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# Actinobacteria for the control of phytopathogenic and/or mycotoxinogenic fungi of cereals

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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Microbiologie et Biotechnologie

École doctorale GAIA - Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau  
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Unité de recherche UMR 095 QualiSud

## ACTINOBACTERIA FOR THE CONTROL OF PHYTOPATHOGENIC AND/OR MYCOTOXINOGENIC FUNGI OF CEREALS

Présentée par Ixchel Campos Avelar  
Le 22 juin 2021

Sous la direction de Sabine Schorr-Galindo

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## ABSTRACT

Wheat is the main cereal crop consumed in Europe. Its contamination with fungal pathogens is one of the main threats, leading to massive yield losses and the potential occurrence of mycotoxins. *Puccinia triticina* causes leaf rust, *Magnaporthe oryzae* causes wheat blast, Septoria nodorum blotch and Septoria tritici blotch are caused by *Parastagonospora nodorum* and *Zymoseptoria tritici*, respectively, Fusarium head blight is caused by *Fusarium graminearum* and *Fusarium verticillioides* emerges as a wheat pathogen. *Aspergillus flavus* and *Penicillium verrucosum*, develop mostly during grain storage. The decrease on chemical fungicides has motivated the search for alternatives for fungal management. *Streptomyces* genus are promising as biocontrol agents (BCAs) due to their wide production of bioactive secondary metabolites and enzymes, but also due to their capacity to detoxify mycotoxins. In this study, the impact of *Streptomyces* isolates on fungal growth and mycotoxin accumulation was evaluated. Maximal decreases of fungal growth varied from 66% (*F. graminearum*) down to 10% of the control (*P. nodorum*). Mycotoxin accumulation was decreased from 35% of the control (DON), to a complete elimination (AFB<sub>1</sub>), while some isolates caused an overaccumulation. Bacterial Cell Free Extracts (CFEs) were in general less efficient at decreasing fungal growth, and DON, AFB<sub>1</sub> and OTA accumulation. Sometimes causing a strong overproduction (at least 8 times more fumonisins than the control). This highlights the importance of assessing the impact on mycotoxins when evaluating BCAs. OTA was strongly degraded by bacterial isolates mainly in liquid medium (down to 38% of the control on average), while AFB<sub>1</sub> was degraded in solid and liquid medium and by bacterial CFEs (33%, 43% and 69% of the control on average, respectively). Moreover, the evaluation of AFB<sub>1</sub> residual genotoxicity indicated an effective detoxification down to 27% of the control. Clustering analysis and Pearson correlations allowed to classify the bacterial isolates according to their performances and to evoke possible antagonistic mechanisms, such as the production of antifungal compounds, mycotoxin degrading enzymes and potential inhibitors of mycotoxin biosynthesis. Bacteria colonized wheat leaves during *in planta* assays and remained viable after 1 week. However, only *S. griseoviridis* from Mycostop®, protected wheat against the tested fungi. This work presents an integrated approach for the screening of potential BCAs, focusing on the global analysis of the results to elucidate antagonistic mechanisms and to identify the most promising isolates.

**Key words:** Fungal phytopathogens, *Streptomyces*, biocontrol, mycotoxins, detoxification, clustering analysis.

## RESUME

Le blé est la principale céréale consommée en Europe. Sa contamination par des pathogènes fongiques est une des menaces principales, provoquant des pertes de rendement et la potentielle apparition des mycotoxines. *Puccinia triticina* produit la rouille brune, *Magnaporthe oryzae* la pyriculariose, Septoria nodorum blotch et Septoria tritici blotch sont provoquées par *Parastagonospora nodorum* et *Zymoseptoria tritici*, respectivement, la fusariose est produite par *Fusarium graminearum* et *Fusarium verticillioides* émerge comme pathogène du blé. *Aspergillus flavus* et *Penicillium verrucosum*, se développent principalement lors du stockage des graines. La diminution des fongicides chimiques a motivé la recherche des alternatives pour le contrôle des champignons. Le genre *Streptomyces* est prometteur comme des agents de biocontrôle (BCAs) grâce à leur importante production des métabolites secondaires bioactifs et des enzymes, mais aussi due à leur capacité à détoxifier des mycotoxines. Dans cette étude, l'impact des isolats de *Streptomyces* sur la croissance fongique et l'accumulation des mycotoxines a été évaluée. Des réductions maximales de la croissance fongique ont varié entre 66% (*F. graminearum*) et 10% du contrôle (*P. nodorum*). L'accumulation des mycotoxines a été diminuée de 35% du contrôle (DON) jusqu'à l'élimination complète (AFB<sub>1</sub>), tandis que certains isolats ont provoqué une suraccumulation. Des surnageants bactériens ont été en général moins efficaces pour réduire la croissance fongique et l'accumulation de DON, AFB<sub>1</sub> et OTA, provoquant parfois des importantes surproductions (au moins 8 fois plus de fumonisines que témoin). Ceci met en évidence l'importance d'évaluer l'impact sur les mycotoxines lors des évaluations des BCAs. L'OTA a été fortement dégradée par des isolats bactériens, principalement en milieu liquide (réduction jusqu'à 38% du contrôle en moyenne), tandis que l'AFB<sub>1</sub> a été dégradée en milieu solide, liquide, et par les surnageants (33%, 43% et 69% du contrôle en moyenne, respectivement). De plus, l'évaluation de la toxicité résiduelle de l'AFB<sub>1</sub> montrait une détoxification efficace, réduction jusqu'à 27% du contrôle. L'analyse du Clustering et des corrélations de Pearson a permis de classer les isolats bactériens en fonction de leurs performances et d'évoquer des possibles mécanismes antagonistes, tels que la production des composés antifongiques, des enzymes dégradatrices de mycotoxines et des potentiels inhibiteurs de la biosynthèse des mycotoxines. Les bactéries ont colonisé les feuilles du blé lors des essais *in planta* et sont restées viables pendant une semaine. Néanmoins, seulement *S. griseoviridis* du Mycostop®, a protégé le blé contre les champignons testés. Ce travail présente une approche intégrée pour le criblage des potentiels BCAs, ciblant l'analyse globale des résultats pour élucider les mécanismes antagonistes et identifier les isolats les plus prometteurs.

**Mots clés :** phytopathogènes fongiques, *Streptomyces*, biocontrôle, mycotoxines, détoxification, clusterisation.



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## PUBLICATIONS AND COMMUNICATIONS

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### Research articles published or under revision

1. Minimizing ochratoxin A contamination through the use of actinobacteria and their active molecules.

Ixchel Campos-Avelar, Alexandre Colas de la Noue, Noel Durand, Blandine Fay, Véronique Martinez, Angélique Fontana, Caroline Strub and Sabine Schorr-Galindo.

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2. *Aspergillus flavus* growth inhibition and Aflatoxin B<sub>1</sub> decontamination by *Streptomyces* isolates and their metabolites.

Ixchel Campos-Avelar, Alexandre Colas de la Noue, Noel Durand, Guillaume Cazals, Véronique Martinez, Caroline Strub, Angélique Fontana, Sabine Schorr-Galindo.

Toxins 2021, 13(5), 340; <https://doi.org/10.3390/toxins13050340>

3. Biocontrol of wheat phytopathogens using *Streptomyces* spp.

Ixchel Campos-Avelar, Alexandre Colas de la Noue, Aurélie Ducasse, Monique Royer, Stéphane Cociancich, Angélique Fontana, Caroline Strub, Jean Benoit Morel, Sabine Schorr-Galindo.

Submitted to Biological Control.

### Scientific Congress (Oral communications and posters)

1. Natural Products and Biocontrol 2018. International Congress. Perpignan, France. Poster.

“Potentialities of actinomycetes from soils for the development of novel biocontrol products to manage cereals fungal diseases and mycotoxins risk”.

I. Campos, A. Colas de la Noue, C. Strub, A. Fontana, N. Durand, S. Schorr-Galindo.

2. Microbiocccitanie 2019. French Congress. Montpellier, France. Poster.

“Biocontrol of fusariotoxins: Strategy of BCA development from a rationalized screening to deciphering mechanisms of action”.

Caroline Strub, Alexandre Colas De La Noue, Angélique Fontana, Lucile Pellan, Ixchel Campos, Stéphane Conciacich, Monique Royer, Jean-Benoît Morel, Florentin Constancias, Noël Durand, Sabine Galindo.



