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MONITORING, SUPPRESSION AND MOLECULAR REGULATION MECHANISMS OF MYCOTOXIN-PRODUCING FUNGI

Asmaa Shaaban Abdo Abbas

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The future belongs to those who believe in the beauty of their dreams
-Eleanor Roosevelt

Ayselille ja Esraalle

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ABSTRACT

Within the group of filamentous fungi, *Aspergillus flavus* is a prominent species because of its capability to produce aflatoxin B1 (AFB1). The consumption of AFB1-contaminated food and feed causes aflatoxicosis, which can lead to serious health risks, particularly liver damage, primary liver cancer, and even death. Research has indicated that over 5 billion people worldwide are exposed to aflatoxins (AFs) through their diet, contributing to an annual occurrence of up to 155,000 cases of liver cancers. Therefore, AFs have been classified as group 1 carcinogens by the International Agency for Research on Cancer. Many countries have set limits for the AFB1 occurrence in food and feed, with more than 75 countries imposing a maximum limit of 5 µg/kg in food products. To comply with food safety regulations and official standards, precise and sensitive analytical techniques for detecting AFB1 are required. Aflatoxin biosynthesis pathway consists of 30 genes and includes transcriptional regulators *aflR* and *aflS*. AflR is a master regulator protein and is essential for the AFB1 production, in contrast, the precise role of AflS as aflatoxin regulator remains unclear. Owing to the adverse effects of AFB1 on human and animal health, the prevention of AFB1 accumulation in food and feed is necessary. Plant-derived compounds are promising biocontrol agents to inhibit the production of aflatoxin by *A. flavus* and deoxynivalenol (DON) by *Fusarium graminearum* fungi.

My doctoral research had three primary objectives. First, I aimed to develop methods to identify aflatoxigenic *Aspergillus* strains and detect aflatoxin contamination in food. I applied a polyphasic approach, integrating data from phylogenetic, sequence, and toxin analyses, to the *Aspergillus* isolates. This enabled to identify the key genomic characteristics, genetic diversity and phylogenetic relationships among the *Aspergillus* isolates. We then developed a new and rapid noncompetitive immunoassay for AFB1 detection in food. This assay utilized a monoclonal capture antibody and a unique anti-immunocomplex (anti-IC) antibody fragment (scFv) derived from a synthetic antibody library. The single-step assay is fast, performed in 15 min, and has a detection limit of 70 pg/mL for AFB1. Second, I investigated the molecular mechanisms governing the DNA binding activity of AflR, and examined how AflS modulates it. Biophysical data confirmed that AflR and AflS directly interact forming a protein complex. AflS was found to moderately reduce the binding affinity of AflR to its target DNA site. Kinetic assays additionally

suggested that two AflR monomers sequentially bound to the palindromic target DNA sequence forming the stable AflR-DNA complex. Third, I investigated and found that antioxidant-rich methanolic extract from *Zanthoxylum bungeanum* (*Z. bungeanum*) plant inhibited the growth and toxin production of *Aspergillus* and *Fusarium* fungi. Transcriptomic analysis indicated that the extract indeed repressed the AFB1 biosynthesis pathway in *A. flavus* and additionally generated significant transcriptional changes in several other secondary metabolite pathways. The effects were apparently mediated by the global regulators of secondary metabolism and cell development, including the velvet complex, instead of pathway specific regulators (AflR and AflS). Furthermore, co-inoculating the extract with *F. graminearum* effectively inhibited the fungal growth and DON production in both laboratory and field conditions.

Taken together, these findings highlight the importance of distinguishing aflatoxin and non-aflatoxin producing fungi, as well as understanding the molecular interactions between the master regulators AflR, AflS, and their DNA binding activities. Additionally, the findings suggest that *Z. bungeanum* extracts may facilitate the development of effective strategies to control AFB1 and DON contaminations.

KEYWORDS: *Aflatoxin, AflR, AflS, Aspergillus flavus, deoxynivalenol, Fusarium graminearum, gene regulation, immunoassay, mycotoxin, secondary metabolism, Zanthoxylum bungeanum*

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TIIVISTELMÄ

Aspergillus flavus on merkittävä homesieni, joka tuottaa homemyrkky aflatoksiini B1:tä (AFB1). AFB1 saastuttaa elintarvikkeita ja rehua, mikä voi edelleen aiheuttaa vakavia terveysvaikutuksia, erityisesti maksavaurioita, primaarista maksasyöpää ja jopa kuoleman. Tutkimuksissa on arvioitu, että maailmanlaajuisesti yli 5 miljardia ihmistä altistuu aflatoksiineille ruokavalion kautta, mikä myötävaikuttaa jopa 155 000 maksasyöpätapauksen syntyyn vuosittain. YK:n alainen kansainvälinen syöpätutkimuslaitos on luokitellut aflatoksiinit ryhmän 1 syöpää aiheuttaviksi aineiksi. Lukuisat maat ovat asettaneet raja-arvoja AFB1:n määrälle elintarvikkeissa ja rehuissa, usein käytetty yläraja elintarvikkeissa on 5 µg/kg. Elintarviketurvallisuusmääräysten ja -standardien noudattaminen edellyttää tarkkoja ja herkkiä analyysimenetelmiä AFB1:n määrittämiseen. Koska AFB1:llä on vakavia terveydelle haitallisia vaikutuksia, AFB1:n muodostumista estävien keinojen kehittäminen on tärkeää. Kasveista eristettyjen bioaktiivisten yhdisteiden lisääminen homeiden kasvupaikoille on yksi lupaava tapa estää *A. flavusta* tuottamasta aflatoksiinia ja *Fusarium graminearum* -punahometta tuottamasta deoksiinivalenolia. AFB1:n biosynteesireitti koostuu 30:stä entsyymigeenistä sekä säätelijägeeneistä *aflR* ja *aflS*. AflR-proteiini on aflatoksiinien biosynteesin spesifinen säätelijä ja välttämätön AFB1:n tuotannon aloittamiseen, kun taas AflS:n rooli AFB1:n tuoton säätelijänä on edelleen osittain epäselvä.

Väitöstutkimuksellani oli kolme päätavoitetta. Ensimmäisenä tavoitteena pyrin kehittämään uusia menetelmiä, joilla erottaa aflatoksiinin tuottoon pystyvät ja kykenemättömät *Aspergillus*-kannat toisistaan. Pyrin myös kehittämään menetelmän, jolla voi nopeasti havaita AFB1:n elintarvikkeissa. Kantojen erotteluun sovelsin monivaiheista lähestymistapaa, jossa yhdistyi fylogeneettinen, sekvenssi- ja myrkkyanalyysi. Tämä data mahdollisti tärkeimpien genomiominaisuuksien, geneettisen monimuotoisuuden ja kantojen sukulaisuussuhteiden tunnistamisen *Aspergillus*-näytteistä. AFB1:n havaitsemiseksi elintarvikeissa kehitimme nopean ei-kilpailevan immunomäärityksen. Menetelmä perustuu monoklonaaliseen AFB1:n sieppausvasta-aineeseen ja uuteen, vasta-ainekirjastosta löydettyyn, anti-immunokompleksi-vasta-ainefragmenttiin (scFv). Määrityksen toteuttaminen kestää vain 15 minuuttia ja sen havaitsemisraja on 70 pg/mL AFB1:tä. Toisena tavoitteena selvitin, miten aflatoksiinin valmistamisen aktivoiva AflR sitoutuu spesifiseen kohteeseen DNA:ssa ja miten AflS muuttaa tätä mekanismia. Biofysikaaliset mittaukseni

osoittivat, että AflR ja AflS muodostavat proteiinikompleksin, jossa AflS vähentää AflR:n sitoutumisaffiniteettia DNA-kohteeseen. Kineettisten määritysten mukaan kaksi AflR-monomeeriä sitoutuu peräkkäin palindromiseen kohdesekvenssiin muodostaen stabiilin AflR-DNA-kompleksin. Kolmantena tavoitteena selvitin ja osoitin, että *Zanthoxylum bungeanum* -kasvista peräisin olevan uutteen avulla voi estää *Aspergillus*- ja *Fusarium*-homesienten kasvua ja homemyrkkyjen tuottoa. *A. flavuksen* transkriptomin kartoitus paljasti, että kasviuute repressoi AFB1-biosynteesireitin ja muutti myös useiden muiden sekundaarimetaboliittien biosynteesiin osallistuvien geenien ilmenemistä. Kasviuutteen aiheuttamia geenien ilmenemisen muutoksia välittivät globaalit sekundaarimetabolian ja solujen kehittymisen säätelijät, esimerkiksi velvet-kompleksi. Tutkimustulokseni viittaavat siihen, että *Z. bungeanum* -uutteet voivat olla hyödyllisiä tehokkaiden homemyrkkyjen tuoton estäjien kehitystyössä.

ASIASANAT: *Aflatoksiini, AflR, AflS, Aspergillus flavus, deoksiinivalenoli, Fusarium graminerum, geenien säätely, homemyrkky, immunomääritys, sekundaarimetabolia, Zanthoxylum bungeanum*

Table of Contents

Abbreviations

AF	aflatoxin
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFG1	aflatoxin G1
AFG2	aflatoxin G2
AP	alkaline phosphatase
bp	base pair
CCR	carbon catabolite repression
ChIP-Seq	chromatin immunoprecipitation sequencing
CYE	czapek's yeast extract
DEGs	differentially expressed genes
DNA	deoxyribonucleic acid
DON	deoxynivalenol
EA	ethylacetate
EFI	europium fluorescence intensifier
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ESI-MS	electrospray ionization mass spectrometry
FAM	fluorescein amidite
FDA	food and drug administration
FHB	fusarium head blight
FLD	fluorescence detection
FPKM	fragments per kilobase of transcript sequence per millions
GC	gas chromatography
GRAS	generally recognized as safe
GSH	glutathione
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin layer chromatography
HR-MS	high resolution mass spectrometry
HSCAS	hydrated sodium calcium aluminosilicates
IC	immunocomplex

IC50	half maximal inhibitory concentration
IgG	immunoglobulin g
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl β-d-1-thiogalactopyranoside
IR	infrared
ISSR	inter simple sequence repeats
ITS	internal transcribed spacer
LB	luria-bertani
LC–MS	liquid chromatography-mass spectrometry
M	methanol
MBP	maltose binding protein
MDA	malondialdehyde
MI	marker index
MP	malt peptone
MST	microscale thermophoresis
ntDNA	non-template DNA
PCR	polymerase chain reaction
PDA	potato dextrose agar
pg	picogram
PIC	polymorphism information content
PPb	parts per billion
PPB	percentage of polymorphic bands
qPCR	quantitative PCR
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
RNA seq	next-generation RNA sequencing
ROS	reactive oxygen species
rRNA	ribosomal RNA
RSD	relative standard deviations
Rti-PCR	real-time PCR
Rt-qPCR	reverse transcription-quantitative PCR
scFv	single chain variable fragment
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SM	secondary metabolite
SMURF	secondary metabolite unique regions finder
SOD	superoxide dismutase
T/C	treated/control fpkm ratio
TAMRA	tetramethylrhodamine
TFC	total flavonoid content

TLC	thin layer chromatography
TPC	total phenolic content
TSS	transcription start site
UV	ultraviolet
v/v	volume / volume
VeA	velvet a protein
Vis	visible
YES	yeast extract sucrose

Abbreviations of aflatoxin pathway

AVF	averufin
AVN	averantin
DH	dehydratase
DHDMST	dihydrodemethylsterigmatocystin
DHOMST	dihydro-o-methylsterigmatocystin
DHST	dihydrosterigmatocystin
DMST	demethylsterigmatocystin
ER	enoyl reductase
FAS	fatty acid synthase
HAVN	5′-hydroxyaverantin
HVN	hydroxyversicolorone
KR	ketoreductase
KS	β-ketoacyl synthase
Mal-CoA	malonyl-CoA
NAA	norsolorinic acid anthrone
NOR	norsolorinic acid
OAVN	oxoaverantin
OMST	o-methylsterigmatocystin
PKS	polyketide synthase
PT	product template protein
SAT	starter unit-acp transacylase
ST	sterigmatocystin
TE	thioesterase
VAL	versiconal
VERA	versicolorin a
VERB	versicolorin b
VHA	versiconal hemiacetal acetate

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Abbas, A.**, Hussien, T., Yli-Mattila, T. A Polyphasic Approach to Compare the Genomic Profiles of Aflatoxigenic and Non-Aflatoxigenic Isolates of *Aspergillus* Section *Flavi*. *Toxins*, **2020**, 12, 56.

II **Abbas, A.**, Prajapati R.K., Aalto-Setälä, E., Baykov, A.A., Malinen A.M. Aflatoxin biosynthesis regulators AflR and AflS: DNA binding affinity, stoichiometry, and kinetics. *Biochem J*, **2024**, 12, 805-821.

III **Abbas, A.**, Wright, C.W., El-Sawi, N. Yli-Mattila, T., Malinen A.M. A methanolic extract of *Zanthoxylum bungeanum* modulates secondary metabolism regulator genes in *Aspergillus flavus* and shuts down aflatoxin production. *Sci Rep,* **2022**, 12, 5995.

IV **Abbas, A.**, Yli-Mattila, T. Biocontrol of *Fusarium graminearum*, a Causal Agent of Fusarium Head Blight of Wheat, and Deoxynivalenol Accumulation: From *In Vitro* to *In Planta*. *Toxins*, **2022**, 14, 299.

V Peltomaa, R., **Abbas, A.**, Yli-Mattila, T., Lamminmäki, U. Single-step noncompetitive immunocomplex immunoassay for rapid aflatoxin detection. *Food Chem*, **2022**, 392, 133287.

List of Contemporaneous Publications

I Yli-Mattila, T., **Abbas, A**., Gavrilova, O., Gagkaeva, T. Molecular Variation and Phylogeny within *Fusarium avenaceum* and Related Species. *Diversity,* **2022**, 14, 574.

II Yli-Mattila, T., Hussien, T., **Abbas, A**. Comparison of biomass and deoxynivalenol production of northern European and southern European *Fusarium graminearum* isolates in the infection of wheat and oat grains. *J Plant Pathol,* **2022**, 104, 1465–1474.

III Yli-Mattila, T., Yörük, E., **Abbas, A.**, Teker, T. Overview on Major Mycotoxins Accumulated on Food and Feed. In Fungal Biotechnology: Prospects and Avenues; Deshmukh, S.K., Sridhareds, K.R., Eds.; Springer: Berlin/Heidelberg, Germany; CRC Press: Boca Raton, FL, USA, **2022**; in press; pp. 310–343.

1 Introduction

1.1 *Aspergillus* and Aspergillosis

Aspergillus is a diverse fungus that has a significant economic and social influence and is the most common category of pathogenic molds isolated from the environment. The agriculture sector is the most harmfully affected by *Aspergillus* infection, as many crops can be infected in the field, harvest, storage, processing, or transport stages, making contamination control challenging (Mendu et al., 2022). *Aspergillus* species have been detected worldwide and are reported as human and animal pathogens (Samson et al., 2014). They can infect humans through direct or indirect contamination. Inhalation of *Aspergillus* spores leads to a respiratory disease called Aspergillosis. Generally, Aspergillosis affects immunocompromised and immunosuppressed humans and other mammals. Aspergillosis epidemics are commonly linked with environmental changes that promote the development of *Aspergillus* and expose individuals to high spore concentrations (Winter et al., 2022). Aspergillosis infections are categorized into three classes; allergic, chronic and invasive. Aspergillosis has been detected worldwide and the number of infected cases increases every year. Allergic aspergillosis affected about 1-4 million people worldwide. Chronic pulmonary aspergillosis affected about 3 million people worldwide. Invasive aspergillosis is uncommon, as it affects specific patient populations. This statistical report was published by National Organization for Rare Disorders (NORD) (https://rarediseases.org/rare-diseases/aspergillosis/).

Several factors influence the pathogenicity of *Aspergillus* species, including their ability to produce heterocyclic toxins (mycotoxins). Another aspect that enhances *Aspergillus* pathogenicity is its ability to produce numerous proteolytic enzymes, such as proteases, which aid fungal colonization in infected host tissues (Pasqualotto, 2009). Unfortunately, *Aspergillus* infections have a high death rate. In untreated Aspergillosis cases, the death rate can reach 100%, and even with treatment, it only drops to 60%. *Aspergillus* species have around 250 different kinds. Pathogenicity has been found in many *Aspergillus* species, including *A. fumigatus*, *A. flavus*, *A. ochraceus*, *A. niger*, *A. versicolor*, *A. parasiticus*, *A. nidulans*, *A. ustus*, *A. glaucus*, *A. clavatus*, *A. sydowii*, and *A. terreus* (Gniadek, 2012). *A. flavus* is the second most

prevalent pathogen responsible for both invasive and non-invasive Aspergillosis, following *A. fumigatus* (Amaike & Keller, 2011).

Aspergillus section *Flavi* contains six species that are morphologically and phylogenetically similar. They are divided into two groups based on whether they produce aflatoxins or not. The first category consists of *A. flavus*, *A. parasiticus*, and *A. nomius*, all of which can produce aflatoxins and cause significant damage to stored food products. The second group consists of non-aflatoxin-producing species: *A. tamarii*, *A. oryzae*, and *A. sojae*. The last two species have lost their potential to produce aflatoxins and are commonly employed as koji mold for the manufacture of fermented foods (Godet & Munaut, 2010). *A. flavus* **(Figure 1A)** is the most common, saprophytic, and opportunistic pathogen in agriculture and medicine. It is a widespread soil inhabitant and a mild plant pathogen that infects a wide variety of essential agricultural crops. In humans, *A. flavus* can induce both direct infection and systemic illness.

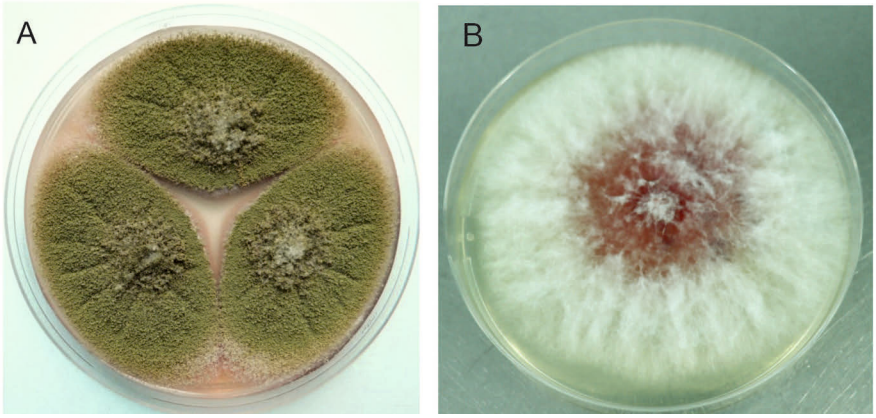

Figure 1. ***Aspergillus* and *Fusarium* species**. (A) *Aspergillus flavus* strain grew on Czapek yeast extract agar at 25℃ for 7 days. adapted from (Pickova et al., 2021). (B) *Fusarium graminearum* grew on potato dextrose agar (PDA) at 25℃ for 5 days.

Aspergillus produces several secondary metabolites, such as mycotoxins. They can change the defense system of the host and work as virulence factors by displaying immunosuppressive properties that facilitate the fungal invasion of host tissues. For instance, *A. flavus* produces aflatoxin, which hampers macrophage function, *A. ochraceus* generates ochratoxin, known for its cytotoxic effects on lymphocytes, and *A. fumigatus* produces various immunosuppressive mycotoxins, including gliotoxin (Kamei & Watanabe, 2005). When fungal receptors detect changes in moisture, temperature, water activity, or the amount of nitrogen, they initiate a signal transduction cascade that regulates the expression of effector genes,

resulting in toxin production. Molds can produce mycotoxins as part of their metabolic process. When fungi grow on organic substrates, they produce natural compounds through a secondary metabolic process that can be harmful to humans and animals. *A. flavus* is particularly known to be a prolific secondary metabolite (SM) producer and it has at least 56 secondary metabolic gene clusters, including those responsible for the synthesis of aflatoxins, kojic acid, cyclopiazonic acid, aspergillic acid, 3-nitropropionic acid, violaxanthin, aspertoxin, and other unknown SMs predicted by a SMURF software (secondary metabolite unique regions finder) (Cleveland et al., 2009; Gniadek, 2012).

1.2 *Fusarium graminearum* and Fusarium head blight

Fusarium species are filamentous fungi that are abundant in the environment and can cause fatal systemic illnesses. One of the most prevalent and aggressive *Fusarium* species is *Fusarium graminearum* sensu stricto **(Figure 1B)**, which is the main species of *F. graminearum* species complex (Hafez et al., 2020). It is a homothallic phytopathogenic fungus, which mainly causes Fusarium head blight (FHB) in several cereals, such as wheat, barley, triticale, and oats. FHB in wheat causes yield and quality losses and overall reduces its economic value. The presence of fungal inoculum from previous crop residues is the most critical driver of the FHB in the field (Malbrán et al., 2020). In maize, *F. graminerarum* causes ear and stalk rot together with other *Fusarium* and *Aspergillus* species including *A. flavus* (Lanubile et al., 2017). *F. graminearum* exists saprotrophically in the soil and on the surface as mycelium, sporodochia, or chlamydospores. It also forms perithecia, that produce wind-dispersed ascospores, infecting the host crop together with rain-spread conidia. FHB incidence is increasing due to global warming and adaptation of agricultural practices aiming to tillage reduction, crop rotation, and the recycling of straw as field fertilizer. *Fusarium* species can also contaminate wheat, barley, oats and maize grains with various mycotoxins, which are harmful to both human and animal health (Y. Chen et al., 2019; Gimeno et al., 2019).

