; Zhang *et al.,* 2004).
Relation of Aflatrem with AF production
-Even if aflatoxins are the most preoccupying mycotoxins produced, other toxic metabolites such as Aflaterm are also produced by *Aspergillus flavus* and little is known about the relation between both clusters. - Aflatrem, Aflatoxin and Cyclopiazonic Acid production were demonstrated to be governed by the same global regulator, *veA* (Duran *et al.,* 2007).
Impact of Aflatrem elements in a AF/ST producer model
-No data of gene observation AF/ST was founded.

- *Kojic acid*

Produced by several species belonging to the *Flavi* section, Kojic acid is one of the secondary metabolites used for industrial processes. This compound is capable to inhibit pigments in plants and animals, which attracted the attention of food and cosmetic industries to preserve or change colors (Varga *et al.,* 2015). Recently, Kojic Acid was also demonstrated as a potential parasite-inhibitor used for medicinal purposes (Rodrigues *et al.,* 2014). The production of this secondary metabolite is governed by a little cluster regulated by *kojR*.

Table 18: Genes involved in Kojic acid synthesis

Kojic Acid	
Genes	Function
kojR	Zn_2-Cys_6 transcription factor
kojT	Major facilitator transporter protein (MFS)
kojA	FAD-dependent oxidoreductase
Relation between genes or corresponding proteins: *kojR* is a transcription factor essential for KA synthesis and is the main and positive regulator of *kojT* and *kojA* (Marui *et al.,* 2011).	
Relation with AF production	
-Several natural inhibitors of AFB1 also seem to modulate KA synthesis, sometimes by increasing its production (Zhang *et al.,* 2014a). Since KA has not been demonstrated as toxic and is a useful compound in industry, mechanisms of action leading to inhibition of AF accompanied with an increase in KA production could be an interesting topic of research.	
Impact of Kojic acid elements in a AF/ST producer model	
Aspergillus flavus - D-glucal inhibits AFB1 production by modulating AF genes (but no *aflR*) while increasing KA biosynthesis (Zhang *et al.,* 2014a). - The same impact between AFB1 and KA was observed with C18:3 acid fractions, this time with a reduction in *aflR* gene expression (Yan *et al.,* 2014). ***Aspergillus parasiticus*** - Dioctatin-A strongly inhibit AFB1 by reducing *aflR's* expression while enhancing the KA production (Yoshinari *et al.,* 2007).	

As demonstrated, AFB1 production is a very complex process that involves a great number of genetic elements. The study of the molecular machinery that is involved in AFB1 production could led to identify essential genes that can be targeted in order to inhibit AFB1 production. Nevertheless, this molecular strategy still requires a lot of information that has to be elucidated.

Since then, and taking in consideration that AFB1 inhibition still remains a priority, the use of other methods including the utilization of pesticide are commonly used.

Within this context, natural compounds can represent an alternative strategy to pesticides (and their own toxicity) since some of them have been demonstrated as effective anti-aflatoxigenic agents. From now, a general overview of the major strategies that are used to inhibit AFB1 production will be presented with a special attention in the use of natural products.

3.5 Use of natural compounds to inhibit AFB1 production

3.5.1 General overview of strategies targeting the reduction of AFB1 occurrence

Nowadays, a great number of strategies have been developed aiming the limitation of AFB1 in food intended for human and animal's consumption.

In general, one of the biggest problems that are related with AFB1's contamination is that, being a very stable molecule, once this mycotoxin is synthesized its elimination from food products is a very difficult process.

In fact, when food commodities are already contaminated with AFB1, those products have to be detoxified and for the moment, such procedures are only allowed for animal feed purposes and cannot be applied to human's food (Adeyeye and Yildiz, 2016).

Thus, in order to avoid crop contamination with mycotoxins it is necessary to develop preventive strategies.

Within these strategies, implementation of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) during the pre- and post-harvest steps are a key point (Bennett, 2003; De Saeger *et al.,* 2016).

The principal Good Agricultural Practices that shall be applied at different stages of crop processing are recapitulated in Figure 23.

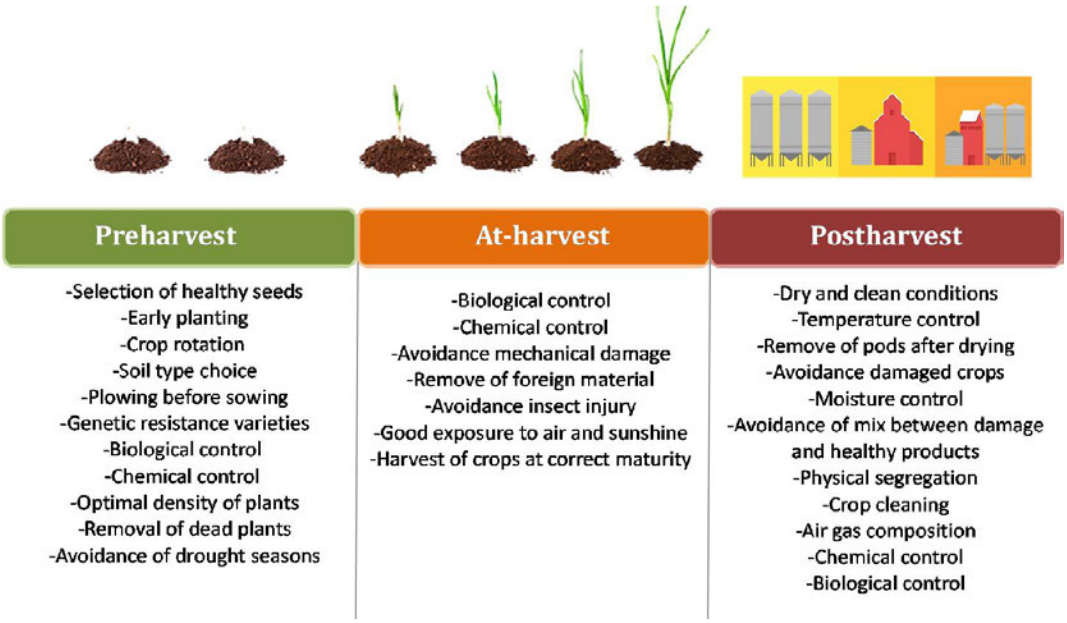


Figure 23: Good Agricultural Practices at different crop processing stages

Aflatoxins can be produced either in the fields or later during crop storage if hydrothermal conditions are favorable for development of toxigenic strains.
GAP during the pre-harvest stage principally aim to avoid mycotoxin appearance by limiting fungal contamination while during the post-harvest stage (when crop is stored) the main goal is to avoid an increase in mycotoxin contamination due to fungal development.
In order to describe more in detail these practices, tables S1-S3 in the annexes of this work are available and summarize the strategies that are nowadays used.
Some of these practices will be further detailed highlighting their advantages and drawbacks.

As an example, within the pre-harvest stages, fungicides are one of the most used strategies to limit fungal contamination and development. Although they are very effective, these compounds also have demonstrated numerous disadvantages concerning their use. In fact, pesticides lead to environmental contamination and subsequent strong impact on microbial biodiversity. (Accinelli *et al.,* 2014; Sakuda *et al.,* 2016; Tola *et al.,* 2016). Moreover, they are more and more suspected to have detrimental effects to human due to repeated and longtime exposure as trace contaminants in many foods. Within this context, a few countries aim to limit the use of pesticides. As an example, in France the "Ecophyto 2" plan, aim to reduce by 50% the utilization of pesticides before 2025 and in Spain, the plan "Possible Mission" also target by 2020 their reduction by 30%.

An alternative to the use of these chemicals products to limit fungal contamination in the fields, is the biological control which is based on the utilization of other microorganisms such as bacteria or non-toxigenic fungal strains in order to compete and limit the development of toxigenic strains.
The great advantage of these biological methods is that most of them have been demonstrated to effectively inhibit AFB1's contamination and to date, some non-toxigenic fungal strains are already marketed and currently used (*e.g.* Aflasafe™, Aflaguard ®) (Adhikari *et al.,* 2016).
However, the use of non-toxigenic strains, especially those belonging to the *Aspergillus* genus, also present some disadvantages that have to be taken in consideration. In fact, in

the last years, it was demonstrated that several strains of *A. flavus* and *A*. *parasiticus* can be developed by sexual combination and since aflatoxin production is a heritable factor, this could challenge their long term efficiency. Therefore, the non-toxigenic strains have to be chosen based on the insurance that they have disrupted functions or lack genes coding for AFB1's production (Horn *et al.,* 2009; Jalili, 2015).

Concerning the post-harvest GAP, appropriate drying techniques, proper storage that maintain low levels of temperature and humidity as well as the segregation of contaminated product have to be promoted (Torres *et al.,* 2014). Indeed, moisture is a key factor that may positively favor fungus development and thus, mycotoxin synthesis.

Mechanic removal and optical sorting aim the elimination of contaminated product by taking away the mouldy, shriveled or insect-infected grains (Zain, 2011).
Even if those techniques have good results in reducing aflatoxin contamination by removing the contaminated particles and reducing fungal inoculum before storage, disadvantages like false positives as well as misclassification of contamination grade occur frequently (IARC, 2015; Moy and Miller, 2016).

Taken together, even if to date numerous strategies have been developed to reduce AFB1's occurrence in food commodities, GAP by themselves are not sufficiently effective to avoid mycotoxin contamination.
Therefore, it is essential to continue investigating other effective procedures that can help to inhibit mycotoxin contamination.
Within this context, the use of natural products represents an alternative strategy that can complement the use of the existent ones or in the best of the cases, that could represent an effective manner to inhibit AFB1 production.

3.5.2 Natural products

Plants, as fungi, produce secondary metabolites that have been used for years as useful compounds for humans.

Plants and their extracts are widely used for medicinal purposes since some of them are known as antioxidants, anti-inflammatory, antimicrobials and cancer preventive molecules, among various other properties (Friedman and Rasooly, 2013; Jeff-Agboola *et al.,* 2016; Orole *et al.,* 2016; Prakash *et al.,* 2015).

Compounds occurring in plants are bio-actives metabolites that can be divided in four major groups. They include terpens (terpenoides, isoterpenoids), phenylpropanoids (flavonoids, tannins, glycosides and lignins), phenolics and nitrogen-containing compounds (alkaloids and heterocyclic aromatics) (Razzaghi-Abyaneh *et al.,* 2010).

Within these compounds, some of them have been demonstrated as effective inhibitors of fungal growth and AFB1 production even if, for most of them, the exact mechanism of action has not been elucidated.

These active compounds can be contained either in essential oils or in aqueous or organic extracts and their anti-fungal or anti-aflatoxigenic effect can be the result of one or several molecules.

For practical purposes, a presentation of some works using natural products against AFB1 production will be described and divided in:

i) Essential Oils
ii) Plant extracts
iii) Isolated Molecules

i) Essential Oils

The term essential oil is reserved to products obtained from vegetable raw material, either by distillation with water or steam, or from the epicarp of citrus fruits by a mechanical process or by dry distillation (Turek and Stintzing, 2013).

Essential oils (EOs) are lipophilic and they are composed by high volatile secondary metabolites of plants.

They are some of the most studied natural compounds since they present numerous useful properties for varied domains such as medicine, biology, cosmetology, agricultural and food industry. EO's are obtained from plants belonging to different families (*e.g*. *Lamiaceae, Rutaceae, Myrtaceae, Zingiberceae, Asteraceae*) and nowadays more than 17,000 aromatic plant species have been identified and represent a vast field of study (Regnault-Roger *et al.,* 2012; Chizzola, 2013).

Hundreds of studies including their possible use as fungal and mycotoxin inhibitors had been reported. Most of them can be found in reviews by Isman, (2000; 2006); Alpsoy, (2010); Friedman and Rasooly, (2013); Prakash *et al.,* (2015); Macwan *et al.,* (2016) and Sakuda *et al.,* (2016). In particular, Alpsoy's review contains detailed information concerning the use of EOs as aflatoxin inhibitors.

In fact, some EOs have been demonstrated to effectively limit AFB1 production in several species such as *A. parasiticus* and *A. flavus* (Bluma and Etcheverry, 2008; Ferreira *et al.,* 2013; Kohiyama *et al.,* 2015).

Moreover, a recent work reported by Shalaby and El-tawil, (2016) showed that a diet supplemented with 400 mg of oregano oil/kg of diet, can have protective effects against aflatoxicosis in Japanese quails due to the antioxidant effects of oregano EO.

The antifungal activity of the bioactive compounds contained in some of the EOs was strongly associated with monoterpenic phenols (Isman, 2000). Examples of EOs that have been used to inhibit *A. flavus* growth as well as AFB1 production are listed in table 19.

Table 19: Effect of some essential oils in *Aspergillus flavus* growth and AFB1 production.

Essential Oil	Concentration	*A. flavus* fungal inhibition	AFB1 inhibition	Reference
Basil (*Acinum sanctum*)	0.1 µg/ml	72.50%	88.40%	
	0.2 µg/ml	90.10%	100%	(Kumar et al., 2010)
Citronella (*Cympopogon citratus*)	0.2 mg/ml	3%	100%	(Paranagama et al., 2003)
Cumin (*Cuminum cyminum*)	0.4 µl/ml	52%	67%	
	0.5 µl/ml	91%	100%	(Kedia et al., 2014)
Rosemary (*Romarinus officinalis*)	450 ppm	0%	100%	(Rasooli et al., 2008)
	0.3 µl/ml	46.90%	75.60%	
Thyme (*Thymus vulgaris*)	0.7 µl/ml	100%	100%	(Kumar et al., 2008)

In general, one of the great advantages of EOs is that they are effective fungal inhibitors, which could serve as pesticide replacement (Prakash *et al.*2015). In addition to this, they are considered as naturals, eco-friendly and some of them are not toxic at controlled doses. As a consequence, some of these essential oils are actually marketed as crop protectors such as E-Rase™ (*Simmondsia californica* EO); Sporan™ (*Rosemarinus officialis* EO), Promas™ (*Thymus vulgaris* EO) and Cinnamite and Valero™ (30% *Cinnamomum* EO) (Isman, 2000).

On the other hand, they also present some disadvantages that make difficult their utilization in food:

-Toxicity: a large number of EOs are reported and regulated as weakly toxic. Nevertheless, their overdose ingestion can trigger negative effects in mammals as well they could drive to morphological damages in plants (Isman, 2000). As an example, several cases of human intoxications to EO's have been reported.
In 1993, Hartool *et al.* reported a case of a 2-years child who ingested among 5-10 ml of clove's EO resulting in coma, coagulopathy and acute liver damage.
In 2008, five cases of poisoning by citronella's EO were also reported and its ingestion also caused a child's death (Temple *et al.,* 2008).
On the other hand, as part of their mechanism of action against fungi, EOs can alter cell membrane which could potentially have the same deleterious effect in plants (Isman and Machial, 2006).

-Modification of organoleptic qualities: It is well-know that EOs are used in cosmetic and pharmacology industries since they contain volatile compounds that can be used for their influence on odor of products (Kumar *et al.,* 2010). Nevertheless, this represents a strong limitation for food industry. As an example, thyme as well as rosemary essential oils at 0.1% and 1% respectively, were effective against the bacteria *Brochothrix thermosphacta* but this concentration was also sufficient to cause negative changes in organoleptic characteristics of meat (Nowak *et al.,* 2012).

- Stability: Essential oils can react to external factors such as heat, light, air, water content or metal contaminants, resulting in chemical changes. They are known to be

converted to other compounds due to oxidation, isomerization, cyclization or dehydrogenation reactions that can also trigger enzymatic changes (Turek and Stintzing, 2013).

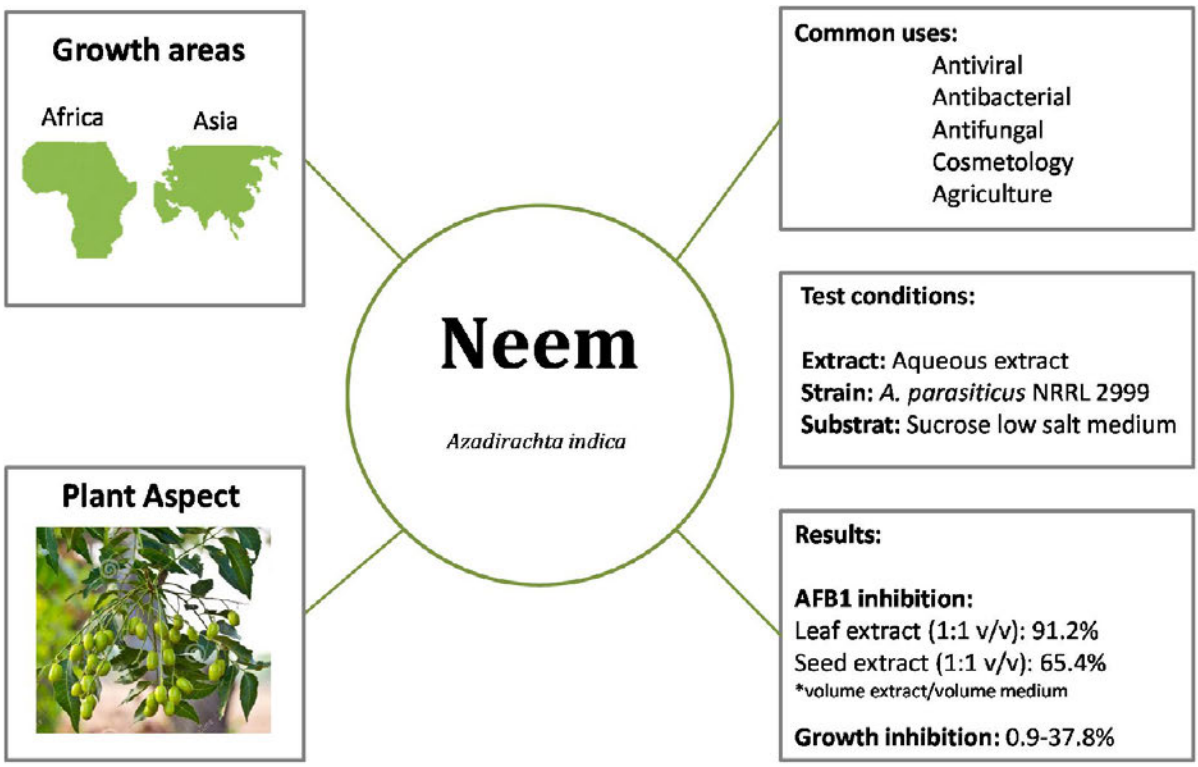
-Finally, there exist some other technical barriers that make difficult their use in food production. It concerns the scarcity of the natural resource, the need of chemical standardization and traceability and quality control.

Since then, the use of essential oils in food is limited to only a few of them and in order to extent the possibilities to inhibit AFB1 production other kind of natural extracts need to be explored.
In fact, the study of other extraction techniques for natural bio-actives compounds relies in the utilization of aqueous or organic extracts of plants that could represent an alternative strategy to inhibit mycotoxin production. Some of them have been already demonstrated as anti-aflatoxigenic agents and their study had increased during last years.
Due to their nature, these kind of extracts can notably display a greater inhibition of mycotoxin production as well as they could have less impact on organoleptic changes in food, among other benefits.
In addition to this, during the hydro-distillation of EOs, the aqueous phase is frequently an industrial waste that could be considered as an option to inhibit mycotoxin's production.
It has to be noted that aqueous extracts are less studied than EOs and in the next section natural products that were only obtained by aqueous or organic extraction with the aim to inhibit AFB1 production will be presented.

ii) Plant extracts

One of the greatest advantages of using plant extracts as mycotoxin inhibitors relies in the bio-diversity that exists all over the world. More interestingly, some of these plants can be developed in different countries. Since then, their identification as anti-aflatoxigenic agents could represent a useful skill.

The next figures recapitulate the principal information of works that had reported the use of natural plants against AFB1 production. Information such as, name of the natural product, countries of development, as well as general information and test conditions are presented.



Razzaghi-Abyaneh et al., 2005

As supplementary information of this study, authors reported that higher concentrations of leaves extract resulted in cell damage with loss of cytoplasmic content. Otherwise, while using seeds extract, cytoplasm vacuolization and deformation of the cell's wall was observed. The authors suggested that aflatoxin inhibition was associated to cell destruction. They compared neem's effect to those of other antifungal agents explaining that chitin and glucans of the *Aspergillus* cell wall might be sensitive to neem's compounds causing wall destruction.

Growth areas

India

Plant names

Acacia catechu
Acacia ferruginea
Adenanthera pavonina L.
Albizia amara
Albizia odoratissima
Albizia saman (Jacq.)
Anogeissus latifolia
Caesalpinia coriaria
Cassia spectabilis
Dodonaea viscosa
Prosopis juliflora
Solanum indicum

Medicinal Plants

Common uses:

Medicinal purposes
(*e.g.* antimalarial, expectorant, antimicrobial, astringent, diuretic)

Test conditions:

Extract: Aqueous/ Methanol/Chloroform
Strain: *A. flavus* MY5
Substrat: Sabouraud Dextrose Agar/Broth

Results:
Using 10% of each aqueous extract in medium
AFB1 inhibition: not determined
Growth inhibition: 11.5 – 42.4%

Using 2 mg/ml of *A. amara; S. indicum* and *A.saman* solvent extract in medium
AFB1 inhibition: completely inhibited
Growth inhibition: 14.3 – 63.5%

Thippeswamy et al., (2014)

Growth areas

Mexico

Plant Aspect

Agave

A. asperrima
A. striata

Common uses:

Manufacture of threads, crop bags, industrial use, sugar, honey, fermented beverages

Test conditions:

Extract: Aqueous/Ethanol/Methanol
Strain: *A. flavus* SRRC 1273/ 1299
A. parasiticus 148/ Si-I
Substrat: A&M broth

Results:
Flower and scape methanol extracts

AFB1 inhibition: From 16-99% *
Growth inhibition: 0-79%*

* Depending on strain and extract concentration

Sánchez et al., 2005

Some other works using natural compounds against AFB1 producer strains have been reported. Even if their action over AFB1 production has not been evaluated, based on their antifungal effect some of them could be considered as interesting sources to limit *A. flavus* contamination and subsequently limit the risk of AFB1 contamination.

For instance, a study performed by Sree *et al.,* (2014) evaluated five plants from Western Ghats of India and tested methanolic, aqueous and ethyl-acetate extracts of (I) *Naravelia zylanica DC*; (II) *Adhathoda zeylanica Medic*; (III) *Cassia auriculata*; (IV) *Vitex negundo L*. and (V) *Orthosiphonstaminus* against 12 fungi including *A. flavus* and *A. parasiticus* strains. Some of these extracts were characterized showing that they are rich in phenol and flavonoid content while they presented interesting antifungal activity.

Another study performed by Satish *et al.,* (2007) tested the effect of fungal inhibition caused by aqueous and solvent extracts of 52 plants against different fungal strains in which *A. flavus* was included. Results showed that *A. flavus* was very susceptible to aqueous extracts as well as solvent extracts from all plants with the exception one of *Polyalthia longifolia*.

Jeff-Agboola *et al.,* (2016) tested ethanol- as well as cold and hot-water extracts of Nigerian plants: *Cymbopogon citratus*, *Moringa oleifera*, *Ocimum gratissimum* and *Clerodendrum volubile* against an *A. flavus* strain isolated from contaminated poultry feed.
These plants were chosen since they are known to have beneficial properties such as flavoring, antioxidant, anti-inflammatory and antifungal among others. Results varied depending on plant and extraction method. In general, all of them with the exception of *Cymbopogon citratus* were effective antifungal agents. Characterization of plant extracts showed that saponins were present in all of them with the exception of *C. citratus* and tannins were presented in all extracts. Authors proposed that these plant extracts could be useful tools to limit fungal infections caused by *A. flavus*.

All together these data demonstrate that plants and their extracts may contain specific molecules that can lead to an effective inhibition of AFB1. Thus, the study of their active molecules may allow a better understanding of the mechanism of action underlying

their biological effect and some of the reported isolated molecules will be further presented.

iii) Isolated Molecules

In the last years, the development of techniques such as q-PCR allowed the study of gene expression upon natural treatments and in particular, with isolated molecules. One of the principal focus is without a doubt, their impact on genes involved in AFB1 synthesis. Within this, *aflR* is one of the principal studied genes based on the fact that this gene is the main regulator of the AFB1 gene cluster.
In the next section, the information of isolated anti-aflatoxigenic natural molecules for which the impact on genes belonging to the AFB1 gene cluster was determined will be detailed. In addition to this, the structures of these isolated anti-aflatoxigenic compounds are presented in the Figure 25 at the end of this section.

Curcumin

Curcumin is a phenolic compound that is classified as safe (GRAS) and is currently found in turmeric. This compound is known as antioxidant and antimicrobial agent.
In *A. parasiticus* NRRL 2999 strain, curcumin inhibited in a dose-dependent manner AFB1's production from 22.6 to 94.9% using concentrations from 125 to 2000 µg/ml. This effect was accompanied with a fungal growth inhibition of 34 to 60.8%. At 250 and 1000 µg/ml, *aflR* gene expression was significantly reduced as well as the same trend was observed for *alfM*, *aflD*, *aflC*, *aflP*.
Authors proposed that AFB1 inhibition was due to the *aflR's* gene reduction or by direct inhibition of all genes (Jahanshiri *et al.,* 2012).

Eugenol

Eugenol is one of the active compounds of cloves, basil and other plants that has antioxidant properties and has been classified as safe by the FAO (daily intake up to 2-5 mg/kg body weight in humans). In 1999, Jayashree T. and C. Subramanyam,

demonstrated that this compound inhibited AFB1 in *A. parasiticus* NRRL 2999 in a dose-dependent manner from 0.75 mM without inhibiting fungal growth.

Afterwards, in 2015, Jahanshiri *et al.,* used the same fungal strain and tested concentrations between 15.62 to 500 µg/ml. They confirmed the AFB1's inhibitory effect and also studied the expression of *alfM*, *aflD*, *aflC*, *aflP* and *aflR.* Results showed that all genes were significantly down-regulated at concentrations of 62.5 and 125 µg/ml.

Authors proposed that toxin inhibition was attributed to *aflR's* gene reduction and to the disruption of lipid peroxidation through microsomal activity reduction.

The same year, Liang *et al.,* reinforced the inhibitory effect of eugenol in an *A. flavus* YC-15 strain at 0.80 mM; q-PCR analysis were performed, demonstrating a down-regulation of *aflP* (the most impacted gene) followed by *aflM*, *aflR*, *aflD* and *aflT* that are five genes that were measured to control aflatoxin production. Experiments were performed over 7 days demonstrating that after 6 days the only down-regulated gene was *aflP*.

Authors suggested that eugenol might directly inhibit *aflP* or *aflR.*

Figure 24 recapitulates the inhibitory effect observed with different doses of curcumin and eugenol that were reported in the above works.

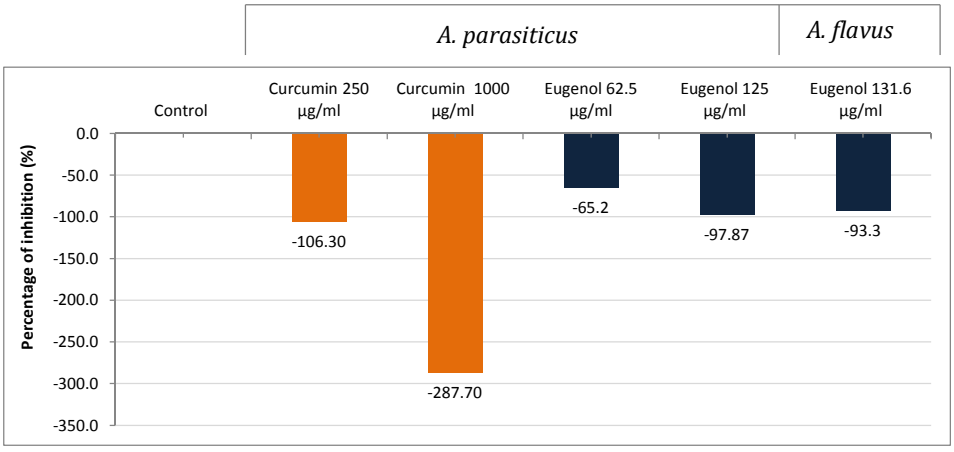


Figure 24: Effect of curcumin and eugenol at different concentrations over the expression of *aflR* detected by Real Time PCR in *A. parasiticus* NRRL 2999 strain and *A. flavus* YC-15 based in the works of Jahanshiri *et al.,* (2012;2015); Liang *et al.,*(2015).

Cinnamaldehyde

This compound is recognized as an antioxidant agent that is also the main component of Cinnamon. It represents a safe component that is legally registered as flavoring agent by the FDA, FAO and WHO organisms. Its effect on AFB1 production was recently analyzed by Sun *et al.,* (2015) in two *A. flavus* strains: (I) CGMCC 8050 and (II) CGMCC 3.6153. They demonstrated that cinnamaldehyde inhibits AFB1's production in a dose-dependent manner and that a total inhibition of toxin and fungal growth was registered at 104 mg/L. Lower inhibitions were registered at 25.4 and 19.9 mg/L for I and II strains respectively.

Authors demonstrated that larger amounts of cinnamaldehyde cause losses of cytoplasmic contents and mitochondrial destruction. These effects were accompanied with redox perturbations in the II strain by increasing enzymatic activities of catalases and glutathione peroxidases while superoxide dismutase activity was slightly reduced.

Liang *et al.,* (2015) also tested cinnamaldehyde at concentrations of 0.40 and 0.80 mM in an *A. flavus* strain. At 0.40 mM AFB1's inhibition was of 68.9% and this effect was dose-dependent with a complete inhibition of both fungal growth and toxin production at 0.80 mM. In addition, an important down-regulation of genes belonging to the AF gene cluster was noted with higher impact on the expression of *aflM* followed by *aflP*, *aflR*, *aflD* and *aflT*.

Authors proposed that:

(i) *aflR* was the principal responsible of AFB1 inhibition

(ii) all genes were directly inhibited by cinnamaldehyde via *aflM* gene.

Caffeic acid

The 3, 4-dihydroxycinnamic acid also named caffeic acid, is a phenolic and antioxidant compound that is widely distributed in flowers, leaves and buds of medicinal plants belonging to a great number of families (e.g. *Umbelliferae, Cricifera, Curcubitaceae, Solanaceae, Leguminosae, Valerianaceae*).

This compound has been shown to inhibit AFB1 production by 95% at a concentration of 12mM in *A. flavus* strain NRRL 3357 without affecting fungal growth. A microarray

analysis demonstrated that upon treatment, all genes belonging to the AFB1 gene cluster were down regulated with the exception of *aflF* and *aflS* that presented lower expression regarding to the control. In addition to this, authors were unable to detect *aflR* expression. Genes coding for molecules involved in lipid metabolism, cell wall integrity, transporter pumps and oxido-reductase/oxygenase were also down regulated while an up-regulation was observed for enzymes, hypothetical proteins and other proteins such as integral membrane protein.

Authors suggested that AFB1 inhibition might be associated with alleviation of oxidative stress response of the fungus (Kim *et al.,* 2008).

Piperine-like compounds

Piperine and piperine-like compounds are currently compounds present in black peppers and they also have been studied as anti-aflatoxigenic agents. In fact, Lee *et al.,* (2002b) demonstrated that piperine, piperlongumine, pipernolaline and piperoctadecalidine isolated from *Piper longum* inhibit AFB1 biosynthesis produced by *A. flavus* WRRC 3-90-42-12 strain and Yazdani *et al.,* (2013) tested fractions of *Piper* crude extract, chloroform and water fractions against *A. flavus* UPMC 89.