3. World Mycotoxin Forum 2019. International Congress. Belfast, Ireland. Poster.  
“Ability of soil actinobacteria to avoid and biodegrade aflatoxin B<sub>1</sub> and ochratoxin A”.  
Ixchel Campos-Avelar, Alexandre Colas de la Noue, Caroline Strub, Angélique Fontana,  
Noel Durand, Sabine Schorr-Galindo.
4. World Mycotoxin Forum 2019. International Congress. Belfast, Ireland. Poster.  
“Biocontrol Agents of Mycotoxigenic Fusarium: Elucidating Mechanisms of Action”.  
Lucile Pellan, Ixchel Campos-Avelar, Caroline Strub, Noël Durand, Angélique Fontana,  
Sabine Schorr- Galindo.
5. PlantBioProTech 2019. International Congress. Marrakech, Morocco. Oral communication.  
“Antagonistic ability of soil actinobacteria to control fungal pathogens and mycotoxins on  
wheat”.  
Ixchel Campos Avelar, Alexandre Colas de la Noue, Angélique Fontana, Caroline Strub,  
Noel Durand, Jean Benoit Morel, Monique Royer, Stéphane Cociancich , Sabine Schorr-  
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6. Journées Mycotoxines 2020. French Congress. Brest, France. Oral communication.  
“Antagonistic capacity of soil actinobacteria against fungal pathogens and mycotoxin  
occurrence”.  
Ixchel Campos Avelar, Alexandre Colas de la Noue, Angélique Fontana, Caroline Strub,  
Noel Durand, Sabine Schorr-Galindo.
7. Journées Mycotoxines 2020. French Congress. Brest, France. Poster and short  
communication.  
“Biocontrol Agents of Mycotoxigenic Fungi: Toolbox for Elucidating Mechanisms of  
Action”.  
Lucile Pellan, Ixchel Campos-Avelar, Caroline Strub, Noël Durand, Angélique Fontana,  
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## List of abbreviations

<b>3-ADON</b>	3-adenosyldeoxynivalenol
<b>15-ADON</b>	15-adenosyldeoxynivalenol
<b>AFB<sub>1</sub></b>	Aflatoxin B <sub>1</sub>
<b>AFB<sub>2</sub></b>	Aflatoxin B <sub>2</sub>
<b>AFM<sub>1</sub></b>	Aflatoxin M <sub>1</sub>
<b>AFG<sub>1</sub></b>	Aflatoxin G <sub>1</sub>
<b>aW</b>	Water activity
<b>BCA</b>	Biocontrol agent
<b>C-</b>	Carbon
<b>CA</b>	Cellulose acetate
<b>CAS</b>	Chrome azurol sulphonate
<b>CFEs</b>	Cell free extracts



<b>CYA</b>	Czapek yeast agar
<b>CYB</b>	Czapek yeast broth
<b>DMIs</b>	Demethylation inhibitors
<b>DNA</b>	Deoxyribonucleic acid
<b>DON</b>	Deoxynivalenol
<b>DOM-1</b>	De-epoxy-deoxynivalenol
<b>ESI</b>	Electrospray ionisation
<b>EU</b>	European Union
<b>FAO</b>	Food Agriculture Organization
<b>FB<sub>1</sub></b>	Fumonisin B <sub>1</sub>
<b>FB<sub>2</sub></b>	Fumonisin B <sub>2</sub>
<b>FDA</b>	Food Drug Administration
<b>FER</b>	Fusarium ear rot
<b>FHB</b>	Fusarium head blight
<b>HPLC</b>	High-Pressure Liquid Chromatography
<b>IAA</b>	Indole-3-acetic acid
<b>IARC</b>	International Agency for Research on Cancer
<b>ISP2</b>	International <i>Streptomyces</i> Project medium 2
<b>ISP4</b>	International <i>Streptomyces</i> Project medium 4
<b>LR</b>	Leaf rust
<b>MoT</b>	<i>Magnaporthe oryzae Triticum</i> pathotype
<b>MT</b>	Million tons
<b>NIV</b>	Nivalenol
<b>OTA</b>	Ochratoxin A
<b>OTB</b>	Ochratoxin B
<b>OTC</b>	Ochratoxin C
<b>OT<math>\alpha</math></b>	Ochratoxin $\alpha$
<b>PES</b>	Polyethersulfone
<b>PDA</b>	Potato dextrose agar
<b>PGPR</b>	Plant growth promoting rhizobacteria
<b>PSU</b>	Percentage siderophore unit
<b>PTFE</b>	Polytetrafluoroethylene
<b>PVK</b>	Pikovskaya's medium
<b>QoIs</b>	Quinone outside inhibitors
<b>RASFF</b>	Rapid Alert for Food and Feed
<b>RNA</b>	Ribonucleic acid
<b>ROS</b>	Reactive oxygen species
<b>SDHIs</b>	Succinate dehydrogenase inhibitors
<b>SEM</b>	Scanning Electron Microscopy
<b>SNB</b>	Septoria nodorum blotch
<b>STB</b>	Septoria tritici blotch
<b>VOCs</b>	Volatile organic compounds
<b>WB</b>	Wheat blast
<b>ZEN</b>	Zearalenone



# 1. General introduction

Cereals are the main staple food for most communities around the world, they represent an important source of energy, fiber, proteins and lipids. Maize, wheat, barley, rice and oat are the main cereals consumed worldwide. Cereals crops suffer from a series of diseases provoked by insects, bacteria and fungi. Fungal contamination in particular leads to massive losses on yield and grain quality, and to the potential occurrence of toxic fungal metabolites that can remain active throughout the production chain, down to the finished products. These toxic metabolites, called mycotoxins, constitute a critical hazard due to their detrimental impact on human and animal health.

As good agricultural practices are insufficient to mitigate plant diseases and mycotoxins risk, the use of chemical fungicides is nowadays the main tool to counter fungal development. However, their negative impacts on the environment and on human health, as well as the pathogenic resistance they generate, have urged the scientific community to develop new strategies for crop disease management. For instance, biocontrol by beneficial microorganisms represents an environmental-friendly alternative to chemical treatments.

*Streptomyces* are soil bacteria with great potential for agriculture due to their capacity to produce a wide variety of secondary metabolites, including antimicrobial agents and enzymes. In addition, they apply a series of antagonistic mechanisms that confer them the ability to combat pathogenic fungi. Furthermore, *Streptomyces* beneficial impacts on plant development have been largely documented, due to their capacity to stimulate plant defense mechanisms, and to promote their growth and fitness. Moreover, their production of enzymes confers them potential as mycotoxin detoxification tools. For these main reasons, *Streptomyces* genus constitutes a promising strategy to handle fungal contamination and mycotoxin risk.

The study on the biocontrol of fungal wheat pathogens by *Streptomyces* isolates achieved during this thesis is presented in two main sections:

The first section includes a comprehensive bibliographical study, which starts by highlighting the nutritional and economical importance of cereal crops. Followed by a description of phytopathogenic and/or mycotoxinogenic fungi, affecting wheat in the field and during storage, targeted during this work. For mycotoxinogenic fungi, a description of their main mycotoxins' biosynthesis, toxicity and regulation is presented. Later, the principal means

for control of plant molds during pre and post-harvest stages are detailed. Decontamination of mycotoxins by chemical, physical and biological methods is described next. Furthermore, a portrayal of actinobacteria, particularly *Streptomyces*, main characteristics and antagonistic mechanisms deployed during the biocontrol of plant pathogens is presented, together with a description of the principles of biocontrol agents' selection and formulation. The last part of this section includes details about the documented potential of *Streptomyces* strains as detoxification tools.

The second main section contains the experimental work, where the obtained results were organized in the form of scientific publications, a complementary research section and a general results synthesis and comparison section.

First, the description of the *Streptomyces* collection constitution, preliminary identification and conservation is detailed. As well as the results of the *in vitro* preliminary evaluation of their plant growth promoting traits.

The second segment comprises the work conducted on storage pathogenic fungi and their mycotoxins, namely *Penicillium verrucosum*, producer of ochratoxin A, and *Aspergillus flavus*, producer of aflatoxins. *Streptomyces* isolates were screened for their *in vitro* capacity to decrease fungal growth and mycotoxin production during dual culture assays. Then, the impact of their cell free extracts (CFEs) on the same parameters was also evaluated. Degradation capacity of *Streptomyces* isolates and their CFEs was further assessed. For aflatoxin B<sub>1</sub>, degradation assays were followed by the evaluation of the residual toxicity. Next, a complementary study on the stimulation of *Streptomyces*' ochratoxin A degrading capacities is also described. Finally, a comparison between the results observed for both storage pathogens is detailed.

The next experimental section presents the evaluation of *Streptomyces* isolates against phytopathogenic fungi, starting by an *in vitro* assessment of the bacterial antifungal activities. Then, active isolates were selected to perform an *in planta* verification of their biocontrol potential against three wheat diseases, under greenhouse conditions. Their impact on the expression on plant defense genes was also measured. Finally, their installation on the surface of wheat leaves was verified by Scanning Electron Microscopy (SEM).

The last experimental procedures consist on a study on the impact of *Streptomyces* and their CFEs on *Fusarium graminearum* and *Fusarium verticillioides* growth and mycotoxin production.

The experimental section concludes with a synthesis depicting the Clustering and correlation analysis of all the outcomes obtained for the different models evaluated, their biological interpretations and the identification of the most outstanding isolates.