1.2.1 Natural control of deoxynivalenol

Based on strain-specific chemotype, *F. graminearum* infections can contaminate cereal grains under field or storage conditions with different trichothecenes; including deoxynivalenol (DON), nivalenol, and zearalenone (Llorens et al., 2006). Dietary intake is considered the most common way of exposure to DON, resulting in acute symptoms including nausea, vomiting, gastroenteritis diarrhea, immune suppression, and reduced daily food consumption, leading to chronic consequences,

such as weight loss. DON binds to the 60S ribosomal subunit, inhibiting translation and causing ribotoxic stress; hence, earning the name "ribotoxin". DON exhibits exceptional thermal stability, allowing it to withstand cooking temperatures. It is also stable in an aqueous medium at low pH and high temperatures. The European Commission has established maximum limits to control the use of DON mycotoxin-contaminated food and feed. According to the European Commission Regulation (EU), the maximum limit for DON in wheat grains is 1 mg/kg (European Commission, 2024).

Bio-pesticides or -fungicides contain plant-derived active compounds, which decompose rapidly and thus have less dentrimental effect on ecological equilibrium. As a result, bio-pesticides and fungicides are recognized as environmentally friendly pesticides. For example, strobilurins are used in the management of FHB by disrupting electron transport in the mitochondrial respiratory chain. This leads to a reduction in aerobic energy production and inhibits fungal growth (Cendoya et al., 2021). Nevertheless, the widespread use of synthetic fungicides such as tebuconazole, metconazole, prothioconazole, and prochloraz is exerting selective pressure on *Fusarium* species and influencing population dynamics. Furthermore, using low concentrations of the aforementioned fungicides may result in an incomplete reduction of fungal development. As a result, alternative management strategies, such as biocontrol agents, the development of resistant cultivars, agronomic practices, and natural plant extracts, have become significant areas of research. In recent years, researchers have focused their efforts on identifying antifungal components produced by plants and using them to mitigate fungal growth and suppress mycotoxins (Buzón-Durán et al., 2020; C. Chen et al., 2018).

Using plant extracts is an efficient method for reducing crop illnesses during post-harvest storage. This is due to the presence of beneficial elements such as flavonoids, phenols, polyphenols, tannins, and alkaloids. Additionally, plant extracts contain antimicrobial compounds and exhibit synergistic effects, which help to inhibit the resistance development among pathogens (Fandohan et al., 2004; Sultana et al., 2013). For example, *Curcuma longa* extract was tested for antifungal activity against *F. graminearum* and found to have strong antifungal activity with an IC50 value of 0.11 mg/mL (C. Chen et al., 2018). Additionally, phenolic compounds extracted from mustard were effective in controlling *F. graminearum* (Drakopoulos et al., 2020). A methanolic extract of three medicinal plants, namely *Leptadenia hastata*, *Barringtonia racemose*, and *Barringtonia asiatica*, was found to exhibit significant inhibition of *Fusarium oxysporum* in a dose-dependent manner (Umaru et al., 2018). Essential oils extracted from medicinal plants have also demonstrated antifungal, antibacterial, and antiviral activities, making them valuable natural fungicides. Due to their potential benefits, they have been used in healthcare and agriculture sectors, and as an alternative treatment for many infectious diseases. For

instance, *Zanthoxylum bungeanum* essential oil significantly reduced cell membrane integrity and inhibited spore germination in *Fusarium sulphureum* (Xing-dong & Hua-li, 2014). Therefore, phenolic and antioxidant compounds extracted from medicinal plant essential oil are promising alternatives to synthetic fungicides.

1.3 Aflatoxins and their toxicity

Mycotoxins are a class of low-molecular weight chemical compounds produced by filamentous fungi as secondary metabolites that have harmful effects on humans and animals. Mycotoxins commonly accumulate in hot and humid climate regions. Exposure to high levels of mycotoxins through diet, respiration, or dermal contact is correlated with mycotoxicosis disease. Mainly, mycotoxins are produced by the genera *Aspergillus*, *Fusarium*, *Alternaria*, *Claviceps*, *Penicillium*, and *Stachybotrys*. There are approximately 400 known mycotoxins (Qu et al., 2024), with aflatoxin being one of the most extensively studied because of its biological importance and severe negative effects (Mendu et al., 2022). Aflatoxins (AFs) are carcinogenic compounds that were first discovered in the spread of Tukey X disease. In the early 1960s, a poultry farm in the UK experienced the death of 100,000 turkeys because of the so-called turkey X disease after being fed contaminated groundnut meal. Extensive field and laboratory studies were conducted, and postmortem examinations revealed that liver lesions were the most common and serious damage observed in all animals and birds (Van Der Zijden et al., 1962). It was demonstrated that toxic metabolites synthesized by *A. flavus* were responsible for the bird deaths (Nesbitt et al., 1962). The toxin was separated and quantified by thin layer chromatography (TLC), and two distinct fluorescent spots were noticed under ultraviolet light (UV). One spot emitted blue fluorescence at 425 nm (referred to B-toxins) and the other emitted green fluorescence at 450 nm (referred to G-toxins). *Aspergillus* species produce four main aflatoxin-related compounds: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). The subscript numbers indicate their relative migration distance on a TLC plate (Norlia, et al., 2019a). The chemical structures of these compounds are shown in **Figure 2**. The most toxic type is AFB1, with a toxicity 68 times that of arsenic and 10 times that of potassium cyanide (P. Li et al., 2009; Mupunga et al., 2017). Chemically, aflatoxins are a polyketide group derived from difuranocoumarin with a bifuran group attached to the coumarin nucleus, and they possess a pentanone ring in AFBs or a lactone ring in AFGs.

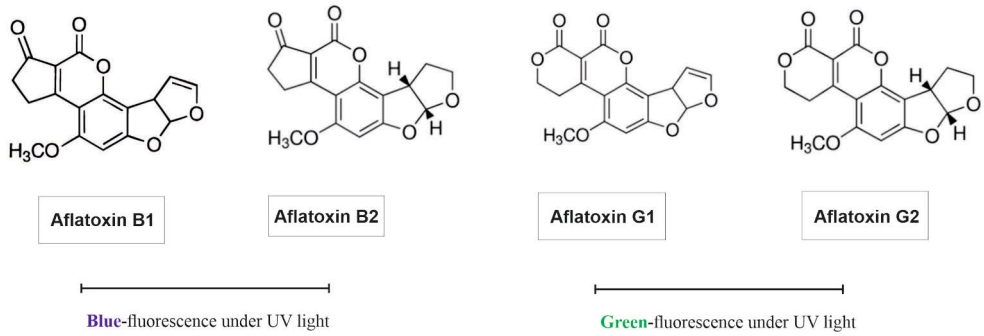

Figure 2. Chemical structures of aflatoxin types; B1, B2, G1, and G2. Aflatoxin B1 is the most toxic type. Aflatoxin B1 and B2 exhibit blue fluorescence under UV light. Aflatoxin G1 and G2 exhibit green fluorescence under UV light.

Because of their remarkable stability under various food processing conditions, eliminating AFs from human diets and animal feeds is exceedingly challenging. Therefore, AFs pose significant health risks and cause substantial financial loss. These toxins can induce carcinogenic, teratogenic, mutagenic, hepatotoxic, or nephrotoxic effects in humans and other animals. AFs are commonly found in a wide range of food and feed stuff, including peanuts, nuts, figs, wheat, maize, corn, rice, spices, milk, meat products, and dried fruits. Researchers assessed the global presence of aflatoxins in raw maize, rice, sorghum, and wheat samples collected since year 2000 by analyzing the published data. Because 38% of tested samples were contaminated with at least one type of AF, it is clear that AFs are a common health risk by consumption of contaminated food and feed (Mahato et al., 2019). Although aflatoxins are typically produced by fungal colonization in preharvest maize, cereals, and nuts, they can also be transported to surface waters with rainfall. Consequently, AFs have been detected in various water sources, including surface water, wastewater, tap water, and bottled water. The precise causes of water pollution in this context remain unknown highlighting a critical research gap that needs to be addressed in future studies (S. Y. Wang et al., 2023).

For human consumption, the mean lower bound (LB) exposure to AFB1 ranged from 0.22 to 0.49 ng/kg bw per day and the mean UB exposure from 1.35 to 3.25 ng/kg bw per day (Schrenk et al., 2020). The European Union has implemented that AFB1 and total AFs not exceeding 2 µg/kg and 4 µg/kg, respectively, in any directly consumed product (European Commission, 2023). Similarly, in the United States, the maximum allowable level for AFs is set at 20 µg/kg (F. Wu, 2006). Aflatoxin concentrations can reach up to 300 µg/kg in cattle feed, as stated by the U.S. Food and Drug Administration (FDA) (Jiang et al., 2021). When food fails to meet regulatory AFs limits for human consumption, it is completely discarded. Consequently, the agriculture industry experiences significant annual losses. Many

researchers have assessed the annual losses attributed to aflatoxin exposure in various regions. For example, estimated annual losses due to aflatoxin contamination is between US$1.7 and US$52 billion in the United States (Mitchell et al., 2016).

Regular ingestion of high levels of AFB1 can cause cancer by interfering with the basic metabolic pathways of the cells and altering important enzymatic processes such as carbohydrate and lipid metabolism, as well as protein synthesis (Quist et al., 2000). Foodborne AFB1 is absorbed in the duodenum and enters the liver, where it is bioactivated by cytochrome enzymes (CYP450). These monooxygenases catalyze the oxidation of the C8 = C9 double bond in the furan ring, resulting in the production of AFB1-exo and -endo 8,9 epoxide stereoisomers. The AFB1-exo isomer exhibits >1000-fold higher toxicity than the AFB1-endo isomer. The specific CYP450 isozymes responsible for the bioactivation of AFB1 vary depending on the host, organ, and sub-cellular component involved. In the human liver, CYP1A2 or 3A4 are the key isoenzymes catalyzing the bioactivation. When aflatoxin B1 epoxide (AFBO) is released, it integrates into the DNA and forms a covalent bond through an alkylation reaction with the N7 atom of the guanine residue creating a stereospecific aflatoxin-DNA adduct called trans-8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 (AFB1-N7-gua). In most cases (60-80%), this adduct is found at the third guanine residue of the codon 249 (5'-AG*G-3') on the p53/PT53 tumor suppressor gene. Because of its positive charge, the AFB1-N7-gua adduct is highly unstable and tends to dissociate, leaving behind an apurinic DNA molecule. However, the imidazole ring can be opened to generate two stable isomers; cis- and trans-AFB1-formamidopyrimidine adducts, often referred to as minor and major AFB1-FAPy adducts, respectively **(Figure 3)**. These three AFBO-induced DNA lesions (apurinic DNA, AFB1-N7-gua, and AFB1-FAPy) are considered the primary precursors of AFB1-dependent genotoxic and carcinogenic effects. Among them, AFB1-FAPy has been identified as the most mutagenic due to its ability to cause persistent DNA damage (Benkerroum, 2020). In addition to its genotoxic and carcinogenic properties, AFB1 exhibits other toxic effects, including malnutrition, growth impairment, and immunomodulation in both humans and animal models (Eaton & Gallagher, 1994; Rushing & Selim, 2019).

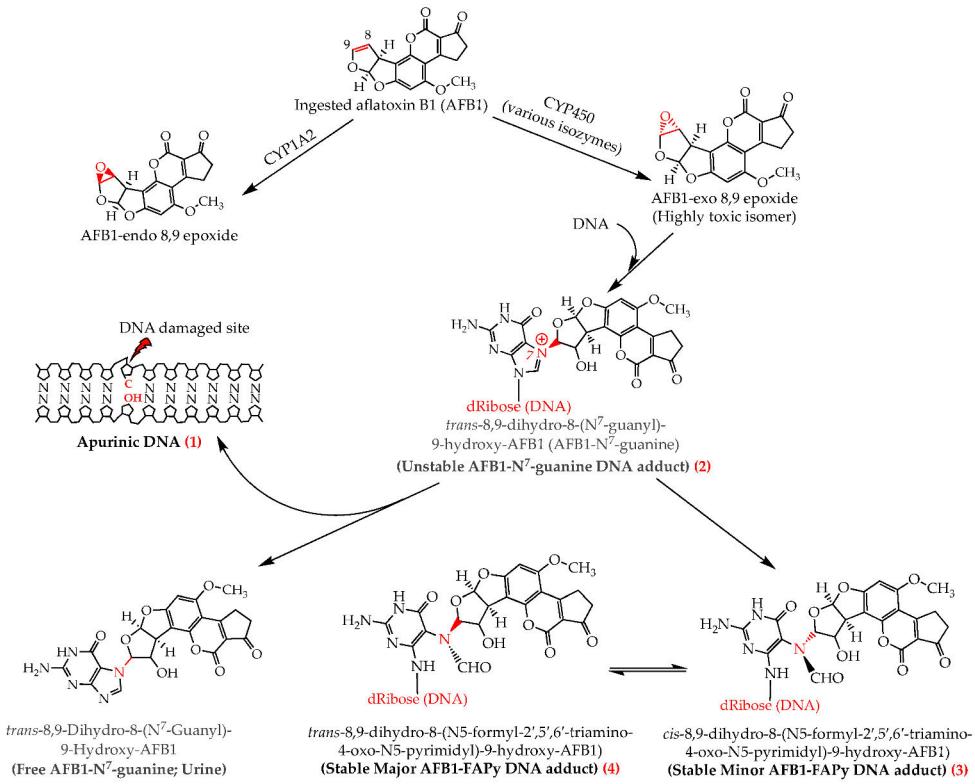

Figure 3. The process of AFB1 activation and DNA damage formation. The primary DNA lesions are (1) apurinic DNA, (2) AFB1-N7-guanine, and (3,4) AFB1-FAPy. The instability of the AFB1-N7-guanine DNA adduct prompts the furan ring to open, leading to the formation of a "cis" (minor) rotamer (3) of the AFB1-FAPy adduct, which subsequently forms equilibrium with "trans" (major) rotamer (4); the major rotamer predominates at a ratio of 2:1 in equilibrium (Benkerroum, 2020).

1.4 Detection methods for aflatoxins and aflatoxin-producing fungi

Numerous approaches have been developed to identify and differentiate aflatoxigenic and atoxigenic *Aspergillus* species. Extensive research has focused on the detection and quantification of aflatoxins in *Aspergillus* species, aiming to establish highly specific, useful, and practical methods. Because of its prevalence and toxicity, several analytical, molecular, and immunological methods have been developed.

1.4.1 Chromatographic methods

All analytical procedures for aflatoxin analysis involve a set of fundamental steps: sample extraction, clean-up, detection, confirmation, and toxin quantification.

Chromatographic methods such as TLC, High-performance Thin Layer Chromatography (HPTLC), High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and Liquid Chromatography-Mass Spectrometry (LC–MS), are based on the physical interaction between a mobile phase and a stationary phase. These chromatographic methods have demonstrated rapidity, sensitivity, and reproducibility (M. Abbas, 2021; Alameri et al., 2023). Among them, reversed phase HPLC is the most extensively employed technique for aflatoxin separation and quantification. The HPLC process involves several key steps, starting with sample preparation. To ensure reliable findings, the aflatoxins must be extracted from the food or feed matrix using an appropriate solvent such as methanol, acetone, chloroform, or acetonitrile. After sample extraction, the material is subjected to a cleaning step to eliminate any interfering chemicals that may affect the analysis. Solid-phase extraction or immunoaffinity columns are frequently used for this purpose. The cleaned and extracted material is then introduced into the HPLC apparatus. For separation, a reverse-phase HPLC column with a mobile phase consisting of methanol, acetonitrile, and water is frequently utilized. Chemical derivatization of aflatoxins B1 is necessary to improve the sensitivity of HPLC analysis, as the natural fluorescence of aflatoxins B1 may not be strong enough to meet the required detection limit. HPLC with fluorescence detection (FLD) can achieve a sensitivity as low as 0.1 ng/kg (Ghali et al., 2009; Wacoo et al., 2014). After liquid chromatographic separation, Papadopoulou-Bouraoui et al. (2002) investigated two post-column derivatization procedures for the measurement of aflatoxin mixtures using fluorescence detection. The results demonstrated that both bromination and UV light irradiation were suitable for determining aflatoxins, yielding comparable results in terms of fluorescence amplification and repeatability. HPLC analysis offers a highly sensitive and accurate method for differentiating between aflatoxin-producing and non-producing fungi.

1.4.2 Molecular-based methods

Both morphological and molecular identification methods are used to discriminate *Aspergillus* species. However, morphological characters might not be enough since the microscopic and macroscopic properties of certain species are comparable. For molecular identification, amplification of the internal transcribed spacer (ITS) within ribosomal DNA is commonly employed. The ITS region, which includes intergenic spacer regions 1 and 2 (ITS1 and ITS2) as well as variable regions in the 5′-end of the 28S rRNA gene, serves as the universal barcode for fungi and is used to identify *Aspergillus* species. Therefore, for accurate discrimination between *Aspergillus* isolates at the species level, a combination of phenotypic (macro- and microscopic characters) and molecular methods is employed (Zulkifli

& Zakaria, 2017). Generally, the conventional PCR technique is employed for DNA amplification. In addition, DNA can be amplified and quantified using monochrome and multiplex real-time PCR (Rti-PCR) assay. Bu et al. (2005) utilized two Rti-PCR duplex tests and one monochrome PCR with SYBR green as the reporter molecule to detect the ITS1 region and the 5.8S rRNA. In the multiduplex Rti-PCR tests, species differentiation was achieved through amplicon melting analysis. The assay exhibited a sensitivity of 0.1 pg of fungal genomic DNA for *A. flavus*, equivalent to three cells. Furthermore, quantitative PCR (qPCR) has been developed for detecting and quantifying *A. flavus* species. The real-time quantitative PCR (Rti-qPCR) assay utilized forward and reverse primers derived from the 5.8S rRNA and 28S rRNA sequences, with a dual-labeled probe with 6-FAM at the 5′-end and TAMRA at the 3′-end in the reporter molecule (Cruz & Buttner, 2017). Sardiñas et al. (2011) employed individual Rti-qPCR assays using SYBR green as the reporter molecule and the primers were designed based on multicopy ITS2 rDNA target sequences.

So far, three distinct molecular methods have been employed to identify fungi capable of producing aflatoxin by focusing on specific genes involved in aflatoxin biosynthesis. These methods include: (1) a multiplex PCR targeting the *nor-1*, *apa-2*, and *omt-1* genes, (2) individual PCR tests targeting the *omt-1*, *nor-1*, and *ver-1* genes, and (3) PCR tests that amplify individual sequences of the *aflR*, *aflJ*, and *omtB* genes. Both traditional PCR and Rti-PCR have utilized these systems (Levin, 2012). These methods provide an alternative approach for distinguishing between aflatoxin producing and non-producing fungi complementing chromatographic techniques. In aflatoxigenic fungi, most of the AF pathway genes are expected to be amplified, whereas representative atoxigenic strains are unable to amplify the majority of genes required for aflatoxin production. The heterogeneity observed in the aflatoxin gene cluster within the *A. flavus* population is valuable for understanding the danger of aflatoxin contamination and selecting biocontrol agents (Norlia et al., 2019b).

Molecular markers play a crucial role in genetics-based research disciplines because of their capacity to discriminate between genotypes. Random Amplified Polymorphic DNA (RAPD) is a prevalent marker that utilizes PCR technology to identify random amplified polymorphisms in DNA. RAPD employs a single primer with a random nucleotide sequence to identify polymorphisms in specific nucleotide sequences, resulting in consistent outcomes. RAPD markers have been extensively employed in various areas such as exploring genetic diversity, characterizing populations, understanding genetic structures, geographic relationships, and mapping genomes. These markers are widely utilized to simultaneous screening of polymorphisms at multiple loci. This technique is rapid, affordable, and effective in generating a substantial number of markers within a

short timeframe, compared to alternative methodologies. Numerous studies have demonstrated the efficacy of RAPD markers in detecting polymorphisms across various populations. They have been extensively used to investigate genetic diversity within economically significant food crops. In addition, they have identified genetic variations among different isolates of *A. flavus* and closely related species. These markers have been employed to distinguish between isolates of *A. flavus* that possess the ability to produce aflatoxins (aflatoxigenic) and those that do not (non-aflatoxigenic) (Al-Wadai et al., 2013; Grover & Sharma, 2016; Mohammed & Mohamed, 2019).

ISSR, which stands for Inter simple sequence repeats, is a marker that relies on the PCR technique using a single primer that complements a specific microsatellite sequence. The ISSR marker operates by targeting two identical regions of microsatellite repeats, typically 16 to 25 base pairs in length, positioned in opposite directions. The ISSR is considered an excellent molecular marker because it combines the desirable characteristics of amplified fragment length polymorphism and simple sequence repeats. The ISSR has gained significant popularity in the field of plant genetics for evaluating genetic diversity and conducting genome analysis due to its simplicity, ease of use, and rapidity. Isolates can be differentiated from one another by observing variations in the number of repeats. PCR amplification of microsatellite markers is straightforward and achieved by utilizing primers designed based on the flanking sequences of the markers. The fragment sizes obtained from PCR amplification provide valuable information regarding the number of repeats present in each marker. By analyzing the repeat numbers of multiple markers, a unique genotype can be established for each individual isolate, facilitating easy comparison between genotypes. The ISSR markers have proven to be highly valuable in investigating the diversity and population structure of *A. flavus* and closely related species. Furthermore, the ISSR markers have been employed to assess the similarity and dissimilarity between isolates of *A. flavus*, distinguishing between those capable of producing aflatoxins and those that do not possess this ability (Al-Wadai et al., 2013; Mohammed & Mohamed, 2019).