In 2016, Moon *et al.,* tested twelve methylenedioxy-containing compounds that are abundant in *Piper* fruits including piperine and 10 piperine-like synthetic compounds against AFB1 produced by *A. flavus* ATCC2254.

In general, they demonstrated that two of the compounds: 3-(benzo-1,3-dioxol-5-yl)-1-(2-methylpiperidin-1-yl) prop-2-en-1-one and 1-(2-methylpiperidin-1-yl)-3-phenylprop-2-en-1-one exhibited antifungal activities. Molecular studies were performed and demonstrated that the latter compound also reduced the expression of *aflR* and *aflS* as well as *aflD*, *aflK*, and *aflQ*.

Author suggested that these compounds might act directly on the AFB1 biosynthesis pathway by inhibiting *aflR* and *aflS*.

Gallic acid

Gallic acid is another compound that has been demonstrated as anti-aflatoxigenic agent and occurs in a great quantity of plants such (*e.g.* clove, origan, *Salvia officinalis*). Other uses of this compound include the creation of transgenic plants with elevated levels of gallic acid that resist *Aspergillus flavus* contamination (Jermnak *et al.,* 2012).
Cary *et al.* (2003) demonstrated that gallic acid was an effective compound to inhibit AFB1. In *A. flavus,* treatment at 0.25% with this compound showed a strong inhibition of *aflD* and *aflM* expression but interestingly, *aflR* levels were only slightly reduced.
Authors suggested that an external transcription factor, involved in oxidative stress and interacting with *aflR* might be down regulated and thus, the efficient transcription of AFB1 genes was disrupted causing the inhibition of aflatoxin production.

Methyl syringate

This compound is a derivative of gallic acid and was isolated from the essential oil of *Betula alba* by Jermnak *et al.,* (2012). It is capable to inhibit AFB1 production in a dose-dependent manner in *A. parasiticus* and *A. flavus* strains. Four different concentrations were tested and, for both strains, AFB1 was no longer detected after exposure at 4 mM. This effect was accompanied with a slight impact on fungal growth.
In addition to this, methyl syringate inhibited, in a dose-dependent manner, norsolorinic acid production with an IC50 value of 0.8 mM. This response indicated that inhibition of aflatoxin occured at earliest stages of the pathway. The mRNA levels of *aflR*, *aflC* and *aflP* were analyzed demonstrating a down-regulation of all of them.
For authors, inhibition of aflatoxin by methyl syringate is a result of the down-regulation of *aflR*.

Citral

Citral (3,7-dimethyl-2,6-octadienal) or lemonal, is a compound present in several plants such as lemon, orange and derivatives. Its anti-aflatoxigenic effect was demonstrated by Liang *et al.,* (2015) and its impact on several genes of the aflatoxin gene cluster was analyzed. Different citral concentrations resulted in AFB1's inhibition ranging from 23.5

to 60.5%. In terms of fungal inhibition, citral significantly reduces *A. flavus* growth by 61.1% at 1.68 mM and this inhibition was complete at 2.80 mM. In addition to this, mRNA levels were measured from day one to day seven in order to observe the kinetics of the effect. Contrary to other compounds, citral induces a complete inhibition of *aflT* at all tested-time. Other genes coding for *aflM*, *aflP*, *aflR* and *aflD* were also significantly different.

Therefore, under citral treatment, *aflR* and *aflD* were only slightly inhibited while other such as *aflM* and *aflP* were more impacted. Moreover, at day 7 *aflR* and *aflD* levels were comparable to the control.

In order to conclude, the structure of each of these isolated compounds that have been demonstrated as AFB1 inhibitors is presented in the Figure 25.

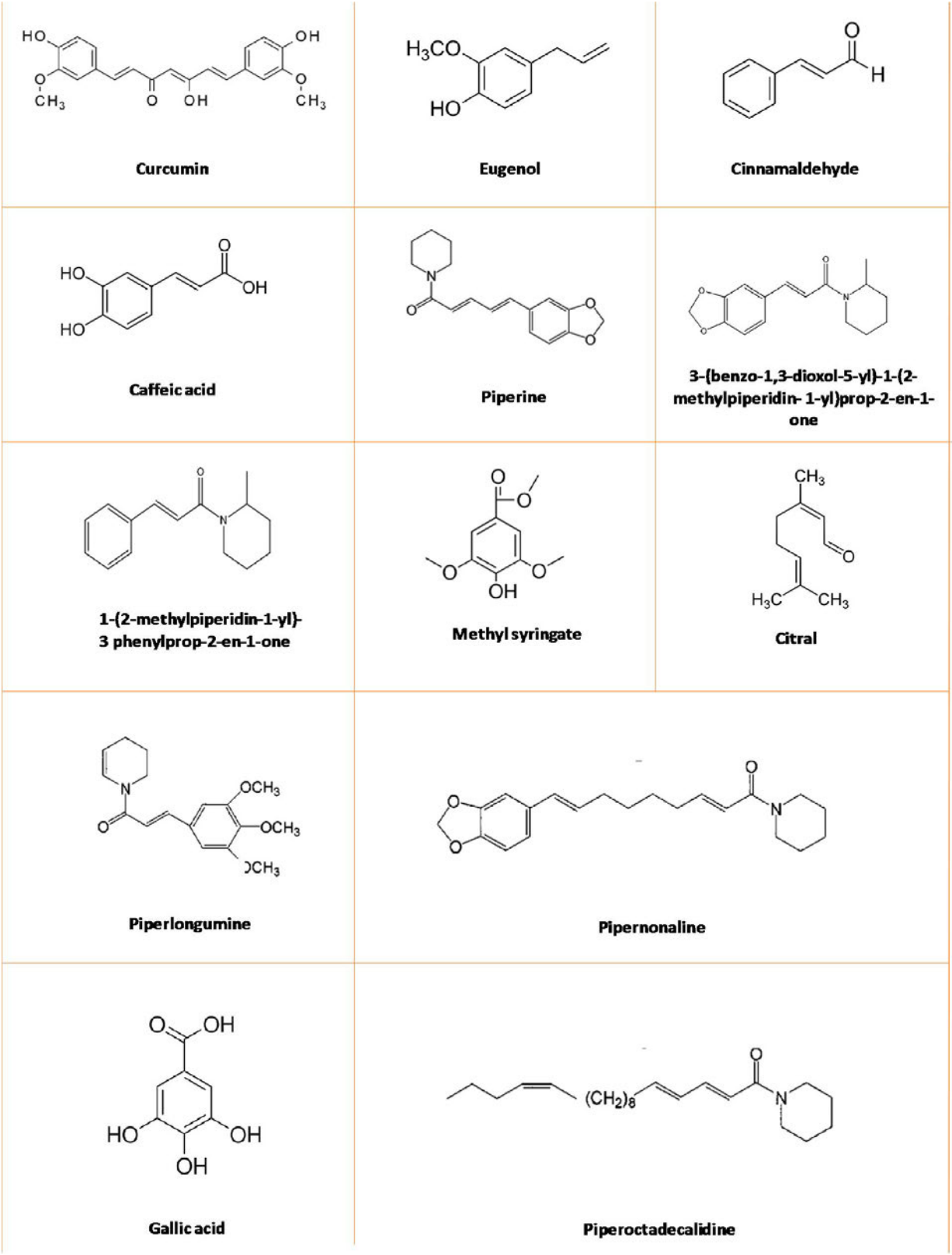


Figure 25: Structures of AFB1 inhibitory molecules

3.6 Conclusion

As demonstrated during the bibliography study, contamination by fungi represents an important issue taking in consideration that not only fungal contamination is a constraint, but that also Aflatoxin B1 production represents a dangerous problem for human and animal's health.

Since then, numerous strategies are used in order to prevent mycotoxin occurrence in food commodities. Within these preventive methods, one of the most preoccupying is the use of pesticides.

In order to offer an alternative strategy, the use of natural products that inhibit mycotoxin production could be taken in consideration. In particular, works based in the properties of these natural products as anti-aflatoxigenic agents have demonstrated that it could be a viable strategy to inhibit AFB1 production.

Nevertheless, the exact mechanism of action by which these natural products prevent AFB1 production lacks of a lot of information.

Since AFB1 production is a complex and not understood mechanism, the molecular study of the genes intervening during AFB1 production is a key element to understand the impact that natural products have over the genetic machinery in charge to produce this toxin.

3.7 Objectives

According to this overall context, the objectives of the present work will be oriented towards two principal axes.

The first one aims the construction of a molecular tool to understand the mechanism of action by which natural products inhibit AFB1 production in *Aspergillus flavus*. In fact, as demonstrated in bibliography, most of the reported works mainly studied the impact of natural molecules over the principal regulator of the pathway *aflR* but little is known about the effect on the rest of the genes belonging to the AFB1 cluster. Thus, the present molecular tool was developed in order to simultaneously measure the mRNA expression levels of the entire AFB1 gene cluster.

In addition to this, while using natural products as AFB1 inhibitors we considered that the study of the external factors that are involved in the regulation of AFB1 can be a helpful skill to elucidate the mechanisms of action of these natural products.
For that, within the construction of the molecular tool some of the principal genes that are involved in AFB1 global regulation were also included.

Several works that had already evaluated the AFB1 inhibition by natural products in a great number of genes mainly use microarrays or RNA-Seq techniques in order to have a wide vision of the inhibitory agent. Even if this technique has great advantages at big-scale, the interpretation of the mechanism of action is a difficult process since the precis role of most of the genes belonging to the *Aspergillus* genome has not been reported. Taking this in consideration, the construction of the present molecular tool uses the q-PCR technique which allows targeting a reduced number of genes with the advantage of include specific genes that have been already reported to directly or indirectly interact with AFB1 production.

The second aim of this study relies in the investigation of new natural sources to inhibit AFB1 production. Since pesticides is nowadays of the most used methods to inhibit this toxin and taking in consideration that pesticides have been demonstrated as hazardous for human and animal's health, alternatives strategies are needed.

For this, the use of natural products represents a strategy that could offer several advantages such as an eco-friendly alternative as well as a potential element to inhibit mycotoxin production. According to this, two isolated molecules (eugenol and piperine) that have been previously identified as anti-aflatoxigenic agents were characterized by using the developed q-PCR molecular tool. In addition to this, Mediterranean and Mexican plants were also evaluated in order to report their efficacy as AFB1 inhibitors as well as their molecular impact was also characterized.

04

EXPERIMENTAL WORK

4.1 Chapter 1

Deciphering the anti-aflatoxigenic properties of eugenol using a large-scale qPCR approach

(Article 1 – Toxins, 8 (Basel), 123)

Introduction

AFB1 production is a complex mechanism that involves numerous genetic elements. Although the final biosynthesis of this mycotoxin is known to occur in the AFB1 gene cluster, little is known about the influence that external regulators may have on the cluster and thus, on AFB1 production.

To date AFB1's inhibition is an important topic of research, the study of this gene network as a global fungal mechanism is of great importance. This could lead not only to understand which of the genes are intrinsically involved during AFB1 production but also, to increase the understanding of the mechanisms by which this toxin is controlled and possibly inhibited.

According to this, an important number of natural compounds have been demonstrated as anti-aflatoxigenic agents. Nevertheless, even if they effectively inhibit toxin production, their precis mechanism of action is yet to be elucidated.

Taking advantage of the great available information that has already been reported for the *Aspergillus flavus* genome, we constructed a q-PCR molecular tool involving a total of 60 genes where the entire AFB1 cluster consisting in 27 genes, but also 33 external regulatory factors were included.

First of all, the present q-PCR molecular tool needed to be tested and validated by using a molecule that was already demonstrated to greatly inhibit AFB1 production.

It has to be noted that within the last years, one of the eco-friendliest strategies against AFB1 production resides in the use of natural compounds and to date, molecules from several spices and plants are used for this purpose.

For instance, Eugenol is a molecule that naturally occurs in species such as clove and cinnamon but also in plants like basil. Moreover, its anti-aflatoxigenic ability is known since 1999. In order to validate our molecular tool, Eugenol seemed to be a good candidate.

Thus, the work below reports in detail the construction of the molecular tool as well as the impact of the inhibitor molecule Eugenol, on the expression of the 60 analyzed genes.

toxins

MDPI

Article

Deciphering the Anti-Aflatoxinogenic Properties of Eugenol Using a Large-Scale q-PCR Approach

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Abstract: Produced by several species of *Aspergillus*, Aflatoxin B_1 (AFB_1) is a carcinogenic mycotoxin contaminating many crops worldwide. The utilization of fungicides is currently one of the most common methods; nevertheless, their use is not environmentally or economically sound. Thus, the use of natural compounds able to block aflatoxinogenesis could represent an alternative strategy to limit food and feed contamination. For instance, eugenol, a 4-allyl-2-methoxyphenol present in many essential oils, has been identified as an anti-aflatoxin molecule. However, its precise mechanism of action has yet to be clarified. The production of AFB_1 is associated with the expression of a 70 kB cluster, and not less than 21 enzymatic reactions are necessary for its production. Based on former empirical data, a molecular tool composed of 60 genes targeting 27 genes of aflatoxin B_1 cluster and 33 genes encoding the main regulatory factors potentially involved in its production, was developed. We showed that AFB_1 inhibition in *Aspergillus flavus* following eugenol addition at 0.5 mM in a Malt Extract Agar (MEA) medium resulted in a complete inhibition of the expression of all but one gene of the AFB_1 biosynthesis cluster. This transcriptomic effect followed a down-regulation of the complex composed by the two internal regulatory factors, AflR and AflS. This phenomenon was also influenced by an over-expression of *veA* and *mtfA*, two genes that are directly linked to AFB_1 cluster regulation.

Keywords: Aflatoxin B_1; *Aspergillus flavus*; aflatoxinogenesis; molecular tool; gene regulation; eugenol

1. Introduction

Aflatoxin B_1 (AFB_1) is a mycotoxin produced by many fungal species belonging to the *Flavi* section of the *Aspergillus* genus, *A. flavus* being the most preoccupying species [1]. AFB_1 is the most potent naturally occurring carcinogen [2], responsible for hepatocarcinoma; it is also an immunosuppressive agent and has been linked to growth impairment in children [3,4].

AFB_1 is problematic in countries with tropical and sub-tropical climates where temperature and humidity conditions are optimal for fungal growth and toxin production [5]. However, this danger has spread beyond its predicted geographical borders and has reached countries previously considered as safe. In fact, in recent years, several surveys demonstrated the contamination of European crops by this

toxin [6,7]. Fungal infection can occur at a pre- or post-harvest stage of cereal production, especially corn, but also oilseeds, nuts, spices and dried fruit [8].

Many strategies have been developed to reduce AFB_1 contamination, either by preventing the fungal development or by blocking the toxin's production after infection [9]. Natural extracts are considered as a possible alternative antimicrobial agent [10]. Indeed, organic and aqueous extracts from plants and spices, as well as essential oils, have demonstrated fungicidal and/or anti-toxinogenic properties [11–13]. For example, eugenol (4-allyl-2-methoxyphenol), the active compound of many anti-toxinogenic essential oils [14,15], blocks AFB_1 production in aflatoxigenic fungi [16,17]. However, little is known about the molecular mechanism of this inhibition and the fungal pathways affected by eugenol remain to be determined.

AFB_1's biosynthetic pathway is well characterized and consists in *A. flavus*, of a cluster of 27 genes whose expression is governed by two internal regulators (AflR and AflS). AFB_1's biosynthesis is also interconnected with developmental genes that play a role in morphology, conidiation, or sclerotia formation [18,19], as well as genes encoding the velvet regulating proteins that coordinate primary and secondary metabolism (SM) [20]. Transcription factors (TFs) such as *mtfA* [21] and *fcr3* (*A. nidulans rsmA* orthologous) [22] and other TFs influenced by environmental factors like pH, nitrogen and carbon, can also interfere with aflatoxin production [23]. In the same way, genes related to oxidative stress regulation [24] as well as genes encoding cellular signal mediators such as *rasA*, [25] G-protein receptors [26] and oxylipins' biosynthetic genes [27] influence toxin synthesis. This demonstrates the complexity of environmental signals and cellular pathways involved or interfering with mycotoxin production.

In the present study, a molecular tool was developed including genes directly implicated in aflatoxin production as well as genes involved in the upstream regulation of this toxin. This tool was used to determine the molecular mechanism of AFB_1's inhibition by eugenol. Hence, we demonstrate that this later compound acts at the transcriptomic level to inhibit AFB_1 production by restricting the expression of its biosynthetic cluster. Moreover, we also reveal that this inhibition is mainly governed by the modification of *mtfA* and *veA*'s expression levels.

2. Results

2.1. Effect of Eugenol on Fungal Growth and Aflatoxin B_1 Production

Five different concentrations of eugenol were tested for their effect on both fungal development and AFB_1 biosynthesis (Figure 1). Fungal growth was only slightly affected and colony diameter was reduced by 11.4% for 0.5 mM and 34.5% for 1 mM eugenol. By contrast, eugenol decreased AFB_1 production in a dose-dependent manner with inhibitions of 19.8, 30.9, 70.2 and 100% at eugenol concentrations of 0.01, 0.05, 0.1 and 0.5–1 mM, respectively.

For subsequent assays, a concentration of 0.5 mM of eugenol was used allowing complete inhibition of AFB_1 production with a limited impact on fungal growth. Since AFB_1 production can be modulated by pH, this parameter was measured in the fungal cultures. Before incubation, pH values were 5.30 ± 0.06 and 5.23 ± 0.08 in control and treated cultures, respectively. After four days at 27 °C, both cultures displayed a mild but statistically significant (p-value = 0.049) acidification with pH means ± Standard Error of Mean (SEM) of 4.7 ± 0.03 for control and 4.4 ± 0.2 for eugenol treated cultures.

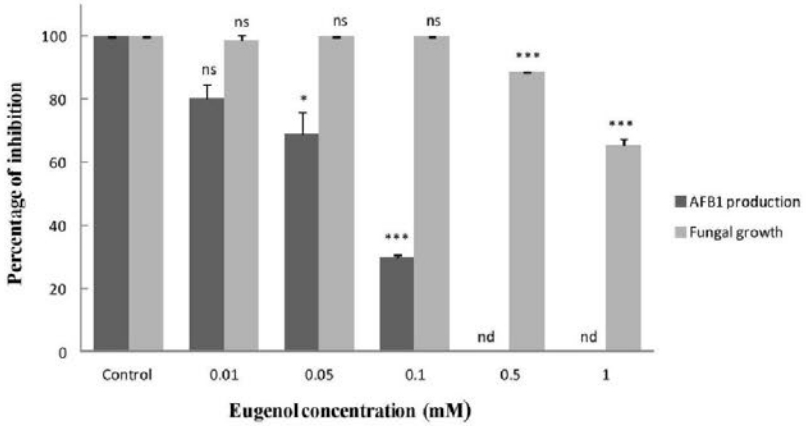

Figure 1. Effect of eugenol on Aflatoxin B_1 (AFB_1) production and fungal growth in *A. flavus* NRRL 62477. Results are expressed as percentage of the control value. AFB_1 was estimated by High Performance Liquid Chromatography (HPLC) and fungal growth by colony diameter. Both measures were taken on day 4 on six biological replicates. ns = no significant changes; nd = not detectable; * p-value < 0.05; *** p-value < 0.001.

2.2. *Effect of Eugenol on Aflatoxin Biosynthetic Pathway*

In *A. flavus*, AFB_1's biosynthetic pathway consists of 27 genes regrouped in a cluster where *aflR* and *aflS* are the two internal regulators. Following the addition of 0.5 mM eugenol, AFB_1 was completely inhibited and all cluster genes with the exception of *aflT* (p-value = 0.8667) were strongly down-regulated. In fact, the expression of 19 out of 27 genes was almost completely inhibited, whereas five others had 10- to 20-fold reductions in expression levels compared to control conditions (Figure 2). Therefore, the extent of down-regulation was mildly different according to the chronological intervention level of the encoded enzyme in the biosynthetic pathway.

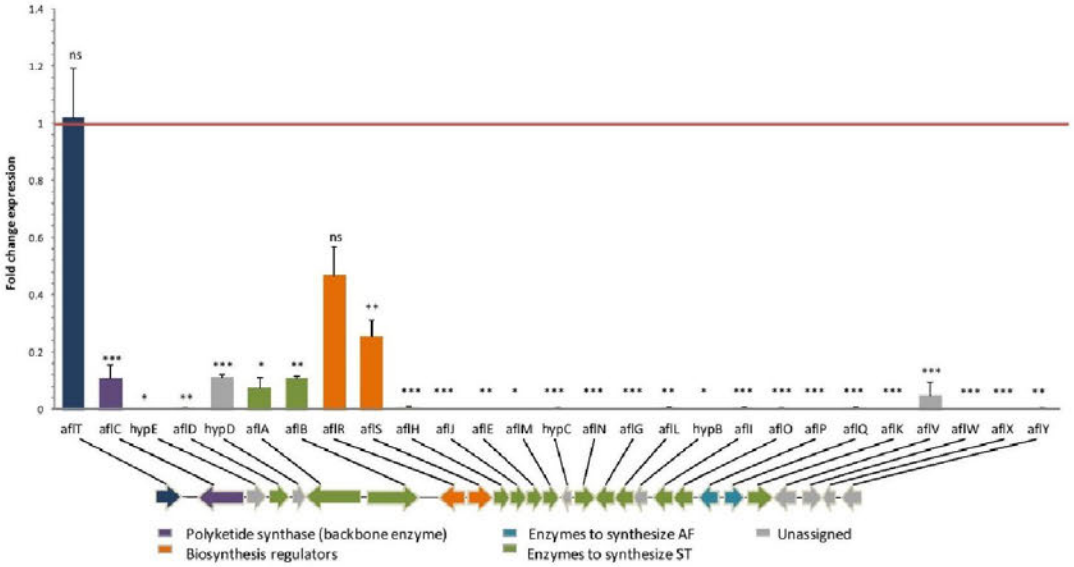

Figure 2. Fold change expression of genes belonging to the cluster responsible for aflatoxin biosynthesis in response to eugenol at 0.5 mM. Red line represents control expression level. Gene cluster organization was adapted from Amaike and Keller [1]; ns = no significant changes; * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

As an illustration, fundamental genes involved in the first steps of AFB_1's enzymatic cascade, such as *aflC*, encoding the polyketide synthase A, and two of fatty acid synthase genes aflA (fas-2) and aflB (fas-1), appeared less affected by eugenol compared to further intermediate genes such as *aflO (omtB)*, *aflP (omtA)* and *aflQ (ordA)*, the latter being an enzyme in charge of the final transformation of AFB_1. For those genes, expression was almost completely inhibited by eugenol (p-values < 0.0001).

The reduced expression of cluster genes went with a decreased expression of the internal regulators. Concerning *aflS*, it saw a 3.9-fold down-regulation (p-value = 0.0030) whereas *aflR*'s expression level was not significantly affected by eugenol addition (p-value = 0.0522), although a diminution averaging at half was observed.

2.3. Effect of Eugenol on Regulatory Factors Linked to AFB_1 Production

Since AFB_1 biosynthesis is strongly interconnected with several other fungal biosynthetic pathways, a large number of genes considered as regulatory factors were analyzed. They included the velvet complex, genes involved in oxidative stress response, environmental and global transcription factors, genes involved in cellular signaling (oxylipins, Ras family and G-protein signaling and receptors) and developmental regulators (Figure 3).

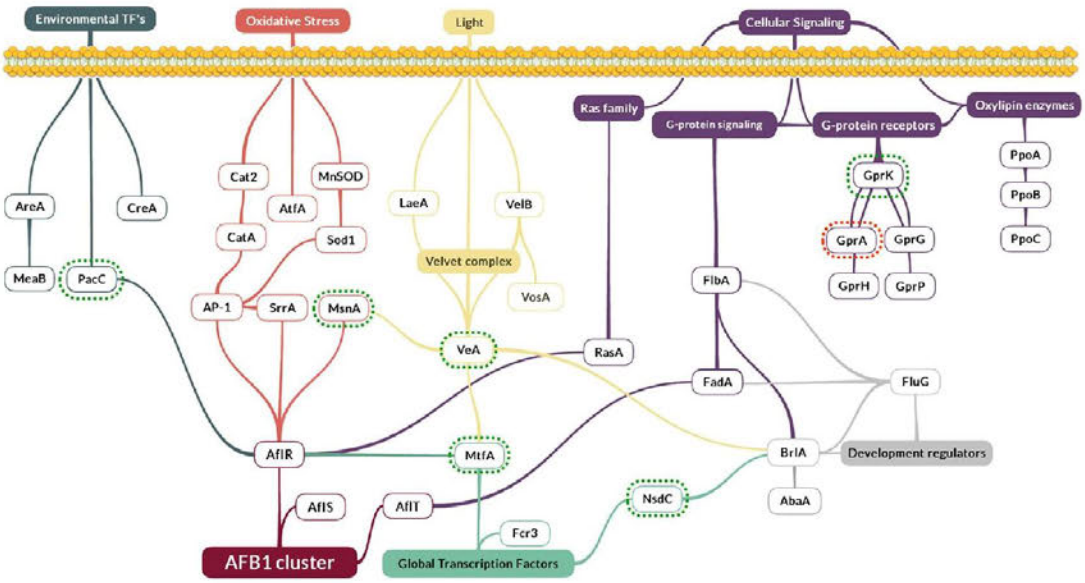

Figure 3. Schematic representation of the 33 selected regulatory factors linked to AFB_1 cluster in *A. flavus*. This hypothetical schema represents a simplified version of the different interactions between the regulatory factors and Aflatoxin's cluster. Schema was constructed based on gene interaction data described by the following works: [10,19–24,26,28–38]. Up- or down-regulation of genes upon eugenol addition is represented by green an red dotted lines, respectively.

As shown in Figure 4, among the 33 tested genes, only seven presented significant modifications of their level of expression upon eugenol exposure.

These genes were:

- The global regulator gene *veA*, belonging to the velvet complex. It was over-expressed with a 3.8-fold change compared to the control (p-value = 0.002);
- *mtfA*, a putative C_2H_2 zinc finger transcription factor. It presented the same up-regulated pattern, increasing its expression by 2.2 times (p-value = 0.0297);
- *nsdC*, of the global transcription factors, whose expression was increased by 1.7 times (p-value = 0.0100);

- *gprK*, which was the most affected gene among the five G-protein coupled receptors analyzed here. This gene was over-expressed by 4.5 times (p-value = 0.0009). By contrast, *gprA* was down-regulated by 0.45 times (p-value = 0.0177);
- The *msnA* gene was increased by 1.9 times (p-value < 0.0001), whereas no significant changes were observed for other genes implicated in the oxidative stress response such as superoxidase dismutases, catalases or oxylipins;
- Finally, *pac*C's expression, a zinc finger transcription factor related to pH, was increased by 2.3 times (p-value = 0.0098).

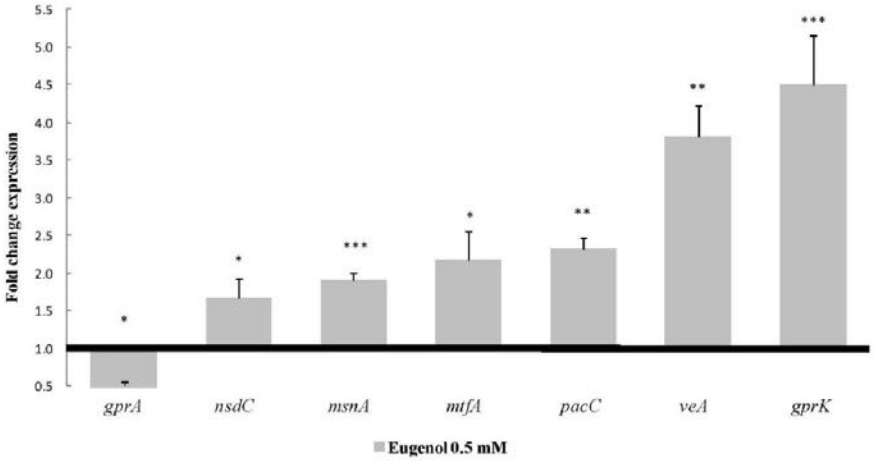

Figure 4. Fold change levels of the seven affected regulatory factors in presence of eugenol at 0.5 mM. Baseline represents control expression level; * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

3. Discussion

A number of natural extracts were identified as being able to down-modulate the synthesis of the carcinogenic mycotoxin AFB_1 in *A. flavus* [39]. The use of such inhibiting compounds could therefore represent an alternative strategy to the use of pesticides to control crop contamination. However, to date, the precise molecular mechanism responsible for this effect is only poorly documented. Indeed, mycotoxin production is a complex phenomenon based on the presence of biosynthetic clusters in toxigenic fungi whose regulation is governed by many environmental and physiological processes.

In order to better understand the inhibition of AFB_1 production by such natural extracts, we developed a molecular tool allowing the simultaneous analysis of the expression of both the AFB_1 cluster and a large number of global regulatory genes involved in different cellular pathways. We used it to characterize the mechanism of action of eugenol, a compound present in many essential oils and that has been previously identified as an AFB_1 inhibitor. Eugenol has been extensively studied for its many biological effects including antimicrobial, anti-inflammatory, anti-oxidant and anticancer activities. However, to date, no precise cellular target was identified even though the interaction with cell membrane may represent a key point in the biological effects of this molecule [40].

3.1. Eugenol Inhibits the Expression of Aflatoxin Cluster Genes in A. flavus

The inhibition of AFB_1 production by a toxigenic strain of *A. flavus* following eugenol addition is accompanied by the down-regulation of all but one aflatoxin cluster genes.

We demonstrated that the expression of 19 out of 27 genes of the cluster was almost no more detectable after eugenol exposure, whereas the others saw 10- to 20-fold reductions in their expression levels. These results are coherent with a very recent work of Jahanshiri *et al.* [16] showing that eugenol decreased the expression of some of Aflatoxins (AF)'s cluster genes in *A. parasiticus*. In that previous

study, only five genes of the pathway were analyzed. Our present work extended this finding to the whole genes involved in the biosynthesis of AFB_1.

AFB_1's cluster is internally regulated by two genes, *aflR* and *aflS*, whose activation is governed, independently one from another, by external regulators. An interaction between AflR and AflS is reportedly required for aflatoxinogenesis [41]. Kong *et al.* [42] reported that the activation by these two proteins led to the formation of a functional activation complex in the proper ratio of four AflS to one AflR. In our study, the expression of *aflR* was not significantly decreased in cultures exposed to eugenol, although a major down-regulation tendency was observed. Such a finding was already described in several studies where even low-level changes of *aflR*'s expression levels were accompanied by a severe decrease of structural genes [25,43,44]. However, levels of *aflS* transcripts were significantly reduced, which might have led to an alteration of the ratio between AflR and AflS and thus the formation of a limited number of active complexes. Consequently, AflR-binding sites were not attained and the transcription of the cluster was not activated (Figure 2). All of the cluster genes regulated by *aflR* had their expression levels severely decreased by eugenol's addition. The genes intervening in the later stages of AFB_1's enzymatic cascade (*aflM*, *aflN*, *aflX*, *aflO*, *aflP*, *aflQ* and *hypB*) were more impacted than those involved in the beginning stages (*aflA*, *aflB*, *aflC*) leading to the polyketide structure [45]. The limited AflR/AflS complexes formed might have been promptly used up at the beginning of AFB_1's synthesis and were no longer sufficiently available for the proper activation of the rest of the cluster genes.

For *aflT*, the expression levels were not significantly different between control and eugenol-treated cultures. In fact, *aflT*, a Major Facilitator Superfamily (MFS) transporter encoding gene, is regulated neither by AflR nor by its co-activator AflS, due to the absence of an AflR binding-site on its promoter, but rather by the FadA-dependent G-protein signaling pathway [46]. Moreover, the expression of none of the genes belonging or affected by the latter pathway, notably *fadA*, *flbA*, *fluG* and *brlA* [34], has been altered by eugenol's addition (Table S1).