A general conclusion resumes the principal results obtained during this work, highlighting the potential of *Streptomyces* as biocontrol agents and as a source of bioactive compounds for mycotoxin decontamination of food and feed. Future research perspectives are also suggested, with a focus on the elucidation of decontamination mechanisms displayed by *Streptomyces* genus and on the verification of the *in planta* protective effects. It is also suggested that the improvement of screening methodologies will accelerate the process for the development of novel agricultural products based on *Streptomyces*.

The diagram on Figure 1 illustrates the context of the study, depicting the critical points where the presence of fungal pathogens represents a risk for cereal production and safety. Measures to avoid fungal contamination, which are currently applied and under development are indicated, particularly those where *Streptomyces* and their derived products could play an essential role.

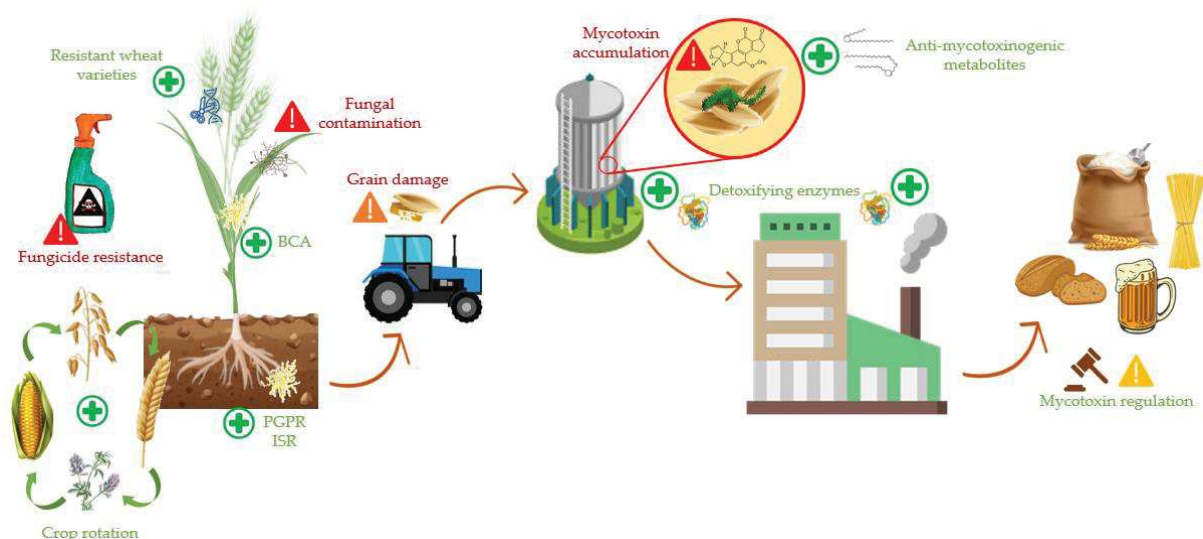


Figure 1. Diagram of fungi/mycotoxins occurrence risks throughout a cereal industrialization chain. Current and under development preventive/curative measures, with potential application of *Streptomyces* and their bioactive compounds, are indicated (icons taken from Freepik.com).



## 2. Bibliographical synthesis

## 2.1 Wheat fungi occurrence and risk

### 2.1.1 Cereals importance

Cereals are the principal source of carbohydrates, proteins, B vitamin, E vitamin, iron and other minerals for the general population, including animal nutrition. Main cereal crops include wheat, maize, rice, oat and barley (Fig. 2). Rice being the major food for Asians, wheat, rye and Barley for Europeans, maize for Americans and sorghum and millet for Africans and Indians. (Mannaa and Kim 2017b; Pereira, Fernandes, and Cunha 2014; Saldivar 2015). The Food and Agriculture Organization (FAO) of the United Nations estimates that 2719 million tons of cereals were produced in the world in 2019, 2.3% more than in 2018 (FAO 2020). Almost two thirds of total cereal production worldwide are destined to animal feed, one third to human consumption and around 3% to biofuel production. Around 300 million tons of cereals are produced in Europe each year, of which near a half is wheat. France is the main cereal producer country in the European Union (EU), generating about 58 million tons (MT), followed by Germany (43 MT), Poland (33 MT) and Spain (26 MT) (European Commission 2020; Kelly 2019). Wheat is considered a major staple food in Mediterranean Europe, main derived food products include bread, breakfast cereals, biscuits, pasta, couscous and bulgur, among others. Its use for human consumption represents about 70% of total wheat utilization. Other uses include animal feed, food additive and biofuel (Elias 1995; González-Esteban 2017; Oleson 1994). As world population increases rapidly, cereals production is expected to escalate in order to meet the predicted demand, preferably without increasing the impact on the already limited resources such as land and water. This implies that assuring cereal grains safety at each stage of their transformation, from growing to storage, will be a major issue (Figueroa, Hammond-Kosack, and Solomon 2018). During their development, wheat cultivars are subject to biotic (diseases, insects, weeds) and abiotic (frost, drought, low rainfall, soil aridity) factors that threat their production yields (Le Gouis, Oury, and Charmet 2020). One of the main risks is the occurrence of pathogenic fungi, which cause



Figure 2. Author's illustration of the main cereal crops.

severe diseases, leading to about 20% of yield losses each year. Therefore, disease control will play a critical role in increasing wheat production. Moreover, several of the fungal pathogens affecting wheat are capable of producing secondary toxic metabolites, with harmful impacts on human and animal health, called mycotoxins. According to recent studies, 60 to 80% of food crops around the world present mycotoxin contamination (Eskola et al. 2020) which leads to a major risk for livestock and humans. A factor increasing fungal contamination exposure is the need to store grains for large periods of time to ensure a sufficient supply throughout the year. If the conditions are not optimal, fungal development and production of mycotoxins can occur. Furthermore, as global warming progresses, climate conditions will facilitate the general widespread of pathogenic fungi that were mainly a concern in warmer ecosystems (Battilani et al. 2016; Moretti, Pascale, and Logrieco 2018). Until now, the major risk of devastating plant diseases and mycotoxin incidence has forced farmers to intensively use chemical products in order to rapidly avoid fungal development. There has been increasing awareness about the harmful impacts of the spread of chemical products in the ecosystem and the documented decrease on their efficacy. This has urged the government to create more initiatives to encourage agronomical research, in order to facilitate researchers' task of developing new and more ecological alternatives for the management of fungal contamination and mycotoxin risk.



Figure 2. Cont. Author's illustration of the main cereal crops.

### 2.1.2 Wheat phytopathogenic non-mycotoxinogenic fungi

As mentioned before, wheat is affected by a variety of diseases that cause important yield losses each year. Main diseases include the rusts, blotches and head blight/scab. The aspect of the lesions they provoke is illustrated in Figure 3. Rust fungi are obligated biotrophs, which depend on the living plant host to acquire the necessary nutrients for their growth and reproduction. There exist three wheat rust diseases: stem rust, stripe rust and leaf rust, caused by the *Puccinia* genus, namely *P. graminis*, *P. striiformis* and *P. triticina*, respectively (Kolmer, Chen, and Jin 2009). Strategies to control wheat rust infection include agricultural practices



such as crop rotation and tillage, in addition to genetical and chemical control. Genetic resistance represented the main fighting tool against these fungi. However, the emergence of new rust races has led to the use of fungicides, which implicate a potential risk of creating pathogenic resistance. Blotch diseases are caused by *Zymoseptoria tritici*, *Parastagonospora nodorum* and *Pyrenophora tritici-repentis* which cause Septoria tritici blotch (STB), Septoria nodorum blotch (SNB) and Tan spot (TS), respectively. These pathogens are also managed through culture practices, chemical control and host resistance. An emerging wheat disease is the wheat blast (WB), caused by *Magnaporthe oryzae* which infects mainly wheat heads, creating elliptical lesions and bleaching of the spikes; foliar lesions by this pathogen have also been reported. *M. oryzae* develops in warm and humid conditions. As an emerging wheat disease, main control strategies are still based on the use of chemical fungicides (Figuroa et al. 2018).