1.4.3 Immunochemical methods

To address issues related to food safety and comply with official regulations, there is a need for precise and reliable analytical techniques to identify and measure aflatoxins in food and feed. Many countries have established measures to screen and regulate the levels of mycotoxin, particularly for agricultural imports from developing countries. Alternative methods such as immunoassays, dipsticks, biosensors, and nondestructive techniques utilizing infrared spectroscopy have

demonstrated significant promise for aflatoxin analysis (Xie et al., 2016). Immunochemical methods offer another approach to detect aflatoxin levels in food and feed samples. These methods rely on the binding specificity between antibodies and antigens. They eliminate the need for highly skilled and extensively trained personnel to address any issues that may arise during the separation process. These methods are less labor-intensive and time-consuming, making them more favorable compared to chromatographic and spectrophotometric techniques (Wacoo et al., 2014). Immunoassays are commonly classified into two functional formats: noncompetitive sandwich type and competitive. In a noncompetitive immunoassay, a target molecule is captured by primary antibody immobilized on a solid support, while a secondary antibody conjugated with signal-producing molecules is used to detect the captured target molecules. The secondary antibody specifically recognizes the complex of the primary antibody and the target molecule enhancing the sensitivity and accuracy of the assay (Kim et al., 2010). Numerous researchers have contributed to improve food safety and quality control by developing immunological methods to specifically recognize and quantify the presence of AFB1. Azri et al. (2018) developed an ultrasensitive electrochemical immunosensor employing an indirect competitive ELISA technique to detect AFB1. The immunosensor exhibited exceptional sensitivity, with a detection limit of 0.3 pg/mL, along with excellent reproducibility and repeatability. Similarly, Pietschmann et al. (2020) used a competitive magnetic immunodetection assay to detect and quantify AFB1 with a detection limit of 1 ng/mL. Therefore, immunoassays and other immunological techniques are exceptionally well-suited for rapid and highly sensitive analysis of mycotoxins and other small molecule contaminants.

1.5 Genetics of the aflatoxin biosynthesis pathway

1.5.1 Aflatoxin biosynthesis cluster

Aflatoxin biosynthesis genes are arranged in a biosynthetic gene cluster that comprises synthases and/or synthetases genes, as well as genes encoding tailoring enzymes (Keller, 2018). The aflatoxin pathway also includes pathway-specific regulators, which function as positive regulators and stimulate the expression of the remaining biosynthesis genes. The aflatoxin biosynthesis cluster (ABC) includes ~30 genes **(Figure 4)**, which are clustered together within a 70-kb region of the *A. flavus* genome on chromosome III, positioned roughly 80 kb away from the telomere. To date, the ABC has been found to involve a minimum of 27 enzymatic reactions, which are detailed in **Table 1**. Norsolorinic Acid (NOR) serves as the initial stable aflatoxin precursor and its synthesis requires four enzymatic reactions

catalyzed by *aflA*, *aflB*, *aflC*, and *hypC*. *aflA*, *aflB*, and *aflC* synthesize the hexanoyl primer by integrating with malonyl-CoA molecules. Subsequently, the hexanoyl primer moves to the β-ketoacyl synthase region and united with malonyl-CoA to form norsolorinic acid anthrone (NAA). Due to its high reactivity, NAA is converted to NOR by oxidase. AflD is a ketoreductase which reduces the 1'-keto group in NOR to the AVN 1′-hydroxyl group. *aflE* and *aflF* are genes homologous to *aflD* and are predicted to encode short-chain aryl alcohol dehydrogenases. AflG, a cytochrome P450 monooxygenase, catalyzes the conversion of the AVN 5′-keto group to the HAVN 5′-hydroxyl group. The conversion of HAVN to Averufin (AVF) occurs through the dehydrogenation of the HAVN 5′-hydroxyl group to the 5′-oxide group of oxoaverantin (OAVN) by *aflH*, followed by the dehydration of the 5′-oxide of OAVN to form the AVF, which is catalyzed by OAVN cyclase (*aflK*). Indeed, *aflK* was initially related with the conversion of versiconal into versicolorin B (VERB) but was also found to transform OAVN into AVF. Sakuno et al. (2005) was the first to reveal that the same enzyme can catalyze two different reactions in the AFB1 pathway. The researchers hypothesized that this phenomenon can be attributed to the evolution of the AFB1 gene cluster, which may previously have consisted of two copies of the *aflK* gene.

The transition from AVF to HVN is catalyzed by the *aflV*, and the conversion of HVN to VHA is aided by *aflW* through a Baeyer-Villiger reaction. *aflI* plays a role in converting AVF to versiconal hemiacetal acetate (VHA) by catalyzing the ring-closure step in the creation of hydroxyversicolorone (HVN). The esterase enzyme *aflJ* promotes the transformation of VHA into versiconal (VAL). Subsequently, *aflK* catalyzes the conversion of VAL into versicolorin B, a critical step in the biosynthetic pathway, as this enzyme closes the bisfuran ring, which is crucial for DNA binding and contributes to the mutagenic effects of aflatoxins. The product of the *aflL* gene modifies the tetrahydrofuran ring of VERB to a dihydrobisfuran ring, resulting in the formation of Versicolorin A (VERA). This step marks the final metabolic change before the major branch leading to the production of B or G-type aflatoxins. *aflM*, *aflY*, *aflN*, and *aflX* are four genes involved in the conversion of VERA/VERB into DMST/DHDMST. In *A. flavus*, *aflM* and *aflY* convert VERA into demethylsterigmatocystin (DMST), whereas in *A. parasiticus*, *aflY* is responsible for converting VERB into DihydroDemethylsterigmatocystin (DHDMST).

aflN codes for a cytochrome P450-type monooxygenase, playing a crucial role in the conversion of VERA to an unidentified intermediate. Additionally, the oxidoreductase *aflX* facilitates the oxidative decarboxylation and ring-closure of the Baeyer–Villiger intermediate. The O-methyltransferase *aflO* transfers the methyl group from S-adenosylmethionine, leading to the production of Sterigmatocystin (ST) from DMST and Dihydrosterigmatocystin (DHST) from

DHDMST in the biosynthetic pathways. Similarly, another O-methyltransferase, *aflP*, converts ST into O-methylsterigmatocystin (OMST) and DHST into Dihydro-O-methylsterigmatocystin (DHOMST). Finally, *aflQ* transforms OMST to AFB1 and DHOMST into AFB2. *hypB* (*hypB2*) is involved in the second phase of HOMST transformation, resulting in a 370 Da 7-ring lactone, and its expression is observed in aflatoxin-tolerant environments. HypD (AflNa) codes for a 129 Da integral membrane-binding protein, although its function remains unknown. *hypE* (*aflLa*) is responsible for the final steps of AFB1 biosynthesis, and its deletion leads to an intermediate preceding deoxyAFB1 synthesis. As illustrated, the synthesis of AFB1 is a complex process involving at least 27 enzymes encoded by genes in the AFB1 cluster (Caceres et al., 2020; Khan et al., 2021; Yu, 2012).

The primary aflatoxin-producing fungi are found in *Aspergillus* section *Flavi*, particularly *A. flavus*, *A. parasiticus*, and *A. nomius*. *A. flavus* exclusively produces B-type aflatoxins, while *A. parasiticus* and *A. nomius* can produce both B- and G-type aflatoxins (Pildain et al., 2008). *A. flavus* lacks the ability to produce G aflatoxins due to a deletion of 0.8 kb (L-strain) and 1.5 kb (S-strain) regions in the aflatoxin biosynthesis gene cluster. This deletion extends from the 5′ end of *aflF* and *aflU* to the entire 279 bp intergenic loci (Ehrlich et al., 2004).

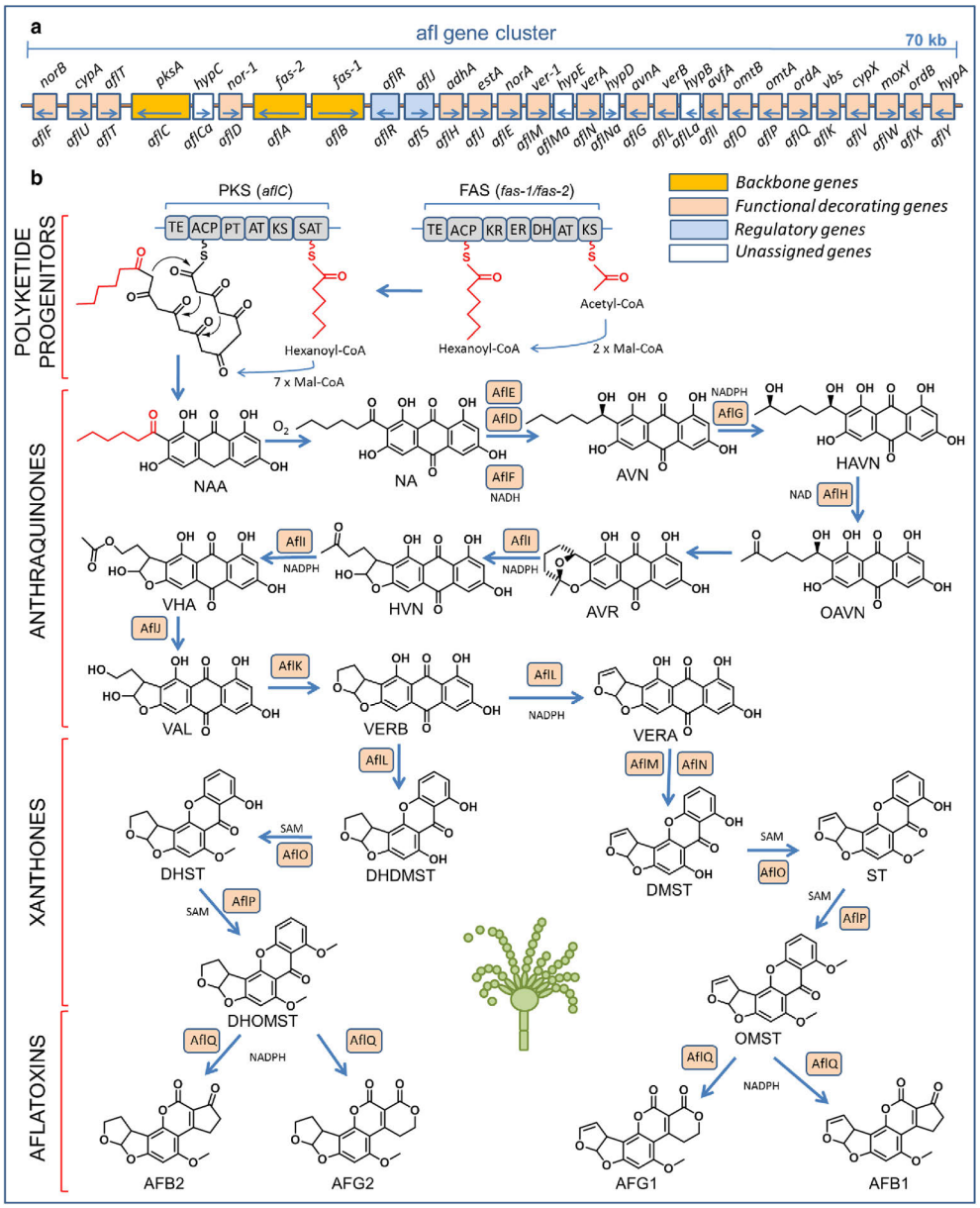

Figure 4. (a) Aflatoxin gene cluster. The order and position of the aflatoxin pathway genes, as well as an *aflR* antisense gene are clustered together in a 70 kb DNA region. Old gene names are indicated at the top of the line, and the new gene names, which have been renamed according to gene convention, are indicated at the bottom of the line. (b) Aflatoxin biosynthetic pathway with detailed chemical structures (Uka et al., 2020).

Table 1. Gene names, codes and their functions involved in the aflatoxin biosynthesis cluster.

Gene name (new)	Gene name (old)	Gene code	Function
aflA	*fas-2*	fatty acid synthase α	malonylCoA → hexaketide
aflB	*fas-1*	fatty acid synthase β	hexaketide → hexanoylCoA
aflC	*pksA*	polyketide synthase	hexanoylCoA → dodecaketide
hypC	*hypB1*	oxidase	NAA → NOR
aflD	*nor-1*	norsolorinic acid ketoreductase	
aflE	*norA*	norsolorinic acid reductase	NOR → AVN
aflF	*norB*	dehydrogenase	
aflG	*avnA*	cytochrome P-450 monooxygenase	AVN → HAVN
aflH	*adhA*	alcohol dehydrogenase	HAVN → OAVN
aflK		OAVN cyclase	OAVN → AVF
aflV	*cypX*	microsomal monooxygenase	AVF → HVN
aflW	*moxY*	cytosolic monooxygenase	HVN → VHA
aflI	*avfA*	oxidase	
aflJ	*estA*	esterase	VHA → VAL
aflK	*vbs*	versicolorin B synthase	VAL → VERB
aflL	*verB*	cytochromeP-450 monooxygenase/ desaturase	VERB → VERA
aflM	*ver-1*	dehydrogenase/ketoreductase	VERA → DMST
aflY	*hypA*	monooxygenase	VERB → DHDMST
aflN	*verA*	cytochrome P-450 monooxygenase	VERA → unknown intermediate
aflX	*ordB*	oxidoreductase	oxidative decarboxylation and ring-closure
aflO	*omtB*	O-methyltransferase B (O-methyltransferase I)	DMST → ST DHDMST → DHST
aflP	omtA	O-methyltransferase A (O-methyltransferase II)	ST → OMST DHST → DHOMST
aflQ	*ordA*	Oxidoreductase	OMST → AFB1 / AFG1 DHOMST → AFB2 / AFG2
hypB	*hypB2*	oxidase	HOMST → 370Da 7-ring lactone
hypD	*aflNa*	integral membrane-binding protein	unclear
hypE	*aflLa*	oxidase	final steps in AFB1 synthesis
aflR	*aflR*	transcription activator	regulate expression of genes
aflS	*aflJ*	transcription enhancer	unclear
aflT	*aflT*	fungal transporter	unclear
nadA	*nadA*	NADH oxidase	NADA → AFG1

1.5.2 Pathway-specific regulators; AflR and AflS

Aflatoxin production by toxigenic *Aspergillus* strains is affected by various factors, including pathway-specific regulators, global regulators and epigenetic modification. AflR and AflS are two pathway-specific regulators that regulate the synthesis of aflatoxin. The *aflR* gene encodes a 47 kDa Cys_6Zn_2 DNA-binding protein of the Gal4-type family, which is exclusively found in the fungus kingdom (Shelest, 2008). AflR acts as a transcriptional activator for most, if not all, aflatoxin structural genes, positively regulating the AF gene cluster. Overexpression of *aflR* in *A. flavus* up-regulates numerous AF genes, resulting in a 50-fold boost in aflatoxin synthesis (Flaherty & Payne, 1997). These findings showed that increasing the AflR concentration in the cell, changed the normal regulation of aflatoxin production. On the other hand, a microarray analysis comparing gene expression in a wild-type strain and *aflR* deletion strain of *A. parasiticus* revealed that some AF cluster genes exhibited no expression in the *aflR* deletion strain, highlighting the essential role of AflR in their transcription (Price et al., 2006). AflR binding motif, 5′-$TCGN_5CGA$-3′, was found in the promoter regions of many AF cluster genes **(Figure 5)**. Electrophoretic mobility shift assays (EMSA) experiments revealed that the genes *nor1*, *pksA*, *adhA*, *norA*, *ver1*, *omtA*, *ordA*, and *vbs* all contain at least one binding site 5′-$TCGN_5CGA$-3′ within 200 bp of the translation start site, with *pksA* and *ver1* having an extra binding site further upstream (Ehrlich et al., 1999). AflR, similar to other Gal4-type proteins that bind to partially palindromic regions, likely binds as a dimer to its recognition site. The *aflR* gene may be self-regulated as well as influenced by negative regulators. Upstream components might be implicated in the negative regulation of *aflR* promoter activity (Bhatnagar et al., 2006). ChIP-Seq analysis identified the AflR binding site in the genome of *A. flavus*, which is an 18-bp palindromic sequence 5′-CSSGGGW**TCGA**W**CCCSSG**-3′. Positions 8–18 of this DNA motif correspond to previously known AflR/AflS complex binding sites, referred to as motif A (bold), whereas positions 1-11 represent motif B (underlined). AflR most likely binds to either one or both of motif A and motif B (Kong et al., 2020).

The nuclear localization domain is located at the N-terminus of *aflR*, and it plays a crucial role in the translocation of AflR from the cytoplasm to the nucleus (Tang et al., 2017). The amino acid sequence of AflR includes a cysteine-rich motif, CTSCASSKVRCTKEKPACARCIERGLAC, near its N-terminus. This domain exhibits homology to the Cys6-Zn2 domains found in yeast GAL4-type transcription factors. It is noteworthy that a mutation of Cys6 to Trp results in the loss of the function of AflR. Furthermore, mutations in the zinc cluster region and specific amino acids within the nuclear localization region led to non-functional AflR. This suggests that these mutations hinder the translocation of the protein to the nucleus, rendering AflR unable to perform its function (Bhatnagar et al., 2003).

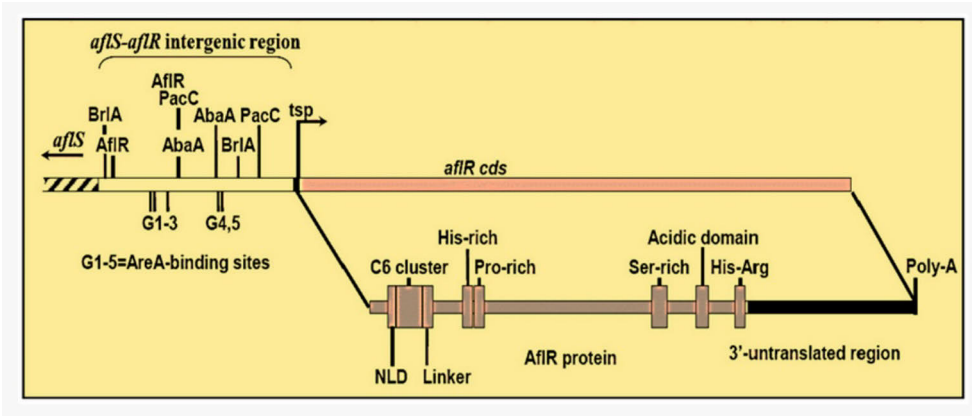

Figure 5. Genetic regulation by *aflR* and *aflS* in aflatoxin biosynthesis (Khan et al., 2021)

Adjacent to *aflR*, there is another pathway-specific gene called *aflS* **(Figure 5)**. This gene encodes a 438-amino acid protein and controls AF synthesis. Although AflS lacks a conserved domain, it exhibits certain similarities to a methyltransferase (W. Wang et al., 2021). It was hypothesized that AflS is membrane-bound protein since it has three transmembrane binding helices and a microbodies C-terminal targeting signal (Meyers et al., 1998). Deleting *aflS* gene in *A. parasiticus*, reduced the expression of some AF pathway genes by 5- to 20-fold. Conversely, overexpression of *aflS* in *A. flavus* led to a 4- to 5-fold increase in AFB1 levels (Caceres et al., 2020).

1.5.3 Global regulation of aflatoxin biosynthesis

Global regulator genes are not part of the aflatoxin gene cluster but play significant roles in the regulation of aflatoxin production. The biosynthesis of AF is controlled by a network of at least 53 genes, including 39 positive regulatory genes (listed in **Table 2**), 12 negative regulatory genes, and 2 other regulatory genes (*nmrA* and *afrmtA*). These global regulators are involved in coordinating the response to various factors, such as nutritional conditions like carbon (CreA, CreB, CreC, RimO) and nitrogen supplies (AreA, NmrA), as well as environmental factors including pH (PacC), light (VelB-VeA-LaeA), oxidative stress (Ap-1, AtfA, AtfB, MsnA, SrrA), and temperature. Epigenetic regulators such as SntB, Rtt109, RmtA also contribute to the regulation of aflatoxin production (W. Wang et al., 2022). Some of the global regulators are shown in **Figure 6**.

Table 2. List of the 39 positive regulatory genes for AF biosynthesis pathway (adapted from Liao et al., 2020).

NO.	Gene	NO.	Gene	NO.	Gene	NO.	Gene	NO.	Gene
1	*aflR*	9	*aflSkn7*	17	*acyA*	25	*afstuA*	33	*hamF*
2	*aflS*	10	*aflGcnE*	18	*pbsB*	26	*sntB*	34	*hamG*
3	*nsdC*	11	*hdaA*	19	*htf1*	27	*spc105*	35	*hamH*
4	*nsdD*	12	*hosA*	20	*dmtA*	28	*ste11*	36	*haml*
5	*creA*	13	*laeA*	21	*rtfA*	29	*norA*	37	*hexA*
6	*afrafA*	14	*dot1*	22	*farB*	30	*afSumO*	38	*aflPex5*
7	*hbx1*	15	*aflSte20*	23	*meaB*	31	*cap*	39	*atfB*
8	*rum1*	16	*gpaB*	24	*afapA*	32	*veA*		

Simple sugars (glucose and fructose), which either pre-exist in the growth environment or are released from polymers by fungal hydrolytic enzymes during seed tissue invasion, are carbon sources that yield higher levels of AF (Klich, 2007). Carbon catabolite repression (CCR) mediated by CreA is carried out through signal transduction. During the CCR process, fungal species conserve energy and adjust carbon catabolism to use the most advantageous carbon source (Roze et al., 2004). Several genes in the AF pathway contain CreA-binding sites near their promoter regions, thereby enhancing AF production (Georgianna & Payne, 2009). The nitrogen source is critical in AF biosynthesis. Generally, nitrate inhibits AF synthesis, whereas ammonium salts promote it. The globally acting transcription factor AreA regulates nitrogen metabolism in fungus. In *A. parasiticus*, AreA binds within the *aflR-aflS* intergenic region (737 bp), which also contains a number of GATA binding sites, as revealed by EMSA (Chang et al., 2000). *A. flavus* strains exhibit diversity in nitrogen regulation, which is often correlated with changes in the number of GATA sites near the transcription start site (TSS) of *aflJ* (Ehrlich & Cotty, 2002). Another external factor that affects AF synthesis is the pH of the media. AF levels are highest in acidic media and decrease as the pH of the medium increases. Indeed, PacC appears to be the major transcription factor implicated in the pH response (Klich, 2007). The influence of light is becoming one of the most well-characterized environmental factors that affect AF biosynthesis. Aflatoxin synthesis by *A. flavus* is regulated by the velvet A protein (VeA). VeA is part of a complex called the velvet complex, which also includes VelB, another velvet-like protein, and LaeA, a global regulator of secondary metabolism. Light can regulate both development and secondary metabolism through these interactions (Georgianna & Payne, 2009).