3.2. Eugenol Alters the Expression of Global Regulation Factors

Eugenol's transcriptomic effect goes upstream of AF's cluster genes, affecting genes encoding general transcriptional regulating factors.

3.2.1. The Pivotal Role of MtfA, VeA and MsnA in Eugenol's Molecular Mechanism

MtfA is a global transcription factor, regulating sterigmatocystin/aflatoxin biosynthesis as well as other secondary metabolites clusters [31]. The deletion as well as the over-expression of *mtfA* has been shown to inhibit the expression of *aflR* and subsequent sterigmatocystin production in *A. nidulans* [21]. Also, in the recent work of Zhuang *et al.* [47] on peanut seeds infected with Δ*mtfA* and over-expressed (OE) *mtfA* gene on *A. flavus* strains, it was observed that the decrease in AFB_1 production was greater when there was a 2.75-fold *mtfA* over-expression compared to the wide-type strain, than deleted, and went with a decreased expression of *aflR*. Although the *mtfA* gene is over-expressed at a similar level as in the above-quoted study [47], this does not mean that MtfA is the direct molecular target of eugenol. A kinetic study of gene expression, using our molecular tool coupled to AF production analysis, could be used in order to determine the time course evolution of modulated genes.

Furthermore, *mtfA*'s expression is highly dependent on that of *veA*. As discussed in the study by Lind *et al.* [31], MtfA interacts with VeA in *A. nidulans* and the expression of *mtfA* (AN8741.2) was decreased by 5 times in an *A. nidulans* Δ*veA*. All of the above findings are in favor of the notion that the over-expression of *mtfA* in *A. flavus* is a result of the increased expression of *veA* upon eugenol addition. The interaction of these two regulators could then be responsible for *aflR*'s down-regulation and the succeeding inhibition of AF's biosynthetic pathway in *A. flavus*. Moreover, VeA is by itself essential for the transcription of both *aflR* and *aflS* and, consequently, the production of aflatoxins [48]. This global transcription regulator plays a key role in secondary metabolite production [49]. Depending on its abundance in the cell, it may act as a repressor or as an activator [50]. For instance, the expression

of fumagillin and fumitremorgin G gene clusters was inhibited in a overexpressing *veA* (OE:*veA*) strain of *A. fumigatus* [51]. Therefore, an over-expression of *veA* (3.8-fold) observed here is compatible with the down-regulation of *aflR* and *aflS* and the subsequent inhibition of aflatoxin production in eugenol-treated cultures. Furthermore, as in many filamentous fungi, SM, and aflatoxin production in particular are often induced as a response to Reactive Oxygen Species (ROS) formation [52]. It has been also demonstrated that VeA plays a critical role in protecting *A. flavus* from oxidative stress. VeA positively regulates the expression of oxidative stress tolerance genes such as *msnA* in *A. flavus* [38] and also in *A. nidulans* [31]. Conversely, levels of ROS as well as AFB_1 increased in Δ*msnA A. flavus* and *A. parasiticus* strains [53]. Furthermore, it has been demonstrated that aflatoxin biosynthesis is itself a source of intracellular ROS, and the over-expression of *msnA* could then also be the outcome of AFB_1's inhibition [54].

Accordingly, an oxidative-stress alleviating condition, such as the addition of eugenol, could lead to an increased expression of *veA* and consequently of *msnA*. In our study, *msnA* transcripts increased by 1.9 times along with AFB_1's inhibition in eugenol-treated cultures, following *veA*'s over-expression. This effect is possibly linked to a decrease of intracellular ROS.

3.2.2. The Putative Implication of Other Regulatory Factors and Signaling Proteins

Eugenol addition also alters the expression of other transcription-regulating factors such as (i) *nsdC* and (ii) *pacC* encoding a pH-dependent transcription factor, as well as (iii) the *grpA* and *grpK* genes involved in the G-protein signaling pathway. Except for *grpK*, these genes have been shown to be closely related to the expression of AF cluster genes and AFB_1 production [19,26,55,56]. However, to date, the exact mechanism of this interaction has to be clarified.

4. Conclusions

In this study, we bring out the transcriptomic inhibition behind eugenol's AFB_1-repressing action. This is the first time, to our knowledge, that the expression of all the 27 genes involved in AFB_1 synthesis has been studied on an inhibitor molecule. In the presence of eugenol, AFB_1 cluster genes were strongly down-modulated following decreased expression of its regulating complex AflR/AflS. We also demonstrated that this went with a modulation of seven regulatory factors. We then highlighted the involvement of *mtfA*, *veA* and *msnA* in this inhibition.

5. Materials and Methods

5.1. Chemicals and Reagents

Aflatoxin B_1 and eugenol standards were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and dissolved in methanol and ethanol, respectively. Stock solutions were stored at 4 °C until use. All analytical grade solvents were purchased from Thermo Fisher Scientific (Illkirch, France).

5.2. Fungal Strain and Culture Conditions

The *Aspergillus flavus* strain NRRL 62477 used in this study [57] was maintened in the dark on a Malt Extract Agar (MEA) medium (Biokar Diagnostics, Allone, France) at 27 °C.

For experiments, 10 µL of a calibrated spore suspension (10^6 spores/mL) prepared from a seven-day culture was used to centrally inoculate the MEA medium covered with sterile cellophane layers (Hutchinson, Chalette-sur-Loing, France) as described by Leite *et al.* [58]. Firstly, five different concentrations of eugenol (0.1 to 1 mM) were tested to determine the concentration able to inhibit AFB_1 with a limited impact on fungal development, as measured by colony diameter. The concentration of 0.5 mM was selected for further experiments. Eugenol dilutions were prepared to add only 20 µL of ethanol in the culture medium, this concentration having been identified as a no-effect dose on both fungal growth and AFB_1 production. Control cultures were performed by adding only 20 µL of ethanol in the medium. Six replicates of each group were prepared and incubated for four days at 27 °C in the

dark. At least three replications of the experiment were performed. The pH of all media was taken before and after incubation using a food pH-meter H199161 (Hanna Instruments, Tanneries, France).

5.3. Aflatoxin B_1 Extraction and Determination by HPLC

For AFB_1 extraction, culture media were mixed with 25 mL of chloroform. Samples were agitated for 2 h on a horizontal shaking table at 160 rpm at room temperature. Chloroform extract was filtered through a Whatman 1PS phase separator (GE Healthcare Life Sciences, Vélizy-Villacoublay, France), evaporated at 60 °C until dry and dissolved in 500 µL of a water-acetonitrile-methanol (65:17.5:17.5; *v*/*v*/*v*) mixture. To eliminate possible impurities, all samples were filtered through a 0.45 µm disk filters (Thermo Scientific Fisher, Villebon-Sur-Yvette, France). The analysis of samples was done with a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Illkirch, France) using a liquid chromatography column, Luna® C18 (125 × 2 mm, 5 µm, 100 Å) (Phenomenex, Torrance, CA, USA) at 30 °C. Separation conditions were adapted from Fu *et al.* [59] with mild modifications. A 20 min isocratic mode was delivered at 82.5% of eluent A: acidified water (0.2% of acetic acid) and acetonitrile (79:21 *v*/*v*); and 17.5% of eluent B: pure methanol. A flow rate of 0.2 mL/min was used and 10 µL of extract was injected. AFB_1 was detected by a fluorescent detector at 365/430 nm excitation/emission wavelengths. Peak identity was confirmed by analyzing absorption spectrum with a diode array detector coupled to the system. Production levels of AFB_1 on media were calculated based on a standard calibration curve.

5.4. Isolation of Fungal RNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

At the end of incubation, mycelia were separated from the medium and ground up under liquid nitrogen. The RNA was purified as recommended by the manufacturer from 100 mg of mycelium using a Qiagen RNeasy PlusMinikit (Qiagen, Hilden, Germany) and including a gDNA eliminator column. The quality of RNA was verified by gel electrophoresis (1.2% agarose) and concentrations were measured using a NanoDrop ND1000 (Labtech, Palaiseau, France). The A_{260}/A_{280} ratio was measured [60], and each sample was adjusted to a final RNA concentration of 300 ng/µL.

First-strand cDNA synthesis was carried out by RT-PCR. Reverse transcription took place in a 20 µL reaction mixture containing 10 µL of RNA, 200 U of RevertAid Reverse transcriptase, 4 µL of 5× Reaction Buffer, 20 U of RNase inhibitor (Thermo Scientific, Illkirch, France), 2 µL of 10 mM dNTP (Euromedex, Souffelweyersheim, France), 1 µL of sterile water, and 1 µL of oligo (dT) Bys 3' Primer: (5'-GCTGTCAACGATACGCTATAACGGCATGACAGTGTTTTTTTTTTTTTT-3').

A first denaturation was done at 70 °C for 5 min and reverse transcription was performed as follows: 5 min at 37 °C; 60 min at 42 °C and 15 min at 85 °C.

5.5. Design and Validation of q-PCR Primers

All primer sets were designed based on the genomic data of the *Aspergillus flavus* strain NRRL3357 (GenBank accession number EQ963478A). A total of 62 genes primer pairs were designed including all 27 AFB_1 cluster genes. It must be noted that *aflF* and *aflU* were not followed in this study, as the promoter regions of both genes are missing in *A. flavus* species, thus their inability to produce type-G aflatoxins. Other regulatory factors directly or indirectly related to AFB_1's production (Figure 3) were included, along with two housekeeping genes, *b-tubulin* and *gpdA*. All primer pairs were designed to amplify a 50–150 bp fragment based solely on the coding sequence of the corresponding genes, with at least one of the primers extending on an exon/exon junction in order to avoid undesirable genomic DNA amplification. Primer-dimer or self-complementarities were evaluated using the PrimerExpress 2.0 software (Applied Biosystems, Courtaboeuf, France). All primers were synthesized by Sigma Aldrich (Saint-Quentin Fallavier, France) and tested with four different concentrations (300/300; 300/900; 900/300 and 900/900 nM) after reception to determine their optimal concentrations in the mix. Primer validation was carried out with the best amplification curve and dissociation curves were used to confirm the good amplification of each gene. At least three biological replicates of

A. flavus NRRL62477 were used to validate the amplification specificity. Negative controls in which no reverse transcriptase enzyme was added and a no template control were included to control reagents contamination. Primer sequences and their concentrations are listed in Table S2.

5.6. Analysis of the Expression of the Genes Linked to Aflatoxin B_1 Biosynthesis

Experiments were carried out using a ViiA7 Real-Time PCR System (Applied Biosystems, Forster City, CA, USA). The 384 well-plates were prepared by an Agilent Bravo Automated Liquid Handling Platform (Agilent Technologies, Santa Clara, CA, USA). Each well contained a final volume of a 5 µL mix: 2.5 µL of Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) used as a fluorescent dye, 1.5 µL of each primer set and 1 µL of cDNA material. Three-step quantitative PCRs were performed as follows: a first one-hold stage at 95 °C for 10 min followed by 45 cycles (95 °C for 15 s and 60 °C for 30 s), and a final extending step (95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s) for melt curve analysis. The results were analyzed with a Quant-Studio Real time PCR software v1.1 (Applied Biosystems, Courtaboeuf, France). Housekeeping genes were analyzed with Normfinder algorithm [61] and the more stable was used as a reference for normalization in the $2^{-\Delta\Delta Ct}$ analysis method [62]. Five distinct experiments were done, each including at least three biological replicates of each condition.

5.7. Statistics

Student's *t*-test was used to analyze the differences between control and treated samples. The differences were considered to be statistically significant when the *p*-value was lower than 0.05.

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Author Contributions: Isabelle P. Oswald, Olivier Puel, Jean-Denis Bailly, Ali Atoui, André El Khoury conceived, supervised and designed the experiments. Isaura Caceres and Rhoda El Khoury performed the experiments, contributed to experiment design. Isaura Caceres, Rhoda El Khoury, Olivier Puel and Jean-Denis Bailly wrote the paper. Ángel Medina contributed to the RNA extraction protocol and culture condition techniques; Yannick Lippi and Claire Naylies contributed to q-PCR performance and data analysis.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

A. flavus	*Aspergillus flavus*
AF	Aflatoxins
AFB_1	Aflatoxin B_1
GPCRs	G-protein coupled receptors
HPLC	High Performance Liquid Chromatography
MEA	Malt Extract Agar
MFS	Major Facilitator Superfamily
mM	Millimolar
nd	Not detectable
ns	No significant changes
OE	Over-expresssed
ROS	Reactive oxygen species
SEM	Standard Error of Mean
SM	Secondary metabolism
TFs	Transcription factors

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Supplementary Materials: Deciphering the Anti-Aflatoxinogenic Properties of Eugenol Using a Large-Scale q-PCR Approach

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Table S1. Gene expression values of regulatory factors that were not impacted upon Eugenol addition. Values are expressed in fold change and compared to a control fixed at 1.

Gene Name	Values	*p*-Value
abaA	1.13	0.8179
ap-1	1.12	0.4589
areA	1.29	0.4578
atfA	2.09	0.0537
brlA	0.77	0.1953
cat2	1.13	0.8072
catA	1.84	0.3623
creA	1.32	0.648
fadA	0.95	0.891
fcr3	0.73	0.0836
flbA	0.55	0.2958
fluG	1.11	0.5914
gprG	1.52	0.1591
gprH	0.84	0.3971
gprP	1.29	0.2462
laeA	1.05	0.7673
meaB	1.43	0.1367
mnSOD	0.84	0.3045
ppoA	1.26	0.2914
ppoB	0.77	0.4413
ppoC	0.89	0.6097
rasA	0.88	0.4225
SOD1	0.95	0.8985
srrA	1.69	0.2001
velB	1.43	0.3543
vosA	1.49	0.1905

Table S2. Primer sequences of the 62 designed genes.

Access Number	Gene	nM	Primers Sequences 5′ to 3′	
AFLA_139380	*aflA*	*300/900*	F: CCAGTCGGTGGTGTGCAAAA	R: CGCAGCACCCCAGAGCTT
AFLA_139370	*aflB*	*300/300*	F: GTCTGCGCAGGCCATTTC	R: AGCTCTTGGCCTTCAACAGTCTC
AFLA_139410	*aflC*	*300/300*	F: CTGACCCCGGCATTTCG	R: TGCCAGATTCCTCATATTCCCG
AFLA_139390	*aflD*	*300/900*	F: AGGCATCTGTGCTCGGATTG	R:TGCCCCGATGTAGTCTCCTTAGT
AFLA_139310	*aflE*	*900/300*	F: CTGCAACACTCGGCTAGAAACA	R: GAAGGGTTCGATCGGCTGT
AFLA_139260	*aflG*	*900/900*	F:CATGCAGAGTAACCGCATCGT	R: TTGTAGACCGACGGATGTCCC
AFLA_139330	*aflH*	*900/900*	F: CCAGCTCCACGGTCGG	R: CTTAAGGTCAAAGATTCCCTCGG
AFLA_139230	*aflI*	300/300	F: GATCGGCTCGTTTGAGGGA	R: GCAAAAATGATATTCAGCTGGTTTGAC
AFLA_139320	*aflJ*	*300/900*	F: GCTCCGTGGCACCAGTT	R: TCGTCACGCTCTCATCGG
AFLA_139190	*aflK*	*300/300*	F: ACAGCGCGAAATGTTTTATATGC	R:GCGCCTTTCGAGCCTCTG
AFLA_139250	*aflL*	*300/300*	F: CCGATTCGTACCCGAAGAGAC	R: TCGGCAAAGTAGGTACCGAGAT
AFLA_139300	*aflM*	*900/900*	F: TCCTGGAGCCATTAAGACTGATATGT	R: GCCAAGCGGCGCACTC
AFLA_139280	*aflN*	*300/300*	F: CCAGGCTCGCCAGGAAGT	R: AGCTTGAACTCTGGCATAGGAAGG
AFLA_139220	*aflO*	*300/900*	F: CCCCAAGAGTATACCTCGAGTGC	R: AAGGTCCCGAGATGTCGAATAGTT
AFLA_139210	*aflP*	*900/300*	F: TATTCTACATGACTATCCCGATGCTG	R: GCGCGACTTGCTTGGGT
AFLA_139200	*aflQ*	*900/900*	F: GGGAGGATCGGACACGACA	R: CATGGCCACGAAAAAGCTAGAC
AFLA_139360	*aflR*	*900/900*	F: GCGGCACAGCTTGTTCTGA	R: CCGGTATCCCTGCTGCATC
AFLA_139340	*aflS*	*900/900*	F: AACGGTCGTGCATGTGGG	R: CGGCCTTAGCTTCTGTCTGC
AFLA_139420	*aflT*	*300/300*	F: GTAGCATGCGCCTCCGTCT	R: GCGAATGGCTTGGAACCAG
AFLA_139180	*aflV*	*300/900*	F: CCTGGTGGTTCCTCGCTGT	R: TGAAATATGCGGTTTGAAGAATCTTG
AFLA_139170	*aflW*	*300/900*	F: CGATGTCTTTGTGCGGACG	R: GTGTTGCCCGCTAGCACTC
AFLA_139160	*aflX*	*900/900*	F: GATCCACGCCTACTGCCGT	R: GATCTCAGGATGCAATCGGTTC
AFLA_139150	*aflY*	*300/300*	F: GGTGTCCCTACGGTCTTAAATGAGT	R: CAATGGGTGGAGAAAGCCAGA
AFLA_139240	*hypB*	*900/300*	F: TCCTGCACTTCAAGCTACCGC	R: GACGCTCATCATGGCTCCG
AFLA_139400	*hypC*	*900/900*	F: GGTCCTTTTTGACGGGAGCC	R: TCTCGATAAGAATGGGAATGGTGA
AFLA_139270	*hypD*	*300/900*	F: TTGCGTTTGCCGACGG	R: GCGCATAACTCCAATGTCCC
AFLA_139290	*hypE*	*900/900*	F: AAGTTCCCGACTTTCCCGC	R:CAAAGTTGATAATAGCAAATCCGTCATA
AFLA_029620	*abaA*	*900/900*	F: TACAAAACTACTGGCAAAAGGAGGTC	R: CCCGTACAAGCCTCTCCCA
AFLA_129340	*ap-1*	*900/300*	F: CTTGCACCAAGATTTGGGACC	R: TCGTACGCAATTCGGAGCA
AFLA_049870	*areA*	*300/900*	F: CAGCCAATCCGGCGAAC	R: CCCATTCCTGACTAGCCCCTT
AFLA_031340	*atfA*	*900/900*	F: GCAGTTACAGGCACCTTAACACATC	R: CGGCGCCTGGGAGTCTT
AFLA_082850	*brlA*	*300/300*	F: GCGTGCCTTCTCCCGC	R: GGAGTCCATCCGGTGTGAGTT
AFLA_068620	*β-tub*	*300/900*	F: AACGTCTACTTCAACGAGGCCA	R: GTACCAGGCTCAAGATCAACGAG
AFLA_122110	*cat2*	*300/900*	F: TGGCAGCAGTGACGGAAAG	R: AGCCCAAGCGGCAACAA

Table S2. *Cont.*

AFLA_056170	*catA*	*300/900*	F: ATTAAGTTCCCGGATTTTGTCCA	R: GGTACCTCGTTGTGCGGCT
AFLA_134680	*creA*	*300/900*	F: CAGCCGGAAGGCCCC	R: CATATCTTGCACCGCGACCT
AFLA_018340	*fadA*	*900/900*	F: TACATCCTCAACCGCTTCGTG	R: GGTTCTCCTGGATGATGATGTCATT
AFLA_133560	*fcr3*	*300/900*	F: GGCACAGAACAGAGCAGCTCAA	R: GCACATGTCGCTCCTTTCGTT
AFLA_134030	*flbA*	*300/900*	F: CTGGCTGCAGCATATGGTCTT	R: CATTGATTCGTCATCCCCTACAG
AFLA_101920	*fluG*	*300/300*	F: CGCCGGTTACCTAGGCGT	R: TTGGCGGCATCGTGGA
AFLA_025100	*gpdA*	*900/900*	F: CGTGTTGTTGACCTCATTGCCT	R: GGTGACCTGATAATCCGGGAAC
AFLA_060740	*gprA*	*900/900*	F: CTTCTGCAGTATCTTCATTATCAAGCTC	R: AATCGAAAGCAGCGCTGG
AFLA_067770	*gprG*	*300/900*	F: CCGCATTCAGCGGAAACTT	R: GGCGTTAGGGTTTGTGAGGAG
AFLA_006920	*gprH*	*300/900*	F: TTGATTGTCACCTGGGTTCCTTC	R: CATAGTTCAGAGCAAAGTTGTGGGA
AFLA_009790	*gprK*	*300/900*	F: CTTGCCATGCAGGTAGTCAAC	R: TCCAAATGGACAGGCAAATCC
AFLA_088190	*gprP*	*300/300*	F: CGATGCAGTCTTTCTAAGGGTGG	R: GCCATCGATTGTACCATGTGTGA
AFLA_033290	*laeA*	*900/900*	F: TGCCAACAAATACCCAGACG	R: TCGCAATTCTTTGGGTGGTT
AFLA_031790	*meaB*	*900/900*	F: CCAAAAGCACTATGATCAGCTTGAA	R: CCCACGGACGACTCATGC
AFLA_033420	*mnSOD*	*300/300*	F: GGTGGCTAATGGACCGGG	R: CATACGAGGCCTTGTTATTGAAGTACTG
AFLA_110650	*msnA*	*900/900*	F: GACGTCCATGCCGAGCTTT	R: AATTGGCGTTGATGAAGACAGG
AFLA_091490	*mtfA*	*300/900*	F: GCATCGACATATGCTGCAAAG	R: GGCGCAAAGGTGGAGTAGG
AFLA_131330	*nsdC*	*300/900*	F: TCCAGGACCACATGACTCCAA	R: GCTGTGCCCTTAAAGCAGCTACT
AFLA_030580	*pacC*	*900/300*	F: CAAGGATGTTCTGAGAAGTGTCCC	R: CGTTCGCAGACGTGCTCATA
AFLA_026790	*ppoA*	*300/900*	F: ATCAAGAGGCTGGCGAAGGT	R: GGGCGCCTTCCATGAAGTA
AFLA_120760	*ppoB*	*300/900*	F: AGTCTCTATGCGTTCCCTATGACTTG	R: CGCTCCCTTCCCGGAAT
AFLA_030430	*ppoC*	*300/300*	F: GTTCACAATCAGGCTCAAATGTTC	R: CAGGCAGTAGCGCATCAACTT
AFLA_132380	*rasA*	*300/300*	F: GCGAGCCGTCACAGTAGAAGAG	R: CGGATGTTTCGATAAATTTGCAG
AFLA_099000	*sod1*	*900/300*	F: CGAGAGCGTACTTGGCCGT	R: TCTTCTTGGACTCCGGGTGTT
AFLA_034540	*srrA*	*300/900*	F: TGTCGTCAGATTGGCGGAA	R: GCCTCGAGTCCGTCGAAAG
AFLA_066460	*veA*	*300/300*	F: CGTCAGCCGGATCACTCG	R: GACGGTCCGCAGAGGACTT
AFLA_081490	*velB*	*300/300*	F: GGCTTCGGCGACAAGGAT	R: TCTGCGTTCCACAAGTCTACCA
AFLA_026900	*vosA*	*300/900*	F: GGGTGTTCCAGATTATGCGATG	R: CCCGTTGAATCCCCCGTAG

Conclusion

We demonstrated that in *Aspergillus flavus*, the impact of the anti-aflatoxigenic molecule Eugenol occurs in a transcriptomic manner and that this molecule modulates some of the external regulatory factors linked to AFB1 biosynthesis.
Analysis of the entire cluster gave an interesting observation of Eugenol's impact. For instance, we observed that with no longer detectable levels of AFB1 production, the gene expression of the main regulators *aflR-aflS* was only moderately inhibited. Nevertheless, this inhibition was sufficient to strongly decrease the expression levels of the other genes belonging to the AFB1 cluster with the only exception of *aflT*.
In addition to this, genes coding for the first steps of the enzymatic cascade pathway were less inhibited than other genes coding for the middle and latter steps.
Since *aflR*/*aflS* genes were not completely inhibited by treatment, this suggests that a remaining AflR/AflS protein complex was still formed and that this complex could be sufficient to activate genes coding for earlier steps of the AFB1 cascade. However, it is likely that the available quantity of complex was not enough to allow the transcription of further genes coding for middle and final steps of the enzymatic cascade.

This study did not allow a complete elucidation of the mechanism of action of Eugenol since among the regulatory factors that were modulated, some of the relationships with AFB1 synthesis are still lacking. Nevertheless, this was an interesting result since it points out the need to study new interactions between genes that could be the target for further works.

We estimate that the molecular approach developed here can represent a useful tool for a first characterization of the molecular impact that natural products have, not only on the entire AFB1 cluster but also in some of the most important transcriptional factors that are involved in toxin production. This could highlight some external stimuli that have direct consequences in mycotoxin production and that could therefore be integrated in a structure-function screening procedure of new anti-aflatoxigenic molecules.

4.2 Chapter2

The anti-aflatoxigenic molecule Piperine modulates aflatoxin's pathway and oxidative stress response in *Aspergillus flavus*

(Article 2 – submitted in Molecular Microbiology).

Introduction

Once the molecular tool was validated we were interested to know more about the impact that other natural products have during AFB1 inhibition.
For that, another pure molecule, originally occurring in black pepper, was investigated. In fact, piperine is the major compound found in black peppers belonging to the *Piper nigrum L*. and *Piper longum L.* species.

As for Eugenol, this molecule was already demonstrated as an anti-aflatoxigenic agent but its mechanism of action has not been identified. In addition to this, piperine has been reported as an antioxidant compound thus, having the capacity to scavenge Reactive Oxygen Species (ROS) (Srinivasan, 2007). Indeed, some of the potent AFB1 inhibitors have also been identified as antioxidant compounds which make interesting their study over the fungal oxidative stress response mechanisms.

In the last years, oxidative stress response in fungi has attired the attention of researches since this factor has been closely related to secondary metabolites production. For instance, it has been demonstrated that several genes coding for oxidative stress are involved in AFB1 production. In addition to this, a co-regulation of some of genes belonging to AFB1 gene cluster and oxidative stress-response elements has also been demonstrated (Hong *et al.*, 2013).

Thus, the principal aim of the next article was to use Piperine to inhibit AFB1 production to observe its impact on the entire AFB1 gene cluster and in some external regulatory factors.
Due to the anti-oxidative properties of piperine, we focused our research in the investigation of its molecular impact on genes coding for oxidative stress response.

The anti-aflatoxigenic molecule Piperine modulates aflatoxin's pathway and oxidative stress response in *Aspergillus flavus*

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Abstract

The presence of the pathogenic fungus *Aspergillus flavus* in food commodities represents a potential danger for humans and animals since it is capable of producing the carcinogenic toxin: Aflatoxin B1. To date, several strategies have been developed to reduce this contaminant. Nevertheless, some of them are non-environmental friendly or even toxic for human's health. Within this context, the use of natural products could represent an alternative strategy to limit toxin production without affecting microbial biodiversity. In the present study, piperine, a natural antioxidant and major compound of black peppers, was evaluated for its anti-aflatoxigenic properties as well as its impact on oxidative stress response using a q-PCR approach. We demonstrated that this molecule inhibits AFB1 production in *Aspergillus flavus* with only a moderate impact in fungal growth. In addition to this, morphological and molecular studies were performed. Piperine inhibits AFB1 production in a transcriptional manner and interestingly, this response was accompanied by a modulation of genes coding for oxidative stress response and fungal enzymatic defense. In fact, contrary to our expectatives, Piperine strongly up-regulates genes belonging to superoxide dismutase and catalase families while catalase enzymatic activity followed the same up-trend. We also emphasize the modulation of different b-ZIP transcription factors involved in stress defense systems such as *atfA* and *ap-1*. Taken together, these results demonstrated that piperine has a transcriptomic impact on Aflatoxin B1 pathway and that this effect was accompanied with an increased modulation of oxidative-stress elements in *Aspergillus flavus*.

Key words: Aflatoxin B1, inhibition, oxidative stress, gene expression, enzymatic activity**,** piperine.

Introduction

Mycotoxins are toxic secondary metabolites produced by some filamentous fungi. According to the Food and Agricultural Organization (FAO 2003) they contaminate about 25% of the world's food commodities leading to important sanitary problems as well as economic and agricultural losses. Some recent surveys reported that worldwide contamination of cereals and agricultural commodities is becoming even more frequent (Streit *et al.,* 2013). To date, the precise role of mycotoxins in fungal physiology has not been clearly elucidated. Nevertheless, these compounds could be involved in fungus defense and interspecies competition (Thippeswamy *et al.,* 2014; Vaishnav and Demain, 2011). Nowadays, more than 400 mycotoxins have been identified and among them, aflatoxins are considered as the most important family. Indeed within Aflatoxin's group Aflatoxin B1 (AFB1) is the most dangerous one (Iram *et al.,* 2016). This mycotoxin is a potent hepatocarcinogenic agent in humans (I.A.R.C. 1993), inducing liver cancer through a mutation in the *p53* gene sequence, which encodes a tumor-suppressor factor (Hsu *et al.,* 1991). AFB1 is synthesized by at least 20 species belonging to the *Aspergillus* genus and more precisely to the *Flavi* section. *Aspergillus flavus* and *Aspergillus parasiticus* are the most frequent toxinogenic fungi in crops, especially in tropical and subtropical zones where climate conditions favor their development on a wide variety of products (Baranyi *et al.,* 2015). In fact, it is estimated that more than 500 million people residing in Africa, Latin America and Asia are exposed to exceeded normative levels of mycotoxins, including aflatoxins (Moy and Miller, 2016). Moreover, AFB1's contamination was also recently reported on crops produced in other geographical regions such as Europe, demonstrating an increase in the geographical distribution of this food contaminant potentially related to climate changes (Battilani *et al.,* 2016; Perrone *et al.,* 2014). Thus, consumption of contaminated products exposes human and animal populations to high sanitary risks. Based on this evidence, limiting AFB1's exposure has become an essential target for research. Different approaches, using physical, chemical and biological methods have been developed for contamination control and reviewed in a recent work (Jalili, 2015). In addition to this, the use of anti-aflatoxigenic natural products such as spices, plant extracts or essential oils could be considered as a complementary or alternative strategy against mycotoxin biosynthesis (Sakuda *et al.,* 2016; Holmes, Boston and Payne 2008). For instance, the biological

properties of piperine, a major active principle of black and long peppers (*Piper nigrum L*. and *Piper longum L.)*, has been investigated in the last decades (Srinivasan, 2007). This molecule was demonstrated as a scavenger of Reactive Oxygen Species (ROS) *in vitro* (Mittal and Gupta, 2000) as well as an effective inhibitor of aflatoxin production in fungi (Lee, Mahoney and Campbell, 2002; Madhyastha and Bhat, 1984). Nevertheless, the exact mechanism of action leading to inhibition of AFB1's synthesis in toxigenic fungi was yet to be elucidated. The understanding of molecule's mechanism of action as well their impact on biosynthetic pathways involved in mycotoxin production, is of great importance. It can be a useful strategy to promote the use of natural compounds but it also allows a better understanding of the nature of environmental stimuli leading to mycotoxin production thus, providing new targets to limit toxin production. This strategy can be reinforced by the fact that several anti-aflatoxigenic compounds do not have an important impact on fungal development contributing in this manner, to preserve natural biodiversity. Therefore, the aim of this study was to investigate the mechanism of action through which piperine inhibits AFB1 production and study its impact on oxidative stress response. For that, gene expression of the AFB1's cluster as well as different transcription factors involved in fungal stress response were analyzed using a previously-developed q-PCR molecular approach (Caceres *et al.,* 2016). We demonstrated that piperine inhibits AFB1 production by down-regulating the expression of almost all genes of the AFB1's biosynthetic pathway. Moreover, this response was accompanied by a modulation of several important oxidative-stress genes as well as an increase of catalase's enzymatic activity.