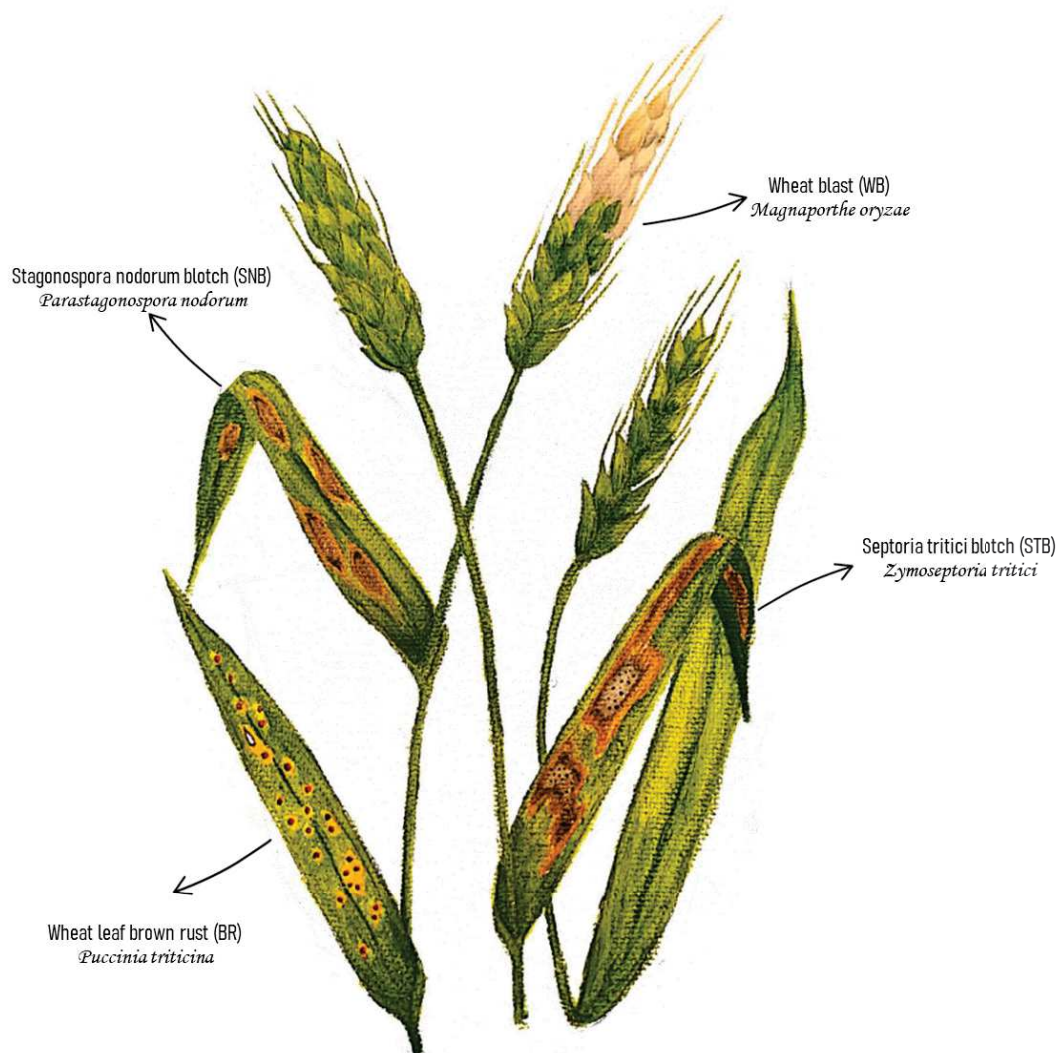


Figure 3. Authors illustration of the aspect of wheat lesions originated by the main foliar phytopathogenic fungi.

### 2.1.2.1 *Puccinia triticina*

#### Generalities

*Puccinia triticina*, the causal agent of rust disease of wheat, is mainly present in areas with temperate climate and high humidity, however, it has shown high adaptability to a large range of climates, which makes it the most common and widespread rust of wheat around the world (Kolmer et al. 2009). The decrease on yield caused by this disease is the result of a reduction in kernel weight and on the number of grains per head. Losses from this infection are calculated at hundreds of millions of dollars in the USA. The most economical way of reducing yield losses due to rust is the use of genetically resistant wheat varieties. Resistance to *P. triticina* is conditioned by 68 *Leaf rust (Lr)* genes being frequently used in wheat cultivars around the globe. Nevertheless, the use of resistant cultivars has caused the selection of virulent leaf rust races that are no longer affected by resistance genes (Figuroa et al. 2018; Kolmer et al. 2009).

#### Life cycle and pathogenesis

The infection by *P. triticina* starts by an uredinial stage of small round, brown to orange pustules on the leaf surface. These uredinia are capable of producing thousands of spores each day, that can be disseminated by the wind through hundreds of kilometers. Then, the urediniospores germinate to produce infection structures comprising appressoria, germ tube and penetration peg, that facilitate the penetration of the plant host stomata (Fig. 4). The uptake of nutrients from plant cells without killing them is achieved thanks to the production of specialized structures: the substomatal vesicle and haustoria. As uredinia mature, the formation of teliospores begins. The production of pycniospores and aeciospores, related to sexual reproduction, occurs only on alternate hosts, not on wheat (Kolmer et al. 2009). Figure 5 illustrates the life cycle of *P. striiformis*, the causal agent of stripe rust.

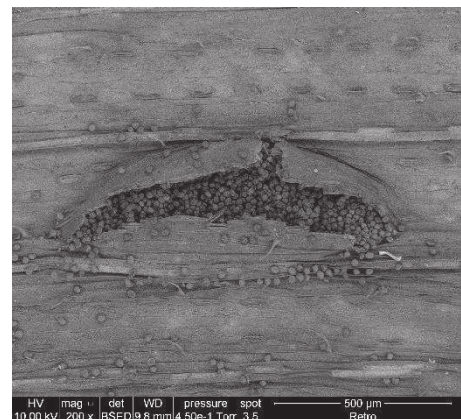


Figure 4. SEM observation of urediniospores of *P. triticina* gathered in the stomata of a wheat leaf (Photo taken during this study).

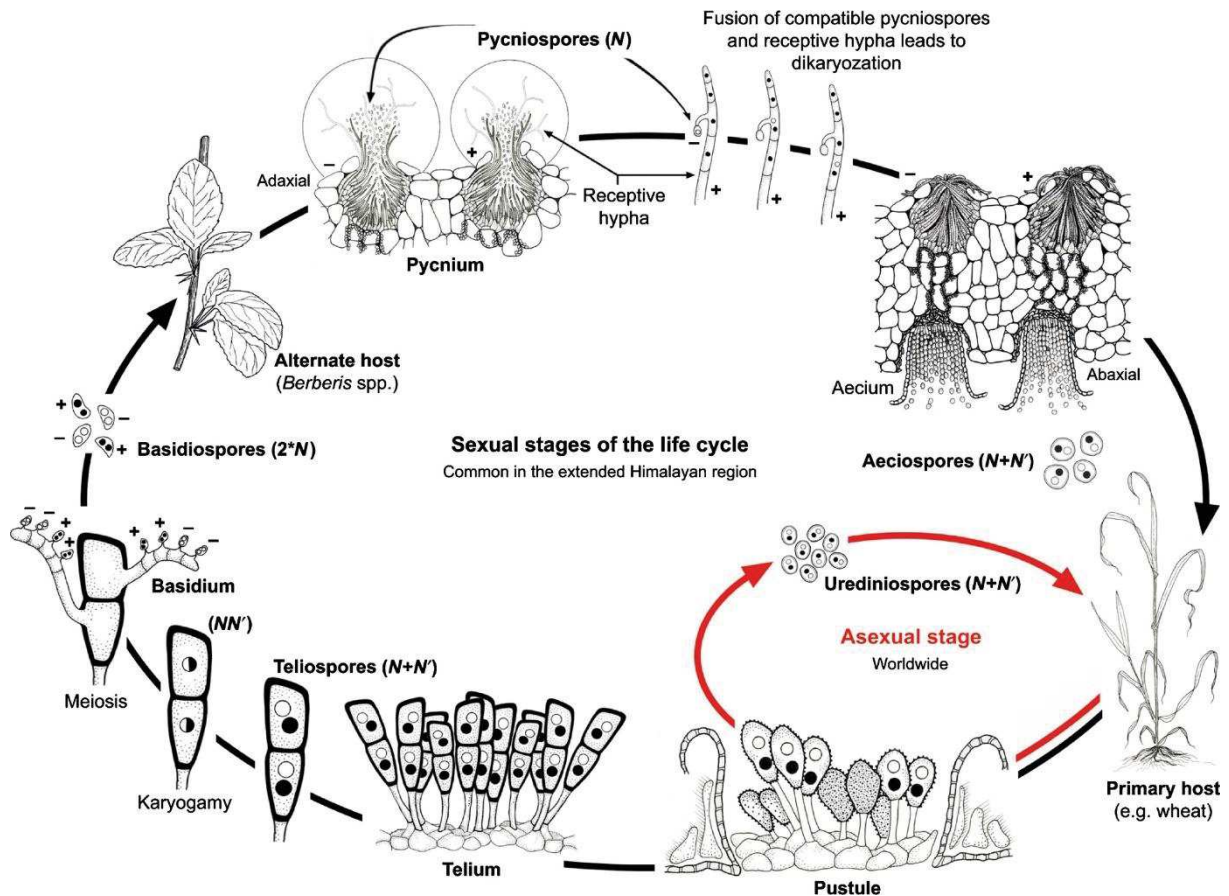


Figure 5. Life cycle of *Puccinia striiformis* f. sp. *tritici* (Schwessinger 2017). Original illustration from Jacolyn A. Morrison at the USDA-ARS Cereal Disease Laboratory, St Paul, MN, USA.