It is well-established that oxidative stress activates AF production, while anti-oxidants have an inhibitory effect on AF production. For example, studies have shown that different antioxidants, e.g., caffeic acid (J. H. Kim et al., 2008), gallic

acid (Mahoney & Molyneux, 2004), Tridham (Ravinayagam et al., 2012), and quercetin (Choi et al., 2010), inhibit AF production. The choice of growth culture media also influences AF production. When *Aspergillus* was tested for growth on Czapek's yeast extract and yeast extract sucrose (YES) media for 14 days, the highest levels of aflatoxin were observed in the YES medium plates.

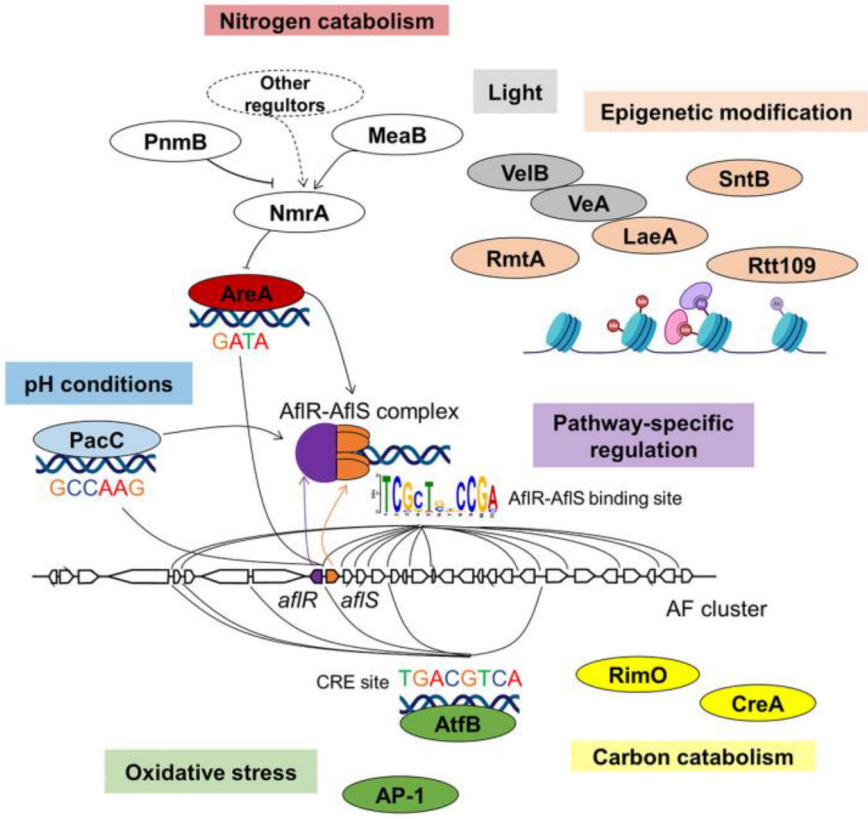

Figure 6. Regulatory mechanism involved in of AF biosynthesis by specific and global regulators (W. Wang et al., 2022).

1.5.4 Effect of climate change on aflatoxin production in Europe

Aflatoxin-producing fungal strains favor specific growth temperatures as AF levels are significantly lower at 35°C compared to 25°C or 30°C. Adequate water availability is essential for fungal development and aflatoxin formation. Highest aflatoxin levels were obtained at high water activities of 0.95–0.96 (Klich, 2007). Climate change exerts a substantial influence on the quality and availability of staple foods, particularly maize. It modifies environmental temperature and water activity,

which in turn affects fungal growth, gene expression, and AF production (Mahato et al., 2019). The development of fungi in the soil is influenced by geographic area, climate conditions (temperature, humidity, and rainfall), the specific crop being cultivated, and the existence of insects. Fungal spores can spread by direct contact with the soil, being transported by soil-containing dust particles, or being carried by insects (Loi et al., 2023).

The intensified use of fossil fuels since the industrial revolution is the primary driver of the climate change. The concentrations of the three primary greenhouse gases, i.e., carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O), are steadily increasing. The most significant outcome of the elevation in greenhouse gas levels is the increase in atmospheric temperature (Kos et al., 2023), which leads to evaporation, and hence intensifying the moisture loss (Vicente-Serrano et al., 2020). Europe is experiencing a more rapid warming trend compared to other regions. Instances of intense rainfall have grown more frequent in northern and eastern Europe (Kos et al., 2023). Concerning mycotoxins, Europe has a strong focus on their prevention and management levels having the strictest and most comprehensive regulations in the world in both food and feed. However, mycotoxin contamination in various food and feed products is on the rise. Annually, mycotoxin contamination significantly impacts crops throughout Europe, influenced by climate change. This leads to shorter crop utilization and processing times, resulting in substantial economic losses. Particularly during extreme weather conditions, crops become more susceptible to mold infestations, which substantially elevate the levels of mycotoxins in food products (S. Luo et al., 2021). Among the various crops, maize and wheat are the most widely cultivated and crucial cereals in Europe. Unfortunately, they also rank as the most susceptible crops to mycotoxin contamination, particularly during periods of extreme weather conditions. For instance, in the case of wheat, elevated levels of precipitation create favorable environments for the proliferation of *Fusarium* species, leading to the presence of the DON mycotoxin. The elevated temperature and humidity are suspected to be the primary factors behind the recent increased occurrence of *Aspergillus* mycotoxin contaminations, particularly in both southern and northern Europe. Of these contaminants, AFB1 in maize has emerged as the most frequently detected mycotoxin (Kos et al., 2023). The influence of climatic change factors on mycotoxin production is shown in **Figure 7**.

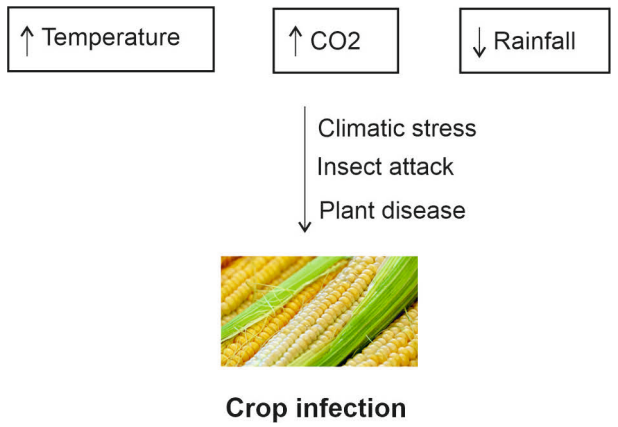

Figure 7. Climatic change factors influence mycotoxin production by *Aspergillus* species (modified from (Kos et al., 2023)).

1.6 Mitigating of aflatoxin contamination

1.6.1 Current control strategies of aflatoxins

Due to the negative consequences caused by the accumulation of AF, researchers worldwide are focusing to develop innovative approaches to detoxify aflatoxins. To date, there are three major approaches employed for this purpose: physical processes, chemical additives, and biological methods. Additionally, in recent years, specific natural phytochemicals have emerged as a promising strategy to tackle contamination issues. Several physical techniques have demonstrated their efficacy in managing or minimizing AF contamination in numerous agricultural crops. These techniques include high hydrostatic pressure and pulsed electric field procedures, UV-C irradiation in combination with rotation, gamma irradiation, extrusion cooking, magnetic carbon, and the use of hydrated sodium calcium aluminosilicates, which is an aflatoxin-selective clay. The proper utilization of specific chemicals has also shown the ability to remove AFs from contaminated crops. For instance, ammoniation has been considered the most advanced and economically viable method for eliminating aflatoxins from food, leading to authorization for its use in crops contaminated with aflatoxins in several countries. Another approach, known as neutral electrolyzed oxidizing water treatment has demonstrated reduced cytotoxic and genotoxic effects of aflatoxins on HepG2 cells in *in vitro* studies. Moreover, a pyrazine derivative called 2-(allylthio) pyrazine has been found to protect the liver against toxins, including AFB1, through inhibiting the formation of AFB1-DNA adducts. Other chemicals, including sodium bisulfite, sodium hydrogen sulfate, sodium hydroxide, hydrogen peroxide, calcium hydroxide, formaldehyde, sodium hypochlorite, sodium borate, and sorbents, have demonstrated notable

effectiveness in reducing AF levels in different food products. However, concerns regarding chemical residues have raised questions about the safety and economic viability of these chemical agents for commercial use (Alameri et al., 2023; Peng et al., 2018).

Biological controls, including organisms and extracts, has also been explored for their effectiveness in reducing AFB1 levels in aflatoxin-contaminated food products. One approach involves introducing bacterial strains into food substrates to reduce AFB1 levels through metabolic processes or direct binding to the bacteria. Several genera of micro-organisms, such as *Lactobacillus*, *Cellulosimicrobium*, *Actinomycetales*, *Mycobacterium*, *Saccharomyces*, *Bacillus*, *Pseudomonas*, *Ralstonia*, *Burkholderia*, and others, have been investigated for their potential in this regard (Palumbo et al., 2006; Rushing & Selim, 2019). Moreover, fungal inoculation has been explored as a potential detoxification method. Biocontrol mechanisms, such as competitive exclusion using non-aflatoxigenic *A. flavus*, have proven highly effective in controlling and managing *A. flavus* strains in maize (H. Abbas et al., 2007). However, similar to other biocontrol methods, these approaches often require a relatively long treatment duration, which may not be practical for large-scale applications. Furthermore, many studies in this field have not thoroughly characterized the degradation products resulting from these treatments. Without a comprehensive understanding of the end products, it becomes challenging to fully assess the safety of treated foods.

1.6.2 Medicinal plants as aflatoxin inhibitors

Plants lack an inherent immune system and rely on alternative mechanisms to protect themselves from fungi and bacteria. One such mechanism involves the production of bioactive substances specifically designed to inhibit fungal growth. These substances include alkaloids, flavonoids, saponins, glycosides, and tannins. Numerous studies have highlighted the significant antifungal properties of various plant extracts, particularly essential oils and organic extracts. Utilizing these natural products as biocontrol agents presents the advantage of avoiding the use of synthetic fungicides. Extensive efforts have been made to discover novel antifungal substances derived from natural origins, aiming to effectively manage AF contamination in food without significant reduction of nutritional content. Several plant extracts have demonstrated remarkable antifungal properties (Tian & Chun, 2017). For instance, methanolic extracts of *Leptadenia hastata*, *Barringtonia racemosa* and *Barringtonia asiatica* exhibited effective inhibition of both *A. flavus* and *A. niger* growth (Umaru et al., 2018). One important consideration in fungal growth suppression is the emergence of resistant strains. Therefore, it may be beneficial to focus on inhibitors of AF production instead of fungal growth inhibitors. Plant-based inhibitors of AF

production hold significant potential due to their dual benefits: high efficacy and the potential for transferring the genes responsible for their biosynthesis into susceptible host plants. This could enable the engineering of transgenic plants that possess inherent resistance to AF contamination by producing AF inhibitors on-site (Tian & Chun, 2017).

Gallic acid, a plant-derived compound, is known to inhibit the production of AF by interrupting the expression of early and late AF biosynthesis genes (Mahoney & Molyneux, 2004) or by decreasing the expression of transcription factors CreA and FarB (X. Zhao et al., 2018). Antioxidant eugenol (4-allyl-2-methoxy-phenol), a phenolic found in clove oil, nutmeg, cinnamon, basil and bay leaf, can diminish AF production by downregulating the AF biosynthesis pathway genes (Jahanshiri et al., 2015). *Capparis spinosa* extract also inhibited AF production by reducing the expression of major AF biosynthetic pathway genes, such as *aflQ*, *aflO*, *aflS*, and *aflR* (Mehraban et al., 2018). One medicinal plant which attracted my interest is *Zanthoxylum bungeanum*. It is a traditional medicinal plant from the *Rutaceae* family. *Z. bungeanum* has been found to contain many bioactive compounds, which have been detected with several techniques, including HPLC, UV-VIS, GC–MS, IR, and TLC. The identified compounds include alkaloids, lignans, coumarin, terpenes, steroids, volatile oils, aliphatics, aromatics, amides, and pyrroles. Among these, alkaloids and lignans are the principal active compounds. *Z. bungeanum* exhibits many claimed pharmacological benefits, including anti-inflammatory, antibacterial, antithrombotic, anti-aging, and anti-tumor properties. The antibacterial activity was studied against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. Additionally, it possessed antifungal activity against *A. oryzae* and *A. niger*. *Z. bungeanum* volatile oil has significant antioxidant activity. A comparison of the antioxidant activities of *Z. bungeanum* and cinnamon volatile oils revealed that both oils had anti-lipid peroxidation effects that increased with increasing concentration. Furthermore, *Z. bungeanum* had higher antioxidant efficiency and free radical scavenging capacity compared to cinnamon, making it an outstanding natural antioxidant (Bao et al., 2023).

2 Aims

The overall objective of this thesis was to study the molecular regulation mechanism of aflatoxin biosynthesis, develop techniques to detect aflatoxin and aflatoxin producing fungal strains, and explore the potential of medicinal plant extracts in controlling the fungal growth and mycotoxin production.

Specifically, the aims were:

I. To compare the genomic profiles of aflatoxigenic and non-aflatoxigenic *Aspergillus Flavi* isolates and develop a rapid immunoassay to detect aflatoxins in food (Articles I and V).
II. To unravel the molecular mechanisms governing the DNA binding activity of transcription factor AflR (specific regulator of aflatoxin biosynthesis pathway), and its modulation by co-regulator AflS (Article II).
III. To study the inhibitory effect of methanolic extract of a medicinal plant; *Zanthoxylum bungeanum* against *Aspergillus flavus* and *Fusarium graminearum* growth and toxin production (Article III and IV).

3 Materials and Methods

3.1 Screening of mycotoxins with HPLC (Articles I, III, IV, V)

Aspergillus isolates were refreshed on PDA media. Triplicate experiments were conducted by inoculating 50 µL of each spore suspension ($1x10^6$ spores/mL) into 500 µL of yeast extract broth (YES), which were then incubated at 25°C for 7 days. The cultures were filtered and aflatoxins were extracted from the supernatant with chloroform and derivatized with trifluoroacetic acid (Article I). To study the inhibitory effect of *Z. bungeanum* extract, culture tubes containing 1 mL YES medium and varying concentrations of M20 fraction (a fraction eluted from the silica column with a mixture of 80% ethyl acetate and 20% methanol) were seeded with *A. flavus* (10^3 spores/mL) and incubated for 48 or 120 h in the dark at 25°C. Control culture tubes without the presence of the plant extract were also included. AFs were extracted and derivatized from the culture medium (Article III). To determine the AFB1 concentration in spiked food samples, one gram of rice flour, maize flour, and hazelnut was extracted using 5 mL of 70% methanol. To prepare the spiked samples, a known quantity of AFB1 was added to the food sample in triplicate. These samples were allowed to equilibrate for 3 days at room temperature, protected from light. The spiked samples were then extracted with methanol and cleaned using AFB1 immunoaffinity column (Vicam), following the manufacturer instructions. The samples were derivatized with trifluoroacetic acid (Article V). The derivatized standard and samples were injected into the HPLC system which was LiChroCART (Agilent Technologies, Waldbronn, Germany) and an Agilent 1100 series device with absorption and fluorescence detectors (Agilent Technologies, Palo Alto, CA, USA). HPLC was equipped with a UV detector and a fluorescence with excitation at 365 nm and emission at 464 nm. The column was C18 reversed-phase (LiChrospher 100, 125x4 mm, 5 µm). The mobile phase consisted of a mixture of water: methanol: acetonitrile in a ratio of 60:30:15 (v/v/v) at a flow rate of 500 µL/min. To determine the concentration of DON, 0.5 g from each ground wheat sample was extracted with acetonitrile: water (80:20 v/v). The samples were filtered and the supernatant was diluted (1:1) with extraction solvent. Samples and standard (10 µL) were injected into the HPLC. The mobile phase consisted of acetonitrile and

water (15:85) (v/v) at a flow rate of 250 µL/min. The HPLC system was equipped with a UV detector and fluorescence with 220 nm wavelength (Article IV).

3.2 Nucleic acid isolation and PCR (Articles I, III, IV)

The molecular studies involved the growth of *Aspergillus* isolates in malt peptone (MP) broth. Approximately 100 mg of mycelia from each isolate were finely ground into powder using tissue-grinding pestles. DNA was extracted from *Aspergillus* mycelia (Article I), 40 mg of ground *Z. bungeanum* plant (Article III), and 100 mg of ground wheat (Article IV) using the GenElute^{TM} Plant Genomic DNA Miniprep Kit (ThermoFisher Scientific), following the manufacturer's recommendations. PCR reactions were prepared by Taq DNA polymerase (F-501L, Thermo Scientific) and High fidelity Phusion DNA polymerase (Thermo Fisher Scientific). For *F. graminearum* isolate, fungal DNA was extracted from pure cultures using a manual DNA extraction protocol (Mitina et al., 2011) (Article IV). To extract RNA, culture tubes contained 1 mL of YES medium supplemented with either 0.25 mg/mL of M20 fraction (solubilized in DMSO) or an equivalent concentration of DMSO (0.5%). A spore suspension of *A. flavus* was added to these tubes and incubated at 25°C for 4 days. Mycelia were collected, frozen in liquid nitrogen, and ground into a fine powder. RNA extraction was carried out using Trizol Reagent (ThermoFisher Scientific), following the manufacturer´s instructions (Article III).

3.3 Quantitative PCR and gene expression (Articles III, IV)

Complementary DNA (cDNA) was produced from 20 ng of total RNA (samples and controls) using gene-specific primers, and the quantification was carried out using a Luna Universal One-Step RT-qPCR Kit (New England BioLabs) following the supplier's protocol. cDNA was quantified based on SYBR Green fluorescence, and the PCR program was performed on an iQ^{TM}5 Real-time PCR apparatus (Bio-Rad). Relative gene expression levels in M20-treated samples compared to control samples were calculated using the $2^{-\Delta\Delta CT}$ method and using beta-tubulin as a reference gene (Article III). The PCR mixture for detecting *F. graminearum* DNA or wheat DNA contained the iQ^{TM} Supermix, primers and a probe oligo labeled with 6-FAM and TAMRA fluorophores at the 5' and 3' end, respectively. The TaqMan quantitative PCR was conducted using an iQ^{TM}5 Real-time PCR apparatus (Article IV).

3.4 Transcriptomes (Articles III)

cDNA libraries were prepared from the total RNA at Novogene company (Cambridge, UK) using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (New England Biolabs) following manufacturer's recommendations and sequenced using the Illumina platform and a paired-end 150-bp sequencing strategy. Raw RNA-seq data (FASTQ format) were processed with fastp to remove any low-quality reads and clean reads were mapped to the *A. flavus* NRRL 3357 reference genome using HISAT2 software. Quantification of gene expression was calculated according to Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM). Differentially expressed genes (DEGs) between control and test samples were analyzed using the DESeq2 R package.

3.5 Protein expression plasmids (Article II)

The plasmid used for overexpressing and producing the AflR protein was pAM068. To produce AflS protein, another plasmid, pAM069, was designed. However, AflS purification resulted in a precipitated protein, so to enhance the solubility of AflS, a gene fragment encoding maltose binding protein (MBP) was ligated to pAM069. The resulting *E. coli* expression construct, pAM073, encoded AflS as a fusion protein with N-terminal His-tagged MBP (AflS(MBP)). As a control the free MBP was produced from pAM074. Molecular cloning steps were carried out using T4 DNA ligase or Gibson assembly. DNA isolations and purifications were performed using the GeneJET plasmid miniprep and GeneJET gel extraction kits (ThermoFisher Scientific). Detailed information regarding the construction of all plasmids, primers, inserts, and restriction sites can be found in Article II.

3.6 Recombinant protein expression, purification and analysis (Article II)

The protein expression plasmids were transformed into *E. coli* BL21 (DE3) and cultured in Luria-Bertani (LB) media. The cells were grown in Erlenmeyer flasks at 37°C until the absorbance at 600 nm reached 0.5. Then, the cells were induced overnight with 0.5 mM IPTG while maintaining the temperature at 16°C. The cells were collected by centrifugation and stored at -80°C. On the day of purification, the cell pellets were thawed and suspended in a lysis buffer R (50 mM Tris-HCl, pH 6.9, 0.5 M $(NH_4)_2SO_4$, and 5% glycerol) supplemented with 1 mM β-mercaptoethanol (β-ME) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was then sonicated and the resulting lysate was clarified by centrifugation. For AflR purification, the cleared supernatant was applied to a Ni-NTA agarose column (PureCube 100, Cube Biotech), and unbound proteins were washed away using the

lysis buffer R. AflR was then eluted using 300 mM imidazole in the lysis buffer R. The eluted protein was concentrated to a final volume of 3 mL using Amicon® Ultra-4 Centrifugal Filter Units and injected into a gel filtration column (HiLoad 16/600 and 26/600 Superdex 200 prep grade) that had been pre-equilibrated with a gel filtration buffer (50 mM Tris-HCl (pH 6.9), 5% glycerol, 0.5 mM EDTA, 0.5 M $(NH_4)_2SO_4$). AflR was eluted at a flow rate of 0.1 mL/min using Äkta Purifier system (GE Healthcare). For AflS(MBP) purification, the cell pellets were resuspended in lysis buffer S (50 mM Tris-HCl, pH 7.9, 0.5 M KCl, 5% glycerol, 1 mM β-ME, 20 μM ZnSO4) supplemented with 0.1 mM PMSF. The mixture was incubated on ice, sonicated, and cleared by centrifugation. The supernatant was precipitated by adding solid ammonium sulfate (300 g/L). The protein pellet was collected by centrifugation and resuspended into lysis buffer S supplemented with 0.1 mM PMSF, and loaded onto pre-equilibrated Ni-NTA column. The column was washed and AflS(MBP) was eluted using 0.3 M imidazole in the lysis buffer S followed by the dilution of KCl concentration in the eluate to 150 mM using buffer A (50 mM Tris-HCl, pH 7.9, 5% glycerol, 1 mM β-ME, and 20 μM ZnSO4). The sample was then loaded onto a pre-equilibrated RESOURCE Q anion exchange column (6 mL column volume, GE Healthcare). AflS(MBP) was eluted from the column using a gradient of 10–100% buffer B (buffer A supplemented with 1 M KCl) at flow rate 1 mL/min. The fractions containing AflS(MBP) were combined and further purified by loading the sample with a syringe pump (1 mL/min flow rate) onto a pre-equilibrated dextrin sepharose column (MBPTrap HP, 1 mL column volume, GE Healthcare). The column was washed followed by the elution of AflS(MBP) with 20 mM maltose in lysis buffer S. MBP protein was also purified to be used as a negative control. The fractions containing the purified proteins were analyzed with SDS-PAGE, combined, concentrated, aliquoted, and stored at -80°C. Expression conditions and purification procedures for AflR, AflS(MBP), and MBP proteins are shown in **Table 3**.