Materials and Methods

Chemicals and Reagents

Lyophilized piperine standard was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Stock and work solutions were diluted in acetonitrile and stocked at 4°C until use. All solvents were analytical grade and purchased from Thermo Fisher Scientific (Illkirch, France).

Fungal Strain and Culture Conditions

The *Aspergillus flavus* NRRL 62477 strain, previously identified as an AFB1 producer, was used in this study (El Mahgubi *et al.,* 2013). All cultures were performed on Malt Extract Agar (MEA) medium (Biokar Diagnostics, Allone, France) and maintained in the dark at 27°C during 4 days. In order to determine the optimal piperine's concentration capable to inhibit AFB1 with a limited impact on fungal development, different concentrations of piperine were tested and in all cases, solvent addition in culture medium did not exceed 25 µL. Control cultures were performed with the same volume of acetonitrile in the medium; this latter concentration having been identified as a no-effect dose on both, fungal growth and AFB1 production. Before inoculation, media were covered with sterile cellophane layers (Hutchinson, Chalette-sur-Loing, France) as described by Leite, Magan and Medina (2012) and then, centrally inoculated using a calibrated spore suspension (10^6 spores/mL) prepared from a seven-day stock culture. Six replicates of each group were prepared and the entire experiment was repeated three times.

Fungal growth, Morphology and Spore Quantification

Fungal growth was evaluated by measuring colony diameters (length and width).
Macroscopic characteristics (colour of conidial areas, thallus margin and texture, aspect of conidial heads and colony reverse, etc.) were observed under stereomicroscope (Olympus SZX9 –X12-120).
Microscopic observation of conidial heads was performed by staining sample material with a lactophenol blue dye solution and fungal structures were observed using an Olympus CX41 microscope with a total magnification of x400. In order to observe the impact of piperine on sporulation, spore quantification was realized at the end of the incubation. For that, cultures were suspended in 50 mL of Tween 0.05% using a stomacher bag where spores were carefully scraped up off the mycelium. Samples were homogenized with a Stomacher Lab-Blender 400 during 90 s and filtered through sterile gauze. To recover the remaining spores, three supplementary rinses were made, each one with 20 ml of Tween (0.05%). Spores were counted on a Malassez cell and at least 2 dilutions were made to define the optimal counting conditions. Spore density was

calculated as follows: $SD = SC/(\pi r^2)$ where SC is the spore count and r is the average colony radius.

Aflatoxin B_1 Extraction and Determination by HPLC

For AFB1 extraction, culture media were mixed with 25 mL of chloroform. Samples were agitated for 2 h on a horizontal shaking table at 160 rpm at room temperature. Chloroform extracts were filtered through a Whatman 1PS phase separator (GE Healthcare Life Sciences, Vélizy-Villacoublay, France), evaporated at 60 °C to dryness and dissolved in 500 µL of a water-acetonitrile-methanol (65:17.5:17.5; *v*/*v*/*v*) mixture. All samples were filtered through a 0.45 µm disk filters (Thermo Scientific Fisher, Villebon-Sur-Yvette, France) to eliminate possible impurities. Sample analysis was done with a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Illkirch, France) using a liquid chromatography column Luna® C18 (125 × 2 mm, 5 µm, 100 Å) (Phenomenex, Torrance, CA, USA) at 30 °C. Separation conditions were adapted from Fu *et al.*(2008) with mild modifications. A 20 min isocratic mode was delivered at 82.5% of eluent A: acidified water (0.2% of acetic acid) and acetonitrile (79:21 *v*/*v*); and 17.5% of eluent B: pure methanol. Flow rate was at 0.2 mL/min with an injection volume of 10 µL. AFB1 was detected using a fluorescent detector at 365/430 nm excitation/emission wavelengths. Peak identity was further confirmed by analyzing absorption spectrum with a diode array detector coupled to the system. Production levels of AFB1 in media were calculated based on a standard calibration curve.

Isolation of Fungal RNA, RT-PCR and q-PCR Conditions

On day 4, mycelia were separated from media and grounded under liquid nitrogen. RNA was purified with a Qiagen RNeasy PlusMinikit (Qiagen, Hilden, Germany). Quality of samples was verified by gel electrophoresis (1.2% of agarose) and A_{260}/A_{280} ratio, while concentrations were measured using a NanoDrop ND1000 (Labtech, Palaiseau, France). First-strand cDNA synthesis was carried out by RT-PCR. A first denaturation step was done at 70 °C for 5 min and reverse transcription was performed as follows: 5 min at 37 °C; 60 min at 42 °C and 15 min at 85 °C. All primer sets were designed based on the genomic data of the *Aspergillus flavus* strain NRRL3357 (GenBank accession number EQ963478A) and pair sequences were adapted from our previous work (Caceres *et al.,* 2016). A total number of 35 genes were tested: the 27 genes of the AFB1's biosynthesis

cluster in *A. flavus* (Ehrlich *et al.,* 2004) and 8 genes coding for external regulatory factors. The choice of these latter was based on their implication in oxidative stress response in fungi, which includes:

-Enzymatic fungal response: *cat2*, *catA*, *mnSOD* and *sod1*.

-Transcription factors involved in oxidative stress response: *srrA, atfA*, *msnA* and *ap-1.*

Experiments were carried out using a ViiA7 Real-Time PCR System (Applied Biosystems, Forster City, CA, USA). The 384 wells' plates were prepared by an Agilent Bravo Automated Liquid Handling Platform (Agilent Technologies, Santa Clara, CA, USA). Each well contained a final volume of a 5 µL mix using Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) as a fluorescent dye. Negative controls, in which no reverse transcriptase enzyme was added, and a no template control were included to control reagents contamination. Three-step quantitative PCRs were performed as follows: a first one-hold stage at 95 °C for 10 min followed by 45 cycles (95 °C for 15 s and 60 °C for 30 s) and a final extending step (95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s) for melt curve analysis.

Gene expression data analysis

Gene expression data was analyzed with a Quant-Studio Real time PCR software v1.1 (Applied Biosystems, Courtaboeuf, France). The housekeeping gene β-tubulin, proved as being the most stable after analysis with the Normfinder algorithm (Andersen *et al.,* 2004) was used as a reference for data normalization. Gene expression values were determined using the $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen, 2001) and final results are expressed based on a control value fixed at 1.

Fungal Enzymatic activity

Sample preparation

At the end of the incubation period, mycelium was separated from the medium and cytosolic superoxide dismutase (SOD) and total catalase (CAT) activities were determined. For each assay, 200 mg of mycelium were suspended in 1 mL of cold buffer: catalase samples were placed in a 50 mM potassium-phosphate buffer (ph 7.0

containing 1 mM EDTA) and SOD samples were placed in a 20 mM HEPES buffer (pH 7.2; 1 mM EGTA, 210 mM mannitol and 70 mM sucrose). Samples were then snap-frozen in liquid nitrogen and kept on ice until homogenization using a Precellys®24 coupled with a Cryolys system (Bertin Technologies, Montigny-le-Bretonneux, France) in order to avoid enzyme degradation.

Superoxide dismutase and Catalase tests

After homogenization, all samples were centrifuged at 9.6 g for 15 min at 4°C and both, enzymes and total proteins were determined in supernatants. Proteins were measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, Villebon-Sur-Yvette, France) and determination of enzymatic activity was performed using commercial kits of Catalase (CAT-707002) and Superoxide Dismutase (SOD-706002) (Interchim, Montluçon, France) according to the manufacturer's instructions. At the end of the experiment, sample absorbance was determined at 540 and 450 nm for CAT and SOD assays respectively, using an ELISA plate reader (Spectra thermo scan, Tecan, NC, USA). All enzymatic activities were normalized according to the protein content. For CAT assays, results were expressed in nmol min^{-1} mg^{-1} ps and for SOD assays in U mg^{-1} ps where one unit defines the amount of enzyme needed to exhibit the dismutation of 50% of the superoxide radical. Experiments for each enzymatic measure were repeated by triplicate.

Statistics

Data analyses were analyzed using GraphPad Prism v4 software. A non parametric Mann-Whitney's test was used to determine the differences between control and treated groups and differences were considered to be statistically significant when *p*-value was lower than 0.05.

Results

Effect of Piperine on AFB1 production and fungal growth in Aspergillus flavus

Five concentrations of piperine were evaluated on both AFB1 production and fungal growth. We observed that piperine inhibits AFB1 in a dose-dependent manner, where a

concentration of 0.006 mM significantly reduces toxin production by 30.21% and AFB1 was no longer detectable at 0.17 mM (Figure 1). This response was accompanied by a moderate impact on the development of *A. flavus*, showing a significant diminution of the fungus growth rate by 11.67% with 0.04 mM of piperine, while a 35.42% of growth inhibition was reached using 0.17 mM. A concentration of 0.04 mM was chosen as the optimal condition for further experiments, since it decreased toxin production by 95% with only a slight impact on fungal growth.

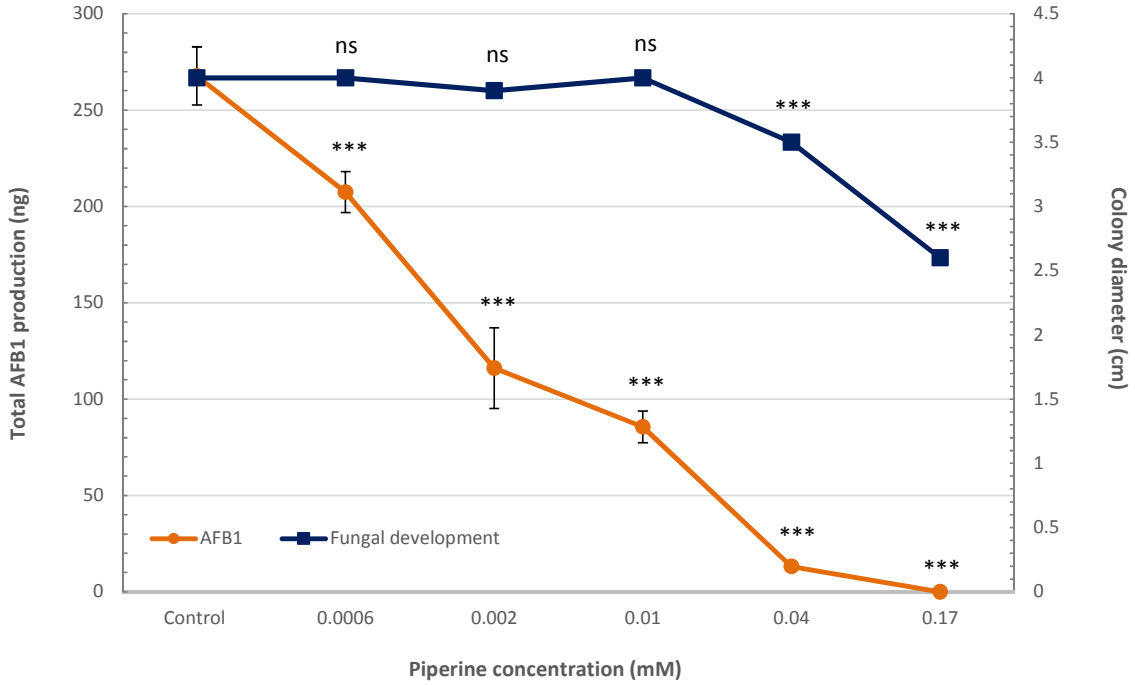

Figure 1. Effect of piperine on Aflatoxin B1 (AFB1) production and fungal growth in *A. flavus* NRRL 62477. AFB1 was estimated by High Performance Liquid Chromatography (HPLC) and fungal growth by colony diameter. Both measures were taken on day 4 on three biological replicates. ns=no significant changes; * *p*-value <0.05; ** *p*-value <0.01; *** *p*-value<0.001.

Morphological changes in presence of piperine

The presence of piperine in the culture medium resulted in a marked reduction of *A. flavus*' aerial mycelium that was left only in the center of treated cultures, as well as the appearance of pronounced ridges in the colony (Figure 2). In addition, basal mycelium's aspect appeared to be more compact, with packed *Aspergillus* heads, compared to that of the control. However, piperine did not have an impact on other morphological aspects of *Aspergillus flavus* such as pigmentation or microscopic features, since conidial heads

maintained the characteristic morphology of the species (data not shown). In order to evaluate piperine's effect on sporulation, spores' quantity and density upon treatment were analyzed. Results showed that quantity of spores was not significantly decreased, with $\log_{10}$ values of 9.100±0.015 and 9.000±0.016 for control and treatment respectively. Similarly, no significant changes were observed for spore density between control and piperine treated groups ($\log_{10}$ values of: 7.837 ± 0.007 *v/s* 7.893 ± 0.036 sp/cm^2 respectively).

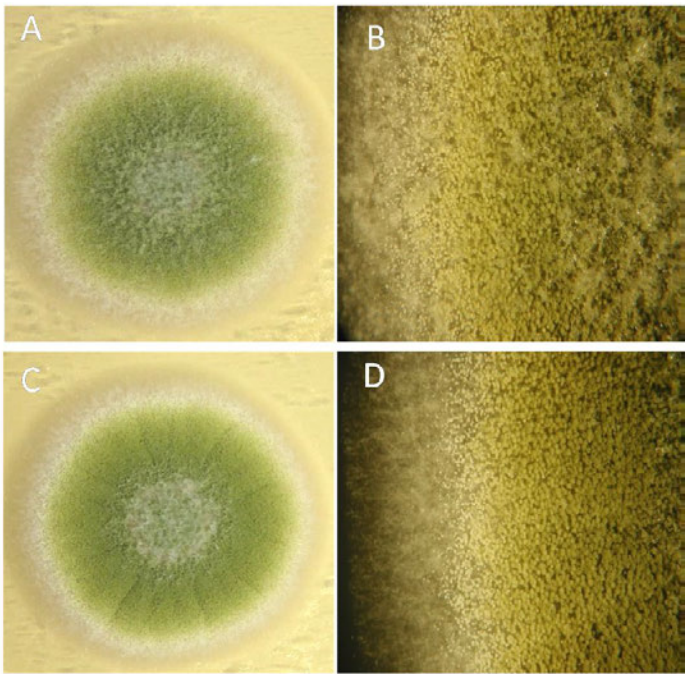

Figure 2. Macroscopic effects of piperine on day 4 of *A. flavus* NRRL 62477 growth in MEA medium. Figures A and B represent control mycelia culture and C and D treated mycelia culture.

Piperine down-regulates gene expression of AFB1 biosynthetic pathway

In *A. flavus,* AFB1's synthesis is the result of an enzymatic cascade involving 27 genes, grouped in a cluster. The impact of piperine on the entire AFB1 biosynthetic pathway is represented in figure 3.

Based on the statistical analysis, 25 out of the 27 genes were significantly down-regulated following piperine exposure compared to control. In a general manner, no significant changes were observed for a*flT* (*p*-value= 0.5653), neither for the principal activator of the cluster *aflR* (*p*-value= 0.4047); nevertheless, the expression of this latter was decreased by 1.11 folds. For genes that were statistically modulated, two presented levels decreased by less than 2 folds (*aflS* and *hypD*); 9 genes were mildly inhibited

between 2 to 4 folds (e.g. *aflC*, *aflD*, *aflQ*) and 13 genes presented inhibition rates from 4 to 8 folds; *aflN*, *aflW* and *aflK* being the most down-regulated genes with respective fold changes of 6.77, 6.82 and 7.25 and *p*-values lower than 0.0001. Gene expression ratios and *p*-values of the test are listed in Table S1.

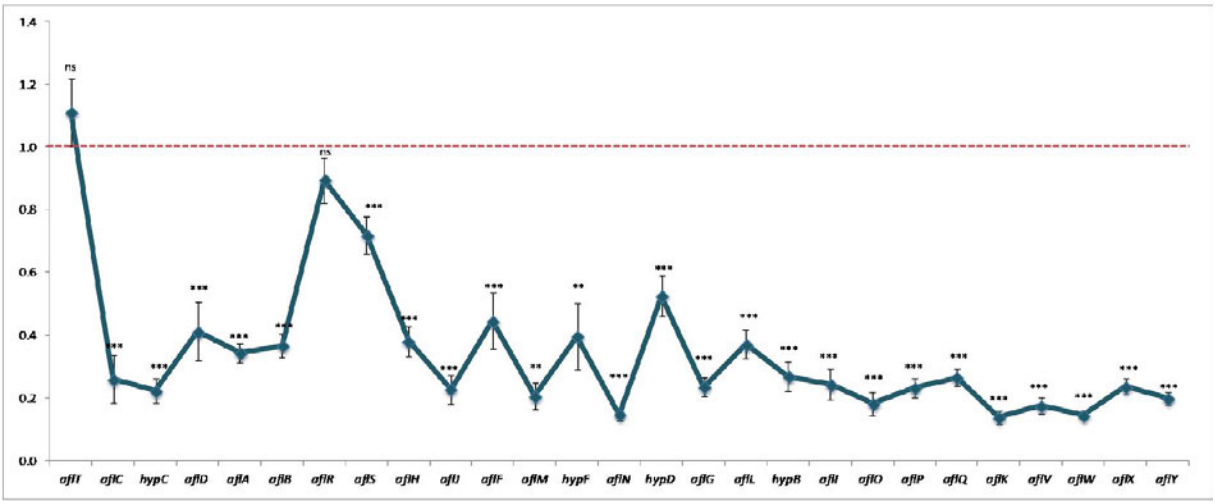

Figure 3. Fold change expression of genes belonging to cluster responsible for aflatoxin biosynthesis in response to piperine at 0.04 mM. Red dotted line represents control expression; error bars represent the standard error of mean. ns = no significant changes; ** *p*-value <0.01; *** *p*-value<0.001.

Effect on oxidative stress genes and enzymatic activities

In order to better characterize the effect of piperine on *A. flavus*, several external regulatory genes involved in oxidative stress response were also tested. Results showed that after 4 days of incubation, 7 out of the 8 analyzed genes were significantly modulated (Figure 4). We observed a down-regulation of gene expression of two transcription factors: *msnA* by 1.35 folds (p=0.0102) and *srrA* by 1.19 folds (p=0.0125). Otherwise, other tested genes were over-expressed following piperine exposure:

- The transcription factor *atfA* was up-regulated by 2.32 folds (p<0.0001) while *ap-1* presented an over-expression of 1.43 folds compared to control (p= 0.004).
- Genes encoding enzymes such as catalases and superoxide dismutases were over-expressed with fold changes of: 1.72 for *catA* (p<0.0001), 2.06 for *cat2* (p<0.0001) and 3.31 for *sod1* (p<0.0001). In addition to this, no significant change was observed for *mnSOD* (p=0.2906). All gene expressions are illustrated in Figure 4.

In order to investigate the impact on final products of the above modulated genes by piperine, total catalases and cytosolic superoxide dismutases activities were measured.

As shown in figure 4-A, enzymatic activity of catalase's activity was significantly increased (+68%, p=0.0165) compared to the control. On the other side, SOD activity did not display significant changes even if a slight increase tendency was observed (Figure 4-B).

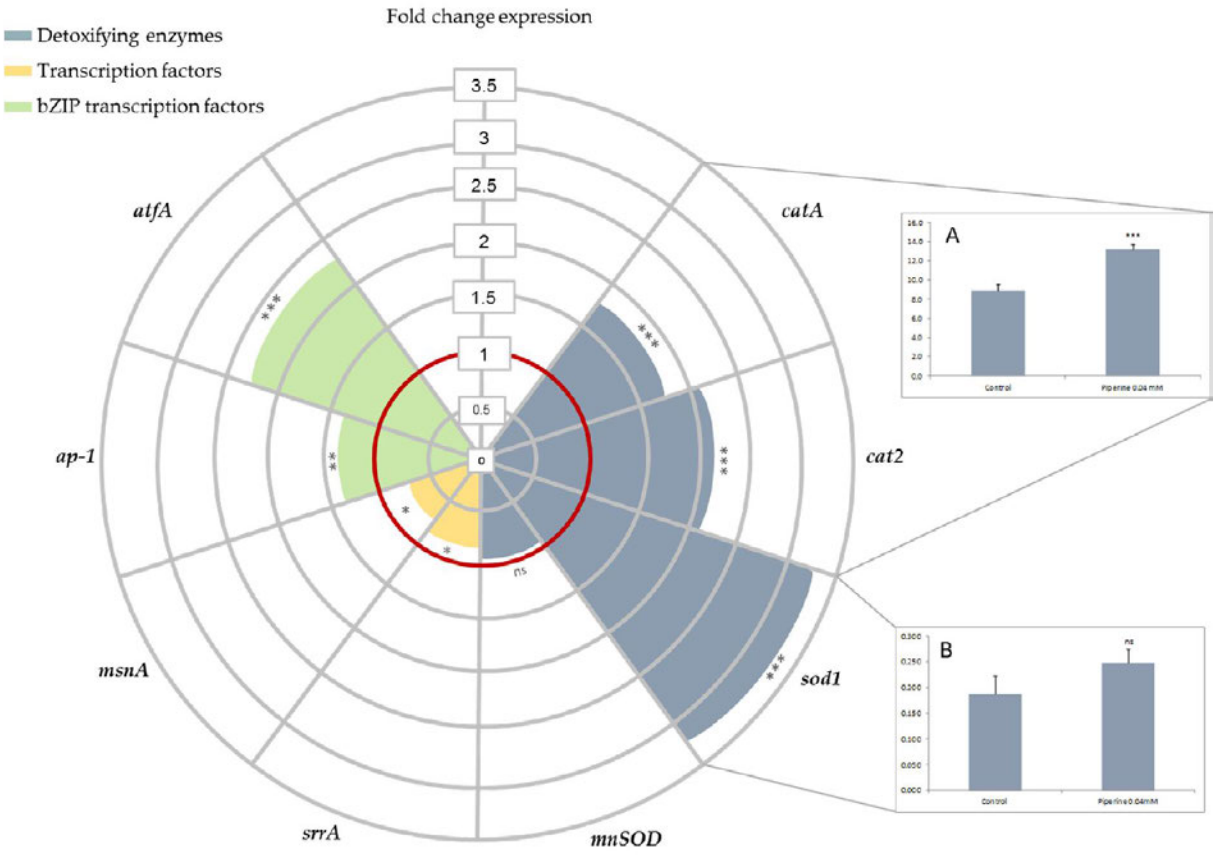

Figure 4. Effect of piperine treatment on gene expression coding for oxidative stress response and enzymatic activity defense in Aspergillus *flavus*. Red circle represents control expression. Figure A and B are representative assays of the activities of antioxidant enzymes including total catalase activity (4-A) and cytosolic superoxide dismutase (4-B). ns = no significant changes; * *p*-value <0.05; ** *p*-value <0.01; *** *p*-value<0.001.

Discussion

Black pepper is widely used for a variety of different purposes such as food condiment but also medicinal, insecticide, among others (Srinivasan, 2007). The Food and Drug Administration (F.D.A.) has classified this product as Generally Regarded as Safe (GRAS) (CITE:21CFR182.10) making of it an interesting agent against AFB1 production. Within the last decades, the identification of the chemical constituents of *Piper* genus has been investigated in order to elucidate their role in the different biological activities of black

pepper. Within this context, piperine, which represents between 2 to 8% of black pepper compounds (Madhyastha and Bhat, 1984), has been demonstrated to be an antioxidant as well as an anti-aflatoxigenic agent but its precise mechanism of action was not elucidated.

Impact of piperine on AFB1 gene cluster

Aflatoxins are furanocoumarins synthesized by an enzymatic cascade involving at least 20 steps (Bhatnagar *et al.,* 2003). Near of the chromosome 3, the 54th gene cluster coordinates this enzymatic response. The genes *aflR* and *aflS* are the main regulators and their activation is governed, independently one from another, by external regulators (Georgianna and Payne, 2009).

In fact, *aflR* provides the gene transcription machinery to activate aflatoxin's pathway and *aflS* acts as its co-activator by enhancing the levels of aflatoxin gene expression (Chang, 2003). As long as both genes are normally expressed, the two corresponding proteins AflR and AflS interact to form a functional activation complex (Kong *et al.,* 2014a). Once aflatoxin-conducive conditions are present, AflR binds to a palindromic site (5'-TCGN$_5$CGA-3') located in the promoter regions of the structural genes inducing the transcription of at least 19 of the AFB1 genes (Bhatnagar *et al.,* 2006; Price *et al.,* 2006). It must be noted that this process represents only one phase of the complex mechanism that leads to aflatoxin B1 production.

In the present study, mRNA levels of both regulators were decreased in piperine-exposed cultures compared to those of the control. Despite the slight down-regulation of *aflR*, its co-activator *aflS* was more inhibited. Thus, the inhibition of both regulatory genes explain the marked negative effect in the expression of other genes belonging to the pathway which demonstrates that piperine induce a transcriptomic effect over the AFB1 gene cluster.

We suppose that dysfunctional complexes of AflR-AflS were formed upon piperine exposure which causes a diminution on the activation of genes regulated by this complex. This is in agreement with the results obtained with other AFB1 inhibitors such as eugenol, D-glucal or Dioctatin A (Yoshinari *et al.,* 2007; Zhang *et al.,* 2014). We also observed that genes localized at the mid and end of the AFB1 cluster, such as *aflN*, *aflK* and *aflW*, presented the most important inhibitions, suggesting that the lack of activation complexes could have a more pronounced effect along the cluster as already

reported for eugenol (Caceres *et al.,* 2016). Interestingly, as for eugenol, we observed that expression of *hypD* was less impacted by piperine. To date, the exact role of this gene is yet to be clarified, but it has been demonstrated to be involved in fungal development as well as secondary metabolite production (Ehrlich, 2009b). Finally, *aflT* was the only gene within the AFB1 cluster that was not modulated by piperine. This observation accords with the fact that this gene is not regulated neither by AflR nor by its co-activator AflS, because of the absence of an AflR binding-site on its promoter (Chang *et al.,* 2004).

Effect of piperine on oxidative stress response

In fungi, oxidative stress is an important factor for secondary metabolite regulation (Narasaiah *et al.,* 2006). In addition to this, a recent study demonstrated that several genes belonging to the AFB1 cluster as well as genes coding for oxidative stress response have conserved motifs and could be co-regulated (Hong *et al.,* 2013).

One of the factors that modulate oxidative stress in fungi is related to changes in the environmental conditions which cause an alteration of normal intracellular ROS levels causing an imbalance between ROS production and scavengers (Apel and Hirt, 2004).

To cope these changes, a phosphorelay pathway coupled to a MAP kinase module is activated (SAPK/MAPK) (Lara-Rojas *et al.,* 2011). Within this pathway, several transcription factors activate enzymatic defenses in order to protect the cell from excessive levels of ROS and thus, damages to DNA, proteins and lipids (Montibus *et al.,* 2013).

Thus, we studied the impact that piperine has over some of these transcription factors coding for oxidative stress response and corresponding to *ap-1, atfA, srrA*, *msnA*, *cat2*, *catA*, *sod1* and *mnSOD.*

Within these genes, *ap-1* and *atfA* which are both bZIP transcription factors were up-regulated. According to this, it is demonstrated that the bZIP-type transcription factors may regulate secondary metabolite production by binding to the promoters regions of the genes involved in the biosynthetic pathways (Yin *et al.,* 2012, Hong *et al.,* 2013).

In regard to the up-regulation of *ap-1*, this gene can be modulated by both pro- and anti-oxidative conditions, where in the latter condition, an up-regulation of the antioxidant enzymes' activity is observed (Gomez del Arco *et al.,* 1997). In addition to this, the study

of some antioxidants in cell systems revealed that these compounds can stimulate AP-1 and by consequence also the expression of genes involved in ROS scavenging such as superoxide dismutases and catalases (Reverberi *et al.,* 2005).

In fact, the corresponding homologous and orthologous of *ap-1* have been characterized in *Aspergilli* species. For instance, its over-expression in *A. nidulans* resulted in lower amounts of sterigmatocystin and this effect was accompanied with a modulation of the enzymatic activity of catalases (Yin *et al.,* 2013). In the present study and using an *A. flavus* strain, our observations demonstrate that an over-expression of *ap-1* was also accompanied with an AFB1 inhibition and that genes coding for catalase activity were up-regulated.

Similar to this, *atfA,* another bZIP transcription factor was also up-regulated. Homologous of this gene in other fungi such as *Botrytis cinerea* and *Fusarium graminearum* have been demonstrated to be involved in secondary metabolite production (Van Nguyen *et al.,* 2013; Temme *et al.,* 2012).

Nevertheless, the role that *atfA* has over the AFB1 biosynthesis remains unknown. In the present study, we demonstrated that the inhibition of AFB1 was accompanied with an over-expression of *atfA* suggesting that this gene plays a role during the biosynthesis of this toxin and further studies are needed.

In 2010, Balázs *et al.* reported that in *A. nidulans*, a modulation of *atfA* was a key element for other genes coding for stress responses such as *catA.* This seems to be in accord with our results since a modulation of *atfA* was accompanied with an up-regulation of *catA*. Nevertheless, it is difficult to attribute this co-regulation only to *aftA* and not to the effect that other regulatory factors could directly have over the *catA* expression.

In addition to this, we also observed that upon treatment *msnA* was down-regulated. Regarding to this, Chang *et al.,* (2011) demostrated that Δ*msnA* in *A. flavus* strain resulted in increased amounts of aflatoxin and in an up-regulation of *catA* expression.

This does not seem to be our case since upon piperine's treatment, a down-regulation of *msnA* was observed during AFB1 inhibition. Interestingly, *catA* expression was still up-regulated.

In fact, *catA*, *cat2* and *sod1* were significantly up-regulated upon treatment; these genes code for the activation of superoxide dismutases and catalases and they are involved in enzymatic fungal defenses.

In general, superoxide dismutases intervene in the dismutation of superoxide radicals

into H_2O_2 while catalases transform H_2O_2 into H_2O molecules and oxygen (Weydert and Cullen, 2011).

In order to observe if the up-regulation of these genes had a final impact over the enzymatic response, the enzymatic activity of the total catalases (CAT) and cytosolic superoxide dismutases (SOD) was measured. We observed that upon piperine, CAT's activity was significantly up-regulated but it was not the case for SOD activity.

According to this, previous studies were performed with other AFB1 inhibitors demonstrating that such enzymatic activities were increased upon treatments. For instance, a study performed with cinnamaldehyde, resulted in AFB1 inhibition in *A. flavus* with significant increased levels of CAT while SOD activity was only slightly decreased (Sun *et al.,* 2015).

In addition to this, Reverberi and co-workers (2005) observed that the utilization of β-glucans of cultures filtrates of *Lentinula edodes* inhibits AFB1 production in *A. parasiticus* and that this response was also accompanied with an enhancement of the antioxidant system. They suggested that an enhancement on the anti-oxidant system could impede AFB1 production and that this response could be related by the stimulation of transcription factors coding for oxidative stress response, as it seems to be our case.

To conclude, piperine inhibits AFB1 production in *A. flavus* by down-regulating the mRNA expression levels of genes belonging to the AFB1 cluster. This response was accompanied by a modulation of genes involved in oxidative-stress response. In addition, an enhancement of catalase's enzymatic activity was also observed.

Taken together, our results demonstrated that in *A. flavus*, piperine impacts the antioxidant machinery involved in fungal defense leading to AFB1 inhibition.