### 2.1.2.2 *Zymoseptoria tritici*

#### Generalities

*Zymoseptoria tritici* (teleomorph *Mycosphaerella graminicola*) causes Septoria tritici blotch (STB), the main leaf disease striking wheat in temperate regions. STB is nowadays the most important wheat disease in Europe, where it causes losses of about 800 to 2400 million of euros each year (Fones and Gurr 2015). Chemical control is the main mechanism for STB control in Europe, together with the use of genetically resistant varieties (Figuerola et al. 2018). It is estimated that about 70% of the volume of fungicides used in Europe is destined to control STB (Fones and Gurr 2015). STB occurs mainly in cool wet weather, under optimal temperatures between 20 and 25°C (Tiley, Karki, and Feechan 2018).

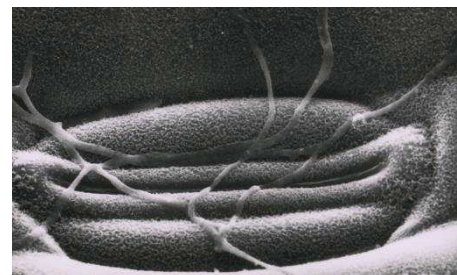


Figure 6. SEM observation of *Z. tritici* mycelium entering the stomata of a wheat leaf (Ponomarenko 2011).

### **Life cycle and pathogenesis**

STB is characterized by necrotic lesions on leaves and stem, the primary entrance site on the leaf is through the stomata (Fig. 6), which eliminates the need for specialized penetration structures such as an appressorium (Brennan et al. 2019; Ponomarenko, Goodwin, and Kema 2011). *Z. tritici* has a hemibiotrophic lifestyle, it starts as a biotroph (latent phase) taking nutrients from the apoplast around living cells, then it kills the surrounding host cells and becomes necrotrophic. During the asymptomatic stage, the fungus produces cellulases, peptidases and xylanases which may help to degrade host cell wall components to release nutrients with minimal damage. It also produces effectors to inactivate the plant immune response and to protect its cell wall from host chitinases (Kettles and Kanyuka 2016; Sánchez-Vallet et al. 2015). The switch from the biotrophic to the necrotrophic phase is still unknown, but it coincides with an abrupt increase in fungal growth rate and biomass (Rudd et al. 2015). STB disease cycle is illustrated on Figure 7. Infection is initiated by air-borne ascospores and rain dispersal of conidia and is carried out following five phases (Ponomarenko et al. 2011):

1. Initial growth of the hyphae on the leaf surface (0-24 hours).
2. Host penetration via the stomata (24-48 hours).
3. Hyphae proliferation in the mesophyll tissue, nutrient uptake from the apoplast (2-12 days).
4. Change to necrotrophic growth, appearance of lesions on the leaf surface (12-14 days).
5. Further colonization of mesophyll tissue, formation of pycnidia with conidia (14-28 days).

To date, 21 different genetic loci of qualitative genes for resistance to STB have been identified (Kettles and Kanyuka 2016). As well as 89 quantitative trait loci (Tiley et al. 2018). In the field, resistance genes lose effectivity rapidly due to fast genetic changes in the pathogen. Besides, wheat cultivars resistant in one region frequently show susceptibility in another (Ponomarenko et al. 2011). *Z. tritici* is commonly found concomitantly with *Parastagonospora nodorum* (Duba et al. 2018; Willocquet et al. 2021).

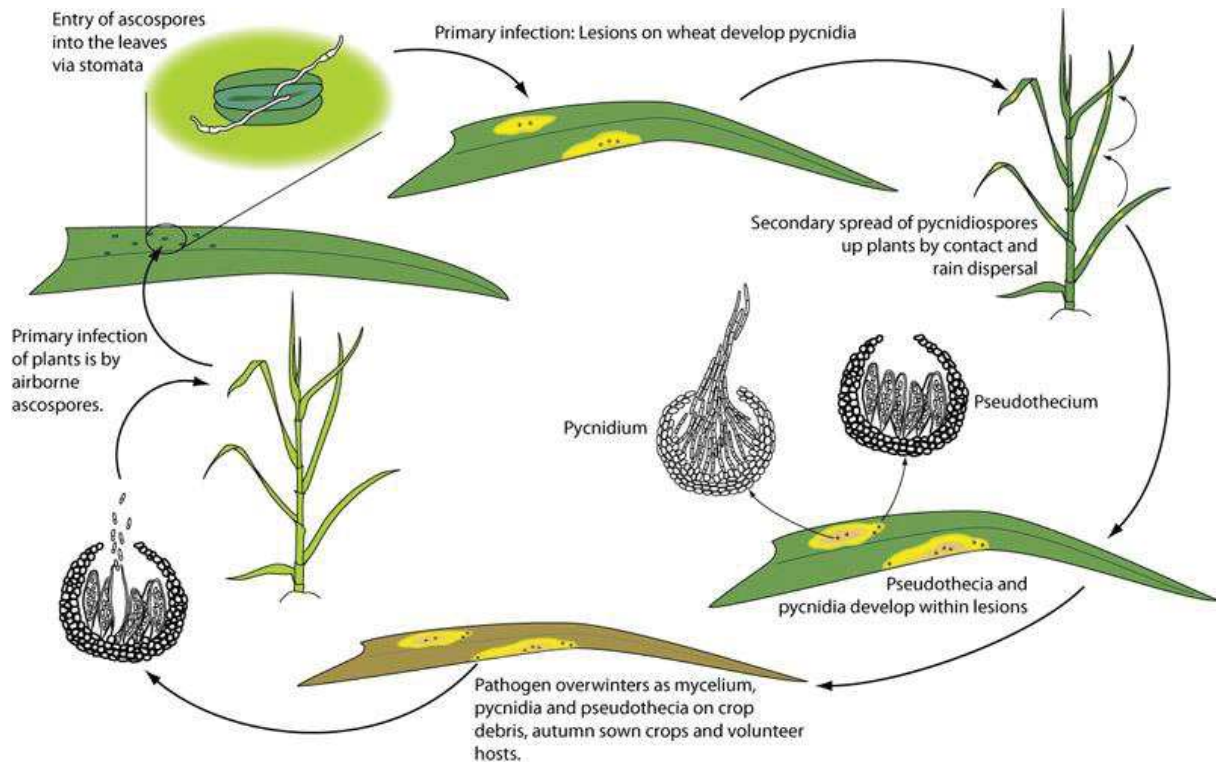


Figure 7. Lifecycle of the wheat pathogen *Zymoseptoria tritici* (Ponomarenko et al. 2011).

### 2.1.2.3 *Parastagonospora nodorum*

#### **Generalities**

*Parastagonospora nodorum* (anamorph *Stagonospora* and teleomorph *Phaeosphaeria*) is a necrotrophic fungus and the causal agent of Septoria nodorum blotch (SNB) in wheat. SNB symptoms are visible at the leaves, stems, nodes, glumes and awns. They appear as small dark-brown lesions with a yellow halo, resulting from diffusible toxins produced by the pathogen (Mehra et al. 2019), and as a discoloration of the head during glume blotch (Solomon et al. 2006). Infected glumes result in shrunken kernels with low quality and quantity of grains, which lead to reduced yield losses going up to 20-50% (Duba et al. 2018). *P. nodorum* is mainly present in Australia, Brazil and USA, but SNB cases have been documented in France and in the Scandinavian countries. Disease incidence can be reduced by good agricultural practices, such as crop rotation and tillage. However, effective management is currently based principally on chemical control and the use of wheat resistant varieties (Figuerola et al. 2018; Mehra et al. 2019).

#### **Life cycle and pathogenesis**

Host colonization by *P. nodorum* is light dependent (Oliver et al. 2012) and starts when hyphae growing from germinating spores penetrate through the leaf cuticle, and opportunistically through stomata, which causes the site of leaf infection to turn yellow. Within the chlorotic areas, small regions of necrosis are often observed and pycnidia will begin to form in the lesion in about a week (Solomon et al. 2006). During the first stage of infection, the pathogen produces toxic necrotrophic effectors that kill host tissue during colonization, such as ToxA, Tox1 and Tox2 (Mehra et al. 2019; Oliver et al. 2012). The production of cell wall-degrading enzymes such as xylanases, glycanases, glucanases and cellulases, induce the host tissue necrosis as well, and is one of the characteristics that differentiates *P. nodorum* from *Z. tritici* (Duba et al. 2018; Solomon et al. 2006). Pathogenic inoculum can originate from various sources, illustrated in Figure 8. Overwintering: the pathogen pseudothecia and/or pycnidia spend the winter in wheat debris. Primary infection: the main inoculum source consists on windborne ascospores released from pseudothecia, or splash-dispersed conidia released from pycnidia. Secondary spread: vertical pathogenic dispersion develops as conidia goes from lower canopy to upper, and then to glumes. Seedborne infection: seedlings can be infected by the dormant mycelium on contaminated seeds from a previous season where glume blotch occurred. Both conidia and ascospores can germinate and cause infection between 5 and 35°C (optimal: 15 to 25°C), favored by rainstorms (Mehra et al. 2019).