Table 3. Expression and purification procedures of proteins.

Protein	Expression vector	Antibiotic resistance	Expression host	Purification procedure
AflR	pAM068:His_6-(TEV cut site)-AflR	ampicillin	*Escherichia coli* BL21 (DE3)	Ni-NTA column Gel filtration column
AflS(MBP)	pAM073:His_6-MBP-(TEV cut site)-AflS	ampicillin	*Escherichia coli* BL21 (DE3)	Ammonium sulphate precipitation Ni-NTA column RESOURCE Q column MBPTrap column
MBP	pAM074:His_6-(TEV cut site)-MBP	ampicillin	*Escherichia coli* BL21 (DE3)	Ammonium sulphate precipitation MBPTrap column

3.7 Protein interactions (Article II)

The interaction between AflR and AflS(MBP) proteins was monitored using MonolithX (MM-078) instrument from NanoTemper Technologies GmbH. AflS(MBP) was labeled with lysine residues through covalent bonding, employing the RED-NHS 2nd generation protein labeling kit from NanoTemper Technologies. A serial dilution of AflR was prepared with 10 µL sample in each tube. Then, 10 µL of the labeled AflS(MBP) in the binding buffer was added to each tube. The samples were incubated at 23°C for 30 min, centrifuged at 12000 rpm, and loaded into capillaries. Sample fluorescence was recorded using Monolith X. The dissociation constant of AflS·AflR complex ($K_{D,S-R}$) was obtained by fitting the data to **Equations 1**, **2**, **3**. Furthermore, the protein-DNA interaction was examined using EMSA assay. The used DNA probes were either *norA* promoter DNA or negative control DNA. The DNA fragments were amplified via PCR and purified using the GeneJET gel extraction kit (Thermo Fisher Scientific). The *norA* DNA segment (369-bp long) encompassed the promoter region from position -291 to +25 relative to the translation start site at +1, along with upstream/downstream linkers containing primer binding sites, and the Atto488 fluorophore positioned on the bottom strand **(Figure 8)**. For the binding assays, a 10 µL reaction mixture was prepared containing 20 nM of DNA in binding buffer, along with varying concentrations of AflR, AflS(MBP), or a combination of both. The mixtures were then incubated at 23°C for 30 min before separating via native polyacrylamide gel electrophoresis (PAGE). A 5% PAGE gel was prepared in 0.5X TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA; Thermo Fisher Scientific) and run at 100 V for 90 min. Subsequently, the gel was scanned using an Azure Sapphire Biomolecular Imager (Azure Biosystems) in the 488 nm channel. The percentages of protein-bound and free DNA were determined by analyzing gel band intensities with ImageJ software. The dissociation constants K_1 and K_2 of the DNA-protein complex, whose dimension is μM^2, were calculated by fitting data to **Equation 4** in the absence of AflS or **Equation 5,** If AflR and AflS are present in equimolar amounts, where Y represents the percentage of protein-bound DNA, [R] is the concentration of AflR.

$$[\boldsymbol{SR}]_{\mathbf{fraction}} = \frac{([\mathbf{S_{total}}]+[\mathbf{R_{total}}]+\boldsymbol{K}_{\mathbf{D,S-R}})-\sqrt{([\mathbf{S_{total}}]+[\mathbf{R_{total}}]+\boldsymbol{K}_{\mathbf{D,S-R}})^{\mathbf{2}}-\mathbf{4}\times[\mathbf{S_{total}}]\times[\mathbf{R_{total}}]}}{\mathbf{2}\times[\mathbf{S_{total}}]} \quad \textbf{(1)}$$

$$[\boldsymbol{S}]_{\mathbf{fraction}} = \mathbf{1} - [\boldsymbol{SR}]_{\mathbf{fraction}} \quad \textbf{(2)}$$

$$\boldsymbol{F}_{\mathbf{total}} = [\mathbf{S}]_{\mathbf{fraction}} \times \boldsymbol{F}_{\mathbf{S}} + [\mathbf{SR}]_{\mathbf{fraction}} \times \boldsymbol{F}_{\mathbf{SR}} \quad \textbf{(3)}$$

$$Y = 100\ /(1 + K_1/[R]^2) \quad (4)$$

$$Y = 100\ (1 + K_1[R]^2/K_{D,R-S}K_2)/(1 + K_1[R]^2/K_{D,R-S}K_2 + K_1/[R]^2) \quad (5)$$

3.7.1 Fluorescence measurements (Article II)

Fluorescence anisotropy and intensity measurements were carried out to analyze the interaction between the protein and DNA(Cy3) or DNA(FAM) using LS-55 fluorimeter (PerkinElmer) at 23°C. The excitation and emission wavelengths were set to 480 nm (excitation) and 520 nm (emission) for the DNA(FAM), and were adjusted to 550 and 565 nm for the DNA(Cy3) samples. A reaction mixture of 200 µL, containing 20 nM labeled DNA in binding buffer, underwent titration with AflR or AflS(MBP), or their combination, with 5-minute incubations after each protein addition. In competitive trials, the fluorescence intensity of 20 nM DNA(Cy3) was assessed before and after the addition of 330 nM AflR, followed by the subsequent addition of 1 µM unlabeled competitor DNA scaffold. Wild type (WT) and mutant sequences are shown in **Figure 8**.

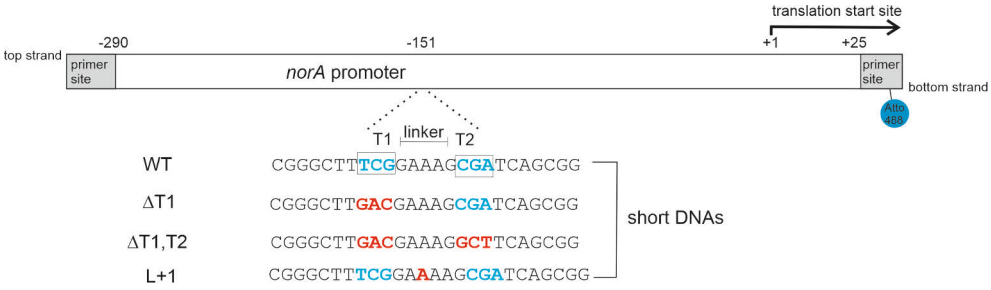

Figure 8. The sequences of non-template strands and unlabeled competitors of wild-type and mutated DNA scaffolds. The triplet sequence motifs T1 and T2 (shown in blue) and linker (L) in the AflR binding region in the wild-type DNA scaffold are highlighted. DNA mutations are shown in red font in the competitor scaffolds with one (ΔT1) or both (ΔT1, ΔT2) base triplets being substituted or the linker extended by 1 bp (L+1). Source: Article II.

3.7.2 DNA binding kinetics (Article II)

Stopped-flow measurements were conducted at 23°C using SFM-3000 stopped-flow instrument (BioLogic Science Instruments). The reaction was initiated by mixing 75 µL of AflR (20−330 nM final concentration after mixing) and 75 µL DNA (20 nM final concentration) in binding buffer. Changes in fluorescence emission intensity of DNA(Cy3) were measured using excitation wavelength 546 nm and 570 nm longpass emission filter. For each concentration of AflR, at least three individual traces were recorded and averaged. A two-phase exponential equation was fit to

stopped flow traces to obtain the apparent rates (k_1 or k_2) and fluorescence amplitudes (A_1 or A_2) at each [AflR], respectively. The dissociation constant for AflR and DNA(Cy3) interaction ($K_{D,SF}$) was derived by **Equation 6** from the AflR concentration [R] dependence of the apparent A_1. The parameter A_1^{ind} refer to the value of A_1 in the saturating concentration of AflR.

$$\mathbf{Y} = \boldsymbol{A}_{\mathbf{1}}^{\mathbf{ind}}/(\mathbf{1} + \boldsymbol{K}_{\mathbf{D,SF}}/[\boldsymbol{R}]) \quad \mathbf{(6)}$$

4 Results and Discussion

4.1 Comparison between aflatoxigenic and non-aflatoxigenic *Aspergillus* isolates (Original publication I)

4.1.1 Molecular identification and aflatoxin analysis

In recent years, advancements in DNA sequencing and the application of PCR techniques have greatly enhanced the effectiveness and affordability of molecular approaches for identifying fungal pathogens. These molecular methods have proven to be more advantageous than morphological culture-based methods (Alshehri & Palanisamy, 2020). In Article I, the objective was to develop a polyphasic approach to differentiate between aflatoxin and nonaflatoxin-producing strains using phylogenetic, sequence, and toxin analyses. Forty *Aspergillus* section *Flavi* isolates (originally from SRRC culture collection (USA), Philippines, and Egypt) were molecularly identified by sequencing ITS1 and ITS2 regions in the ribosomal RNA gene repeat. Among them, the majority (32 isolates, or 80%) belonged to *A. flavus* followed by three isolates (7.5%) of *A. parasiticus*, three isolates (7.5%) of *A. nomius*, and one isolate (2.5%) of *A. tamarii*. ITS1 and ITS2 sequences of thirty *Aspergillus* isolates were found to have a 100% identity with GenBank sequences. The sequences with no perfect match were submitted to the GenBank with new accession numbers **(Table 4, Figure 9A)**. HPLC analysis detected the aflatoxin profile in all *Aspergillus* isolates. Twenty-two out of forty samples were aflatoxigenic. Six out of the eleven isolates from the SRRC culture collection were AF producers. Eleven out of the twenty-one isolates from the Philippines were aflatoxigenic. Among the eight Egyptian isolates, five were found to produce aflatoxin **(Table 4, Figure 9B)**. Our data showed also the general phenomena that *A. flavus* isolates produce only B-type AFs, *A. parasiticus* and *A. nomius* produce both B- and G- type AFs, and *A. tamarii* is non-aflatoxigenic species. The ecological conditions in the Philippines, characterized by high temperatures and humidity, create favorable circumstances for the growth of mycotoxigenic fungi and the production of mycotoxins in agricultural crops (Sales & Yoshizawa, 2007). Literature research revealed that seven *Aspergillus* species, namely *A. flavus*, *A. parasiticus*, *A. carbonius*, *A. japonicus*, *A. ochraceous*, *A. niger*, and *A. westerdijkiae*, have been identified in the

Philippines (Balendres et al., 2019). Among these species, *A. flavus* is the predominant aflatoxin producer. We identified the majority of the isolates from the Philippines as *A. flavus*, and we also discovered a new species, *A. nomius*, in the Philippines. Previously, *A. nomius* isolates were morphologically identified as *A. parasiticus* (AboDalam et al., 2020), highlighting the importance of molecular methods in the fungal identification. *Aspergillus nomius* isolates were found to secrete significant quantities of all four types of aflatoxins. This increases the health risks associated with the consumption of contaminated commodities by humans and animals.

Table 4. Geographical locations of *Aspergillus* section *Flavi* isolates, molecular identification, and aflatoxin production. Source: Article I.

GEOGRAPHIC ORIGIN	SOURCE	IDENTIFICATION	GENBANK ACCESSION NUMBER	TOTAL AFLATOXINS (ppb)
SRRC CULTURE COLLECTION	Cottonseed, USA	*A. flavus*	MH752568	31
	Karnataka, India	*A. flavus*	KF432854	68
	Pistachio, USA	*A. flavus*	MH752566	40
	Peanut, Australia	*A. flavus*	MH244421	ND
	Dried fish, Indonesia	*A. flavus*	MN511750	ND
	Dead termites, China	*A. flavus*	FN398160	ND
	Lung tissue, USA	*A. flavus*	AY510451	ND
	Corneal ulcer, USA	*A. flavus*	KY630136	2339
	Peanuts, Uganda, Africa	*A. parasiticus*	MN511749	ND
	Rice, USA	*A. parasiticus*	KC769508	2311
	Peanut, Australia	*A. parasiticus*	MH752575	932
PHILIPPINES	Soil	*A. tamarii*	MN511748	ND
	Soil	*A. flavus*	LN482489	ND
	Soil	*A. nomius*	MH752557	4714
	Soil	*A. nomius*	AY510454	14,416
	Soil	*A. flavus*	KX426971	ND
	Soil	*A. flavus*	MN511747	ND
	Soil	*A. flavus*	KF432854	ND
	Soil	*A. flavus*	KY630136	13
	Soil	*A. flavus*	MF094441	33
	Soil	*A. flavus*	MG720232	7
	Soil, peanuts	*A. flavus*	FN398161	51
	Soil	*A. flavus*	MH595954	ND
	Soil	*A. flavus*	FN398157	5
	Soil	*A. flavus*	MK791661	ND
	Soil	*A. flavus*	KX426971	ND
	Soil	*A. flavus*	MN511746	ND
	Soil	*A. flavus*	LN482481	ND
	Soil, coconut	*A. flavus*	MN511745	68
	Soil, Coconut	*A. nomius*	MN511744	292
	Soil	*A. flavus*	KU561938	5
	Peanuts	*A. flavus*	MN511743	72

GEOGRAPHIC ORIGIN	SOURCE	IDENTIFICATION	GENBANK ACCESSION NUMBER	TOTAL AFLATOXINS (ppb)
EGYPT	Maize	*A. flavus*	MN511742	38
	Maize	*A. flavus*	MH752568	772
	Maize	*A. flavus*	JF729324	121
	Maize	*A. flavus*	MG554234	1158
	Soil	*A. flavus*	MH595954	ND
	Soil	*A. flavus*	MH595954	ND
	Bench sample	*A. flavus*	FN398156	66
	Air sample	*A. flavus*	MH595954	ND

Aflatoxin concentration (ppb) was calculated by the average of three replicate cultures per isolate, ND = Not Detected, ppb = part per billion, SRRC = Southern Regional Research Center, New Orleans, LA, USA.

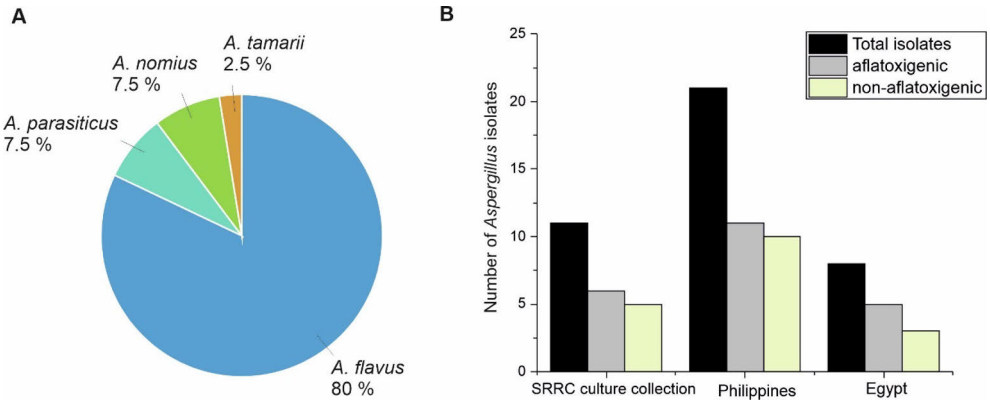

Figure 9. Molecular identification and aflatoxin analysis of *Aspergillus* section *Flavi* isolates originally from SRRC culture collection (USA), Philippines, and Egypt. (A) The percentages of *A. flavus* (80%), *A. parasiticus* (7.5%), *A. nomius* (7.5%), and *A. tamarii* (2.5%) occurrences were calculated relative to the total number of isolates obtained through molecular identification. (B) Aflatoxin profile for the forty *Aspergillus* isolates based on their geographic locations.

4.1.2 Genetic diversity among *Aspergillus* section *Flavi* isolates

We used three random amplified polymorphic DNA (RAPD) and three inter-simple sequence repeats (ISSR) markers to evaluate the genetic diversity and phylogenetic relationships among the forty *Aspergillus* isolates. Both markers have been effectively utilized in phylogenetic and diversity studies due to their simplicity, cost-effectiveness, and reliance on standard thermal cycler and gel electrophoresis systems. Despite using only six primers, our study yielded significant genetic variation among the isolates. The percentage of polymorphic bands (PPB) revealed by the RAPD and ISSR primers was 81.9% and 79.4%, respectively. The PPB for

both markers is comparable to each other in this study and much higher than that found by Valadez-Moctezuma et al., (2015), where, the PPB was 58.3% and 44% for RAPD and ISSR primers, respectively. We also detected greater PPB of RAPD than ISSR and this agreed with the data reported by Bezerra, D.C et al., (2022) and Valadez-Moctezuma et al., (2015). We used the polymorphism information content (PIC) and marker index (MI) values to summarize the banding patterns of RAPD and ISSR-PCR results. Polymorphism refers to the occurrence of different variations among individuals in a population. A PIC value greater than 0.5 indicates that the primer used is an effective marker for assessing genetic diversity (Z. Luo et al., 2019). Our findings indicated that both RAPD and ISSR primers had average PIC values exceeding 0.5. However, ISSR primers exhibited higher average PIC and MI values compared to RAPD primers **(Table 5)**. This disparity can be attributed to the longer length of ISSR primers, which allows for annealing at higher temperatures, resulting in greater diversity (Ganie et al., 2015). Analysis of banding patterns and gene diversities clearly indicates that ISSR-PCR outperforms RAPD-PCR in terms of providing clearer and more successful data for genetic diversification. Agarose gel electrophoresis photo for 27 *Aspergillus* isolates amplified with (GTG)5 primer is shown in **Figure 10**.

Table 5. Summary of banding patterns of RAPD and ISSR results. Source: Article I.

PRIMERS	NUMBER OF BANDS	NUMBER OF POLYMORPHIC BANDS	PPB (%)	PIC	MI
RAPD MARKERS					
RAPD 1	7	6	85.7	0.76	0.55
RAPD 2	5	5	100	0.75	0.48
RAPD 5	5	3	60	0.45	0.39
AVERAGE	5.67	4.67	81.9	0.65	0.47
ISSR MARKERS					
(GTG) 5	6	4	66.7	0.77	0.57
(GACA) 4	7	5	71.4	0.79	0.55
(AGAG) 4G	8	8	100	0.81	0.56
AVERAGE	7	5.67	79.37	0.79	0.56

PPB: percentage of polymorphic bands, PIC: The Polymorphism Information Content and MI: Marker Index.

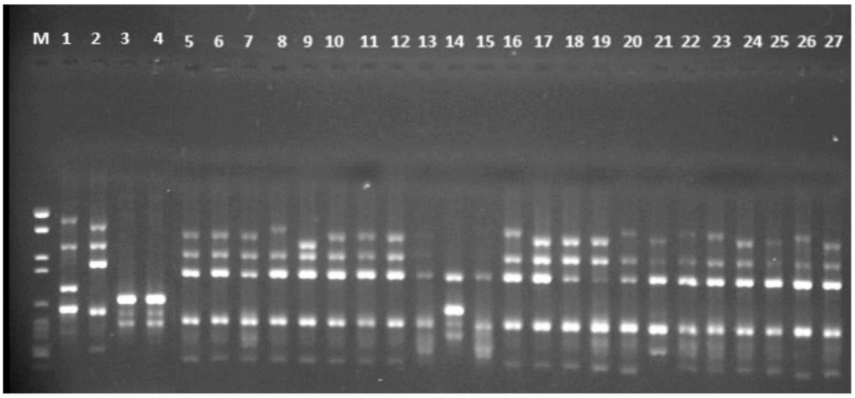

Figure 10. Representative ISSR profiles generated with (GTG)5 primer for 27 *Aspergillus* isolates. M = DNA marker, 1 = AF 144, 2 = AF 1305, 3 = AF 1554, 4 = AF 2041, 5 = AF 2118, 6 = AF 2525, 7 = AF 2649, 8 = AF 2653, 9 = AP 143, 10 = AP 1311, 11 = AP2040, 12 = 2P, 13 = 3P, 14 = 7P, 15 = 9P, 16 = 23P, 17 = 25P, 18 = 32P, 19 = 34P, 20 = 41P, 21 = 42P, 22 = 45P, 23 = 47P, 24 = 51P, 25 = 58P, 26 = 64P and 27 = 81P. Source: Article I.

4.2 Molecular mechanisms of aflatoxin pathway specific regulators AflR and AflS (Original publication II)

4.2.1 Protein purification and protein-protein interaction

In order to thoroughly investigate the recognition mechanism by which AflR identifies its target promoter DNA, as well as the role of AflS in this process, it was crucial to obtain highly purified recombinant AflR and AflS proteins. To achieve this, synthetic vectors containing the codon-optimized genes were utilized to express N-terminal fusion proteins with a histidine tag. Initially, the *aflR* expression plasmid (pAM068) was transformed into *Escherichia coli (E. coli)* T7 express, but the resulting recombinant protein accumulated in the inclusion bodies (cell pellet) when expressed at 37°C for 3 h. To mitigate this issue, pAM068 was transformed into *E. coli* strain BL21 (DE3) and expression was induced overnight at 16°C and shaking set to 160 rpm. Successful overexpression of AflR was achieved. Subsequently, AflR was purified from the cell lysate using immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). The expected size of the AflR protein is 50 kDa. Protein fractions eluted from the gel filtration column were analysed by SDS-PAGE, and one of the observed peaks corresponded to AflR. Western blotting using anti-His-tag antibody further confirmed the identity of the purified AflR **(Figure 11A)**. In order to improve the solubility of AflS in the cytoplasm of *E. coli* BL21, we added His-tagged MBP (maltose binding protein) to

the N-terminal end of AflS. This fusion protein was named as AflS(MBP). The initial capture step in the large-scale purification involved ammonium sulfate precipitation, followed by intermediate purification using IMAC and anion exchange (using RESOURCE Q column). Anion exchange chromatography was selected due to the isoelectric point of AflS(MBP), which was calculated to be 5.8. The final polishing step in the protocol employed amylose affinity chromatography, specifically a MBPTrap column. The presence of AflS(MBP), with calculated molecular mass of about 90 kDa, in the fraction eluted from MBPTrap was confirmed using SDS-PAGE and western analyses **(Figure 11B)**. Noteworthy, the MBP moiety appears to be essential for AflS solubility because the removal of MBP by site-specific TEV protease led to the precipitation of AflS. Since MBP could not be cleaved off from AflS, we separately purified the MBP protein to serve as a negative control in the AflS functional assays. A two-step purification procedure consisting of ammonium sulfate precipitation and MBPTrap yielded a relatively high quantity of MBP protein **(Figure 11C)**.