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Author Contributions: I.P.O, O.P. and J.D.B. conceived, supervised and designed the experiments. I.C. performed the experiments and wrote the paper. R.E.K. contributed to q-PCR molecular tool. S. B. contributed to the morphological and enzymatic tests.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Data

Access number	Gene	Fold change	p-value
AFLA_139340	*aflS*	1.39	0.0008
AFLA_139270	*hypD*	1.91	P<0.0001
AFLA_139310	*aflE*	2.25	0.001
AFLA_139390	*aflD*	2.43	P<0.0001
AFLA_139290	*hypE*	2.52	0.0025
AFLA_139330	*aflH*	2.63	P<0.0001
AFLA_139250	*aflL*	2.69	P<0.0001
AFLA_139370	*aflB*	2.73	P<0.0001
AFLA_139380	*aflA*	2.91	P<0.0001
AFLA_139240	*hypB*	3.72	P<0.0001
AFLA_139200	*aflQ*	3.76	P<0.0001
AFLA_139410	*aflC*	3.83	0.0007
AFLA_139230	*aflI*	4.11	P<0.0001
AFLA_139160	*aflX*	4.22	P<0.0001
AFLA_139260	*aflG*	4.25	P<0.0001
AFLA_139210	*aflP*	4.29	P<0.0001
AFLA_139320	*aflJ*	4.39	0.0008
AFLA_139400	*hypC*	4.46	P<0.0001
AFLA_139300	*aflM*	4.83	0.0043
AFLA_139150	*aflY*	5.03	P<0.0001
AFLA_139220	*aflO*	5.49	P<0.0001
AFLA_139180	*aflV*	5.66	P<0.0001

Table S1. Fold change expression and *p*-values of aflatoxin cluster genes in response to 0.04 mM of piperine.

Conclusion

In general, the study of the entire AFB1 gene cluster under piperine exposure, confirmed that, as previously described for eugenol, this molecule inhibits AFB1 production in a transcriptional manner.
Regarding to this, only a slight inhibition of *aflR* was detected but a stronger impact in the co-activator *aflS* was observed.
As for Eugenol, we can hypothesis that the decreased mRNA levels of the main regulators in the pathway conducted to an inefficient formation of the regulatory protein complex AflR-AflS that can be the principal reason to explain the down-regulation of the subsequent genes belonging to the cluster. As a consequence, this lead to the diminution of AFB1 production.

Concerning the study of the oxidative stress response, Piperine treatment resulted in an increased modulation of key stress elements such as superoxide dismutases and catalases either at gene expression as well as at enzymatic level. Transcription factors such as *ap-1* and *atfA* also seem to play an important role in the piperine's mechanism of action.
These results, compared to other works that reported a similar effect, demonstrate that piperine can positively modulate oxidative stress response while inhibiting AFB1 production.
Such a response is an unusual phenomenon since some antioxidants compounds are mainly related to inhibit AFB1 production due to the alleviation of oxidative stress response (Huang *et al.*, 2009). Thus, further studies are needed to understand and identify the principal factor(s) that are involved in this phenomenon.

The analysis of piperine effects compared to eugenol data, reinforce the idea that natural products can trigger different pathways leading to AFB1 inhibition. It confirms that AFB1 biosynthesis is connected to several different cellular processes and that it can be modulated by different external stimuli. It seems now necessary to analyze more in depth these different pathways since it is possible that one gene could be the common "key" that has to be targeted to inhibit AFB1 production.

4.3 Chapter 3

Identification of an anti-aflatoxigenic aqueous extract from *Micromeria graeca* and elucidation of its molecular mechanism *in Aspergillus flavus*
(Article 3 – submitted in Food Chemistry).

Introduction

One of our goals was to identify new plants extracts that could be used as AFB1 inhibitors without affecting fungal development in order to ensure food safety and preserve biodiversity.

For that, we started screening plant extracts, taking benefit of international collaborations of the laboratory and the local frequent use

of some plants in traditional medicine due to their known benefits.

We also focused our attention mostly in aqueous extract from plants. Indeed, some studies compared the efficacy of both essential oils and aqueous extracts to limit fungal development and/or AFB1 production and demonstrated that aqueous extracts were at least as efficient as essential oils (Kumar *et al.,* 2010; Sarikurkcu *et al.,* 2010).

Moreover, aqueous extracts could present several practical advantages compared to essential oils such as:

- lower toxicity: most of medicinal plants are ingested as decoction or infusion so aqueous extracts may be not toxic as essential oils
- limited impact on organoleptic qualities of treated foods since these extracts do not contain lipophilic volatile compounds which are the responsible for this detrimental effect in essential oils.

In the next article the study of a medicinal plant from the Mediterranean region and identified as *Micromeria graeca* family (commonly known as Hyssop) was investigated.

This plant is principally known to serve as expectorant and anti-inflammatory agent. Nevertheless, to date its utilization as antifungal or anti-aflatoxigenic agent has not been reported.

First of all, it was demonstrated that hyssop's aqueous extract greatly inhibits AFB1 synthesis from 10 mg/ml of hyssop solution and that this effect was accompanied with only a mild reduction of fungal development.

On the other side, hyssop also generated important morphological changes in the *Aspergillus flavus* strains.

Since the extract of this plant was demonstrated to be an effective AFB1's inhibitor, its mechanism of action was investigated using our q-PCR approach.

Once again, both regulators of AFB1 gene cluster *aflR* and *aflS* were inhibited. Concerning the study of the external regulatory factors, Hyssop's aqueous extract modulated a total of 15 genes such as *veA*, *mtfA* but also *msnA*. In addition to this, a negative modulation of genes coding for oxidative stress response was also observed.

Identification of an anti-aflatoxigenic aqueous extract from *Micromeria graeca* and elucidation of its molecular mechanism in *Aspergillus flavus*

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Abstract

Amongst all of the food-contaminating mycotoxins, aflatoxins, and mainly aflatoxin B1, emerge as the most toxic and economically-costly. As green agriculture is thriving to replace fungicides, natural preventive strategies are developed to limit crop contamination by these toxic fungal metabolites. In this study, we demonstrated that an aqueous extract of the medicinal plant *Micromeria graeca* - known as hyssop - completely inhibits aflatoxin production by *Aspergillus flavus* without reducing fungal growth. The molecular inhibitory mechanism was explored by analyzing the expression of 61 genes including 27 aflatoxin biosynthesis cluster genes and 34 secondary metabolism regulating genes. This analysis revealed a 3-fold down-regulation of *aflR* and *aflS* encoding the two internal cluster co-activators, resulting in a drastic repression of all aflatoxin biosynthesis genes. Hyssop also targeted fifteen regulating genes including *veA* and *mtfA*, two major global-regulating transcription factors. The effect of this extract is also linked to a transcriptomic variation of a number of oxidative stress-defense genes such as *msnA*, *srrA*, *catA*, *cat2*, s*od1*, *mnsod* and *stuA*. In conclusion, hyssop inhibits AFB1's synthesis at the transcriptomic level. This aqueous extract constitutes a promising natural-based solution to control AFB1 contamination.

Keywords: Aflatoxin B1, *Aspergillus flavus,* hyssop, inhibition, oxidative stress.

1. Introduction

Aspergillus flavus is a saprophytic fungus developing on many crops and mostly maize, oilseed crops such as peanuts, as well as dried fruit and spices (Klich, 2007). It is the main producer of Aflatoxin B1 (AFB1), the most potent naturally occurring carcinogen. AFB1 is also associated with several health-detrimental pathologies mainly targeting the liver (Cano *et al.*, 2016). This mycotoxin has major economic repercussions by increasing agricultural losses and threatening animal productivity (Bryden, 2012), thus endangering food and feed safety. These damages are further exacerbated by the globalization of food trade (Wu and Guclu, 2012) and the global climate changes (Medina *et al.*, 2014).

Many strategies have been developed to limit AFB1 contamination in crops. Implementing good agricultural practices is undoubtedly a key point to limit an undesirable fungal incidence. Fungal growth and mycotoxin production closely depend on temperature and humidity (Magan and Aldred, 2007) and since these meteorological parameters are impossible to control, contamination cannot be completely avoided. Use of fungicides has witnessed a major drawback for their toxic chemical residues accumulate in food products. Their excessive use in crops over the last decades also resulted in the development of resistant pathogen populations as well as the accumulation of toxic chemical residues in water and soil (Da Cruz Cabral *et al.*, 2013).

Since physical degradation after production remains impossible and decontamination attempts are, to date, restricted to animal feed (Huwig *et al.*, 2001), the attention has been rather shifted towards more natural ways to prevent AFB1's contamination. For example, a biocontrol approach was developed and consists on the use of micro-organisms, such as strains of soil or lactic acid bacteria and atoxinogenic fungi. Such strains displayed the ability to inhibit aflatoxin production or fungal growth to a certain extent (Dalié *et al.*, 2010; Jane *et al.*, 2014). Natural preventive strategies could also rely on the use of plant extracts or essential oils. Plants produce many metabolites as part of their development or as a defensive strategy in response to a number of environmental stresses. Therefore, plant extracts have long been studied as protective bioactive agents and some were characterized as having antifungal or anti-toxinogenic properties (Chulze, 2010; Kohiyama *et al.*, 2015).

M. graeca - hyssop - is an herbaceous plant, belonging to the *Lamiaceae* family. It is commonly found in the Mediterranean region and frequently used for medicinal and condimental purposes (Abu-Gharbieh *et al.*, 2013). The purpose of this study was to test the

aqueous natural extract from *Micromeria graeca* for its ability to prevent aflatoxin's biosynthesis. We observed that it inhibits AFB1's production by *A. flavus* strains without interfering with fungal growth. The molecular mechanism of action of this extract resulted in a down-regulation of all of AFB1's biosynthetic cluster as well as a modulation in the expression of 15 secondary metabolite regulating genes.

2. Material and Methods

2.1. Solvents and standards. All solvents were HPLC grade and purchased from ThermoScientific Fisher (Villebon-Sur-Yvette, France). Lyophilized aflatoxin B1 standard was purchased from Sigma Aldrich (St. Louis, Missouri, USA). Stock solutions of each of the standards were prepared in methanol and stored at 4°C in the darkness. Calibration curves were prepared before use by diluting stock solutions with mobile phase used for HPLC analysis.

2.2. Preparation of the aqueous solution of hyssop. Dried hyssop (*Micromeria graeca)* was commercially purchased from Tyr, Lebanon. Hyssop's species was kindly confirmed by Pr Marc Beyrouthy (Department of Agricultural Sciences, USEK - Lebanon). Leaves were grinded with an electrical grinder and ten grams of ground hyssop were added to 80 ml of bi-distilled water and placed on a horizontal shaking table at 220 rpm for 24 hours. Extracts were then filtered through cotton gauze before being centrifuged for 10 min at 3,500 rpm. Filtrates were centrifuged once again, at 4,700 rpm for 30 min and autoclaved at 121°C for 15 min. Final sterile extracts were stored at +4°C until their use.

2.3. Fungal strains and growth conditions. A referenced toxinogenic *Aspergillus flavus* strain NRRL 62477 isolated from paprika samples harvested from Moroccan market (EL Mahgubi *et al.*, 2013), was used to evaluate aflatoxin inhibition by hyssop's aqueous solution as well as the molecular mechanism of inhibition. Further analysis of total aflatoxin inhibition by the aqueous hyssop solution were conducted on two other *A. flavus* strains (E28 and E71) that were previously isolated by our team from white pepper and paprika samples from Morocco (EL Mahgubi *et al.*, 2013). Strains were cultivated on a malt extract agar (MEA) medium (30 g malt extract and 15 g agar-agar per liter) (Biokar Diagnostics, Allone, France), supplemented at 2% v/v with an aqueous hyssop (*M. graeca*) solution whereas water-supplemented media were used as control cultures. Media for RNA isolation and dry weight measurement were layered with 8.5 cm diameter cellophane disks (Hutchinson, Chalette-sur-Loing, France) before inoculation in order to allow separation of mycelium for

culture medium. Spore suspensions were prepared in Tween 80 (0.05% in water) from a one-week-old MEA culture. Spores were counted on a Malassez cell and 10^3 spores were inoculated in the centre of the medium. Cultures were incubated at 27°C and those destined for RNA isolation were incubated in 6 replicates for four days. For AFB1 quantification assays, cultures were incubated for 8 days and were in triplicates. Media pH was measured before and after inoculation and after incubation using a H199161 food pH-meter (Hanna Instruments, Tanneries, France).

Examination of cultural parameters.

Effect on growth. The final growth mean was estimated by the measurement of culture diameters in length and width at day 4.

Mycelium dry weight. Following a 4-day incubation period, cellophane disks were peeled off and placed in new petri dishes that were incubated for 48 hours at 60°C. Dried mycelium films were allowed to cool in a desiccator before being weighed on an analytical balance. Final weight was calculated by subtracting the mean weight of 4 desiccated control cellophane disks.

Total spore quantification. Colonies were cut out of MEA media, 1 mm beyond the mycelium border, placed in a stomacher bag with 50 ml of Tween 0.05% and spores were gently manually scraped off of culture without tearing the media. The bag was then placed in a stomacher for 90 s. The supernatant was filtered through cotton gauze that was then rinsed with 3x20 ml Tween 0.05%. Spore solutions were homogenized by thorough vortex and subsequent dilutions were prepared in Tween 0.05% for counting on a Malassez cell in order to determine the total spore count (SC). Spore density (SD) was calculated as $SD = SC/(\pi r^2)$, r = average colony radius.

Delay to germination. Two hundred spores were inoculated in the centre of media and germinating spores were counted after a 16-hour incubation period at 27°C by stereo-microscopic examination.

Morphological features of fungi. Macroscopic (e.g. colour of conidial areas, thallus margin and texture, aspect of conidial heads and colony reverse) and microscopic (e.g. conidiophore, shape of vesicles, number of sterigmata, shape of conidia and ornamentation) characters were observed under stereomicroscope (Olympus SZX9 –X12-120) and optical microscope (Olympus CX41 – X400 and X1000) respectively.

2.5. RNA isolation and reverse transcription. Cellophane disks along with the 4-day mycelium were peeled off from medium, finely grinded with liquid nitrogen and a maximum of 100 mg were used for total RNA purification through a RNeasy Plus Minikit (Qiagen, Hilden, Germany) that includes an on-column genomic DNA clean-up, following the manufacturer's instructions. RNA integrity and purity were checked with agarose gel electrophoresis and a NanoDrop ND1000 (Labtech, Palaiseau, France) that also determined its concentration. First-strand cDNA synthesis reaction was primed using RevertAid Reverse Transcriptase (MBI Fermentas, UK), RNase Inhibitor (Applied Biosystems, Warrington, UK) and an anchored oligo(dT) Bys 3' Primer (5'-GCTGTCAACGATACGCTAACGTAACGGCATGACAGTGTTT TTTTTTTTTTTTTT-3'). An RT minus sample, where no reverse transcriptase reaction takes place, and a sterile water sample were added as negative controls to verify the absence of undesirable genomic DNA contamination and primer complementation, respectively.

2.6. Real-time PCR expression profile analysis of genes regulating the aflatoxin B1 biosynthesis in *A. flavus*. The genome of *A. flavus* strain NRRL 3357 (GenBank accession number EQ963478) served as a matrix for all of the primer used in this study. All of the primer pair sequences were adapted from a previous work (Caceres *et al.*, 2016) and primer sequences of the *stuA* gene (AFLA_046990) were added in this study (stuA_F: GATAAACGGAACCAAACTGCTCAA; stuA_R: CACGCTCAAATGGGATCCAA). Primer pairs design was based on the coding sequence of the corresponding genes, with at least one of the primers extending on an exon/exon junction in order to avoid undesirable genomic DNA amplification. Primer-dimer or self-complementarities were evaluated using the PrimerExpress 2.0 software (Applied Biosystems, Courtaboeuf, France). All primers were synthesized by Sigma Aldrich (Saint-Quentin Fallavier, France). Following RNA extractions and reverse transcriptase reactions, real-time PCR assays were performed on 15 ng cDNA in a 5 µl reaction volume per well, using Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) as a fluorescent dye for cDNA quantification. Master mixes and diluted cDNA samples were prepared separately on 96-well Sorenson plates (VWR, Pennsylvania, United States) and mixed in 384-well plates by an Agilent Bravo Automated Liquid Handling Platform (Agilent Technologies, Santa Clara, CA, United States). All real time amplification reactions were carried out on a ViiA7 Real-Time PCR System (Applied Biosystems, Warrington, UK), as described by Tannous *et al.,* 2014. In total, the expression

of 61 genes was simultaneously analyzed, 27 of which corresponded to AFB1 cluster and 34 to regulatory factors.

2.7. Aflatoxin extraction and HPLC quantification. Media of 4 and 8-day cultures were entirely retrieved and their AFB1 content determined after extraction with 25 and 40 ml chloroform respectively. Extracts were held for 2 hours on a horizontal shaking table at 200 rpm and were then filtered through a Whatman 1PS phase separator filter (GE Healthcare, UK, 150 mm diameter). Filtrates were evaporated to dryness and dissolved in 1 ml of a water-acetonitrile-methanol mixture (65:17.5:17.5; v/v/v). Extracts were filtered using 0.45 µm porosity disks (Thermo Scientific Fisher, Villebon-Sur-Yvette, France) before analysis. HPLC analysis was performed using a Dionex Ultimate 3000 UHPLC (Thermo Scientific, France) using a 125 x 2 mm, 5 µm, 100 Å, Luna® C18(2) LC column (Phenomenex, Torrance, CA, USA). Aflatoxins were separated using the program described by Fu, Huang, & Min, 2008, with minor modifications. A mixture of water (acidified with 0.2% acetic acid)-acetonitrile (79:21, v/v) is eluent A and methanol is eluent B. Separation program consists of a 30 min A:B (82.5:17.5) isocratic flow at 0.2 ml/min. Aflatoxins were detected using a fluorescent detector at wavelengths of 365/430 nm (excitation/emission). UV Spectra were confirmed by an additional diode array detector (DAD) coupled to the apparatus. Sample concentrations were calculated based on a standard calibration curve.

3. Results and discussion

3.1. Effect of hyssop's aqueous extract on *A. flavus* development and AFB1 production. When *A. flavus* strain NRRL 62477 is grown in a hyssop-supplemented medium a dose-dependent decrease in AFB1 production was observed. The downward trend started at the lowest concentration used (0.078 mg/ml) and was significant at 28.5% starting 0.156 mg/ml (*p*-value 0.0032). Another recent study of Omidpanah *et al.*(2015), has evoked the fungicidal effect of some aqueous extracts including thyme and mint on *A. flavus* at concentrations of 0.2 and 0.8 mg/ml respectively yet without determining aflatoxin inhibition at any of the concentration range used. In this study, hyssop inhibited AFB1's production by 52% at 0.625 mg/ml without altering *A. flavus*' growth. This inhibition reached 99.2% at 10 mg/ml and AFB1 was no longer detectable at 15 mg/ml (**Figure 1**). Further experiments were then conducted using hyssop at 10 mg/ml in the medium, this concentration being the lowest to present a quasi-total AFB1 inhibition.

At 10 mg/ml, aflatoxin inhibition by hyssop was accompanied by a mild increase of the colony diameter (4.4 ± 0.03 *vs* 4.25 ± 0.03 cm for treated and control respectively, *p*-value 0.0213). However, no significant increase in the total spore count neither in the spore density was observed following hyssop's addition. Besides, no further change was observed for mycelium weights or for the germination delay in the presence of hyssop in medium (**Table 1**).

Following hyssop's addition in medium, *A. flavus* colonies presented numerous macro and microscopic modifications. The major noticeable macroscopic morphological change is the development of an abundant aerial mycelium layering the entire surface of the colony. This latter also displayed a fasciculated edge. The presence of these numerous floccose tufts also increased the depth of the colony (**Figure 2**).

Under microscope, classic *A. flavus* structures were present in the basal mycelium of hyssop-treated cultures: long, coarse, un-branched conidiophores and radiate biseriate conidial heads. However, in the aerial mycelium, conidiophores, vesicles and conidia presented an atypical morphology and organization: i) an increased number of short conidiophores bearing small columnar heads in relation with the abundant aerial mycelium, ii) phialides developing anarchically on hyphae and on conidiophores in the absence of a vesicle (**Figure 3**), iii) presence of conidiophores with two, and less frequently, three fully-sporulated vesicles.

A single previous study has described the modification of the aerial hyphae in an AF-inhibiting profile in *A. parasiticus* in the presence of n-decyl aldehyde, a corn-derived volatile compound (Wright *et al.*, 2000). Nevertheless, another study conducted on *A. flavus* mutant strains described the appearance of morphological abnormalities, notably on phialide formation, associated to a cessation of AFB1 production (Jeffrey W Cary *et al.*, 2012).

At the dose of 10 mg/ml we observed a 77.7 and 70.8% inhibition of AFB1 production in E28 and E71 *A. flavus* strains respectively, without alteration of fungal growth. Similar morphological changes were also observed on these two strains (data not shown).

3.2. Hyssop's aqueous extract down-regulated the expression of AFB1 cluster genes. In *A. flavus* AFB1's biosynthesis is the result of a coordinated cascade of enzymatic reactions. The enzymes catalyzing these reactions are encoded by 27 genes and grouped in a cluster located in the telomeric region of the 3rd chromosome of aflatoxigenic species (D.

Ryan Georgianna and Payne, 2009). Inhibition of AFB1's production in hyssop-supplemented media was accompanied by a decrease in the expression of both of *aflR* and *aflS* genes by 3.2 and 2.8 times respectively (p-value < 0.0001). A down-regulation of these two latter genes has been associated with the repression of the entire AF cluster genes (Holmes *et al.*, 2008). Apart from *aflT* (the MFS transporter encoding gene), which is not regulated by the AflR/AflS complex and was down-regulated only by 2.3 times, the expression of all of the other cluster genes was severely repressed. The extent of this repression varied between the different genes and was not linked with the intervention level within AFB1's enzymatic cascade (**Figure 4**).

Genes undergoing the most drastic inhibitions were *hypC*, *aflI* and *aflO* encoding enzymes respectively intervening at the beginning, middle and end of the biosynthetic pathway and with corresponding fold-changes of 167.2, 60.7 and 468.8 with p-values < 0.001. For the genes encoding enzymes involved in the first steps of the cascade leading to the polyketide structure, *aflA*, *aflB* and *aflC*, expression was decreased by 12.2, 12.3 and 14.7 respectively. Genes least impacted are *aflM*, *aflG* and *hypD* with expression levels decreasing by 8.4, 9.3 and 9.4 times respectively. For the rest of the AFB1 cluster genes, a same downward trend was observed with expression levels decreasing by 14 to 50 folds (Table S1). This repression of the entire aflatoxin gene cluster is then directly responsible for inhibiting toxin production upon the presence of hyssop.

3.3. Transcriptomic effect of hyssop's extract on secondary metabolism regulating genes. The expression of AFB1 cluster genes is linked to the presence of regulating factors, encoded by genes outside of the cluster. In order to further investigate the mechanism behind the cluster's inhibition, we conducted a study on the regulatory network affecting secondary metabolism, including 34 genes involved in several fungal functional pathways. Among these, a total of 15 genes involved in diverse cellular mechanisms were modulated by hyssop's addition to culture medium (**Figure 5**):

- Global regulating factors such as *veA*, *mtfA*, *nsdC* were affected with expression levels respectively increasing by 3.8, 1.9 and 1.5 folds (p-values<0.0001, 0.0001 and 0.0122).
- Genes encoding enzymes involved in cellular protection from oxidative stress such as superoxide dismutases (*sod1* and *mnsod*) and catalases (*catA* and *cat2*) had their expression decreased by 1.6, 2, 2.2 and 3 folds respectively (p-values 0.013, 0.0007, 0.004 and <0.0001). As for other genes intervening in the oxidative stress response and

encoding transcription factors, notably *msnA* and *srrA*, their expression levels increased by 3.2 and 1.4 times with *p*-values of 0.0126 and 0.0017 respectively.

- *gprK* and *gprH*, encoding G-protein receptors involved in relaying external signals were also affected with *gprk*'s expression increasing by 2 folds (*p*-value <0.0001) and *gprH*'s decreasing by 2.1 folds (*p*-value 0.0006).
- The expression of the conidial developmental factor *stuA* was 1.8 times increased (*p*-value 0.0012).
- A couple of genes encoding environmentally-influenced transcription factors, *areA* and *pacC* were also triggered by the addition of hyssop in medium and their expression levels were respectively increased by 1.7 and 1.6 folds with *p*-values of 0.0215 and <0.0001. *ppoC* encoding a fatty acid oxygenase involved in oxylipin production also presented an expression decrease by 1.5 times (*p*-value 0.003).

VeA is a global regulating transcription factor involved in primary and secondary metabolism (Calvo, 2008a) and recruiting other factors such as LaeA and VelB to form the trimeric velvet complex. The activity of this complex affects fungal development, conidiation and secondary metabolism (Bayram and Braus, 2012). In hyssop-treated cultures, transcripts of *laeA*, *velB* and *vosA*, the latter being an interacting partner of *velB* (O., Bayram *et al.*, 2008), were not affected. This result further highlights the independent role of VeA in multiple other cellular mechanisms (Baidya *et al.*, 2014). The presence of VeA is necessary for the expression of secondary metabolite genes however it can also act as a repressor of some of these genes and thus inhibits the production of the concerned metabolite. VeA is essential for the transcription of AF cluster genes, including the transcription factor *aflR* and others (*aflD*, *aflM* and *aflP*) regulating aflatoxin production. Deletion of the *veA* gene led to the repression of AFB1 cluster genes in *A. flavu*s (Duran *et al.*, 2007). However according to our current study and to another recent one (Caceres *et al.*, 2016) a repression of all AFB1 cluster genes can also coincide with a *veA*-over-expression profile. Such is the case of penicillin, produced by *A. nidulans* where an OE:*veA* led to the repression of *acvA*, the penicillin biosynthesis gene and subsequent inhibition of penicillin production (Sprote and Brakhage, 2007).

VeA can also interact with another conserved global transcription factor, MtfA. The latter has a major role in regulating development and secondary metabolism in filamentous fungi (Ramamoorthy *et al.*, 2013) and it is linked to AFB1's biosynthesis and to *aflR*'s expression. An over-expression of *mtfA* in *A. flavus* has drastically inhibited the production of

AFB1 following a down-regulation of *aflR* whereas the effect of an *mtfA* deletion was less important (Zhuang *et al.*, 2016). The over-expression of *mtfA* in our conditions could result from that of *veA* and the interaction between these two factors would then be responsible for down-regulating AF-cluster genes and inhibiting aflatoxin production.

Besides its role in secondary metabolite regulation, VeA is also a developmental factor regulating morphogenesis as alterations in its expression levels can result in morphological abnormalities. For example, a reduction in fungal aerial hyphae was noted in both *A. flavus* and *Fusarium graminaerum veA* deleted strains (Duran *et al.*, 2007; Jiang *et al.*, 2011). Therefore, the modulation of *veA*'s expression could contribute to morphological abnormalities observed upon hyssop exposure.

In the presence of an environmental stress, fungi may establish several defense lines for limiting cellular damages. It has been demonstrated that VeA contributes to a positive transcriptomic modulation of stress-tolerance genes such as *msnA* and *srrA* under induced oxidative stress conditions (Baidya *et al.*, 2014). The expression of these two transcription factors is then highly dependent on that of *veA*. Therefore, their over-expression in a hyssop-treated medium might be the outcome of an over-expressed *veA*. The developmental factor StuA has also been associated to stress-response in fungi yet without a clear view on its contribution (Linz *et al.*, 2013). However, evidence exists on its dependence upon *msnA* since its expression levels were modulated in both *A. flavus* and *A. parasiticus msnA*-deletion strains (Chang *et al.*, 2011). MsnA is also known for regulating the expression of the catalases (CAT)- and superoxide dismutases (SOD)-encoding genes (Chang *et al.*, 2011). Those antioxidant enzymes along with aflatoxin formation are suggested as part of the fungus defense mechanism against reactive oxygen species (ROS) damages (Hong *et al.*, 2013). When medium was supplemented with *M. graeca*'s extract, *A. flavus* responded by decreasing the expression of SOD- and CAT-encoding genes such as *sod1*, *mnsod*, *catA* and *cat2* as levels of *msnA* increased thus resulting in an AF-biosynthesis repression, possibly related to an alleviation of environmental oxidative stress.

As for NsdC and PacC, more data is yet to be collected on their individual and possibly collaborative roles in secondary metabolism regulation. NsdC is known to be a developmental regulator which alteration caused several morphological aberrances such as shorter-stipe conidiophores presenting abnormal conidial-head formations. Similar to VeA, NsdC's modulation could participate to the morphological modifications observed in hyssop-

treated cultures. It has also been linked to aflatoxin cluster-gene expression (Jeffrey W Cary *et al.*, 2012) as well as being a global regulator of secondary metabolism (Gilbert *et al.*, 2016). As for PacC, a pH-dependent factor, its over-expression in aflatoxin-repressive conditions is to be investigated especially that pH conditions are neither alkaline thus suitable for its activation nor varying between control and hyssop-treated media (data not shown).

Alterations in morphology, such as aerial hyphae development, were also associated to an imbalance in the G-protein signal transduction pathway (Han *et al.*, 2004; Yang and Borkovich, 1999). This pathway is governed by the binding of signaling molecule to G-protein coupled receptors (GPCR) such as those encoded by *gprK* and *gprH* and tuned by regulators of the G-protein signaling cascade (RGS), which roles and implication in AFB1's synthesis are being investigated in *A. flavus* (Affeldt *et al.*, 2014). G-protein signaling pathway is also linked to oxylipins that are hormonal-like signaling molecules (Brodhagen and Keller, 2006) produced by fatty-acid-oxygenases such as PpoC. Moreover, oxylipins' regulation has also been described as VeA-dependent (Calvo, 2008a). However, fungal signal perception and transduction pathways is a very complex loop due to the diversity of signals that might be initiating it and most importantly to the numerous acting factors involved downstream any signal perception. Furthermore, since *M. graeca*'s extract is a complex extract containing many signal-provoking agents such as polysaccharides, amino acids, minerals, phenolic compounds and many others, it is possible that morphological modifications have no direct link to AFB1's inhibition.

4. Conclusion

This study demonstrates the efficiency of *Micromeria graeca*'s aqueous extract to limit AFB1 contamination without altering fungal growth. Such an effect could ensure food safety without affecting biodiversity. Indeed, *A. flavus* is a very competitive crop-contaminating agent; therefore, use of fungistatic agents could favor the emergence of other and possibly uncontrollable microorganisms. According to our results, inhibition by hyssop's extract occurs at a transcriptomic level as expression ratios of all of aflatoxin cluster-genes were severely decreased. Nonetheless, hyssop's extract triggered a response in several fungal cellular mechanisms including cellular signaling, global transcription factors, conidial development and factors intervening in the oxidative stress response. Being as complex as it is, this extract may shelter several bioactive compounds (Atoui, 2005) complementarily contributing to its anti-aflatoxigenic activity. For a more accurate determination of the

inhibitory mechanism of action, the content of this extract needs to be deciphered in order to determine and purify its active molecules and the inhibition extent of each of the isolated compounds.

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Conflict of Interest Disclosure. The authors declare no competing financial interest.