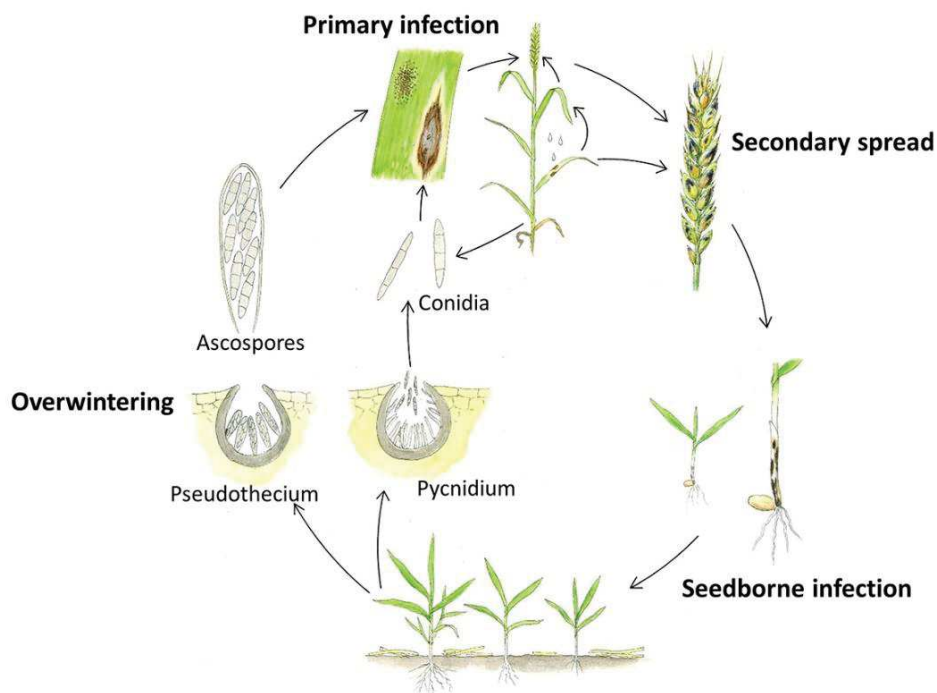


Figure 8. Disease cycle of *Septoria nodorum* blotch (SNB), caused by *Parastagonospora nodorum* (Mehra et al. 2019) (Original drawing by Hermod Karlsen).

#### 2.1.2.4 *Magnaporthe oryzae*

##### **Generalities**

Wheat blast (WB) is a disease caused by the fungus *Magnaporthe oryzae* *Triticum* pathotype (*MoT*), which is a seed-borne pathogen. *M. oryzae* was mainly known as a rice pathogen but it was discovered as a wheat pathogen in Brazil in 1985. It was subsequently disseminated to Bolivia, Argentina, Paraguay and more recently to Bangladesh (March 2016), which represents a risk of dissemination through Asia, the world's greatest wheat producer (Sadat and Choi 2017). Yield losses of 40%-100% have been reported in the affected countries. Warm and humid environments favor fungal development. The optimal temperature ranges between 25 and 30 °C, with spike wetness lasting between 25 and 40 hours, both factors favor wheat blast intensity (Cruz and Valent 2017). Nowadays, the main fighting strategies are based on plant genetic resistance and the use of chemical fungicides (Figueroa et al. 2018). Furthermore, *MoT* resistance genes appear to be rare in wheat. Until now, only four have been identified: *Rmg2*, *Rmg3*, *Rmg7* and *Rmg8*, which conferred resistance against early 90's *MoT* strains. Unfortunately, their effectivity has decreased against recent isolates. Currently, the best sources of resistance are cultivars containing a 2NS translocation segment from *Aegilops ventricosa* (Cruz and Valent 2017). Concerning pesticides, an early application might reduce *MoT* inoculum from basal leaves and subsequently lower the risk of head blast. Another management strategy is the treatment of seeds with chemical fungicides (Cruz et al. 2015).

##### **Life cycle and pathogenesis**

WB is mainly a head disease, but lesions can be observed on leaves, where the pathogen produces high amounts of conidia increment the risk of spike blast development (Cruz et al. 2015). Symptoms on heads go from small elliptical lesions to complete bleaching and empty spikes, as the pathogen can block the translocation of photosynthates and kill the upper parts of the spike, causing partial or total spike sterility (Figueroa et al. 2018). Thus, *MoT* can be transmitted from spikes to seeds, and from infected seeds to seedlings (Cruz and Valent 2017). Seeds produced by infected spikes are usually small, wrinkled, deformed and have low test-weight (Cruz et al. 2015). *M. oryzae* produces both conidia (asexual cycle) and ascospores (sexual cycle) (Fig. 9). Fully fertile strains are self-sterile hermaphrodites, mating occurs by

alternate alleles *MAT1-1* and *MAT1-2*. Conidia are pyriform-shaped, hyaline to pale gray-colored, and are considered the main source of inoculum, as they can remain on crop residue and alternate hosts (Xue et al. 2018). *M. oryzae* is an hemibiotroph, as it initiates as a biotroph and then, upon causing the host cells death, it switches to a necrotrophic lifestyle (Syed Ab Rahman et al. 2018). Both conidia and ascospores germinate and form appressoria and a penetration peg to penetrate the host leaf surface and develop invasive hyphae, which cause the development of lesions (Cruz and Valent 2017; Fernandez and Orth 2018).

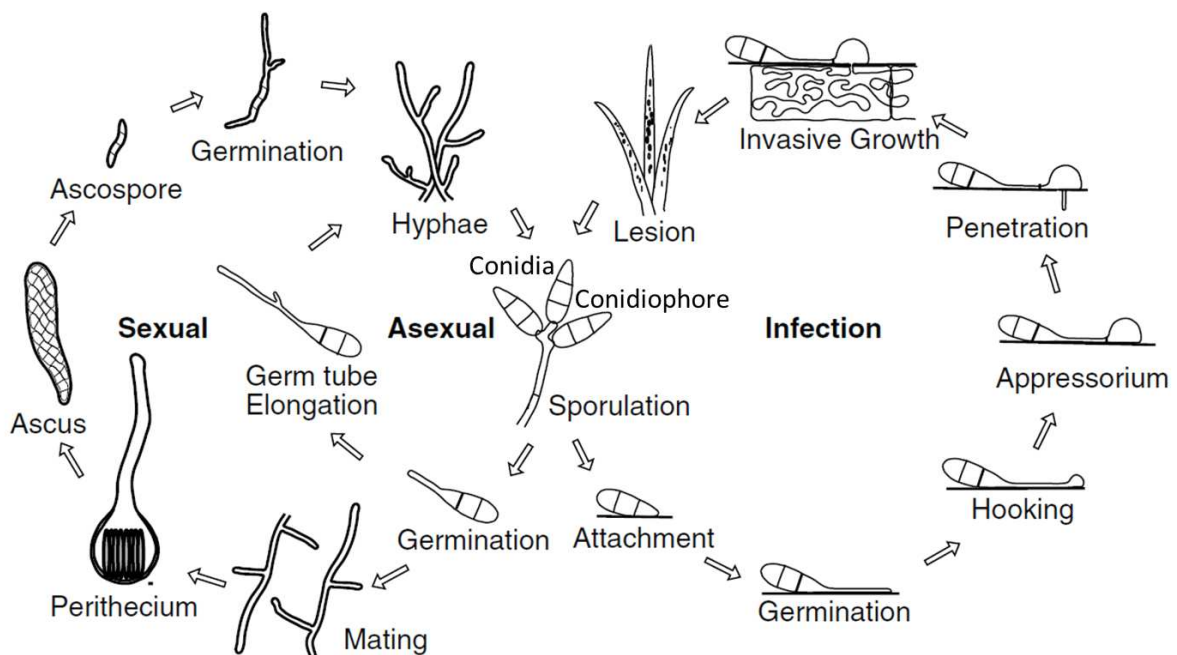


Figure 9. Life cycle of *Magnaporthe oryzae* (Xue et al. 2018).

Wheat is one of the most important staple foods worldwide. Its consistent production has to be guaranteed in order to supply the future generations. Plant pathogenic non-mycotoxinogenic fungi of wheat cause important yield losses and the decrease on grain quality. Fungal pathogens have distinctive lifecycles, which determine the infecting mechanism and the repercussion on plant's health. Main fungal management strategies include plant resistance and the use of chemical fungicides. Climate change is provoking the spread of fungal diseases to previously unaffected regions.