The possible interaction between the two purified AflR and AflS(MBP) was monitored using an *in vitro* experimental system called microscale thermophoresis assay (MST). This assay detects biomolecular interactions by observing changes in the brightness and motion of fluorescent molecules within a microscopic temperature gradient, which are indicative of the formation of protein complexes (Sparks & Fratti, 2019). We therefore labeled AflS(MBP) with a fluorophore dye, which covalently binds to lysine residues. After removing any unbound dye, we mixed the labeled AflS(MBP) with varying concentrations of non-labeled AflR and analyzed the mixture using the MST mode of the MonolithX instrument (NanoTemper Technologies GmbH). Our data clearly demonstrates a significant increase in fluorescence upon the addition of AflR **(Figure 11D)**. By fitting **Equations 1**, **2**, **3** to the data in **Figure 11D**, we estimated the dissociation constant (K_D) for the AflR-AflS(MBP) complex to be 24 ± 6 nM (S.E.), indicating a relatively strong interaction between AflR and AflS(MBP). Similarly, when we performed an interaction analysis between AflR (labeled) and MBP (non-labeled), we observed a constant measurement signal, confirming that these proteins do not interact **(Figure 11E)**. In a previous study, Ehrlich and colleagues investigated the interaction between AflR and AflS in *A. parasiticus* using a yeast two-hybrid assay. They employed an *A. parasiticus* cDNA library as the target and used *aflS* as the bait. The confirmation of this interaction was established by the growth of yeast colonies on specialized media (Ehrlich et al., 2012). The formation of AflS·AflR complex, provided that it is essential for the activation of AF biosynthesis, could explain why *A. flavus* strain lacking *aflS* was unable to produce aflatoxin or its precursors, as reported by Du et al., (2007) and Meyers et al., (1998).

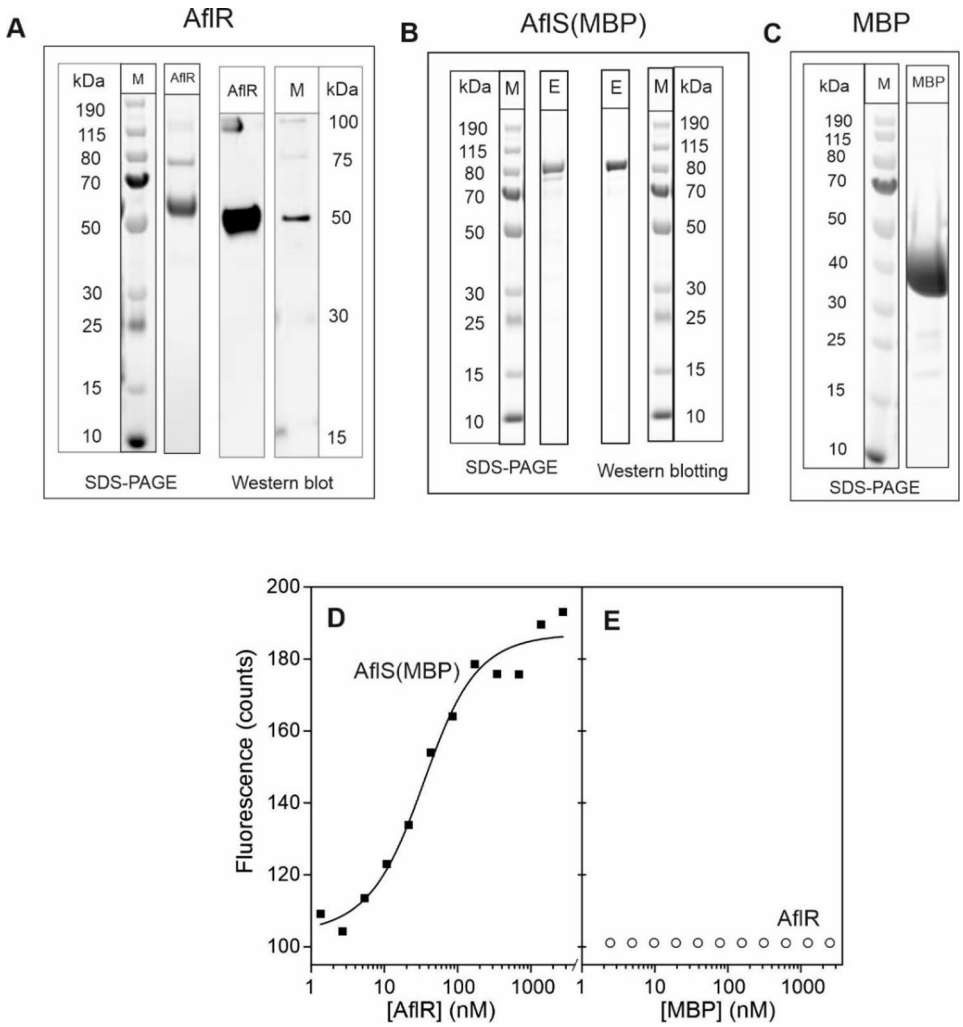

Figure 11. Protein purification and protein-protein interaction. (A) SDS-PAGE and western blot analyses for the purified AflR (50 kDa). (B) SDS-PAGE and western blot analyses for the purified AflS(MBP) (90 kDa). (C) SDS-PAGE for the purified MBP (40 kDa). (D) The binding affinity of labeled AflS(MBP) was titrated with varying concentrations of non-labeled AflR. (E) The binding affinity of labeled AflR was titrated with different concentrations of MBP (in the absence of AflS). Source: Article II.

4.2.2 Effect of AflS on the DNA binding affinity of AflR

We next investigated the DNA binding affinity of AflR, AflS(MBP) and their mixtures on different models of *norA* promoter. We employed EMSA, a technique in which protein·DNA complexes migrate slower than the free DNA. Our findings revealed that the labeled *norA* promoter exhibited high electrophoretic mobility in the absence of proteins. However, when increasing concentrations of AflR were

introduced to the *norA* promoter, we observed a slower-moving band on the gel, accompanied by a reduction in the amount of rapidly migrating DNA fragment **(Figure 12A)**. Importantly, this mobility shift did not occur when we tested the negative control DNA, which lacks known AflR binding motifs **(Figure 12B)**. This outcome strongly suggests that the AflR protein is fully functional and specifically recognizes its target sites within the DNA sequence. Conversely, when we mixed AflS(MBP) with the *norA* promoter or the negative control DNA, we did not observe a significant shift in mobility. This indicates that AflS(MBP) does not possess specific or non-specific binding sites on either DNA. In experiments where we introduced different concentrations (0-980 nM) of a 1:1 mixture of AflR and AflS(MBP) to a 20 nM *norA* promoter, we did observe a mobility shift. However, it is worth noting that the percentage of slowly migrating DNA was consistently lower than when the same concentration of AflR was added as the sole protein. The concentration of the AflR·*norA* promoter complex, in relation to the protein concentrations, appeared to be reasonably explained by assuming the presence of two interacting AflR binding sites within the DNA molecule. When considering the combined dissociation constants derived from EMSA data, it can be concluded that AflS(MBP) reduces the binding affinity of AflR to the *norA* promoter by two-fold [AflR: $K_1 = 0.25 \pm 0.03\ \mu M^2$; AflR+AflS(MBP): $K_2 = 0.54 \pm 0.03\ \mu M^2$].

To confirm the role of AflS(MBP) as a modulator of the DNA binding activity of AflR, we used a short double-stranded DNA oligo scaffold that contained the AflR binding motif and a FAM fluorophore label. This enabled us to monitor protein-DNA interactions directly in solution using fluorescence anisotropy. In the absence of proteins, this DNA(FAM) scaffold displayed low fluorescence anisotropy, approximately 0.15. Such small molecules tend to rapidly rotate, relaxing most of the orientation bias in their fluorescently excited state **(Figure 12C)**. However, upon the addition of AflR, we observed an increase in anisotropy to approximately 0.25. This change is in line with the formation of a AflR·DNA(FAM) complex, which rotates more slowly due to its increased size. Importantly, when we introduced a ten-fold molar excess of unlabeled DNA scaffold, which was otherwise identical, as a competitor in the reaction buffer, it effectively blocked the formation of the high anisotropy AflR-DNA complex (as seen in the AflR+competitor column in **Figure 12C**). When both AflR and AflS(MBP) were simultaneously added to the DNA(FAM), we observed a smaller increase in anisotropy, approximately 0.20. This result is consistent with our hypothesis that AflS(MBP) negatively affects the DNA binding affinity of AflR. Notably, when we added AflS(MBP) alone, it did not induce a significant change in the anisotropy of the DNA(FAM) scaffold. This observation confirms that AflS(MBP) does not possess DNA binding functionality.

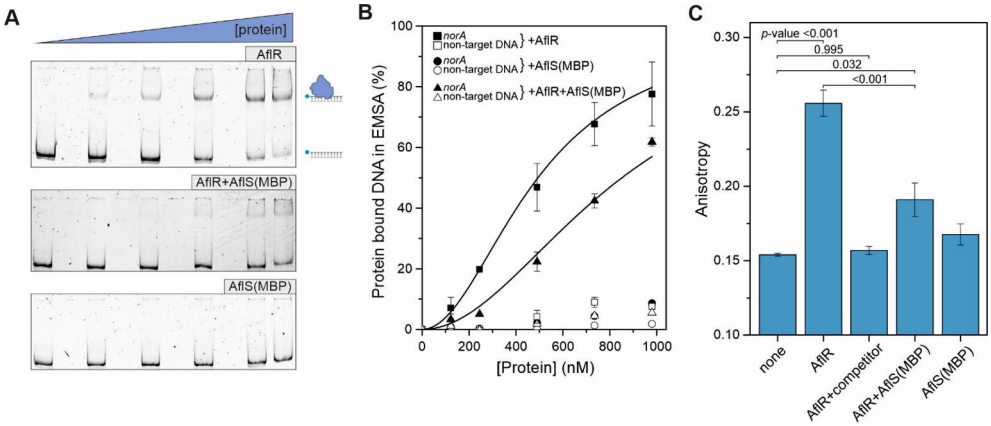

Figure 12. The impact of AflS on DNA binding activity of AflR. (A) EMSA gels show the formation of AflR–*norA* complex specially at high protein concentrations, while AflS(MBP) does not bind to DNA. In all EMSA experiments, 20 nM of *norA* or non-target DNA labeled with Atto488 was used, along with varying concentrations of AflR, AflS(MBP), or equimolar concentrations from AflR and AflS(MBP). (B) The percentage of *norA* DNA (filled symbols) or non-target DNA (open symbols) associated with the protein–DNA complex in the EMSA assays. (C) Anisotropy measurements of 20 nM DNA(FAM) under different conditions. The "sample none" represents the anisotropy of DNA(FAM) only. Anisotropy was measured 10 min after the addition of various combinations of proteins, including 150 nM AflR (*AflR*), 150 nM AflR with 200 nM unlabeled competitor DNA (*AflR+competitor*), 150 nM AflR with 350 nM AflS(MBP) (*AflR+AflS(MBP)*), and 150 nM AflS(MBP) (*AflS(MBP)*). Source: Article II.

4.2.3 AflR recognized the two specific base triplets and the connecting linker in the promoter DNA

We then investigated the kinetics and sequence determinants of the formation and stability of the AflR-DNA complex. To do this, we utilized the same DNA(FAM) scaffold with the AflR binding motif. However, we made a change in our experimental approach to collect more frequently fluorescence intensity data, by using fixed vertical and horizontal polarizers for excitation and emission, respectively. This modification allowed us to increase the data collection rate of the LS55 fluorometer (PerkinElmer) by ten-fold, reaching 2 Hz. This was a significant improvement over the full anisotropy measurements, which required switching between horizontal and vertical emission polarizers. We measured the fluorescence intensity after separately adding four different unlabeled DNAs to the AflR·DNA(FAM) complex, i.e., wild-type DNA, L+1, ΔT1 and ΔT1,T2. The fluorescence intensity of DNA(FAM) alone was initially relatively high, however upon adding 330 nM AflR to 20 nM DNA(FAM), the fluorescence intensity immediately decreased by approximately 15%, indicating AflR·DNA(FAM) formation, an increase in mass, and a significant reduction in the rotational diffusion of the fluorophore **(Figure 13)**. Nevertheless, AflR-DNA(FAM) complex formation happened too quickly to be resolved kinetically, mainly because of

a delay of about 20 s introduced by the steps involving protein addition and sample mixing. Afterward, the addition of wild-type (WT) competitor DNA, which was structurally identical to DNA(FAM) but lacked a fluorophore, led to a gradual increase in fluorescence intensity. This pattern was consistent with the dissociation of AflR from DNA(FAM) and its subsequent binding to the competitor DNA (**Figure 13**, WT trace). The dissociation kinetics exhibited a two-phase behavior with two lifetime parameters, specifically 51 ± 7 s (S.E.) and 575 ± 16 s. The corresponding dissociation rate constants, determined by the reciprocal of the lifetimes ($k = 1/t$), were 0.02 ± 0.003 s^{-1} and 0.0017 ± 0.0001 s^{-1}. When we considered the sum of two amplitude parameters (*A1* and *A2*), we observed that the fluorescence returned to its initial level, similar to the state without the presence of the protein, upon using the WT competitor. This outcome was anticipated because we used a substantial excess (50-fold) of WT competitor. Then, we assessed the binding effectiveness of mutant competitor DNAs. Specifically, we introduced modifications to two base triplets (T1 and T2 in **Figure 8**) and the linker (L) connecting them. Our measurements showed that when both base triplets were replaced, the DNA lost its ability to act as a competitor (**Figure 13**, ΔT1, T2 trace). When only one of the triplets was replaced, the DNA still functioned as a competitor, but its potency was significantly reduced. This was evident from the fact that the fluorescence only recovered to 24% of the initial intensity after the addition of the ΔT1 competitor (**Figure 13**, ΔT1 trace). Extending the linker by 1 base pair was less detrimental to the binding efficiency, as this competitor DNA recovered 75% of the initial fluorescence intensity (**Figure 13**, L+1 trace).

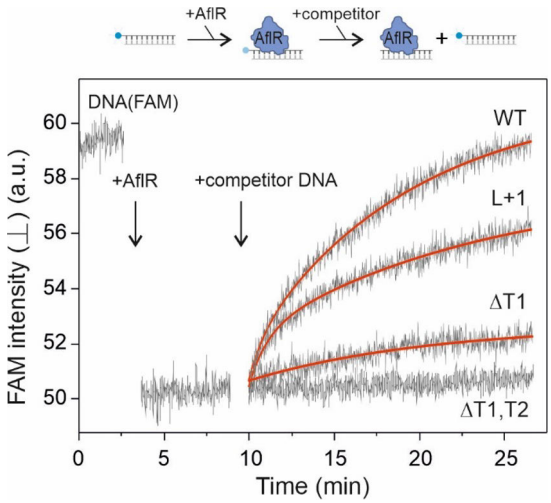

Figure 13. Kinetics and sequence determinants of AflR–DNA complex formation and stability. Fluorescence intensity decreased after AflR addition indicating the formation of AflR– DNA(FAM) complex. The addition of WT, L+1 or ΔT1 competitor DNA caused full or partial disruption of AflR–DNA(FAM) complexes as indicated by the gradual increase in intensity. Source: Article II.

4.2.4 The sequential binding of two AflR molecules to the recognition site in the DNA

We utilized a stopped flow (SF) apparatus to automate the combination of AflR from the first syringe and DNA (labelled with Cy3) from the second syringe. This method substantially reduced the mixing dead time, allowing for fluorescence intensity measurements as early as 2 ms after the reaction initiation **(Figure 14A, B)**. Various AflR concentrations (20–330 nM) were employed, while the target DNA concentration remained constant at 20 nM. The SF data exhibited a rise in Cy3 fluorescence intensity shortly (~0.1 s) after the mixing of DNA with AflR, peaking around 30 s **(Figure 14B)**. Notably, an increase in AflR concentration led to a greater fluorescence intensity, but not a faster intensity change. The two-phasic character of the SF data suggests the involvement of two molecular processes in fluorescence enhancement. The fitted parameters include two apparent rate constants (k_1^{app} and k_2^{app}; **Figure 14C**) and two amplitude factors (A_1^{app} and A_2^{app}; **Figure 14D**). The first two types of parameters are particularly informative for understanding the molecular mechanisms. The apparent rate constants exhibited different dependencies on AflR concentration **(Figure 14C)**; k_1^{app} remained constant under all tested conditions, while k_2^{app} decreased with increasing [AflR]. Notably, none of the rate constants increased with [AflR], ruling out direct reporting of the AflR binding step to the target DNA. Instead, the stepwise fluorescence enhancement results from conformational changes after the formation of the initial AflR–DNA complex. The decrease in k_2^{app} at high [AflR] supports the interpretation that k_1 and k_2 represent conformational changes triggered by the sequential binding of the first and second AflR monomer to the same DNA molecule, respectively. Alternative mechanisms, such as two distinct isomerization steps or the formation of two structurally different AflR·DNA complexes, would predict k_2^{app}, like k_1^{app}, to be independent of [AflR]. To assess the binding affinity of the first AflR molecule to the DNA(Cy3), i.e., parameter $K_{D1,SF}$, we applied **Equation 6** to the AflR concentration dependence of k_1^{app} **(Figure 14C)** and A_1^{app} **(Figure 14D)**. The two datasets, for the reasons not completely understood at the moment, yielded significantly different estimates for $K_{D1,SF}$: 40 ± 7 nM (based on k_2^{app}) and 820 ± 250 nM (based on A_1^{app}).

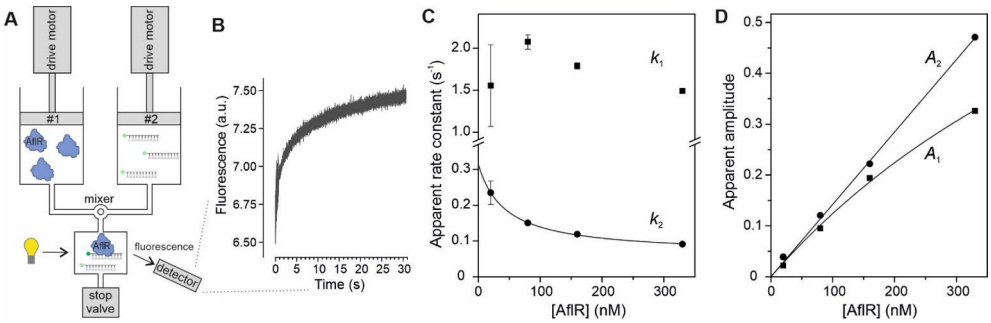

Figure 14. Pre-steady state kinetic analysis of AflR–DNA complex formation. (A) The experimental setup in the stopped flow instrument. (B) An exemplary trace demonstrates the increase in the fluorescence intensity after mixing 20 nM DNA(Cy3) with 330 nM AflR at 23°C. The apparent (C) binding rate constants, k_1^{app} and k_2^{app}, and (D) fluorescence change amplitudes, A_1^{app} and A_2^{app}, were determined by fitting a 2-phase exponential equation to the measured fluorescence intensity traces. The theoretical curves for k_2 and A_1 were generated from the fits using **Equation 6**, enabling the estimation of the dissociation constant of DNA(Cy3) in complex with one AflR molecule. The trend of A_2 is derived from a linear fit and is presented solely for visualization purposes.

4.3 Inhibition of aflatoxin production by a methanolic extract of *Zanthoxylum bungeanum* (Original publication III)

4.3.1 Antioxidant and anti-aflatoxigenic profiles of *Zanthoxylum bungeanum*

Owing to the hazardous impact of aflatoxins on human food and animal feed, it is necessary to seek alternatives to the chemical additives. One such alternative involves the utilization of plant-derived products and their formulations. These products are classified by the FDA as 'generally recognized as safe' (GRAS). They can be employed as antimicrobial agents in pre- and post-harvest applications, as well as food and feed additives. Furthermore, these plant-based products have gained significant acceptance among consumers in Europe and the USA (Pinto et al., 2023). In Article III, we used a methanolic extract of the food additive *Zanthoxylum bungeanum* to inhibit the aflatoxin production by *A. flavus*. We subsequently measured the total phenolic content (TPC), the total flavonoid content (TFC), and the total antioxidant activity of the fractions. Among the fractions, F7 and F9, denoted as EA80 and M20, respectively contained the greatest levels of TPC and TFC **(Figure 15A)**. The results from the ABTS radical scavenging assays, represented by IC50 values, also indicated that the most abundant active antioxidant compounds were found in the M20 and EA80 fractions **(Figure 15B)**. *Z. bungeanum* extracts have been reported to have extensive pharmacological effects, such as anti-inflammatory, antioxidant, anti-tumor, anti-fungal and antibacterial effects (M. Zhang et al., 2017). Also, it was discovered that the ethanol extracts derived from *Z. bungeanum*, as well as its sub-fractions (including the ethyl acetate, acetone, and methanol fractions), demonstrated high antioxidant effects on scavenging DPPH radical activity with low IC50 values (Y. Zhang, Luo, et al., 2014; Y. Zhang, Wang, et al., 2014). We next employed HPLC-MS analysis to determine the chemical constituents present in the EA80 and M20 fractions. The molecular weights of these compounds, obtained by ESI-MS, allowed preliminary identification of quercetin, epicatechin, and kaempferol-3-O-rhamnoside in both fractions. Additionally, the M20 fraction specifically contained hyperoside (quercetin-3-galactoside). To confirm the identities of these compounds, we used high-resolution mass spectroscopy (HR-MS) to determine their molecular weights with an accuracy of up to four decimals. These results confirmed the identity of all four compounds **(Figure 15C)**. Previous research conducted on *Z. bungeanum* isolated and identified over 140 chemical compounds, including alkaloids, terpenoids, flavonoids, free fatty acids, and a trace of inorganic elements. Among these compounds, flavonoids have attracted considerable attention due to their diverse pharmacological activities,

particularly their antioxidant properties. To date, more than 25 flavonoids, such as quercetin, rutin, and quercetin 3-O-α-l-rhamnoside, have been discovered in *Z. bungeanum*. The abundance of flavonoids in *Z. bungeanum* probably explains its potent antioxidant properties and, perhaps, also the positive impact of *Z. bungeanum* consumption on human health (L. C. Yang et al., 2013; M. Zhang et al., 2017; Y. Zhang, Wang, et al., 2014).