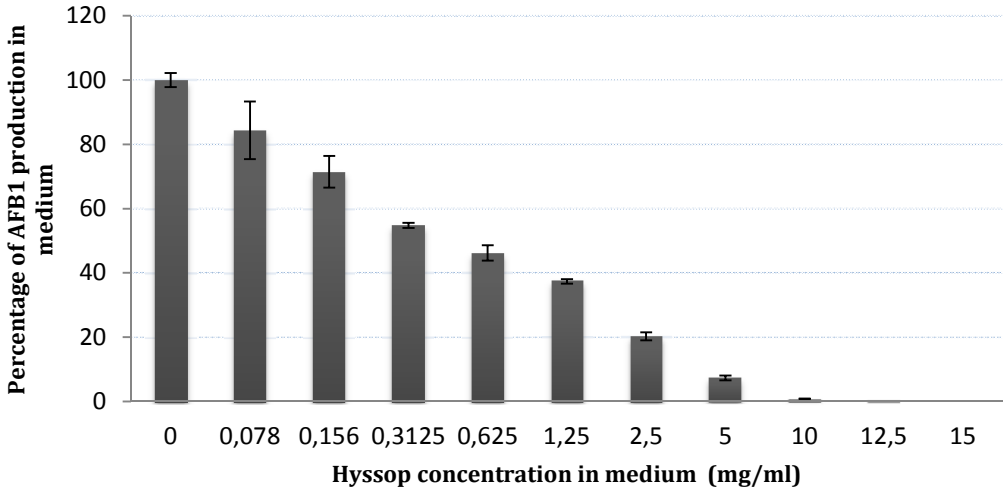

Figure 1. AFB1 production as a function of hyssop concentration. MEA medium was supplemented with increasing concentrations of hyssop's extract ranging between 0.078 -15 mg/ml and cultivated at 27°C, in the dark, for 8 days. AFB1 concentrations were quantified through HPLC/FLD analysis. Results are expressed as mean % ± SEM (n=3). * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

Table 1. Effect of 10 mg/ml of hyssop on the development of *A. flavus* i) colony diameter was measured in length and width, ii) weight was measured after a drying step at 60°C for 48 h, iii) germinating conidia were counted by observation under stereo-microscope after 16h incubation at 27°C, iv) total spore count is estimated following a complete wash of conidia and a Malassez-cell count of proper dilutions and v) spore density was calculated based on the total spore count reported to the colony surface. Results are expressed as mean ± SEM (n=3).

		Control	Hyssop
Growth	Colony diameter (cm)	4.25 ± 0.03	4.4 ± 0.03
	Mycelium dry weight (g)	0.16 ± 0.03	0.15 ± 0.02
Sporulation	Germinating conidia after 16h	193 ± 17	203 ± 8
	Total spore count	8.1E+08 ± 4.5E07	1.1E09 ± 9.9E07
	Spore density (conidia/cm^2)	5.7E07± 2.6E06	7E07 ± 5.6E06

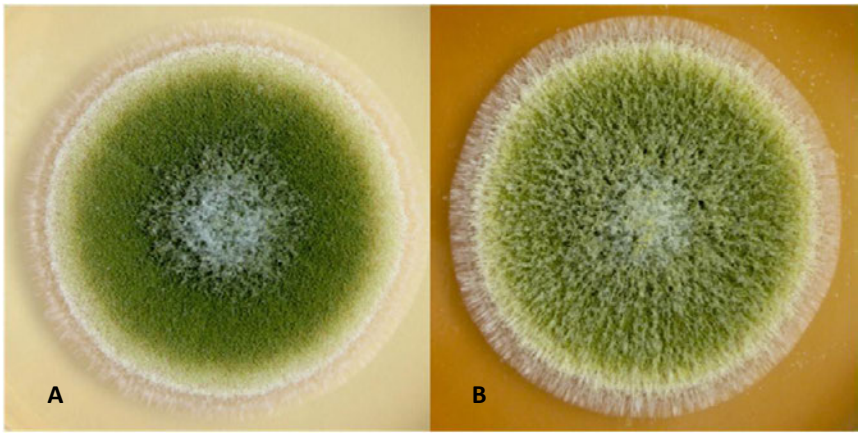

Figure 2 Four-day *A. flavus* strain NRRL 62477 grown in the dark at 27°C. **A.** Control culture grown on a regular MEA medium. **B.** MEA medium was supplemented with hyssop's aqueous solution at 10 mg/ml.

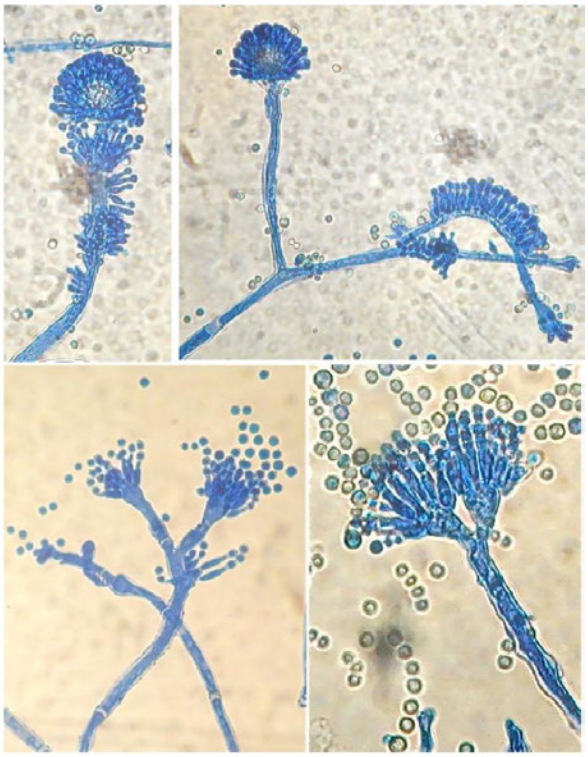

Figure 3. Microscopic views (x400) of conidiophores in the aerial mycelium of *A. flavus* NRRL 62477 showing the development of anarchic philalides when strain was grown on a hyssop-treated MEA medium.

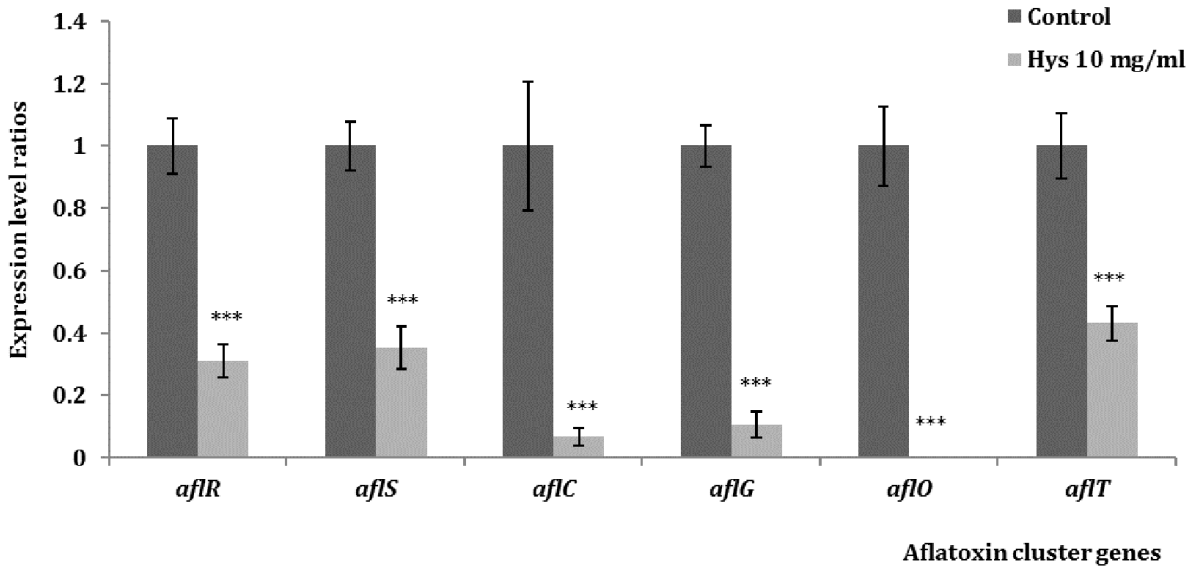

Figure 4. Normalized gene expression ratios of the two internal AF cluster-regulating genes *aflR* and *aflS* as well as *aflC*, *aflG* and *aflO* intervening at the beginning, middle and later stages of the enzymatic cascade, and *aflT* in the presence of hyssop extract (10 mg/ml). *** *p*-value < 0.001. Results are expressed as mean ± SEM (n=6).

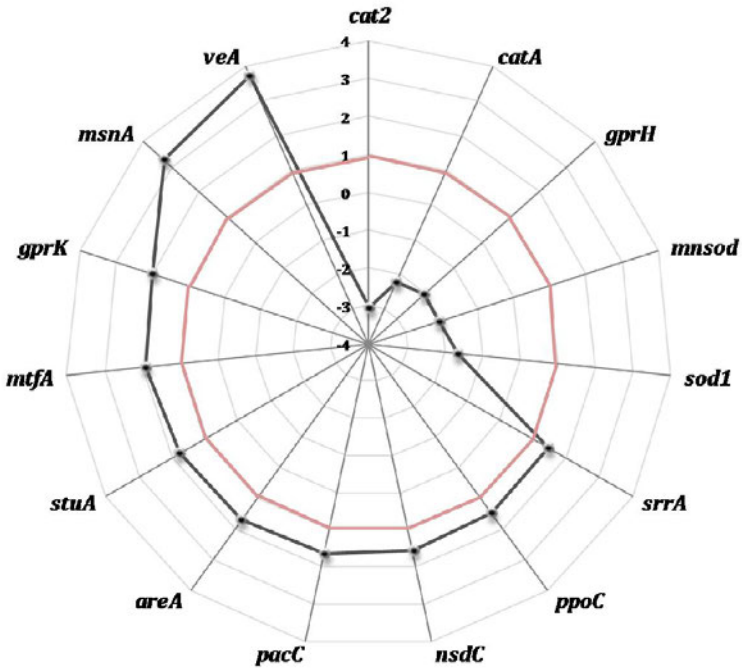

Figure 5. Schematic representation of fold-change ratios of the different regulatory genes affected upon hyssop supplementation of MEA media. The red line represents the expression level of genes set for control cultures and the black one indicates genes' fold change in treated cultures.

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Supplementary Materials: Identification of an anti-aflatoxinogenic aqueous extract from *Micromeria graeca* and elucidation of its molecular mechanism in *Aspergillus flavus*

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Table S1. Gene expression ratio values of AFB1 cluster genes upon hyssop addition. Ratios are obtained in comparison to control values.

Gene	Gene expression ratio	SEM	Fold change	*p*-value
aflR	0.31	0.053	3.2	< 0.0001
aflS	0.35	0.069	2.8	< 0.0001
aflT	0.43	0.055	2.3	< 0.0001
aflA	0.08	0.019	12.2	< 0.0001
aflB	0.08	0.018	12.3	< 0.0001
aflC	0.07	0.029	14.7	0.00058
hypC	0.01	0.003	167.2	< 0.0001
aflD	0.06	0.022	16.8	< 0.0001
aflE	0.06	0.019	17.8	< 0.0001
aflG	0.11	0.042	9.3	< 0.0001
aflH	0.04	0.011	24.8	< 0.0001
aflI	0.02	0.007	60.7	0.00043
aflV	0.05	0.012	19.8	< 0.0001
aflJ	0.06	0.020	18.0	< 0.0001
aflK	0.07	0.019	14.9	< 0.0001
aflL	0.05	0.015	19.2	< 0.0001
aflM	0.12	0.053	8.4	0.00120
aflN	0.03	0.010	34.0	< 0.0001
aflX	0.02	0.008	50.4	< 0.0001
aflO	0.00	0.000	468.8	< 0.0001
aflP	0.03	0.010	29.7	< 0.0001
aflQ	0.07	0.027	13.4	< 0.0001
hypB	0.03	0.016	35.8	0.00085
aflW	0.07	0.019	14.7	< 0.0001
aflY	0.02	0.009	45.8	< 0.0001
hypD	0.11	0.024	9.4	< 0.0001
hypE	0.05	0.011	18.5	0.00018

Conclusion

In this study, we reported, for the first time, the capacity of Hyssop's aqueous extract to inhibit AFB1 production in an *Aspergillus flavus* strain.

The elucidation of its mechanism of action was relied with a marked down-regulation of the genes belonging to the AFB1 gene cluster, which demonstrate that Hyssop's mechanism of action also occurs in a transcriptomic manner via the inhibition of the protein complex AflR-AflS.

Several genes involved in fungal response to cope oxidative stress were modulated which seems to be a key pathway of Hyssop's action.

In fact, we observed that fungal stress response defense elements coding for superoxide dismutases and catalases response were down-regulated.

On the other side, upon Hyssop treatment, important changes in fungal morphology were observed. Although these changes occurred during toxin inhibition, it is possible that they result from the action of other factors than those responsible for the AFB1 inhibition.

Indeed, aqueous extract of Hyssop, is a complex extract that is certainly constituted by more than one molecule which could explain by one hand, the morphological changes and by the other hand the toxin inhibition. As previously mentioned in the article, works by Wright *et al.,* (2000) and Han *et al.,* (2004) reported that an alteration of morphology can be accompagnied with an AFB1 inhibition.

However, some few studies reported such association so it could also be of interest to include in our molecular tools some markers of conidiogenesis or fungal development to evaluate their behavior when AFB1 inhibitors are present in the medium. Such modifications could be either the cause of consequence of fungal metabolic changes upon hyssop (or other inhibitors) exposure.

4.4 Chapter 4

Inhibition of Aflatoxin B1 biosynthesis by aqueous extracts of Mexican plants: *Mimosa tenuiflora* and *Larrea tridentata* (Article 4 – in preparation).

Introduction

In Mexican culture a great number of plants are known as cure to specific diseases making that their use persists from generation to generation.

This remedy is still a common practice and the potential of the active compounds occurring in these plants have demonstrated medicinal properties such as antiprotozoal, antiseptic, antivirial, but also antifungal effects (Arteaga *et al.*, 2005; Quintanilla-Licea *et al.*, 2014). Thus, these plants represent a vast research topic that could be considered whit potential efficacy over mycotoxin inhibition.

Indeed, in countries were mycotoxin contamination is a common problem, the greater the number of anti-aflatoxigenic agents, the better the opportunities to use local sources as an alternative to inhibit AFB1 production.

In fact, in tropical regions AFB1's contamination can often occur during storage since air humidity and storage facilities make difficult to maintain moisture contain (and subsequent water activity of crops) under required limits. Such difficulties are even more frequent in small farmer facilities due to the cost of effective preventive measures. Therefore, the possible identification of a local and abundant resource that could be used to limit AFB1 risk during storage could represent a very interesting and sustainable strategy to help developing countries to improve food safety.

In addition to this, the screening of plants that have anti-aflatoxigenic effect is of great utility. Indeed, some plants that are widely distributed in their native region can also be developed in other countries, offering in this manner an alternative strategy to various populations.

In order to characterize two of the native plants of Mexico, experiments were performed to study their anti-aflatoxigenic effect against *Aspergillus flavus* and especially against AFB1 production.

In the next part, *Mimosa tenuiflora* and *Larrea tridentata* better known as Tepezcohuite and Gobernadora were tested and their molecular impact was characterized.

Several studies have been performed in order to investigate their chemical composition showing that they are rich in antioxidant compounds and in the case of *Larrea tridentata*, the effect of its aqueous extract against AFB1 was already demonstrated.

Thus, we used it as a positive control and contributed with the analysis of its molecular impact.

For *Mimosa tenuiflora,* this is the first time that the aqueous extracts of this plant is tested against AFB1 production.

Inhibition of Aflatoxin B1 biosynthesis by aqueous extracts of Mexican plants: *Mimosa tenuiflora* and *Larrea tridentata*

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Abstract

Contamination by fungi is a worldwide problem in foodstuff causing important economic losses in agricultural sector but also, endangering human and animal's health by consumption of contaminated products. Indeed, some of these fungi are capable to produce dangerous toxins as is the case of Aflatoxin B1 (AFB1), a carcinogenic mycotoxin mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Many studies have been conducted to find out strategies able to limit food contamination with mycotoxins. Recent works demonstrated that natural plant compounds could have an anti-aflatoxigenic effect. Within this context, we studied the effect of two aqueous extracts prepared from Mexican native plants: *Larrea tridentata* and *Mimosa tenuiflora* against AFB1 production in an *Aspergillus flavus* strain. Both extracts were capable to inhibit toxin production with only a slight inhibition on fungal growth. A common concentration of 6.25 mg/ml was sufficient to inhibit AFB1 with ranges between 90-95%. The mechanism of action was investigated by the impact that these extracts have on the expression of the genes involved in AFB1 biosynthetic pathway. Results demonstrated that toxin inhibition occurs in a transcriptomic manner induced by an inhibition of the mainly pathway regulators *aflR* and *aflS* expression.

Keywords: Aflatoxin B1, anti-aflatoxigenic, natural compounds.

1. Introduction

Along food chain process, from crops to foodstuff storage, contamination by fungi represents one of the main causes of agricultural and economical losses worldwide. Moreover, some of these fungal microorganisms are capable of produce toxic secondary metabolites called mycotoxins (Baranyi, Kocsubé and Varga 2015). Aflatoxin B1 (AFB1) is one of the most important mycotoxins. Indeed, it has been demonstrated that a long term exposure to this toxin causes liver cancer and depression of immune system (Warburton and Williams, 2014) leading to the classification of AFB1 in group 1 "Carcinogens" by the International Agency for Research on Cancer (IARC) (Marin *et al.,* 2013). This mycotoxin is produced by several species of *Aspergillus* genus of the *Flavi* section and mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. Its production occurs in a 70 kB gene cluster with at least of 21 enzymatic steps for its formation from acetate molecules (Bhatnagar *et al.,* 2003). Occurrence of *Aspergillus* genera and thus, AFB1 production, is a frequent problem in tropical and sub-tropical zones where climatic conditions are very favorable for fungal growth and toxin production. AFB1 can be present in many products such as corn, peanuts, rice and tree nuts (BIOMIN 2014; Kong *et al.*2014). In Mexico, corn and its by-products are staple foodstuffs with a consumption of 120 kg per capita per year, making of Mexican people the biggest consumers of corn in the world. Unfortunately, climate conditions of this country result in infected crops and it is estimated that 60% of corn produced in Mexico could be contaminated and thus, hazardous for consumption (Ortega-Beltran, Jaime and Cotty 2014; García and Heredia 2006). Because of this, different strategies to inhibit AFB1 production have been developed and the most commonly used is based on pesticides. Nevertheles, the consumption of these compounds can be also toxic on their own.

Within this context, natural compounds occurring in some essential oils and plants extracts could display anti-fungal or anti-mycotoxin properties that could be used as an alternative strategy to reduce food and feed contamination (Gemeda *et al.,* 2014; Samapundo *et al.,* 2007). A large number of these extracts are used by several cultures as an alternative to traditional medicine due to their composition rich in phenolic compounds. In fact, several of them have already been identified as natural aflatoxin's inhibitors (Thippeswamy *et al.*2014; Sánchez, Heredia and García 2005; Sree *et al.*2014). In Mexico, *Larrea tridentata* and *Mimosa tenuiflora* are common plants locally called as "Gobernadora" and "Tepezcohuite" respectively, and their utilization within the

traditional Mexican medicine is frequent. Several studies have been performed in order to investigate their chemical composition showing that they are rich in antioxidant compounds (Lü *et al.*2010; Gardner *et al.*2011). In addition to this, the aqueous extract of *Larrea tridentata*, has been demonstrated as an effective inhibitor of mycelia growth in *Aspergillus flavus*. However, it mechanism of action is yet to be elucidated (Galva *et al.,* 2014). In this study, we demonstrate the effect of the aqueous extracts belonging to traditional Mexican medicinal plants: Tepezcohuite (*Mimosa tenuiflora*) and Gobernadora (*Larrea tridentata*) on *Aspergillus flavus* growth and toxigenesis. The mechanism of action was investigated at the transcriptional level by analyzing their effect on the expression of 4 genes coding for different functions in the aflatoxin biosynthesis pathway: *aflC, aflR, aflS* and *aflQ*.

2. Material and methods

2.1. Plant extracts preparation

Larrea tridentata and *Mimosa tenuiflora* dried plants were bought in Sonora's market in Mexico City. Twenty five grams of each plant were stirred with 200 ml of distilled water during 30 minutes at 60°C. Extracts were filtrated using a Whatman paper no. 1 (Vélizy-Villacoublay, France) in order to obtain aqueous phase extract. Then, aqueous extracts were sterilized at 121°C during 20 min and added by mixing to agar medium before solidification.

2.2. Fungal strain and culture conditions

In this study, the *Aspergillus flavus* strain NRRL 62477 was used (El Mahgubi *et al.,* 2013) and all cultures were performed in 20 ml of Malt Agar Medium (MEA).
For experiments 10 µl of a calibrated spore suspension (10^6 spores/ml) prepared from a 7-day culture was used to centrally inoculate the MEA medium covered with a sterile cellophane layer (Hutchinson, Chalette-sur-Loing, France) as described by Leite *et al.*(2012). Firstly, different concentrations of each extract were mixed with MEA medium and were tested to determine the impact on fungal development as well as AFB1 production.

A final concentration of 6.25 mg/ml of each extract was used for further experiments. Controls cultures were prepared with an equivalent sterile water volume and six replicates of each group were prepared and incubated 4 days at 27°C in the dark.

2.3. Aflatoxin B1 extraction and quantification by HPLC

For AFB1 extraction, culture media were mixed with 25 ml of chloroform and samples were agitated for 2 hours on a horizontal shaking table at 160 rpm at room temperature. Chloroform extract was filtered through a Whatman 1PS phase separator (Vélizy-Villacoublay, France), evaporated at 60°C until dryness and dissolved in 500 µl of a water-acetonitrile-methanol (65:17.5:17.5; v/v/v) mixture then, filtered through a 0.45 µm disks (Thermo Scientific Fisher, Villebon-Sur-Yvette, France). Analysis of samples was done using a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, France) with a LC column, Luna® C18 (125 x 2 mm, 5 µm, 100 Å) (Phenomenex, Torrance, CA, USA) at 30°C. Separation conditions were adapted from Fu *et al.*(2008). Briefly, a flow rate of 0.2 ml/min was used and 10 µl of extract were injected. AFB1 was detected by a fluorescent detector at 365/430 nm excitation/emission wavelengths. Production levels of AFB1 on media were calculated based on a standard calibration curve.

2.4. Isolation of fungal RNA and RT-PCR

At the end of incubation, mycelia were separated from the medium and grounded up under liquid nitrogen. RNA was purified as recommended by the manufacturer using a Qiagen RNeasy PlusMinikit (Qiagen, Hilden, Germany). Quality of RNA was verified by gel electrophoresis and concentrations were measured using a NanoDrop ND1000 (Labtech, Palaiseau, France). The A_{260}/A_{280} ratio and values were compared according to Zeng and Yang method (2002). First-strand cDNA synthesis was carried out by RT-PCR.

2.5. Design and validation of q-PCR primers

Primer sets were designed based on the genomic data of the *Aspergillus flavus* strain NRRL3357 (GenBank accession number EQ963478A). All of the primer pair sequences were adapted from a previous work (Caceres *et al.*, 2016). Primer pairs design was based

on the coding sequence of the corresponding genes, with at least one of the primers extending on an exon/exon junction in order to avoid undesirable genomic DNA amplification. Primer-dimer or self-complementarities were evaluated using the PrimerExpress 2.0 software (Applied Biosystems, Courtaboeuf, France). All primers were synthesized by Sigma Aldrich (Saint-Quentin Fallavier, France) and validated using mRNA product of a no-treated control of *Aspergillus flavus* strain.

2.6. Analysis of the expression of the genes linked to Aflatoxin B1 biosynthesis

Experiments were carried out using a ViiA7 Real-Time PCR System (Applied Biosystems, Forster City, CA, United States). The 384 well-plates were prepared by an Agilent Bravo Automated Liquid Handling Platform (Agilent Technologies, Santa Clara, CA, United States). Each well contained a total volume of a 5 µl mix: using Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) as a fluorescent dye. Three-steps q-PCR were performed as follows: a first one-hold stage at 95°C during 10 min followed by 45 cycles (95°C for 15 s and 60 °C for 30 s) and a final extending step (95°C for 15 s, 60 °C for 1 min and 95°C for 15 s) for melt curve analysis. Results were analyzed with a Quant-Studio Real time PCR software v1.1 (Applied Biosystems, Courtaboeuf, France). Housekeeping genes were analyzed with Normfinder algorithm (Andersen *et al.,* 2004) and the more stable was used as a reference for normalization in the $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen, 2001).

2.7. Statistics

Student's *t*-test was used to analyze the differences between control and treated samples. Differences were considered to be statistically significant when p-value was lower than 0.05.

3. Results and Discussion

3.1. Effect of aqueous extracts on fungal growth and AFB1 production.

Aqueous extracts of Tepezcohuite (*Mimosa tenuiflora*) and Gobernadora (*Larrea tridentata*) were tested against *A. flavus* strain. Treated cultures were macroscopically

observed on day 4 and pigmentation of mycelia presented differences comparing to control. Interestingly, both treatments leaded to yellow colored mycelia but no other important modification of culture aspect was observed (data not shown). This modulation related to pigment could be a first signal on changes occurring in sporulation process that are, in fungi, linked with secondary metabolism (Bayram and Braus, 2012). Fungal growth and AFB1 were also measured in this study. As shown in table 1, mycelia diameter as well as toxin production were inhibited in a dose-dependent manner by the extracts. The highest mycelia inhibitions were observed with *Larrea tridentata*'s extract. For AFB1 production, both aqueous extracts showed a significant inhibition ranging from 90% to no-detectable levels with a p-value of ≤0.001.

Table 1.- Effect of different concentrations of natural aqueous extracts on fungal growth and AFB1 production by *Aspergillus flavus* NRRL 62477 strain.

Extract concentration (mg/ml)	Mycelial inhibition %		AFB1 inhibition %	
	Larrea tridentata	*Mimosa tenuiflora*	*Larrea tridentata*	*Mimosa tenuiflora*
6.2	24.4	10.7	95.3	90.7
9.3	28.9	19.4	97.1	98.9
12.5	48.2	23.3	99.1	100.0

Several studies have been conducted to investigate the active compounds as well as the antifungal activity of *Mimosa tenuiflora*. A recent study performed by Thippeswamy *et al.*(2014) using extracts of *Acacia ferruginea* DC and *Adenanthera pavonina L*, both belonging to *mimosacea*'s family, were tested on *Aspergillus flavus.* Results confirmed a slight inhibition on fungal growth followed by a negative effect on AFB1 production upon addition of 10% for each aqueous extract. In parallel, Gardner *et al.*(2011) analyzed *Mimosa tenuiflora*'s composition by LC-MS technique demonstrating the presence of two major alkaloids identified as N,N-dimethyltryptamine (DMT) and 2-methylcarboline. This would not be the first time that an alkaloid compound demonstrates activity against AFB1; in fact, Lee *et al.,* (2002) performed a study with four different alkaloids isolated from *Piper longum* showing an anti-aflatoxigenic effect on *A. flavus* for all of them. Concerning *Larrea tridentata*, different parts of the plant were yet examined and found to be rich in lignans, especially in methyl-nordihydroguaiaretic acid (methyl-NDGA) and nordihydro-guaiaretic acid (NDGA) as

well as in triterpene glycosides (Jitsuno and Mimaki, 2010; Kumoro *et al.,* 2009; Vargas-Arispuro *et al.,* 2005). The two isolated compounds (methyl-NDGA and NDGA) were tested against *A. flavus* and *A. parasiticus* showing that, in both strains, the highest inhibition of AFB1 production was accompanied by an important fungal growth inhibition. Specifically, for *A. flavus,* a total percentage of 86% of toxin inhibition corresponded to 85.8% of fungal reduction. These results differ from ours, suggesting that other compounds occurring in *Larrea tridentate'*s aqueous extract could be involved in AFB1 inhibition without an important impact in fungal growth.

3.2. Effect of aqueous extracts on AFB1 biosynthetic pathway

In order to characterize the mechanism of action of both extracts, we analyzed their impact on the expression of genes involved in AFB1 biosynthesis. Four genes were tested and chosen due to their importance in AFB1 transformation: *aflC, aflR, aflS* and *aflQ*. Aflatoxins are synthesized by a polyketide metabolic pathway in which *aflR* is the main regulator in charge of activating almost all genes involved in cluster (Bhatnagar *et al.,* 2006; Brakhage, 2012). It has been recently demonstrated that *aflR* interacts with *aflS* forming a functional activation complex, which is absolutely necessary to activate AFB1 biosynthesis (Ehrlich, 2009b; Kong *et al.,* 2014a). In addition to this, a P-450 polyketide-synthase coded by *aflC* gene is also necessary for the first steps of the pathway. A final enzyme, *aflQ*, is involved in the last steps of toxin formation and more precisely in the conversion of B-type toxins. This enzyme is responsible for the conversion of sterigmatocystin into AFB1 (P., K., Chang, Yu, *et al.,* 2004; Ehrlich *et al.,* 2004). Results in this study showed that a transcriptional inhibition occurs upon addition of 6.25 mg/ml of each extract, affecting negatively the expression of the 4 genes involved in this pathway. A highest impact on all gene expressions was observed with *Mimosa tenuiflora*'s extract compared to *Larrea tridentata* (Figure 1).

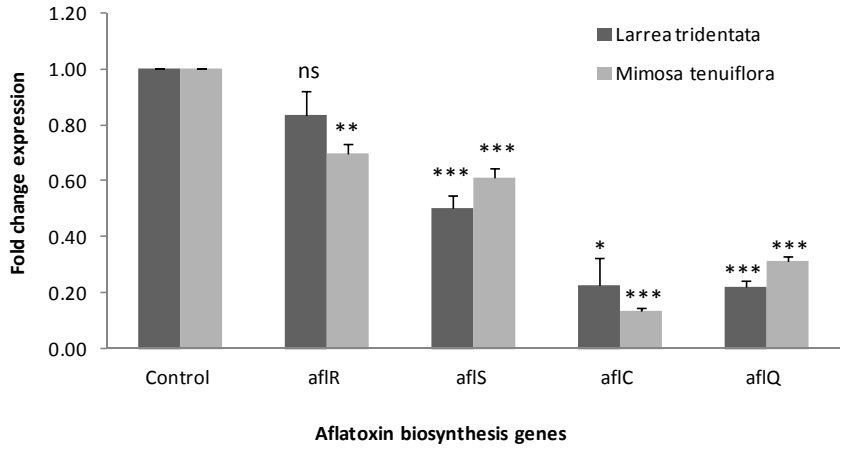

Figure 1.- Fold change expression of genes belonging to cluster responsible for aflatoxin biosynthesis upon addition of 6.25 mg/ml of aqueous extracts. ns= no significant changes; * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

Expression of *aflR* was no significantly different upon *Larrea tridentata* treatment even if a diminution tendency is observed (Fig.1); this effect has been demonstrated in other natural inhibitors of AFB1 (Kim *et al.,* 2006; Yoshinari *et al.,* 2007b). On the other hand, *Mimosa tenuiflora* showed a significantly inhibition on *aflR* expression. Concerning *aflS* gene, both extracts affected significantly its expression with a p-value ≤0.001. A down-regulation of AFB1 synthesis regulators led to a strong inhibition of subsequent genes such as *aflC* and *aflQ.* These results demonstrated that compounds occurring in *Mimosa tenuiflora* and *Larrea tridentata* aqueous extracts are capable to block AFB1 production by affecting the appropriate mRNA stability of genes involved in biosynthesis pathway.

4.0. Conclusion

We demonstrated the anti-aflatoxigenic effect of aqueous extracts of *Larrea tridentata* and *Mimosa tenuiflora* on *Aspergillus flavus* strain. The expression of four genes involved in toxin production was tested and results showed a transcriptomic inhibition by both extracts. This study demonstrates that natural extracts from Mexican plants could be used as an alternative strategy to avoid Aflatoxin B1 contamination. Further studies are needed to identify the active compounds involved in AFB1 inhibition in order to improve their extraction and purification.