### 2.1.3 Wheat mycotoxinogenic fungi

#### 2.1.3.1 Mycotoxins

Mycotoxins are secondary metabolites of low molecular weight produced primarily by filamentous fungi, mainly of the genera *Aspergillus*, *Fusarium* and *Penicillium*. They are toxic to humans and animals, as they provoke a range of disorders going from gastroenteritis to cancer. Mycotoxins are one of the most toxic contaminants in the food chain. These metabolites are frequently found on food products such as cereals, fruits, nuts and meat, among others. According to the Rapid Alert for Food and Feed (RASFF), in the European Union their presence constitutes the major source of risk notifications (20%) (Gil-Serna et al. 2014). Main mycotoxins found in food and feed are aflatoxins, ochratoxins, deoxynivalenol, fumonisins and patulin (Mannaa and Kim 2017b).

In order to manage mycotoxin risk, most countries have established maximum limits for mycotoxin content in food, based on exposure data and toxicity assessments (Karlovsky et al. 2016). The established limits vary from one country to another, and although an homogenization of regulations would be preferable, limitations have to be adapted to ensure that they do not jeopardize the limited food supply of developing countries, neither make basic commodities excessively expensive (CAST 2003). Limitations for raw materials are usually less strict, as processing usually inactivate a fraction of the mycotoxins. However, some of them are resistant to thermal processes (Bullerman and Bianchini 2007; Raters and Matissek 2008).

##### 2.1.3.1.1 Environmental and molecular factors influencing mycotoxin production

The presence of mycotoxinogenic fungi in a food matrix does not automatically indicates the presence of mycotoxins. Inversely, the absence of toxinogenic fungi does not guarantee that the commodity is free of mycotoxins, as toxins may persist long after the fungi have lost viability (Pitt et al. 2000). Indeed, the conditions that favor fungal growth are not always conducive for mycotoxin production, and optimal conditions variate greatly for each fungal specie and mycotoxin. Several factors can affect fungal development and mycotoxin production, such as temperature, water availability, pH, light, nature of substrate and the

presence of reactive oxygen species (ROS), as well as the interaction with other microorganisms and with the food matrix (Mannaa and Kim 2017b; Reverberi et al. 2010) (Fig. 10), which makes difficult to define a particular set of factors that will promote mycotoxin appearance.

For instance, temperature and water availability can compromise fungi's ability to produce hydrolytic enzymes, such as  $\beta$ -D-galactosidase, which are fundamental for the fungal establishment on substrates (Keller et al. 1997). Studies have shown that a low temperature stress provokes the increase on toxin production on certain *Fusarium* spp., which could be used by the fungus to increase competitiveness (Mannaa and Kim 2017b). Additionally, pH was found to greatly impact aflatoxin and fumonisin production and stability. In fungi, transitions from conidia to hyphal growth, and to mycelial growth to conidiogenesis are characterized by an oxidative burst, a factor also associated with the beginning of aflatoxin biosynthesis. In fact, oxidant stressors were found to stimulate aflatoxin production, while the addition of antioxidants inhibit aflatoxin synthesis without affecting fungal growth. The same phenomenon was observed for other mycotoxins such as trichothecenes, ochratoxin A and patulin. A regulation of mycotoxin biosynthetic genes is behind these effects (Reverberi et al. 2010). Light is also a generator of ROS. This was found to have an impact in mycotoxin production by *Penicillium verrucosum*, which can switch from producing ochratoxin A to producing citrinin when exposed to light (Stoll et al. 2014). Nitrogen and carbon sources are known regulators of aflatoxin and ochratoxin biosynthesis. For example, lactose increases ochratoxin production, whilst glucose causes a repressive effect. Furthermore, the addition of suboptimal concentrations of fungicides can increase mycotoxin biosynthesis (Cendoya et al. 2020; Ferrigo, Raiola, and Causin 2016). Plant organic compounds, such as methyl salicylate and oxylipins were found to modulate mycotoxin biosynthesis in pathogenic fungi (Gao and Kolomiets 2009). Finally, global factors defining climate change (high temperature, water stress and elevated CO<sub>2</sub>) can also have an impact on the amount of mycotoxins produced by fungi during crop infection (Medina, Rodriguez, and Magan 2014).

The described factors, affecting the activation of secondary metabolism genes in fungi, are handled molecularly by a series of global regulators. Among them, the most studied have been the velvet protein complex VelB/VeA/LaeA, involved on sexual development and secondary metabolites production in response to light; PacC, the key factor for pH regulation;

CBC, which regulates the response to redox status and iron stresses; CreA, implicated in the regulation of carbon metabolism; and AreA and AreB, involved in nitrogen metabolism (Chen, Kistler, and Ma 2019; Edwards 2011; Gallo, Ferrara, and Perrone 2017).

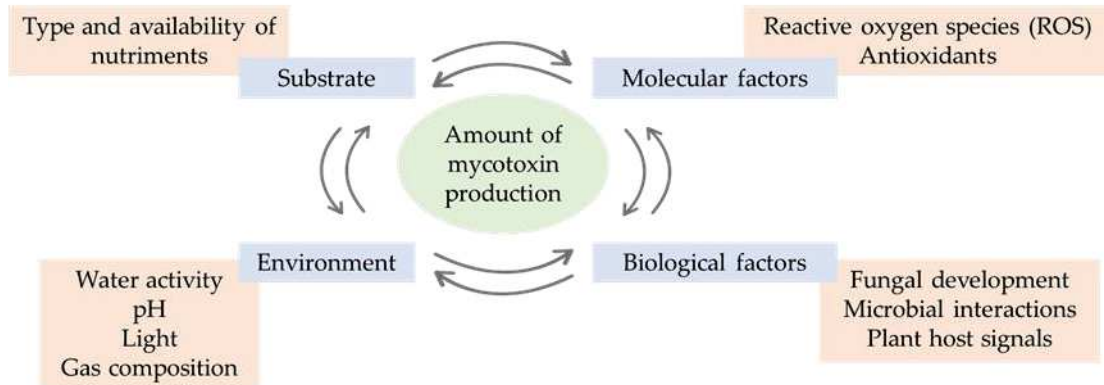


Figure 10. Factors having an impact on mycotoxin production (Modified from Manna and Kim 2017).

#### 2.1.3.1.2 Biological function of mycotoxins

The role of mycotoxins remains enigmatic. Several years ago, Janzen hypothesized that toxin-producing fungi compete with large organisms for resources such as fruit or seeds by making them unpalatable (Janzen 1977). Nowadays, proclamations about their use by the fungus to infect the plant host (virulence factors) or to adapt to hostile environments (defense mechanism) are the most widespread. Laboratory and field experiments have demonstrated that trichothecenes play an important role in wheat head blight caused by *Fusarium graminearum*, while fumonisins are not necessary for ear rot development caused by *F. verticillioides* (Desjardins et al. 2002). However, they seem to lower maize defenses during infection by inhibiting the  $\beta$ -1,3-glucanase activity (Galeana-Sánchez et al. 2017). As mentioned above, mycotoxins production is triggered by the presence of ROS, and recent research has suggested that they may contribute to the detoxification of fungal cells (Reverberi et al. 2010). For instance, aflatoxin production has been linked to the regulation of oxidative stress responses, decreasing oxidative stress in *A. flavus* (Fountain et al. 2020). Moreover, OTA production by *P. verrucosum* and *P. nordicum* was suggested to contribute to their adaptation to NaCl rich environments, by ensuring chloride homeostasis in the cell through the excretion of OTA, that carries a chlorine in its molecule (Gallo et al. 2017). In fact, NaCl rich environments lead to a shift production from citrinin to OTA (Schmidt-Heydt et al.

2015). Alternatively, mycotoxins represent an important part of microbial interactions, where they may protect the producing fungi from competing or invading microbes. For example, fusaric acid produced by *Fusarium* spp., was found to interfere with the quorum sensing mechanism of the bacterial biocontrol agent (BCA) *Pseudomonas chloraphis* (van Rij et al. 2005). In the same way, patulin and penicillic acid produced by *Penicillium* inhibited quorum sensing and blocked chitinase production from bacteria (Rasmussen et al. 2005). Zearalenone, fumonisin, penicillic acid, citrinin and patuline also inhibited the quorum sensing in *Chromobacterium violaceum*, producer of the antibiotic violacein (Bacon, Hinton, and Mitchell 2017). Lastly, studies indicate that mycotoxins may be synthesized in response to microbial signals, while also serving as interspecies signals themselves (Venkatesh and Keller 2019).