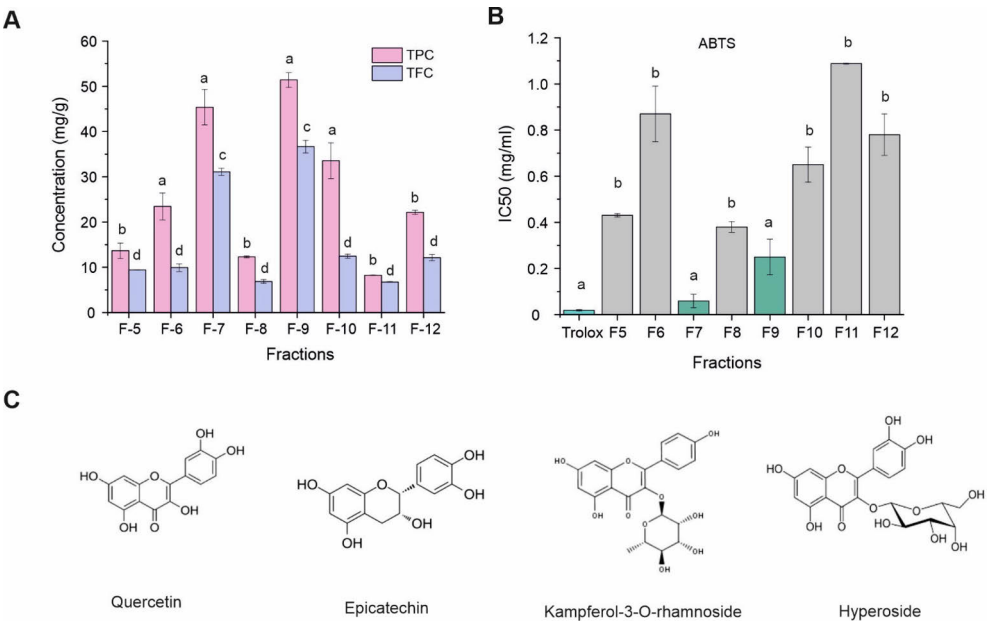

Figure 15. (A) Total phenolic content (TPC) and total flavonoid content (TFC) of *Z. bungeanum* fractions. (B) Antioxidant activities of Trolox standard and *Z. bungeanum* fractions are exhibited as IC50 values. Data are expressed as means ± S.D (n = 3). Columns indicated with the same letter are non-significant ($p > 0.05$). (C) Identification of the major compounds in M20 and EA80 fractions using ESI-MS. Based on mass-to-charge ratios (m/z), the compounds were identified as quercetin (m/z = 303.2, [M+H]+), epicatechin (290.2, M+), kaempferol-3-O-rhamnoside (433.5, [M+H]+), and hyperoside (464.4, M+). Source: Article III.

For the aflatoxin inhibition experiments, we selected the M20 fraction, which exhibited the highest phenolic and flavonoid contents. The application of various concentrations (ranging from 1.95 µg/mL to 250 µg/mL) of *Z. bungeanum* fraction on *A. flavus* spores demonstrated a dose-dependent inhibition of aflatoxin B1 synthesis. Notably, the complete suppression of aflatoxin B1 production was achieved at concentrations as low as 125 µg/mL of the M20 fraction **(Figure 16)**. In our data, the major component in M20 fraction, quercetin, has been shown to effectively suppress the production of AFB1, potentially by lowering oxidative stress levels and reducing the generation of reactive oxygen species (ROS) (X. M. Li et al.,

2019). We propose that the ability of the M20 fraction to inhibit aflatoxin production can be attributed to the presence of flavonoids in general and quercetin in particular. Castano-Duque et al., (2022) investigated the impact of three flavonoids (apigenin, luteolin, and quercetin) on the *A. flavus* growth and aflatoxin production. The findings revealed that flavonoids disrupt the integrity of the fungal cell wall and may localize in vesicle-like structures. At low concentrations, flavonoids may operate as potential signaling molecules and affect the oxidative state of the microenvironment. These effects, either individually or in combination, could contribute to the suppression of fungal proliferation and aflatoxin reduction. Furthermore, phenyl lactic acid is a natural phenolic acid, which has been found to decrease the production of AFB1 by downregulating the expression of key genes involved in AFB1 synthesis. Through a combination of transcriptomic and metabolomic analyses, it was discovered that phenyl lactic acid induced damage to the cell membrane and disrupted energy metabolism (C. Zhao et al., 2023).

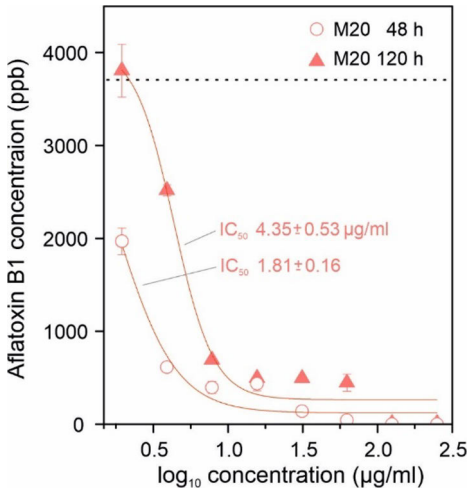

Figure 16. Effects of the M20 fraction on AFB1 production by *A. flavus* after 48 or 120 hour-culture. M20 was used at a range of 1.95-250 μg/mL concentrations. Dashed line indicates AFB1 accumulation level in the control group (without M20). Source: Article III.

4.3.2 Effect of the *Z. bungeanum* extract on the *A. flavus* gene transcription profile

Next, we tried to explore the molecular mechanisms through which the M20 extract inhibits the synthesis of AF in *A. flavus*, utilizing RNA-seq. We extracted the RNA from three control cultures (without extract) and treated cultures with 250 μg/mL of M20. At this concentration, M20 extract did not impact the proliferation of *A. flavus* but it effectively suppressed AF production. Among the total of 13,485 genes present

in the *A. flavus* reference genome, 11,062 genes (82%) were considered expressed with FPKM values ≥ 1. Of these expressed genes, 96% (10,615) were observed in both the control and test groups, while 2.3% (251) were exclusively expressed in the control group and 1.8% (196) in the test group **(Figure 17A)**. Employing criteria of FPKM fold-change ≥ 2 and a corrected *p*-value < 0.05, we identified 950 differentially expressed genes (DEGs) between the control and test groups **(Figure 17B)**. Among these DEGs, 515 genes were downregulated, and 435 genes were upregulated in the M20-treated samples. *A. flavus* contains 55 predicted secondary metabolite (SM) gene clusters (Georgianna et al., 2010) detected by SMURF software (Khaldi et al., 2010), including at least one non-gene cluster SM pathway responsible for Kojic acid production (Uka et al., 2020). Under closer examination, we analyzed the expression patterns of key "backbone" enzymes for each SM gene cluster, which typically catalyze an early crucial step in SM synthesis (Khaldi et al., 2010; X. Zhao et al., 2018). We found that 20 SM clusters showed negligible transcriptional activity in both control and treated samples (FPKM < 1 for the backbone gene). The expression of eleven SM gene clusters displayed moderate changes (**Figure 17C**, orange spheres; treated/control (T/C) ≈ 0.5–2, *p-adj* < 0.05). Three pathways were significantly upregulated (**Figure 17C**, red spheres; T/C > 2, *p*-adj < 0.05). Moreover, four pathways and the kojic acid pathway were significantly downregulated (**Figure 17C**, blue spheres; T/C < 0.5, *p-adj* < 0.05). In line with the fact that M20 extract prevented the accumulation of AFB1 in *A. flavus* cultures, M20 exhibited a suppressive effect on all genes involved in the AFB1 biosynthesis pathway (**Figure 17D**). Out of the total 19 biosynthetic enzyme genes present in the reference genome, nine showed a statistically significant decrease in the expression. Using RT-qPCR, we re-analyzed four of these genes (*hypC*, *alfW*, *aflN*, and *aflQ*), and the results indicated a significant downregulation of all four genes, consistent with RNA-seq data.

Aflatoxin biosynthesis pathway contains two specific transcriptional regulators; AflR and AflS, which are essential for AF production. Indeed, *aflR* mutant strains cannot produce AF (Price et al., 2006). Surprisingly, we found that M20 treatment did not have a significant impact on *aflR* expression, but it led to a decrease of approximately 20% in *aflS* expression. These findings suggest that rather than the pathway-specific regulators AflR or AflS, there may be involvement of global transcriptional regulators responsible for the downregulation of the AF gene cluster. VeA is a global transcriptional regulator that forms a trimeric complex called the Velvet complex with LaeA and VelB, plays a crucial role in fungal development, conidiation, primary and secondary metabolism, as well as oxidative stress responses (Lin et al., 2013). Our data showed that M20 treatment decreased the expression of both *veA* and *laeA*, resulting in a downregulation of the Velvet complex. This is likely the primary reason for the lack of AF synthesis in M20-treated cultures.

Additionally, VeA is known to regulate the expression of *brlA*, which is a regulator of early fungal development (Lin et al., 2013). Consistent with the observed downregulation of *veA* and Velvet complex, we observed a substantial reduction of 80-90% in *brlA* expression. Previous studies have shown that treatment with benzenamine led to the downregulation of *brlA* in *A. flavus* (M. Yang et al., 2019) while treatments with eugenol (Lv et al., 2018) or 5-Azacytidine (Lin et al., 2013) resulted in the upregulation of the *brlA* gene. Collectively, we suggest that the *Z. bungeanum* extract shuts down the AF synthesis by depriving the essential transcription co-activation functions of global regulators.

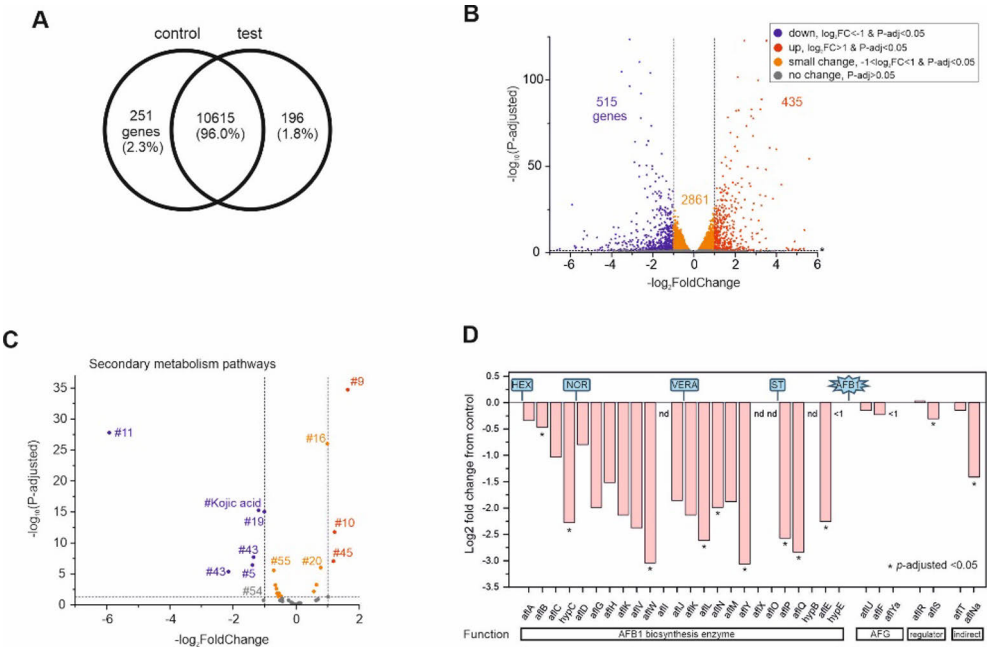

Figure 17. (A) Venn diagram exhibits the number of genes expressed in both control and treated groups (intersection) or in only one of the two groups. (B) Volcano plot categorises genes as downregulated, upregulated, small change, or no change based on their log2FoldChange and *p*-adj values. (C) M20-effect on SM pathways was assessed by analyzing the expression of pathway "backbone enzyme" genes. Volcano plot categorises SM pathways as downregulated (blue), upregulated (red), small change (orange), or no change (grey) based on the log2FoldChange and *p*-adj values. #54 denotes the AF pathway. (D) Aflatoxin biosynthetic enzymes after M20 extract treatment based on RNA-seq data. AFG genes are those that have a special role in the production of G-types. nd denotes genes that are not annotated in the reference genome. Genes having expression levels less than 1 (FPKM < 1) are denoted by <1. Source: Article III.

4.4 Biocontrol of *Fusarium graminearum* (Original publication IV)

4.4.1 *In vitro* effect of M20 extract on mycelium growth

Fusarium head blight (FHB) is a severe plant disease that causes significant declines in both yield and quality of wheat. *F. graminearum* is considered the predominant pathogen of FHB. In Article IV, we continued our investigation by studying the suppression effect of *Z. bungeanum* methanolic extract (M20) on *F. graminearum* growth and deoxynivalenol production. Furthermore, we expanded the investigation from the laboratory bench to the field conditions. In the laboratory, we used a simple *in vitro* bioassay to investigate the antifungal activity of the extract and monitored the *F. graminearum* growth in the absence **(Figure 18A)** and presence **(Figure 18B)** of 150 µg/mL of M20 extract. We used two Finnish and two Russian *F. graminearum* strains. The hyphal growth of the Finnish strains (Fg 2 and Fg 5) was inhibited by 24–25% whereas the Russian strains Fg 13 and Fg 15 were inhibited somewhat stronger by 36 % and 49 %, respectively **(Figure 18C)**.

Antioxidant effects of phenolic and flavonoid chemicals are well known, since they have the potential to scavenge free radicals and reduce the risk of chronic disorders. Among these substances is ferulic acid, a well-known phenolic acid with antifungal action against *Fusarium* species (Dykes & Rooney, 2007). Additionally, phenolic extracts derived from Spirulina have demonstrated potent antifungal properties against *Fusarium* fungi (Pagnussatt et al., 2014). A recent study has found that the limonene displayed fungicidal properties against *F. graminearum*, with an EC50 value of 8.7 µg/mL. It effectively reduced the quantity and length of conidia and caused damage to various cellular components within the hypha, including the cell membrane, cell wall, vacuoles, and organelles. Furthermore, the limonene exhibited a significant inhibitory effect on the production of DON which was correlated with a decrease in the expression of genes involved in trichothecene biosynthesis, as well as several energy metabolism pathways (Jian et al., 2023). We suggest that the antifungal activity of M20 extract against *Fusarium* species can be attributed to its increased flavonoid levels, which could potentially bind to the cell wall and activate a broad defense mechanism against plant pathogens (Schöneberg et al., 2018).

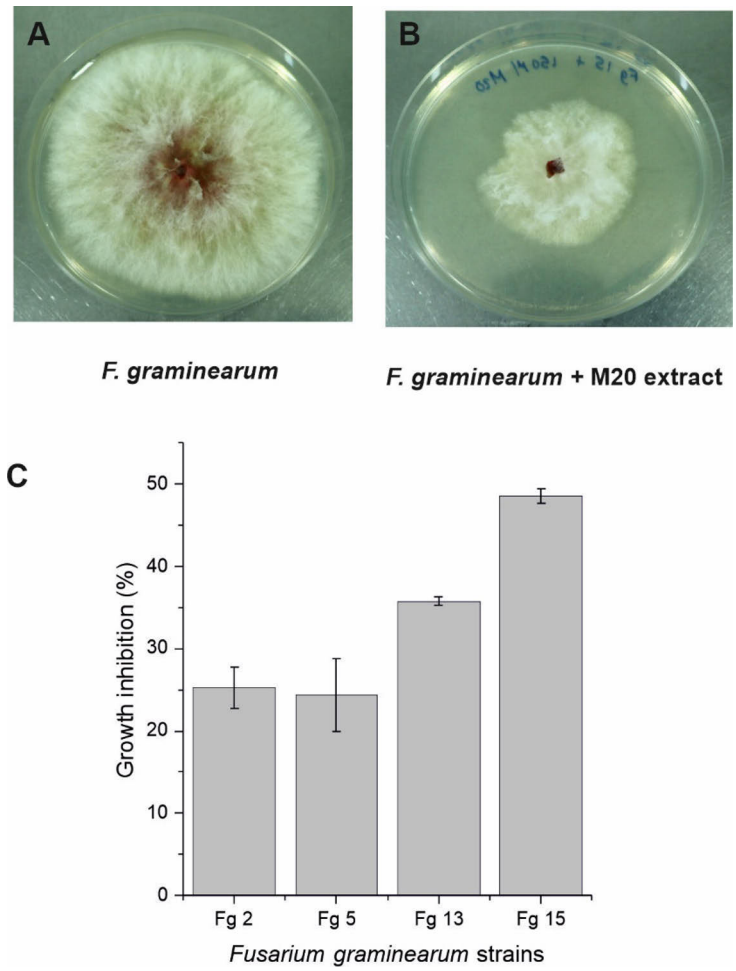

Figure 18. Antifungal activity of *Z. bungeanum* methanolic extract (M20) on *F. graminearum* species. (A) *F. graminearum* strain grew on PDA in the absence of the *Z. bungeanum* extract. (B) *F. graminearum* strain grew on PDA in the presence of 150 µg/mL of M20 extract. Both were incubated in the dark for five days. (C) Percent of growth inhibition (%) in the four tested strains. Control PDA plates were inoculated with DMSO instead of the extract. All experiments were performed in triplicates. Data are expressed as mean ± standard deviation. Fg: *Fusarium graminearum*. Fg 2 and Fg 5 are Finnish strains, Fg 13 and Fg 15 are Russian strains. Source: Article IV.

4.4.2 M20 extract significantly controlled *F. graminearum* growth in the field

The field experiment was carried out in the Southwest Finland. The point inoculation method was employed using a combination of conidia from four *Fusarium graminearum* (Fg) isolates (Boshoff et al., 2019). This study consisted of four groups: the control group received DMSO, treatment I received the mixture of Fg

conidia, treatment II received the mixture of Fg conidia followed by the application of 100 µg/mL M20 extract, and treatment III received only 100 µg/mL M20 extract. The amount of *F. graminearum* DNA was measured using qPCR, targeting the TMFg12 gene. TMFg12 assay enabled the quantification *F. graminearum* DNA. This TaqMan assay was considered to be sensitive, reproducible and highly specific for *F. graminearum* (Sarlin et al., 2006). Our qPCR results indicated that treatment group I displayed TMFg12 gene dose level above the detection limit, with amplification occurring between 23 and 37 cycles. Control group, which did not receive M20 extract or fungal treatment, exhibited minimal levels of *F. graminearum* DNA, suggesting that the natural infection level was low. The point inoculation method successfully cultivated *F. graminearum* fungi in treatment I, where a significant difference in fungal DNA quantity was observed between the control and treatment I (Fg group). The abundance of *F. graminearum* in treatments II and III did not differ significantly from the control. However, after inoculating Fg-infected wheat samples with M20 extract (treatment II), the abundance of *F. graminearum* was decreased up to 86% compared to treatment I, resulting in a significant reduction in the pathogen development in treatment II. Amount of *F. graminearum* DNA in the four treated groups is shown in **Figure 19A**. Similarly, Skadhauge and colleagues (Skadhauge et al., 1997) illustrated that the flavonoid dihydroquercetin impeded the hyphal penetration of *F. graminearum* and *F. culmorum* into the grain testa of barley. Moreover, *Lactobacillus acidophilus* ML14-synthesized selenium nanoparticles (SeNPs) which have potent antioxidant and antifungal properties, effectively controlled the growth of *F. culmorum* and *F. graminearum*, and hence mitigating drought and heat stress in wheat plants. These biological activities were associated with the small size of biological SeNPs and the phenolic content in their suspension. (El-Saadony et al., 2021). Another flavonoid, 5-hydroxy-7,4'-dimethoxyflavone, extracted from *Combretum erythrophyllum* leaves, demonstrated inhibitory effects against various *Fusarium* species, including *F. graminearum*, with a minimum inhibitory concentration (MIC) value of 0.63 mg/mL (Seepe et al., 2021). *Tetradenia riparia* crude extract which was known to contain terpenes, anthocyanins, flavonoids, tannins and phenolic acids, showed antifungal activity against five different fungal species including *F. graminearum* (Scanavacca et al., 2022).