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Supplementary Data

Since the present study is still in preparation, we presented some supplementary information that principally involves the study of other genes belonging to the AFB1 gene cluster as well as the study of genes belonging to regulatory factors that were modulated by the addition of *Larrea tridentata* and *Mimosa tenuiflora*.

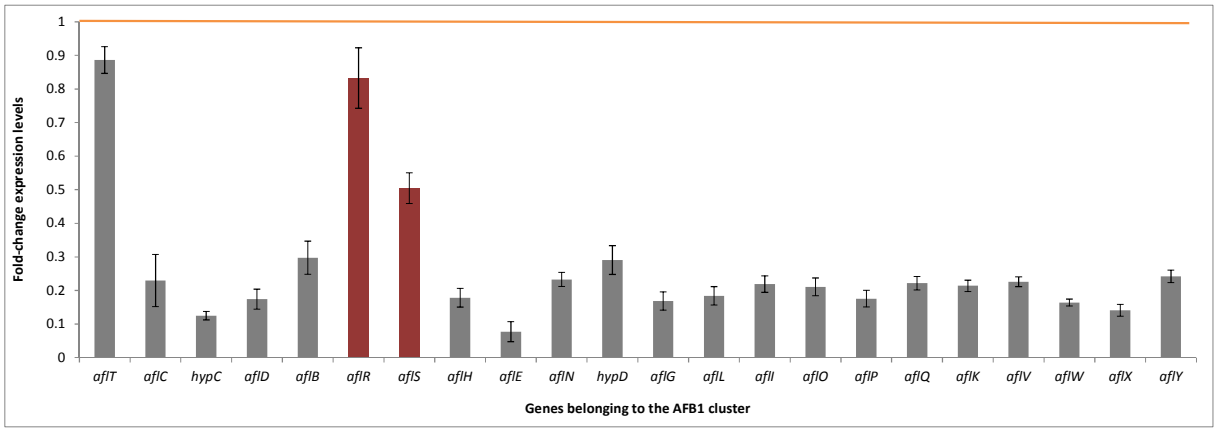

Figure 1. Normalized gene expression ratios of the two internal AF cluster-regulating genes *aflR* and *aflS* (in red) as well as genes belonging to the AFB1 gene clusterin the presence of *Larrea tridentata* (6.25 mg/ml). Results are expressed as mean ± SEM (n=6). The orange line represents the expression level of genes set for control cultures.

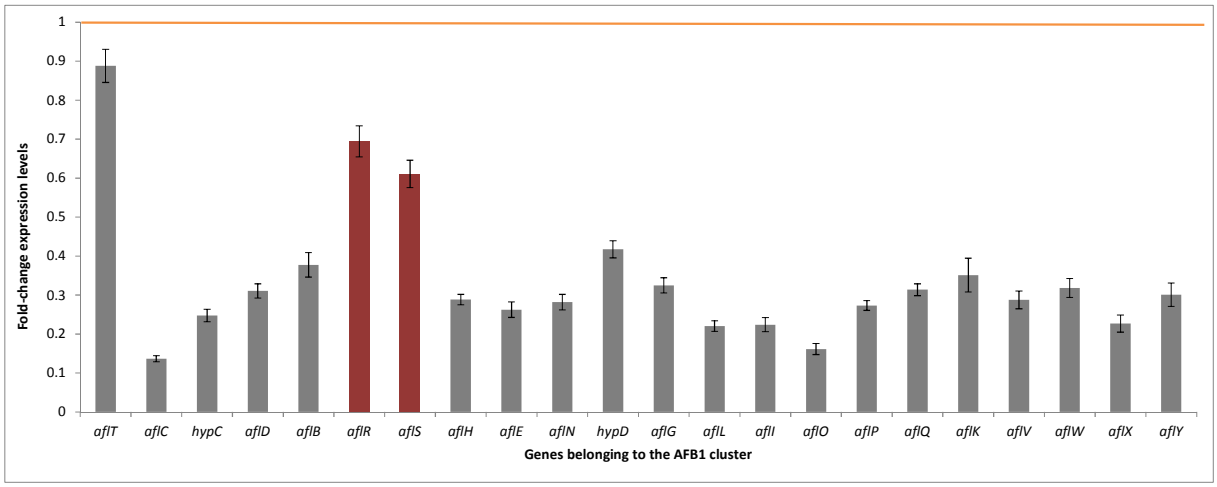

Figure 2. Normalized gene expression ratios of the two internal AF cluster-regulating genes *aflR* and *aflS* (in red) as well as genes belonging to the AFB1 gene cluster in the presence of *Mimosa tenuiflora* (6.25 mg/ml). Results are expressed as mean ± SEM (n=6). The orange line represents the expression level of genes set for control cultures.

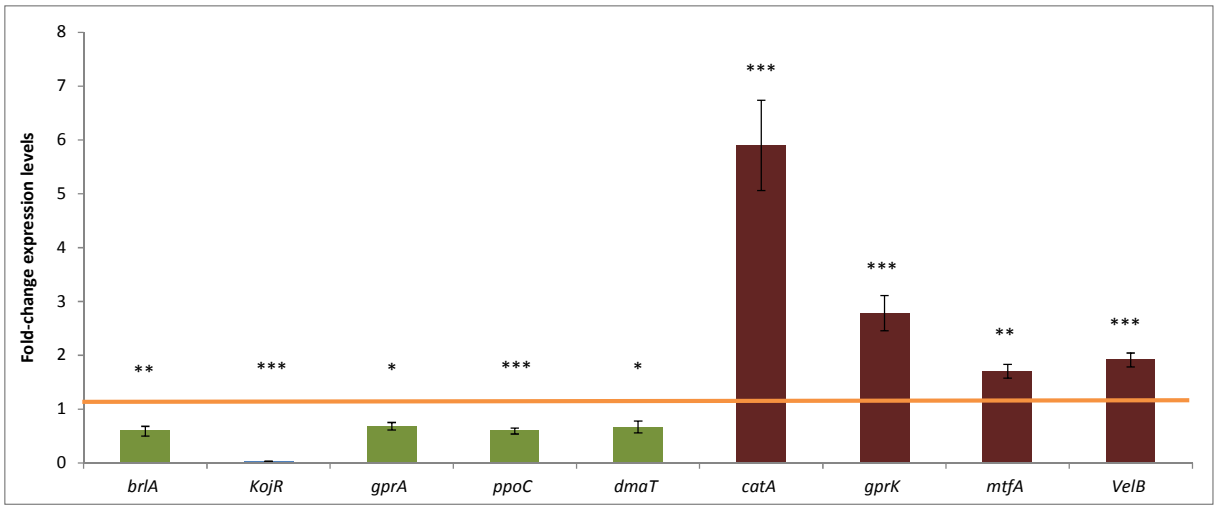

Figure 3. Schematic representation of fold-change ratios of the different regulatory genes affected upon *Larrea tridentata* supplementation of MEA media. The orange line represents the expression level of genes set for control cultures. * *p*-value <0.05; ** *p*-value <0.01; *** *p*-value < 0.001. Results are expressed as mean ± SEM (n=6).

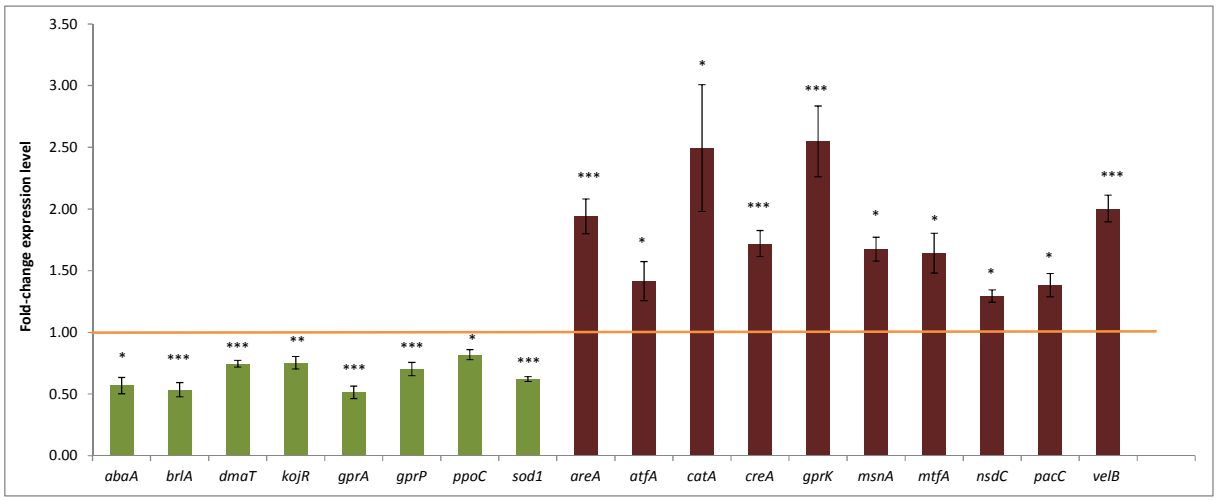

Figure 4. Schematic representation of fold-change ratios of the different regulatory genes affected upon *Mimosa tenuiflora* supplementation of MEA media. The orange line represents the expression level of genes set for control cultures. * *p*-value <0.05; ** *p*-value <0.01; *** *p*-value < 0.001. Results are expressed as mean ± SEM (n=6).

Conclusion

Two endemic plants from Mexico, *Mimosa tenuiflora* and *Larrea tridentata* were analyzed in order to investigate their anti-aflatoxigenic effect against AFB1 production. Results demonstrated that both extracts were capable to greatly inhibit toxin production in *Aspergillus flavus* with a low to mild effect on fungal growth. Within the study of their molecular effect we demonstrated that both plant extracts inhibit AFB1 production in a transcriptomic manner. In fact, reduced levels of *aflR* and *aflS* were also registered similarly to other inhibitors tested in this work and many others reported in the literature.
The inhibitory effect of these plants might be due to a single or even more probably, to several molecules contained in the aqueous extract. It is thus understood that further studies are needed to identify the active molecules.

In addition to this, with this study we demonstrated that *Mimosa tenuiflora* is a new source of AFB1 inhibitor and we determined the dose that allows this inhibition in *in vitro* tests.

Due to its climatic conditions, Mexico is one of the countries that are the most impacted by mycotoxins and especially AFB1. In fact, maize is a staple food for Mexican people and also one of the most frequent sources of AFB1 (García and Heredia, 2006).
On the other side, the same climatic conditions allow the growth of native plants that could serve as AFB1 inhibitors. The demonstration of the anti-aflatoxigenic effect of these extracts could represent a new and useful way for Mexican small producers to fight against mycotoxin production.

05

GENERAL DISCUSSION AND PERSPECTIVES

During the present work different aspects regarding Aflatoxin B1 production were approached.
Based on the vast genetic information of the *Aspergillus* genus, and more specifically on the *Aspergillus flavus* species, two important subjects were the major aims of this work.
By one hand the identification and study of natural products that are capable to inhibit the synthesis of AFB1 production and by the other hand, the study of the molecular impact that these natural products have over the genetic machinery in charge to produce this toxin.

Aiming to better understand the mechanism of action by which natural products inhibit AFB1 production, a molecular tool was created and a total of 60 genes that are known to participate in AFB1's formation were included. The final aim of this molecular tool was to observe simultaneously the impact that these natural inhibitors may have over the 27 genes belonging to the aflatoxin gene cluster as well as on the 33 external regulators that are directly or indirectly involved in AFB1's production.

The choice of using natural products as AFB1 inhibitors relies in an approach that targets the reduction of pesticides use and thus, that represent a sustainable and ecofriendly alternative to reduce mycotoxin contamination.
In the present work, five natural products including two pure molecules and three plant extracts were analyzed against AFB1 production.
The pure molecules (Eugenol and Piperine) and the *Larrea tridentata* plant extract were selected due to their already known anti-aflatoxigenic effect. In addition to this we also demonstrated that two new natural extracts from plants that are native of the Mediterranean and American regions are also effectives against AFB1 production. These extracts correspond to *Micromeria graeca and Mimosa tenuiflora.*

It has to be noted that, during the experimental work of the present study, a special attention was given to the fact that the use of natural products greatly inhibits AFB1 production without a strong impact in fungal development. This factor was chosen since AFB1 inhibitors that proceed in this mode of action can assure the respect of natural biodiversity while improving food safety.

Finally, the purpose to evaluate several natural AFB1 inhibitors with the same molecular tool was important first of all, to characterize the impact of these products on the molecular machinery of *Aspergillus flavus* during toxin inhibition and secondly, to compile the information of each natural product in order to search similitudes among the modulated genes. These observations might help us to better understand their mechanism of action.

Based in the results presented during this work, a general discussion taking in consideration the five natural products is proposed. It has to be noted that some aspects that were already discussed in the corresponding articles will be omitted in this part.

5.1 The analysis of the Aflatoxin B1 gene cluster

Within the already known mycotoxins, AFB1 is one of the most dangerous one. Since that, a great research leading to the characterization of its entire genome has been made. Based in this information and aiming AFB1 inhibition, several research works using natural molecules have been performed in order to understand the mechanism of action by which they inhibit toxin production.
Moreover, since the development of recent techniques such as q-PCR, a new branch of study dedicated to the investigation of the impact that these natural products have over the aflatoxin gene expression has increased during the last years.

As an example, in the present work, the development of a molecular tool that could serve to the study of natural AFB1 inhibitors was a priority.
This idea was based in the fact that the current research works that analyze natural AFB1 inhibitors, principally recurred to analyze only a few genes such as *aflR* or *aflS* that are the mainly regulator of the pathway and in some cases few other genes like *aflC*, *aflD*, *aflM* and *aflP* coding for earlier and later steps in the enzymatic cascade pathway.

It is so that in a first instance we were wondering to know more about the molecular impact that natural inhibitors have over the entire AFB1 gene cluster to have more information about their mechanism of action but also to collect new data.

After having analyzed the five natural products that inhibit AFB1 production from 90% to no detectable levels, we noticed several points.

First of all, comparing all results, a particular gene namely *hypD* was less impacted independently of the treatment. This is an interesting observation since it has been demonstrated that between *aflN* and *hypD* there exists an *aflR* binding site. Nevertheless, within the transcriptomic inhibition caused by natural products, *hypD* do not presented the same inhibition that other genes regulated by *aflR*. Unfortunately, to date little is known about *hypD.* Although deleted mutants of this gene have demonstrated to be involved in development and metabolite production, its exact function still remains unclear (Ehrlich, 2009). Since then, deeper studies in the characterization of this gene in order to known its exact role could be useful.

In addition to this and independently of the treatment, all natural products inhibited AFB1 production in a transcriptomic manner. The report of Kong *et al.,* (2014) proposed that when the principal cluster regulators *aflR* and *aflS* are normally expressed, their corresponding proteins form a protein complex that is essential for a correct transcription of the genes regulated by the AflR-AflS complex. As demonstrated in the corresponding articles, our results are in agreement with this statement since, in all cases, the AFB1 inhibition resulted in down-regulated levels of *aflR* and *aflS.* However, when they were compared, different levels of down-regulation between both genes were observed.
To illustrate this, the impact that the five natural products had over the mRNA levels of genes belonging to the AFB1 gene cluster is presented in the figures 26-28 and associations according to their inhibition tendency over the AFB1 cluster were made.

In fact, we observed several inhibition similarities between Eugenol and Gobernadora and between Tepezcohuite and Hyssop while Piperine presented several particular differences.

First of all, Eugenol and the Gobernadora's extract seem to have a very similar inhibition tendency over the AFB1 gene cluster that is presented on Figure 26.

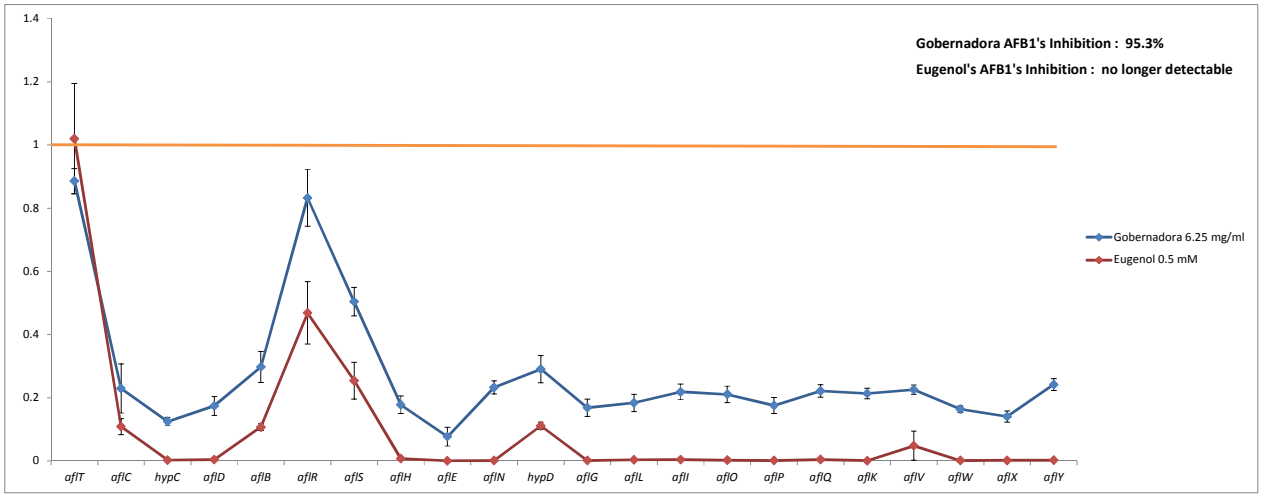

Figure 26: Effect of the inhibitory tendency of Eugenol and Gobernadora over the genes belonging to the AFB1 gene cluster; orange line represents the control levels.

While using both inhibitors, a greater negative impact was observed for *aflS* compared to *aflR*. As a consequence of the main regulators inhibition, the other genes belonging to the pathway were also significantly down regulated with a notable inhibition on the latest genes belonging to the cluster.
Contrary to this, the only exception in both cases was for *aflT*, which confirms the study of Chang *et al.,* (2004b) reporting that this gene is not regulated neither by *aflR* nor by its co-activator *aflS.* Moreover, we demonstrated that neither Eugenol nor Gobernadora's treatment have a significant modulation in the expression of this gene.

In addition to this, several genes coding for the first steps of the enzymatic cascade were also down-regulated. Either with the use of Eugenol and Gobernadora, *aflB, aflC, hypC* and *aflD* were down-regulated. Nevertheless, slight differences between the expression levels of *aflB-aflC* and *hypC-aflD* were observed. In general, the first two one were less impacted that the others ones.

Within the enzymatic cascade, *aflB-aflC* code for the transformation of the hexanoate units into polyketide structures while *hypC-aflD* are involved in Norsolorinic acid transformations (Zhou and Linz, 1999) as shown in Figure 27.

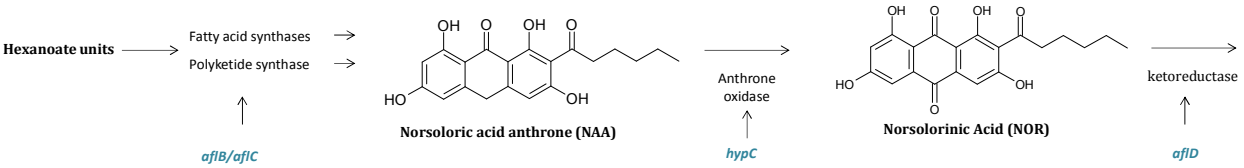

Figure 27: First steps of the AFB1 biosynthetic pathway

We supposed that since the *aflR-aflS* genes were not completely inhibited few amounts of AflR-AflS protein complex were still formed and allowed the activation of the *aflB-aflC* genes, but that the available quantity of active complexes may not be enough to stimulate transcription of further genes such as *hypC-aflD*.

Regarding the intervention of these genes within the enzymatic cascade, these little differences of inhibition could suggest that the use of Eugenol and Gobernadora's extract could cause an AFB1 inhibition may occurs at very early steps of the pathway.
In fact, differences between the expressed levels indicated that maybe no further or only little production of Norsoloric Acid Anthrone (NAA) was achieved.

To illustrate this, and taking the example of Eugenol (where no further AFB1 was detected) and organizing the genes according to their intervention in the enzymatic cascade, a marked diminution between *aflC* and *hypC* is observed even if the expression levels are very low (Figure 28).

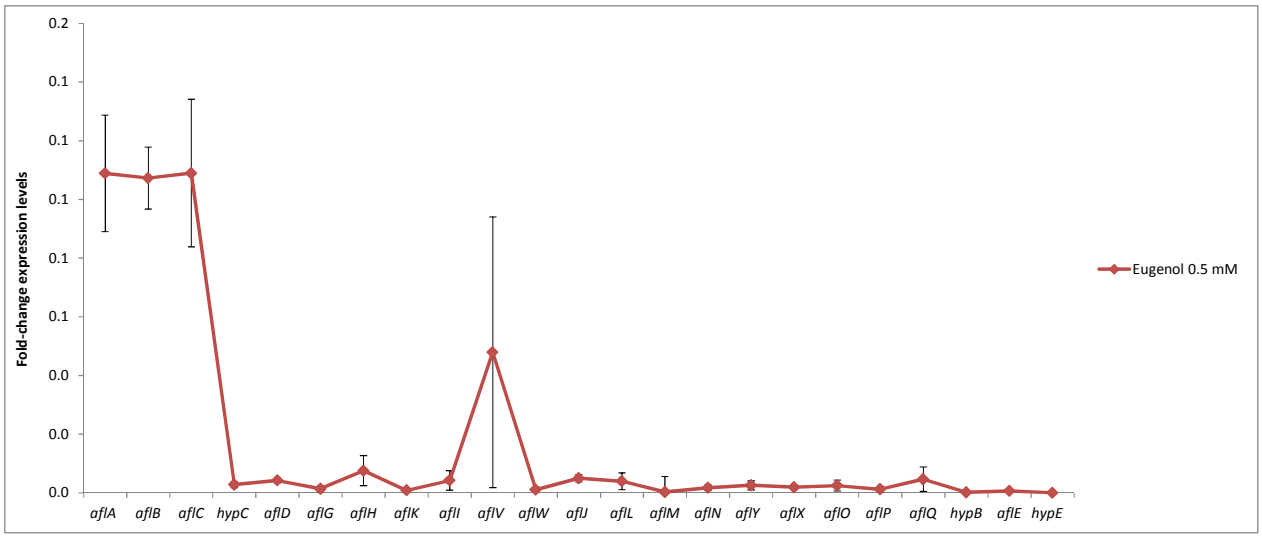

Figure 28: Impact of 0.5 mM of Eugenol in genes intervening for the AFB1enzymatic cascade

To test this hypothesis, either Eugenol or Gobernadora's extract could be tested in *nor-1* mutants of *Aspergillus* strains. This modified strain produces lower amounts of aflatoxins but can accumulate higher amounts of norsolorinic acid (Yabe *et al.,* 1991). Since norsolorinic acid is a stable metabolite during AFB1 biosynthesis, this compound could by measured by techniques such as HPLC in order to observe if effectively, the inhibition of these compounds occurs at earlier steps of the AFB1 enzymatic cascade. One of the advantages that could present an inhibition at early steps of the pathways is to avoid the production of other stable intermediates that appear later in the cascade and that are toxic, such as sterigmatocystin.

On the other side, the characterization of compounds occurring in different parts of the Gobernadora's plant has been already performed. Results reported that this plant contains lignans compounds and notably a major presence of an antioxidant compound known as nordihydroguaiaretic acid (NDGA) (Vargas-Arispuro *et al.*, 2005). Since the latter has molecule is commercially available, further studies could be performed using this isolated molecule to compare its effect whit that of the whole extract in AFB1 cluster.

To continue, figure 29 shows the inhibition tendencies of Tepezcohuite and Hyssop's aqueous extract that were also compared.

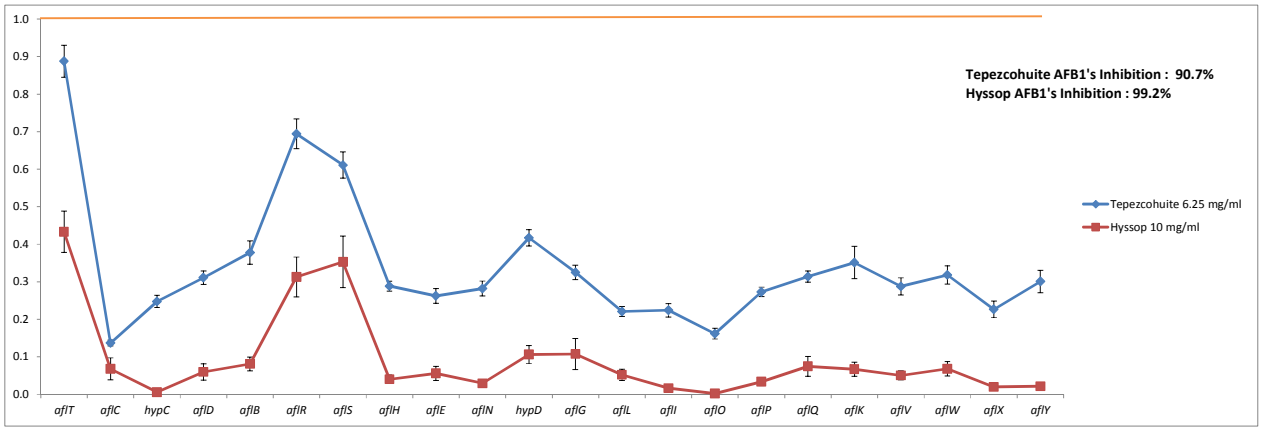

Figure 29: Effect of the inhibitory tendency of Tepezcohuite and Hyssop over the genes belonging to the AFB1 cluster; orange line represents the control levels.

Contrary to Eugenol or Gobernadora in this case, *aflS* inhibition seems to be comparable to that of *aflR* or even lower in the case of Hyssop's treatment. Moreover, both extracts seem to have lower impacts on the latter steps of the AFB1 cluster and since then, it is more difficult to hypothesize at which steps of the enzymatic pathway the AFB1 inhibition occurs.

On the other side, while *aflT* levels were not significantly modulated by Tepezcohuite it was the case using Hyssop's extract. According to this, Chang *et al.,* (2004b) affirm that since *aflT* is not regulated by *aflR* or *aflS* and they supposed that this gene was instead regulated by the *fadA* dependent G-protein. Interestingly, under Hyssop exposure, *fadA* was not significantly impacted. This could suggest that molecules occurring in the Hyssop's extract may directly modulate *aflT* expression or maybe that this gene could be also regulated by another external regulatory factor.

A study performed by Liang *et al.,* (2015) using cinnamaldehyde and citral as AFB1 inhibitors in an *Aspergillus flavus* strain, demonstrated that similarly to Hyssop, at day 4, *aflT* was inhibited by both products. Moreover a study performed by Kim *et al.,* (2008) using caffeic acid also demonstrate reduced level of expression of this gene.

As a useful perspective, the characterization of the Hyssop and Tepezcohuite extracts by techniques like high performance liquid chromatography (HPLC) and mass chromatography could be of great help in order to identify the molecules occurring in these plants. Moreover, due to the fact that their inhibitory tendencies are similar, this could suggest that maybe compounds occurring in these plant extracts are structurally similar or have similar mechanism of action against toxin production.

Finally, one of the compounds that were also characterized using the molecular tool was piperine.

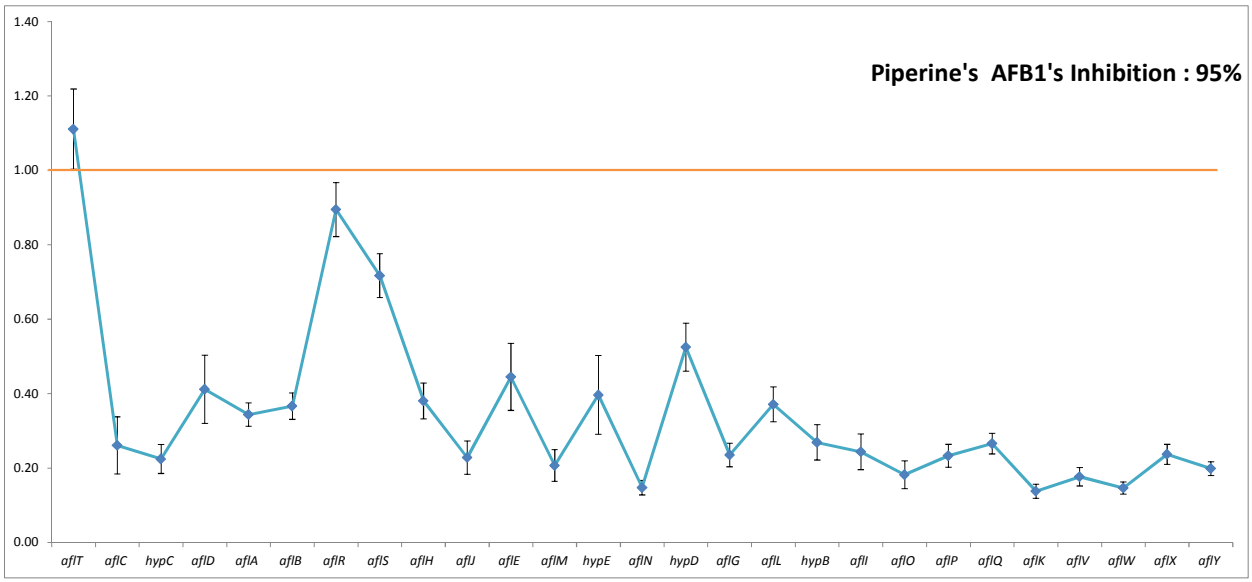

Figure 30: Effect of the inhibitory tendency of Piperine over the genes belonging to the AFB1 cluster; orange line represents the control levels.

In this case while using this pure molecule, we observed that *aflS* was more impacted than *aflR*. Even if this inhibition is similar to those of Eugenol or Gobernadora, slight differences on the impact on the rest of the cluster were observed.

It was notably the case of genes that were less impacted than others genes belonging to the cluster such as *aflE*, *hypE* and *aflD* coding respectively for the final steps of AFB1 production and for the conversion of Norsolorinic Acid into Averantin (first steps in the AFB1 enzymatic cascade) (Ehrlich *et al.,* 2009; Zhou and Linz, 1999).

It is difficult to know why these genes were less impacted. It may be the result of the remaining AFB1 that is still produced but taking in consideration that the rates of inhibition with the other compounds were similar, this seem to be weakly probable.

Another indice could be the highly down-regulation of *aflK* that was one of the genes that were most down-regulated upon piperine's treatmeant. This gene codes either for the conversion of 5'oxoaverantin into Averufin but also for the closure of the bisfuran ring that gives AFB1 their mutagenic effect (Yu *et al.*, 2004b; Sakuno *et al.,* 2003).

Otherwise, *aflR*/*aflS* inhibition levels were recently reported by Moon *et al.,* (2016) after exposure to a piperine-like compound 1-(2-Methylpiperidin-1-yl)-3-phenylprop-2-en-1-one in *Aspergillus flavus* strain. Results were comparable to those obtained with piperine, the tested compound inhibited AFB1 production in a dose dependent manner.

At comparable doses: 11.4 µg/ml for piperine (0.04mM) and 10 µg/ml for 1-(2-Methylpiperidin-1-yl)-3-phenylprop-2-en-1-one, the AFB1 production was inhibited at 95 and 53% respectively. Results using the piperine-like compound demonstrated that at this level of AFB1 inhibition, both genes were not inhibited compared to the control so no possible comparison could be made between both compounds at similar doses.