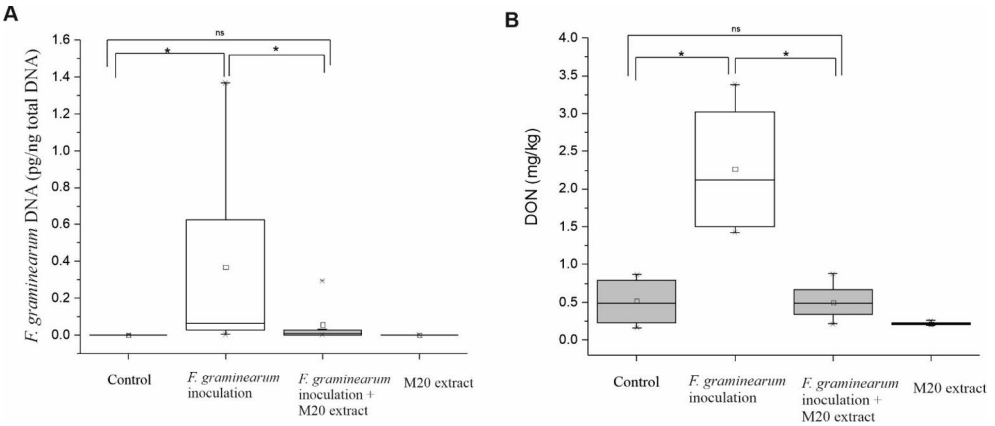

Figure 19. Inhibitory effect of M20 extract on *F. graminearum* DNA and DON levels in the four treated groups; control, treatment I (*F. graminearum* inoculation), treatment II (*F. graminearum* inoculation followed by M20 extract), and treatment III (M20 extract). (A) Suppression effect of M20 extract on *F. graminearum* DNA level (pg/ng total DNA) in wheat grains. (B) Effect of M20 extract on DON (mg/kg) levels in wheat. ANOVA analysis was performed and Tukey test used to recognize treatments significantly different from treatment group I. Asterisk indicates statistically significant differences ($p < 0.05$) between groups. ns; non-significant ($p > 0.05$). Source: Article IV

4.4.3 Inhibition of deoxynivalenol synthesis by M20 extract

Next, we tried to explore whether co-incubation of M20 extract would supress deoxynivalenol (DON) production by *F. graminearum* under field conditions. Acetonitrile - water mixture (v/v, 80:20) was used to extract DON from the ground wheat samples. Wheat heads in treatment I (which exposed to *F. graminearum* mix) exhibited the highest quantity of DON (2.3 ± 0.8 mg/kg) and it was significantly higher than the control group (DMSO group; 0.5 ± 0.3 mg/kg). Conversely, there were no notable differences between the control group and treatments II (administered with a mixture of Fg conidia and M20 extract) and III (received M20 extract). The application of M20 extract led to a significant 73% reduction in DON content in treatment II compared to treatment I. Generally, treatment III (exposed to M20 extract only) exhibited lower DON levels compared to the other experimental groups. DON levels for all treated groups are shown in **Figure 19B**. These findings suggest that the M20 extract suppresses fusarium head blight by inhibiting DON biosynthesis. Previous studies have proposed that the antioxidant action of phenolic/flavonoid compounds may be associated with inhibiting mycotoxin production (Jayashree & Subramanyam, 1999). For instance, Schöneberg et al., (2018) reported that quercetin had a significant reducing effect on neosolaniol and diacetoxyscirpenol mycotoxins. A recent *in vivo* study found that quercetin had a suppressive effect on intestinal inflammation induced by DON as it inhibited the TLR4/NF-κB signaling pathway. Also, quercetin reduced the oxidative stress caused

by DON by increasing superoxide dismutase (SOD) and glutathione (GSH) levels, and decreasing the levels of malondialdehyde (MDA) (Ye et al., 2023). Furthermore, gallic, caffeic, and p-coumaric acids were shown to lower mycotoxin levels produced by toxigenic fungal species (Gauthier et al., 2016; Pagnussatt et al., 2014). Moreover, plant extracts from cinnamon, clove, lemongrass, oregano, and palmarosa were found to decrease the accumulation of DON in grains infected with *F. graminearum* (Marín et al., 2004). The biosynthesis pathway of DON involves a series of steps. Initially, the cyclization of the sesquiterpene ring takes place, a process facilitated by the enzyme trichodiene synthase. Subsequently, eight oxygenation reactions and four esterification reactions occur, leading to the formation of DON and its acetylated intermediates (Garda-Buffon et al., 2010; Kolawole et al., 2021). The inhibition of DON biosynthesis by phenolic substances might be related to metabolic repression, which could need the development of a network of regulatory genes. Overall, our findings suggest that M20 is a potential biocontrol agent for managing FHB and DON in wheat.

4.5 Novel method for AFB1 detection (Original publication V)

4.5.1 Noncompetitive immunoassay

Study V developed a rapid noncompetitive immunoassay for aflatoxin detection in just 15 min and with a detection limit of 70 pg/mL. Aflatoxin B1 is traditionally considered as a small molecule, which cannot be simultaneously be bound by two antibodies in the classic noncompetitive immunoassay. However, significant advancements in the antibody engineering have led to the discovery of antibody binders, which made it possible to use the noncompetitive assays even for small molecules. For instance, anti-immunocomplex (anti-IC) antibodies exhibit a distinct ability to selectively bind to antibody-antigen complex. This unique characteristic facilitates the creation of two-site noncompetitive sandwich-type immunoassays (Y. Li et al., 2018). Here, we employed the synthetic antibody library to choose scFv (single chain variable fragment) antibody that can detect the immunocomplex (IC) formed by the monoclonal anti-AFB1 capture antibody and AFB1. These anti-immunocomplex (anti-IC) binders were prepared by phage display method. Biotinylated anti-AFB was immobilized on the surface of magnetic beads coated with streptavidin and saturated with AFB1. In order to prevent an increase in non-specific binders to streptavidin or biotin, anti-AFB was directly linked to magnetic beads coated with epoxy. These beads were also saturated with AFB1. Furthermore, a screening was carried out to eliminate antibodies that solely targeted anti-AFB, and non-specific soluble mouse IgG was introduced to hinder the enrichment of binders that could recognize anti-AFB in the absence of AFB1. All scFv binders fused with bacterial alkaline phosphatase (scFv-AP) were tested for their binding toward the anti-AFB and AFB1 complex. The most promising binder exhibited the highest signal-to-background ratio (in presence vs. absence of AFB1) of 14 in the assay. This binder was cloned, produced in a large scale, purified with metal affinity chromatography using the His-tag included in the scFv-AP fusion and employed as anti-IC binder for the development of AFB1 detection assay.

The single-step noncompetitive immunoassay was based on a biotinylated monoclonal capture antibody (anti-AFB) that was immobilized on a microtiter plate coated with streptavidin. In the same single step, the immunocomplex, comprising the anti-AFB and the target AFB1, was identified by the anti-IC scFv-AP binder, which was subsequently detected through the Europium (Eu)-labeled anti-AP antibody **(Figure 20A)**. After the incubation period, the wells were washed, the europium fluorescence intensifier (EFI) solution was added, and the time-resolved fluorescence signals were recorded. The amount of antibodies was optimized and finally 50 ng of biotinylated anti-AFB antibody and 1 µg of scFv-AP were used in

the experiments. In addition, with the 10-min incubation, the EC50 value for AFB1 was determined to be 3.5 ng/mL based on a four-parametric logistic fit. The sensitivity of the assay in terms of the limit of detection (LOD) of AFB1 was 70 pg/mL (0.22 nM) calculated from blank signal + 3 × the standard deviation of the blank **(Figure 20B)**. The sensitivity of this novel immunoassay is similar to immunoassays for aflatoxins that have been documented previously. While certain techniques in the literature, such as those relying on photoelectrochemical detection (Pei et al., 2021), have demonstrated markedly lower detection limits, they frequently depend on long assay procedures, long incubation durations, or steps involving signal amplification. In contrast, our approach was based on a simple and rapid assay protocol.

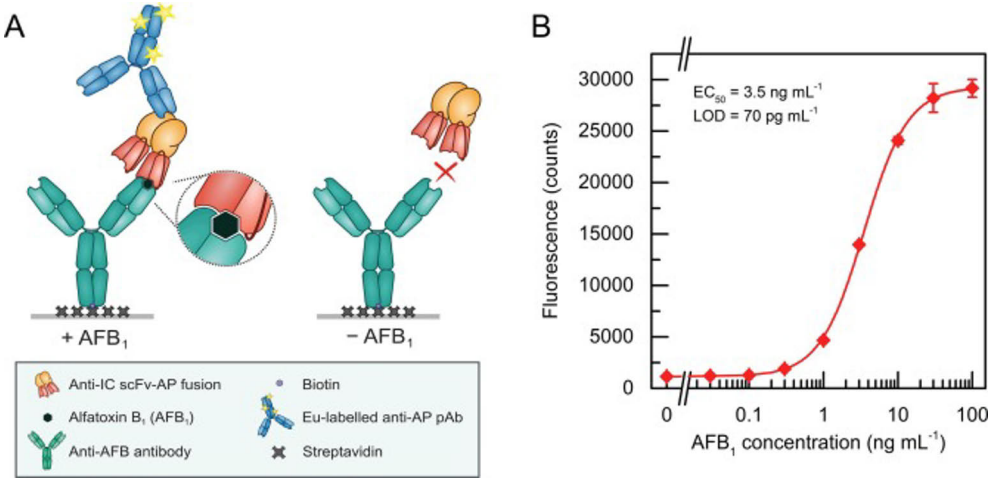

Figure 20. Single-step noncompetitive assay for AFB1 determination. (A) Schematic illustration of the assay based on the anti-immunocomplex antibody in fusion with alkaline phosphatase (anti-IC scFv-AP) that recognizes the monoclonal anti-AFB antibody and AFB1 complex. The detection is based on the time-resolved fluorescence of the Eu-chelate labelled anti-alkaline phosphatase polyclonal antibody (anti-AP pAb). (B) Standard curve of the immunoassay. The measured time-resolved fluorescence signals are represented as the average of three replicates ± standard deviation. The EC50 value was determined by the four-parametric logistic fit and the LOD was calculated as the signal of blank + 3 × standard deviation of the blank. Source: Article V.

4.5.2 Assay validation for food samples

To demonstrate the utility of our immunoassay for quantitative detection of AFB1 in real food samples, three kinds of samples, including maize flour, rice flour, and hazelnut, were spiked with four different concentrations of AFB1. Natural non-spiked control and AFB1 spiked samples were subjected to methanol extraction to

extract AFB1. Control samples were free of aflatoxins as determined by HPLC. In the spiked samples, the AFB1 concentrations and recoveries ($[AFB1]_{determined}/[AFB1]_{spiked}$) were determined by two methods, the novel single-step immunoassay and reference HPLC method. As shown in **Table 6**, the recoveries of AFB1 ranged from 83% to 129% with relative standard deviations (RSD) of 1–4%. These results indicated that both the immunoassay and HPLC were accurate for the AFB1 analysis in the three kinds of samples. Validation with HPLC has also been used in literature (Zou et al., 2022).

Table 6. Recoveries of AFB1 from spiked maize flour, rice flour, and hazelnut samples with the single-step immunoassay and HPLC. Source: Article V.

SAMPLE	SPIKED AFB1 (µG/KG)	IMMUNOASSAY		HPLC	
		Detected AFB1 ± SEM (µg/kg)	Recovery	Detected AFB1 ± SEM (µg/kg)	Recovery
MAIZE FLOUR	20	25.7 ± 0.7	129%	21 ± 7	106%
	30	30.9 ± 0.7	103%	31 ± 3	105%
	50	47 ± 1	94%	54 ± 17	107%
	100	112 ± 9	112%	113 ± 9	113%
RICE FLOUR	20	19.4 ± 0.4	97%	16 ± 4	82%
	30	28 ± 1	94%	30 ± 13	101%
	50	51 ± 2	101%	67 ± 31	135%
	100	99 ± 1	99%	92 ± 14	92%
HAZELNUT	20	16.6 ± 0.1	83%	28 ± 9	140%
	30	28.5 ± 0.5	95%	32 ± 2	106%
	50	45 ± 1	90%	52 ± 5	103%
	100	91 ± 4	91%	91 ± 6	91%

SEM, standard error of the mean (n = 3).

4.5.3 Cross-reactivity with other mycotoxins

Aflatoxin B1 is commonly the most abundant aflatoxin among the different aflatoxin types. However, agricultural products can contain aflatoxins B1, B2, G1, and G2, making it advantageous if our method can detect these aflatoxins too. The specificity of the immunoassay was assessed by determining its cross-reactivity with related aflatoxins. Cross-reactivity with various aflatoxin types varied from 3% (AFM1) to 89% (AFG1). The dose–response curves for AFG1 obtained by the immunoassay showed excellent cross-reactivity and were not very different from that for AFB1. AFM1 is the hydroxylated aflatoxin metabolite present in the milk of mammals consuming aflatoxin-contaminated feed (Rushing & Selim, 2019), but it is not

typically found in grains or nuts. In terms of carcinogenic potential, AFB1 and AFG1 are considered equally potent. Comparatively, AFG2 and AFB2 are less toxic than AFB1, occurring less frequently in agricultural products and generally being absent in the absence of AFB1. Furthermore, other tested mycotoxins, ochratoxin A (OTA) and deoxynivalenol (DON), exhibited negligible cross-reactivity **(Figure 21)**, affirming the high specificity of the assay for aflatoxins. A previously reported noncompetitive ELISA method was able to detect the four types of AFs (AFB1, AFB2, AFG1 and AFG2) by employing the existing cross-reactivity of an anti-AFB1 antibody (Acharya & Dhar, 2008). On the contrary, the noncompetitive immunoassay developed by Zou et al., (2022) found negligible cross reactivity between AFB1 and other aflatoxin types. The cross-reactivity of AFB2, G1, G2 and M1 was 18.03%, 19.57%, 7.83% and 25.97%, respectively.

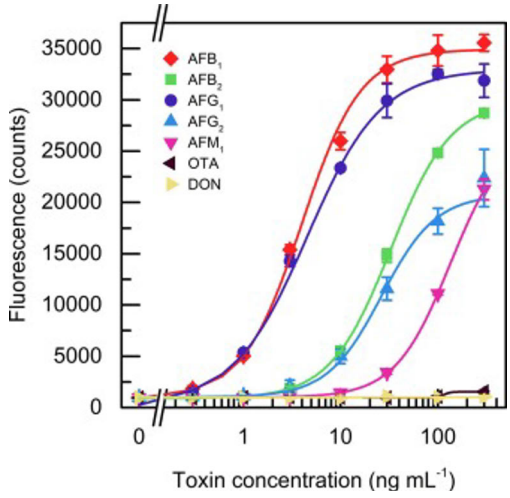

Figure 21. Cross-reactivity of the single-step immunoassay with different aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1) and other, ochratoxin A (OTA) and deoxynivalenol (DON). The measured time-resolved fluorescence signals are showed as the average of three replicates ± standard deviation. Source: Article V.

5 Conclusion and Future Perspective

Aflatoxins are cancerous secondary metabolites mainly produced by *Aspergillus* fungi under certain conditions of nutrients, temperature and humidity. Consumption of aflatoxins is toxic to both animals and humans as they can cause liver lesions, liver cancer, or even death. *Aspergillus* section *Flavi* can be divided into two groups based on their toxicity. First group comprises the aflatoxigenic species, specifically *A. flavus*, *A. parasiticus* and *A. nomius*, while the second group includes the non-aflatoxigenic species such as *A. tamarii*, *A. oryzae* and *A. sojae* (Norlia et al., 2019a). Some *Aspergillus* species share morphological similarities, which can lead to misidentification. Therefore, molecular-based methods have been used to validate the morphological identification because the precise identification of aflatoxigenic *Aspergillus* strains is needed to trigger timely control strategies against fungal growth and aflatoxin production during the crop cultivation and storage, as well as to develop improved versions of the control procedures. Here, we used a polyphasic approach comprises of phylogenetic, molecular and toxin analyses, and applied it to forty *Aspergillus* isolates with the aim of differentiating between aflatoxin producing and non-producing fungi. We found the molecular pattern of RAPD and ISSR markers to be beneficial in evaluating the genetic diversity and phylogenetic relationships of the *Aspergillus* isolates.

Maximum allowable aflatoxin quantities in food and feed stuffs have been established worldwide (M. Abbas, 2021). Because these raw materials and products are used in large quantities in very many manufacturing sites, the analytic methods used to monitor aflatoxin levels to ensure regulatory compliance must be simple and have high-throughput. To facilitate progress towards improved applications, we developed a new noncompetitive immunoassay for rapid detection of aflatoxins in food products. The assay is based on the specific recognition of the immobilized capture antibody–aflatoxin complex by a specific binder protein (anti-IC binder with alkaline phosphatase fusion protein) and finally the binding of the europium labelled secondary antibody to the AP part of the anti-IC. As a key step, we developed a novel anti-IC binder using phage display for binding specificity and affinity selection. This final assay involves only a single incubation step, during which all the three antibody reagents (biotinylated monoclonal antibody, anti-IC binder, and europium-labelled

secondary antibody) are simultaneously added. The assay takes only 15 min to perform and has high specificity and low AFB1 detection limit (70 pg/mL).

The aflatoxin biosynthesis gene cluster comprises of at least 30 genes, organized within an 80-kilobase genome region. The cluster contains two cluster-specific transcriptional regulators, AflR and AflS, that stimulate the expression of the other genes essential for the synthesis of aflatoxins (Price et al., 2006). Understanding the function of AflR and AflS is pivotal for targeted interventions to mitigate toxin accumulation in food and feed. We developed protein purification protocols for AflR and AflS to study their function in detail at the molecular level. We observed *in vitro* protein-protein interaction between AflR and AflS. We found that AflR binds to predicted target sites but AflS does not have a significant DNA binding affinity by its own. However, in the presence of AflS, the binding affinity of AflR to DNA consistently decreased by two-fold. AflS thus appears to act as the apparent negative modulator of the DNA binding of AflR. One speculative consequence is that down-tuning of the DNA binding affinity of AflR by AflS would preferentially release AflR from unspecific binding sites in the chromatin DNA and thus target higher fraction of AflR–AflS complexes in the cell to the promoters of AF gene cluster. Future studies should also investigate whether AflS can facilitate AflR to recruit additional regulatory TF's and/or core components of RNA polymerase II apparatus to promoters and thus promote the expression of AF biosynthetic genes.

There are many strategies to deactivate *Aspergillus* growth and disturb aflatoxin biosynthesis; irradiation, chemical agents, resistant varieties, and microorganisms (Ma et al., 2017; Moon et al., 2018; Y. Wu et al., 2021; Xing et al., 2017). However, these methods can be expensive and cause side effects, including resistant strains, nutrition loss and toxic residues. Medicinal plant extracts have obtained high attention in controlling fungal growth as well as mycotoxin contamination due to their high efficiency. We demonstrated that simple organic extracts derived from *Z. bungeanum* are rich in flavonoids and possess the ability to inhibit AF production. The application of this extract led to significant alterations in the transcriptional activity of *A. flavus* SM pathways, including suppression of the AF pathway, oxylipin-dependent cellular signaling, cellular development, and the oxidative stress pathway **(Figure 22)**. The repression of the AF pathway was found to be closely associated with the downregulation of global transcriptional regulators, including the Velvet complex. Our result suggests that the *Z. bungeanum* extract effectively disrupts the AF pathway by impeding *A. flavus* of the crucial transcriptional co-activation functions provided by global regulators.

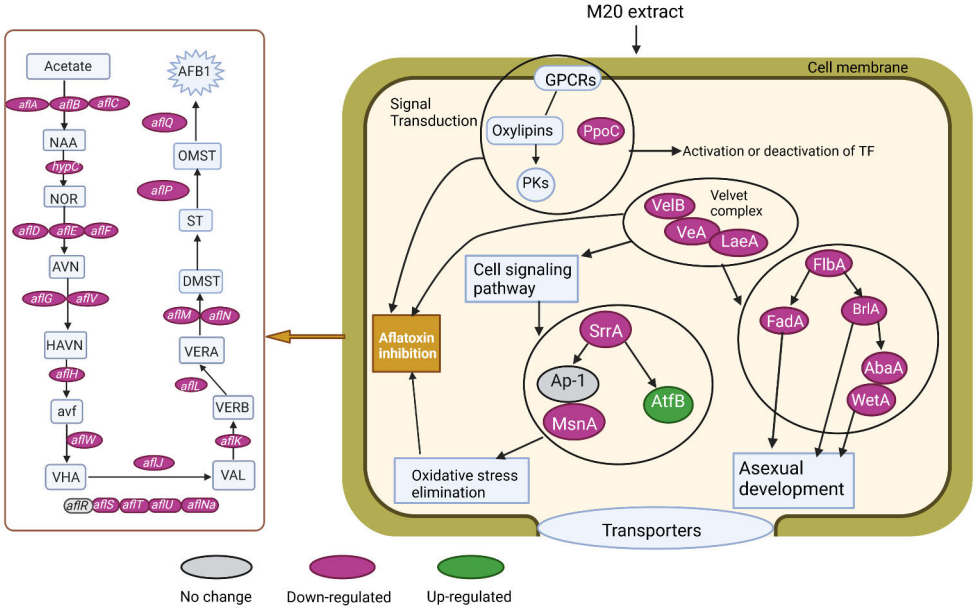

Figure 22. The proposed mechanism by which the methanolic *Z. bungeanum* extract (M20) counteracts aflatoxin production. The extract diminishes the activity of oxylipin-dependent cellular signaling, cellular development, the secondary metabolism regulator Velvet complex, and the oxidative stress pathway. These alterations induced by M20 result in the deactivation of the genes involved in aflatoxin biosynthesis, thereby disrupting the enzymatic processes required for aflatoxin production. PKs, protein kinases; TF, transcription factor. Source: Article III.

The efficacy of *Z. bungeanum* extract is not limited just against *Aspergillus*. We found in a follow-up study that the extract inhibited the growth of different *F. graminearum* strains in the laboratory and, importantly, also in the field conditions. The inhibition of DON mycotoxin accumulation was even stronger (by about 1.5-fold) than fungal growth inhibition. Altogether, our findings suggest that natural extracts sourced from *Z. bungeanum* hold promise for the development of cost-effective and safe strategies aimed at limiting fungal growth as well as mycotoxin production. Future studies may also fractionate the *Z. bungeanum* extract further and identify which exact compounds or compound combinations are responsible for the antifungal and toxin suppression effects.

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