Nevertheless, if comparing results observed when reaching a similar AFB1 inhibition and therefore suing the dose of 100 µg/ml of the piperine-like compound where no longer AFB1 was detected, the *aflR*/*aflS* ratios were comparable to those observed after piperine exposure, showing that *aflS* was more impacted that *aflR*.

In addition to this, authors also tested 1000 µg/ml of the piperine-like compound and results demonstrated that compared to *aflR*, *aflS* still remained more inhibited. This could suggest that piperine and piperine-like compounds may have a greater inhibition on the *aflS* expression that on the main regulator *aflR*.

As demonstrated, the effect of different natural products may show similarities regarding to their molecular impact on the AFB1 biosynthetic pathway. In addition to this the behavior of several external regulatory factors that are directly or indirectly related with the AFB1 production was also analyzed.

5.2 The analysis of the external regulatory factors

Genes that were commonly down-regulated by four of the five natural products are shown in figure 31. Indeed, all the tested products, with the exception of piperine, presented a significant common inhibition of the expression of some genes.

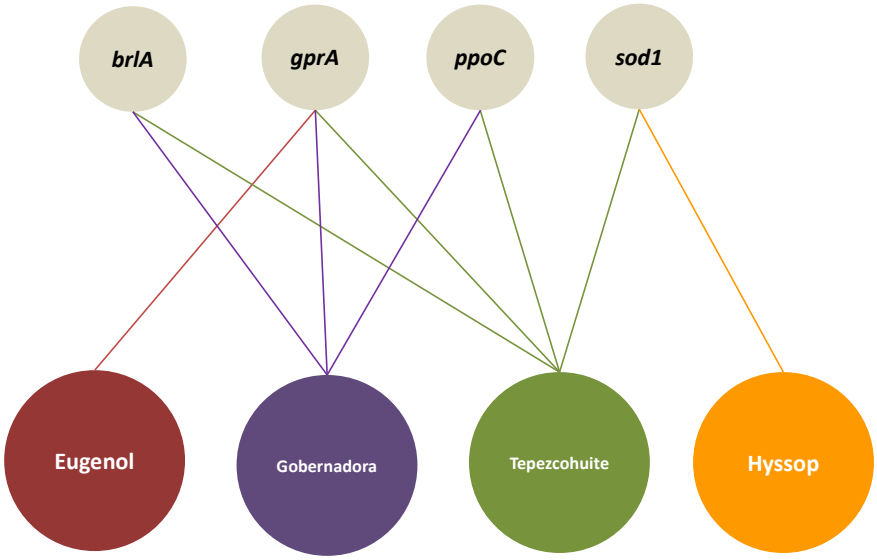

Figure 31: Genes commonly down-regulated by several natural products

As shown, four genes were the main targets of natural products and they are involved in the next functions:

-***brlA*** is a key element for conidiophore development (Tsitsigiannis *et al.*, 2004).

-***gprA*** **is** a G-Protein Coupled receptor (Affeldt *et al.,* 2014).

-***ppoC*** is a fatty dioxygenase predicted to be responsible of oxylipins production (Georgianna and Payne, 2009).

-***sod1***, a Cu/Zn a superoxide dismutase, is involved in fungal enzymatic mechanism of defense against ROS (Apel and Hirt, 2004).

Otherwise, within the genes that were up-regulated, a total of ten genes were the principal targets of the different natural compounds. All of them are shown in Figure 32 and in order to organize them, they were classified in:

1. Oxidative Stress group
2. Velvet complex
3. Cellular signalization
4. Environmental transcription factors
5. Global transcription factors

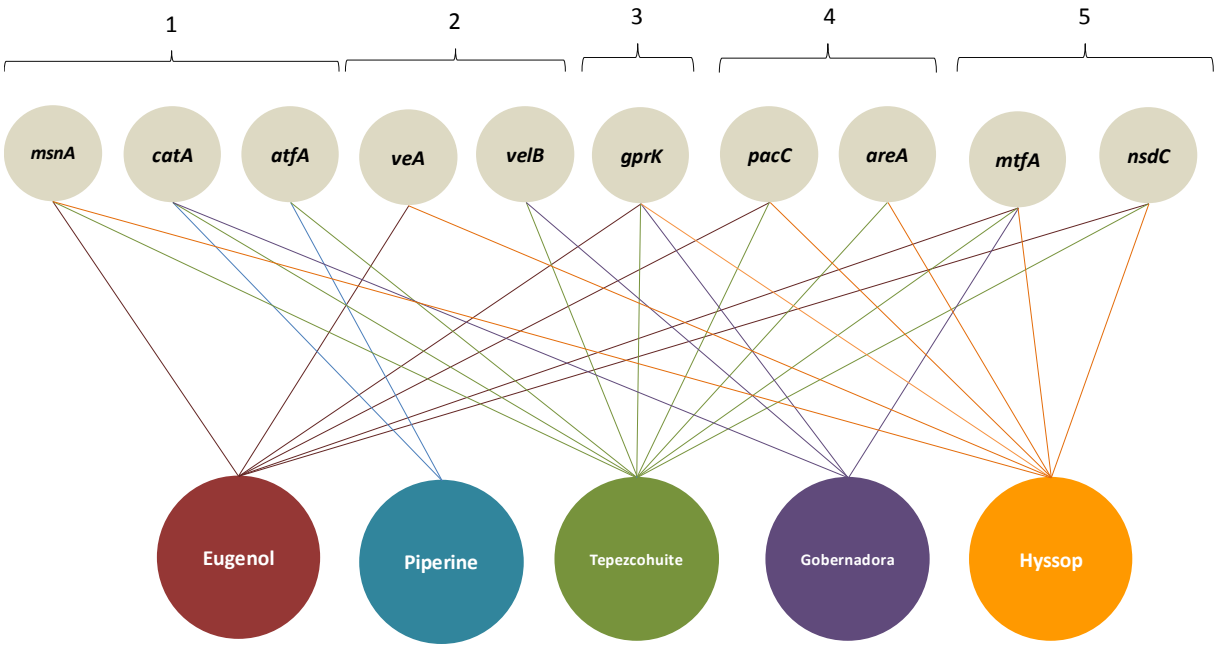

Figure 32: Genes commonly up-regulated by several natural products

Finally, the effect of the different natural compound over the modulated gene-expression is summarized on table 20.

Table 20: Comparative table of up- and down-regulated genes by the different natural products tested during this work.

	Hyssop	Gobernadora	Tepezcohuite	Eugenol	Piperine	Total
		brlA	*brlA*		?	2
Down-regulated		*gprA*		*gprA*	?	3
		ppoC	*ppoC*		?	2
	sod1		*sod1*		?	2
	gprK	*gprK*	*gprK*	*gprK*	?	**4**
	mtfA	*mtfA*	*mtfA*	*mtfA*	?	**4**
	msnA		*msnA*	*msnA*	?	3
	areA		*areA*		?	2
Up-regulated		*velB*	*velB*		?	2
		catA	*catA*		*catA*	3
	nsdC		*nsdC*	*nsdC*	?	3
	pacC		*pacC*	*pacC*	?	3
	veA			*veA*	?	2
			atfA		*atfA*	2

The interest of comparing all the modulated genes was to track information that led us to know if one of the tested genes could be commonly modulated using different AFB1 inhibitors.

Even if some of them could be essential keys within the different mechanism of actions triggered by natural products, two genes seem to be good candidates in order to continue further studies. It is the case of *gprK* and *mtfA* that, independently of the treatment, were always significantly up-regulated.

Concerning the *gprK*, G-Protein Coupled Receptors (GPCR) are known to serve as signal detectors of environmental stimuli that transmit information within the cell and generate some kind of cellular response. As discussed in the corresponding articles, *gprK* is a GPCR that has been recently studied in *Aspergillus flavus* (Affeldt *et al.,* 2014). It has to be noted that since little is known about GPCRs within this species, in the cited work, 15 of them were deleted in order to observe their impact in fungal strain.

One of the most controverting observations was that within the deletion of these GPCRs', Δ*gprA* and Δ*gprP* were reported to have an impact on Aflatoxin production but it was not the case for Δ*gprK*.

This seem to depend on species, since another recent work of Jung *et al.,* (2016) using *Aspergillus fumigatus* demonstrated that null mutants of *gprK* resulted in inhibition of gliotoxin production. Even if the predicted role of *gprK* in *Aspergillus flavus* was not

exactly determined, its deletion demostrated that this gene is involved in germination, carbon sensing, cell wall, osmotic and acidic stress response and deletion resulted in impaired growth on different sources of carbon.
Interestingly, the null mutant of *gprK* was also tested with a compound, named Methyl Jasmonate (MejA), that was previously demonstrated to inhibit AFB1 production in *Aspergillus flavus* (Goodrich-Tanrikulu *et al.*, 1995). Results showed that, in presence of MejA, the Δ*gprK* mutants increased Aflatoxin production.

This could be an interesting point, since the natural products tested in this work inhibit AFB1 production with an over-expression of *gprK*. Thus, even if the Δ*gprK* strain by itself did not showed effect on AFB1 production in *Aspergillus flavus*, the effect of the over-expression of *gprK* in *Aspergillus flavus* using non-conductive AFB1 conditions has not been previously reported.

Since then, one the next perspectives of this work could be to study in detail the over-expression of *gprK* in *Aspergillus flavus*. The construction of an *OE:gprK* strain could led us to understand its impact in fungal growth, developmental processes and notably its impact on the AFB1 production.

Moreover, the construction of such a mutant strain could allow studying the possible relationships between *gprK* and other genes that are involved in AFB1 production, especially *aflR*.
It could therefore help to better understand if this gene is one of the direct targets of the natural inhibitors or if its over-expression is the consequence of other fungal mechanisms.

On the other side, *mtfA,* considered as the second gene target has been demonstrated to be a master transcription factor that is involved in secondary metabolite production of *A. nidulans*, *A. fumigatus* and *A. flavus* (Ramamoorthy *et al.*, 2013; Smith and Calvo, 2014; Zhuang *et al.*, 2016). As described in the corresponding articles, in *A. nidulans*, changes in the expression levels of this gene, either down- or over-expressed, led to *aflR's* inhibition, and thus sterigmatocystin production (Ramamoorthy *et al.,* 2013). It was one of the genes that could be a main target but its expression is related to other genes. It

could be interesting to investigate in detail the relations with the other genes that were modulated within AFB1 inhibition.

In addition to this, in order to complete the present study, experiments will be performed to extend the study of the regulatory factors for piperine (the only compound for which only genes coding for the oxidative stress response were studied) aiming to know if the genes *gprK* or *mtfA* are also modulated.

In addition to this, it could be of interest to perform a kinetic study in order to measure gene expression during the time course of tratmeant exposure.
In fact, studies using eugenol as AFB1 inhibitor, demonstrated that after 7 days of exposure, among 5 genes belonging to the AFB1 pathway that were initially modulated, four of them retrieved their normal expressions and in some cases, they were instead up-regulated compared to the control (Liang *et al.*, 2015).
If a natural inhibitor is destined to be applied in real conditions, it has to be taken in consideration that it needs to keep the capacity to inhibit AFB1 production during medium to long periods, corresponding for instance to crops' storage duration.

Moreover, since mycotoxin contamination co-occurred with other fungal species, the natural extracts can be tested on several other fungal species/mycotoxins to determine if they have an inhibitory effect in other toxic contaminants.
According to this, and beyond *Aspergillus flavus* or *A. parasiticus*, several species of *Fusarium spp*. are major potent toxigenic contaminants of cereals that would be an interesting subject of study. For instance, *F. verticillioides and F. proliferatum* can produce Fumonisin B1 that is another currently mycotoxin that is found as a co-contaminant with AFB1 (Hove *et al.*, 2016).

Moreover in order to consider the use of the natural products as potential AFB1 inhibitors in real conditions, it has also to be considered that *in vivo* tests have to be performed to observe it these natural products can still inhibit mycotoxin production on complex matrixes such as maize grains, that is one of the principal source of AFB1 contamination (Woloshuk and Shim, 2013).

According to this, one of the long-term perspectives is to identify a natural compound or extract that present the above-described characteristics targeting its utilization to inhibit mycotoxin production during grain's storage.

However, it is obvious that, since moisture is a key parameter to ensure proper storage of cereals, the aqueous extract would not be usable as it. Thus, formulation of active compounds has to be modified to allow an application that do not modify the initial water activity of grains but that could lead to a release of active molecules in case of moistening during storage.

For this, several techniques have been proposed and one of the most convincing for natural compounds can be the micro-encapsulation.

5.3 Micro-encapsulation

Encapsulation is a technique by which a core material can be coated with another material or system. The covering material is called "wall material" or encapsulant and will be degraded within time-exposure or by external conditions that allow the liberation of the active compounds to the exterior (Madene *et al.*, 2006).

This encapsulation technique can be made, among others, by spray-dryer that is one of the techniques that offers the advantage of eliminate the water (in the case of aqueous extracts) in addition to protect the bio-actives molecules for their storage and further utilization.

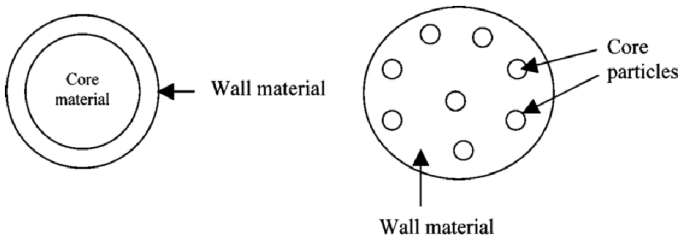

Figure 33: Microencapsulation principle with 2 types of microcapsules. This schematic diagram was integrally taken from the work reported by Desai and Jin Park, (2005).

This encapsulation technique was discovered within the 50's decades and has been developed in several branches of food, cosmetology and pharmaceutical industries. The

use of this technique increased during the last years due to its advantages since it offers an eco-friendly option. Indeed, some of the materials that can be used to encapsulate compounds are biodegradable materials fabricated with carbohydrates, cellulose, gum, lipid and proteins (Desai and Jin Park, 2005).
Thus, micro-encapsulation can be a useful technique to conserve active molecules from natural extracts and to allow their conservation within the time.

One of the disadvantages of using natural compounds is that some of them could change the sensorial properties of food commodities. Such effect has been demonstrated even by using essential oils that usually lead to major sensorial modifications of food commodities (Ayala-Zavala and González-Aguilar, 2010). For this, micro-encapsulation has been demonstrated to reduce these impacts.
In addition to this, water-extracts or antioxidant compounds have been already demonstrated to be micro-encapsulable with good yields while using the use of micro-encapsulation by the spray-drying technique (Şahin Nadeem *et al.*, 2011; Sansone *et al.*, 2011).

Finally, before the industrial application of these microcapsules, some considerations have to be taken in order to characterize the materials that can be used for encapsulate the active compounds or molecules.

One of the most essential tests can be the kinetics studies using different kind of encapsulation materials as well as different relative humidity levels in order to determine the liberation rate of the encapsulated compound. Since micro-capsules can be disintegrated under different conditions, the choice of the material is a key element.
Besides impact of moisture on the dissolution of capsules, other parameters such as pH conditions, temperature, effect of mechanical destruction, morphology, encapsulated yield rates and release mechanisms have to be also considered.

5.4 Conclusion

To conclude, the analysis of gene clusters that are in charge of the secondary metabolite production can represent a useful approach to understand mycotoxin biosynthesis but also to understand the evolution of the fungal species.

In the case of the AFB1's cluster, the genes in charge to synthesize this mycotoxin as well as its precursor sterigmatocystin are shared between fungal species and notably between *A. flavus*, *A. parasiticus* and *A. nidulans*.

In 2009, Ehrlich *et al.,* compared six different gene clusters that code for different secondary metabolites production and demonstrated that some of the aflatoxin genes have similar functions, not only between AFB1 producer strains, but also with other fungal species (Figure 34).

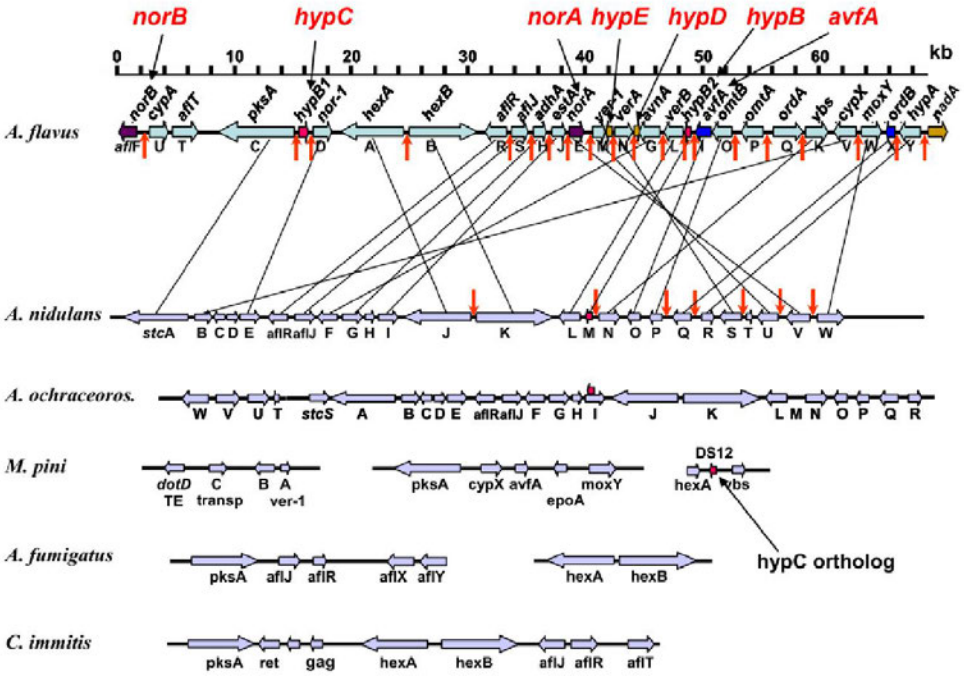

Figure 34: Comparison of gene clusters coding for secondary metabolites in several species

With this image, it is shown that since several species can contain genes related to the aflatoxin production, the impact of natural compounds can lead to consider their use in other species.

In addition to this, the identification of their mechanism of action using molecular techniques could be helpful to identify not only their effect within the gene cluster but also, to study the effect that the external regulatory factors have over toxin production. Such understanding could allow the identification of natural stimuli that directly lead to inhibition of mycotoxin production and that could be subsequently used to limit food contamination at different steps of food chain.

In parallel, this work also demonstrated that, the use of natural products can represent a promising alternative strategy to inhibit mycotoxin contamination and thus, improve food safety. It can offer an eco-friendly and sustainable option to the use of pesticides, especially in regions where natural resources are abundant and climatic conditions are favorable to mycotoxin production.

06

ANNEXES

Annexes

6.1 Physical Methods to reduce AFB1 incidence

Table S1: Physical Methods to reduce AFB1 incidence

Cleaning				
Method	**% Reduction of AFB1**	**Food matrix**	**Advantage**	**Disadvantages**
Washing	3-15	Black pepper	Little efficiency but can represent a complementary alternative to AFB1 elimination	By nature, AF's are not water-soluble Cost of seed drying after washing Increase of moisture levels Jalili, 2015
Mechanical Remove	Average 40	Various	Easy method to eliminate contaminated product	Only used when a partial contamination exists Not practical for maize and cottonseed Peraica, 2002
Optical Sorting	50-60 98	Maize Peanuts	Primary tool to reduce mycotoxin contamination	False positives and sampling error Misclassification grade of contamination IARC, 2015; Moy and Miller, 2016

Fluorescent Detection	90-100	Various	Effective for maize and cottonseed	Not effective for peanuts Other metabolites such as Kojic Acid are also fluorescent (false positives) Peraica, 2002
Irradiation				
Method	**% Reduction of AFB1**	**Food matrix**	**Advantage**	**Disadvantages**
20 kGy	100	Yellow corn and peanut	Non-ionizing radiation (microwaves, visible light) can rise temperature without hazardous molecular changes in products Gamma radiation, depending on A_w of product can form free radicals that attack AF's	Ionizing radiation (X-rays, gamma rays and UV rays) depends on temperature and could produce molecular changes in products Could these free radicals be hazardous for food safety? Radiation can be effective to reduce AF's only into thinner lays of grains
15 kGy	19.25	Almond		
10 kGy	55-74 81.1 87.8 86 84 81.1 68.8	Peanut Maize Rice Barley Bran Corn Peeled Pistachio		
8 kGy	60.26 64.24 64.68	Maize Wheat Rice		
6 kGy	32.39	Maize		
5 kGy	46	Maize		

4 kGy	15.54 22.25 27.46	Maize Wheat Rice	Effective against mold growth and toxin production	Adeyeye, 2016; Peraica,2002;Stoev, 2013; Jalili, 2015
2 kGy	68.9	Maize		
Heating				
Method	**% Reduction of AFB1**	**Food matrix**	**Advantage**	**Disadvantages**
Microwave	50-60 32.3	Peanut Poultry feed	Heat treatment still provides reduction of AF's in maize products and some of them are current and easy to use	High decomposition temperatures of AF Other mycotoxins are also heat-stable Efficacy depends on food constituents and of AF contamination amount Over-heat food can produce undesirable changes Jalili,2015
Boiling Baking Steaming	50-70	Maize		
Roasting (90-150°C)	78.4 17-63	Peanut meal Pistachio nuts		
Roasting (150°C)	70 95	Peanut seed Peanut		
Roasting (140°C)	58.8	Peanut seed		
Hot air oven	57.6	Feed		
Heating (180°C)	62.5 40	Ginger Curry powder		
Ordinary cooking	31-36 34	Rice Polished rice		

Biomass Dryers	lower than 20 ppb	Maize	Protection to insect injury (reduction of insecticides) Fungi can not growth in dry products	Some of them have high costs Kaya and Kyamuhangire, 2010; IARC, 2015
Sun drying	90-100	Wheat	Good cost-effective method	Moisture control Not practical during rain and wet season IARC, 2015 Peraica, 2002
Adsorption agents				
Method	**% Reduction of AFB1**	**Food matrix**	**Advantage**	**Disadvantages**
Bentonite (*e.g.*Mycofix®)	>95%	Various	Mycofix® is already authorized by the European Union	Some adsorbents could not be effective for other mycotoxins
Zeolite	80	Juice fruits	Useful to prevent Aflatoxicosis	Several works *in vitro* but its effect in *in vivo* tests is not predictive
MgO-SiO_2	80-100	Wheat flour	Promising alternative for livestock feed	Active charcoal is expensive and might lead to deficiency of minerals and vitamins in domestic animals
Active Charcoal	>99	Various	HSCAS also reduce AFM1 in cow and goat's milk	
HSCAS	98-100	Various		Peraica, 2002; Nortaa, E., 2016;Jalili, 2015; Biomin; Gallo and Masoero, 2010; Stoev, 2013

Solvents				
Method	**% Reduction of AFB1**	**Food matrix**	**Advantage**	**Disadvantages**
Ethanol Isopropanol Methoxy-metane	90-100	Maize Peanuts	Remove of AF from different types of foods products	High cost of organic solvents Hazardous for industrial use Peraica, 2002

6.2 Chemical Methods to reduce AFB1 incidence

Table S2: Chemical Methods to reduce AFB1 incidence

Method	% Reduction of AFB1	Food matrix	Advantage	Disadvantages
Citric Acid	96.7 86	Maize Barely	Under alkaline and acid conditions, lactone rings of AF's could be open leading to a beta-keto acid compound that is water-soluble. For nixtamalization, not only a greatest reduction of AF's are observed but also of Fumonisins	Almost all experiments have been tested under impractical conditions Jalili, 2015
Nixtamalization	94 94 85 79	Maize Tortilla Tortilla chips Corn chips		Not been adapted in other regions of Africa and Asia Requires adequate water Inhibition rates change depending on method Moy and Miller, 2016; IARC, 2015; García and Heredia, 2006; Jalili, 2015
Extrusion+Lime (0.3%) Extrusion+Lime (0.3%)+H_2O_2 Extrusion+Lime (0.5%)	74 100 85	Corn Tortilla	Several conditions of extrusion are very effective against AFB1	Negative effect on taste and aroma of tortillas Inhibition depends on temperature and pH García and Heredia, (2006); Stoev, 2013; Jalili, 2015

Ammoniation (2%) Ammonia with atmospheric pressured and increased temperatures	88 90-100	Maize Peanut meal	Ammoniation is one of the most current industrial processes to eliminate AF's for animal feeding. Gaseous or liquid Ammonia is allowed by the FDA in the USA. Ammonia could convert AFB1 into a non-toxic compound AFD1.	Long period of aeriation leading to high costs Ammonia reduces nutritional value in feed Peraica, 2002; Jalili, 2015
Ozonization (33 mg/l) (66 mg/l)	 80 93	Flanked and chopped peppers	Could also represent an effective method for AFB1 degradation in corn and peanuts Efficiency increases with higher temperatures and longer exposure time	Not allowed in European Community (EU) for foods destined to humans Torres, 2014; Stoev, 2016; Jalili, 2015

Chemoprevention in humans

Method	% AFM1 Reduction	Test	Advantages	Disadvantages
Oltipraz (500 mg/week)	50	Human Urine	Reduction of disease risk	Needs more investigation Age and genus influence the results IARC, 2015

Green Tea Polyphenols 500 mg / 3 months 1000 mg / 3 months	 42 43	Human Urine	Reduction of toxic metabolites in humans	Needs research in other countries to determine which local plant contain compounds with these effects Tang *et al.,* 2008; IARC, 2015

Pesticides: Fungicides/Insecticides

Examples of Pesticides	Advantages	Disadvantages
Fungicides Copper-Based (*Cooper oxychloride, Cuprous oxide*) Dithiocarbamates (*Mancozeb, Maneb, Zineb, Thiram*) Phtalimides (*Captan, folpet*) Benzimidazoles (*Benomyl, Carbendazim, Thiabendazole*) Phenylamides (*Metalalxyl, Oxidixyl*) Dicarboximides (*Iprodione, procymidone*) Strobilurin Analogues (*Azoxystrobin, Pyraclostrobin*) Azoles (*triadimefon, epoxyconazole, propiconazole*) Benzonitriles (*Chlobenthiazone*)	Some of them are specific Aflatoxin inhibitors Effective against fungal infection Reduction of insect damage in crops which reduce risks	Environmental Contamination Biodiversity changes Toxic Compounds Human allergies Microorganism resistance Pest-tolerance crops Economic issues Food safety issues Resistance mutations Different efficacy depending of fungi Accinelli *et al.,* 2014; Sakuda *et al.,* 2014; Torres *et al.,* 2014; Zain, 2011; Tola *et al.,* 2016;Brent and Hollomon, 2007
Insecticides Spinosad Thiamethoxam Imidacloprid Indoxacarb		

6.3 Biological control to reduce AFB1 incidence

Table S3: Biological Methods to reduce AFB1 incidence

Fungi **Non toxinogenic *Aspergillus* Strains** (Spread Spectrum including AFB1 but also other AF's)				
Fungal strain	**% AF Reduction**	**Food matrix**	**Advantages**	**Disadvantages**
A. flavus (AF36) (USA origin)	>80	Cottonseed Corn Pistachios Figs	First bio pesticide regulated in USA that is still used and approved by the Environmental Protection Agency (EPA) AF36 has a polymorphism near to the coding sequence of the polyketide synthase (*pksA*/*aflC*) required to AF's synthesis	In several regions is only effective from late May through June Strains can still produce CPA Efficiency depends of substrate Ehrlich and Cotty, 2004a; Adhikari *et al.,* 2016
Aflasafe™ (4 atoxigenic *A. flavus* strains of Nigerian origin)	80 90	Maize harvest Storage Maize	Used in pre-harvest control with post-harvest gains Potential utilization on small-scale farmers of maize/cassava in Kenya and sub-Saharan Africa	A sexual stage was recently described for *A. flavus* and sexual recombination in nature and its consequences have to be explored Okike *et al.,* 2015; Marechera and Ndwiga, 2015

Aflaguard ® (*A. flavus* NRRL 21882 strain of USA origin)	9-75 85-88 85.2	Maize Maize Peanut	Commercial use in USA and also tested in Australia and Argentina with good results The entire AF gene cluster and CPA clusters are deleted which is preferable than only to an aflatoxigenic strain	Its efficacy in multi-environment and multi-state conditions and under longer time horizons has yet to be understood Adhikari *et al.,* 2016;Dorner and Lamb, 2006; Torres, 2014; Jalili, 2015
K94 (USA origin)	83-98	Maize	In addition to be atoxinogenic, this strain does not produce CPA Efficiency in maize crops within the years	Need to be tested in other products Jalili, 2015
Aspergillus niger (ND-1)	58.2	Coumarin	Rapid detoxification of AFB1 (24 h of fermentation) Enzymatic degradation	Treatment is affected by heat, pH and metal ions Zhang *et al.,* 2014b
Supplementary Information: There exist other strains such as *Penicillium raistrickii* (NRRL 2038) that is capable to convert AFB1 into AFB2 as well as *Dactylium dendroides* (NRRL 2575); *Mucor griseocyanus* (NRRL 3359); *Mucor alternans* (NRRL 3358); *Absidia repens* (NRRL 1336); *Helminthosporium sativum* (NRRL 3356); *Mucor ambiguous* (I.M.M. 115) and *Trichoderma viride* (ATCC 13233) that could transform AFB1 into a compound also blue-fluorescent: AFR_0 (Ji *et al.,* 2016).				

Bacteria				
Fungal strain	**% AFB1 Reduction**	**Test**	**Advantages**	**Disadvantages**
Actinobacteria (gram positive)				
Corynebacterium rubrum *Mycobacterium smegmatis*	99 >99	Liquid culture Liquid culture	Great efficiency	Needs further tests to understand its mechanism of action and its effect in *in vivo* test (Ji *et al.,* 2016)
Ɣ-Proteobacteria (gram negative)				
Pseudomonas aeruginosa N17-1	82.8	Nutrient Broth medium	Enzymatic degradation, great potential in industrial use	Needs further tests to understand its mechanism of action and its effect in *in vivo* test Sangare *et al.,* 2014
Bacillus (gram positive)				
B. subtilis ANSB060	81.5	*in vivo* Broilers	Antimicrobial activities against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*	Need to be tested in food products
B. megaterium	30.6 36.6-41.6 >98	PDA culture Peanute kernels Liquid culture	Biocontrol effectiveness in *in vivo* and *in vitro* tests Reduce expression of *aflR*/*aflS* and CPA genes	Needs further tests to understand its mechanism of action Kong *et al.,* 2014; Kong *et al.,* 2010; Fan *et al.,* 2015
Streptomyces griseochromogenes (gram positive)				
Blasticidin S	95	Liquid medium	New inhibitor compound Reduce expression of *aflR*, *pksA* and *omtA*	Partial inhibition of CPA Yoshinari *et al.,* (2013, 2010)

Lactobacillus (gram positive)				
L. rhamnosus *L. lactococcus* *L. plantarum*	80 5.6-59.7 15-60	Liquid culture PBS solution Liquid culture	Some of them have been also tested *in vivo* with good results	Undesirable effect in foods (Jalili, 2015)

Yeast				
Fungal strain	**% AFB1 Reduction**	**Test**	**Advantages**	**Disadvantages**
Saccharomyces cerevisiae	40	PBS solution	*S. cerevisiae* is a microorganism very well studied that could facilitate the understanding of a possible mechanism of action against AFB1	Undesirable fermentation in products (Jalili, 2015)
Saccharomyces and *Candida* strains	15-60	PBS solution		
Saccharomyces cerevisiae cell wall	81.6	Feed contaminated		

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