#### 2.1.3.2 Wheat phytopathogenic fungi: The *Fusarium* genus

Among phytopathogenic fungi producing mycotoxins, the genus *Fusarium* is one of the main groups that contaminate a wide diversity of crops in various climatic zones around the world. In addition to affecting crop yields, these fungi produce several mycotoxins endangering human and animal health (Summerell 2019). *Fusarium* spp. are mainly considered as field fungi, however, they are adapted to a wide range of habitats so they can proliferate and produce mycotoxins on stored moist grain under inadequate storage conditions (Samapundo et al. 2007). *Fusarium* head blight/scab (FHB) disease, caused mainly by *Fusarium graminearum*, provokes the premature senescence of wheat heads, which leads to a decrease on grain yield and quality, compromising the harvest and marketability. FHB comes with the presence of mycotoxins called trichothecenes, such as deoxynivalenol (DON), which represents a primary food safety risk. *Fusarium verticillioides* is mainly a maize pathogen, however, its emergence as a contaminant of wheat has been assessed, together with the presence of fumonisins (Deepa and Sreenivasa 2017). Indeed, recently published results revealed the presence of *F. verticillioides* on winter wheat in Finland (Gagkaeva and Yli-Mattila 2020). The expansion on the hosts targeted by fungal pathogens could be influenced by the impacts of climate change (Magan, Medina, and Aldred 2011). Genetic resistance, fungicides, crop rotation and tillage of the infected crop residues are the principal strategies to the control of *Fusarium* diseases, together with the elimination of damaged grains to reduce mycotoxin risk (Figuroa et al. 2018).

### 2.1.3.2.1 *Fusarium graminearum*

#### **Generalities**

*Fusarium graminearum* (teleomorph *Gibberella zeae*) (Fig. 11) is the main causal agent of Fusarium head blight (FHB) in several regions of the world, notably in North America, Europe and Asia. FHB is favored by moderate temperatures and high humidity. Rainfall during and after flowering also promotes FHB development (Ferrigo et al. 2016). *F. graminearum* is known to produce the mycotoxins deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEN/ZEA/ZON) (Pitt et al. 2000). The optimal temperature for *F. graminearum* proliferation is 20-25°C, with an optimal and minimal  $a_w$  of 0.98-0.995 and 0.90-0.91, respectively. Germination occurs at an optimal  $a_w$  of 0.88 (Mannaa and Kim 2017b). A minimal pH of around 3 is necessary for its growth (Pitt et al. 2000). FHB infection appears as dark brown, water-soaked spots and later a reddish mold develops on the kernels, cob, husks and shank (Nourozian, Etebarian, and Khodakaramian 2006).



Figure 11. Author's illustration of microscopical observation of *Fusarium graminearum* perithecia.

#### **Life cycle and pathogenesis**

*F. graminearum* can remain as a saprophyte on crop residues. It then produces ascospores that get dispersed through the air and constitute the main inoculum source during flowering (anthesis). Macroconidia, produced during the asexual phase can be water splashed onto adjacent plant spikes (Haile et al. 2019).

The disease cycle of *F. graminearum* in wheat is illustrated in Figure 12. The whole process of wheat head colonization and bleaching takes around 14 days (Figueroa et al. 2018). The infected pale yellow spikelets are easily detectable among the bright green healthy ones. If the weather is humid, *F. graminearum* can infect the entire head and produce a pinkish mycelium. This pinkish crust on the surface of the grain is the reason why this disease was named "scab" (CAST 2003). *Fusarium*-damaged wheat is typically characterized by thin or shrunken chalk-like kernels. Infected grains are poorly filled so they have a low kernel weight. Genetic resistance is a cost effective and environmentally safe way of managing FHB. However, there is no bread or durum wheat cultivar currently available on the market that

has complete resistance or immunity. The main quantitative trait locus (QTL) of bread wheat resistance for FHB is *Fhb1*, which contribute 25 to 42% of the resistance (Haile et al. 2019).

*F. graminearum* Infection starts as a biotroph by the direct entry into open florets by two routes, the first is spore landing on the emerged silks that then infect the ear by the silk channel, and the second is through wounds caused by birds, insects or extreme weather. As infection advances through the xylem and pith, no disease symptoms are observed (Figueroa et al. 2018). However, during this stage, various *Tri* genes, responsible for trichothecene production are activated, which has been found to aid plant infection. Indeed, DON is the only mycotoxin shown to be a virulence factor in wheat (Trail 2009). Symptoms start to appear when the fungal hyphae enter the wheat cells and start provoking the death of the plant tissues which leads to the necrotrophic phase of the infection. Symptoms at this stage include water soaking, mainly of the chlorenchyma, followed by the bleaching of the colonized tissue (Trail 2009).

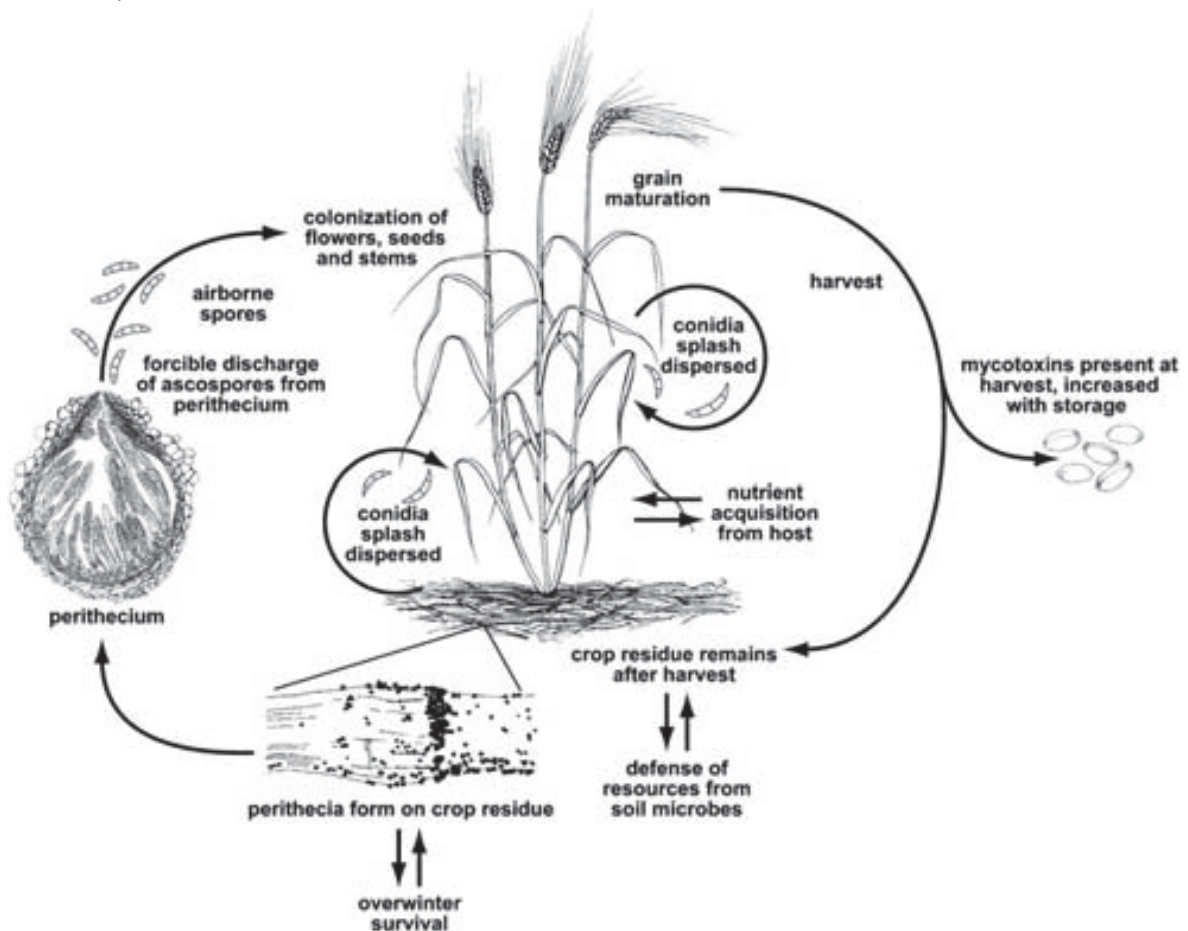


Figure 12. *Fusarium graminearum* life cycle (Trail 2009).

#### 2.1.3.2.1.1 Synthesis of DON and ZEN

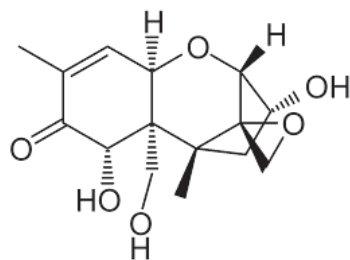


Figure 13. Chemical structure of deoxynivalenol (DON)

During the infection process, *Fusarium* species can produce several trichothecene mycotoxins, such as DON and NIV (Haile et al. 2019). The presence of DON and NIV has been reported in maize, oat, barley and wheat (Gil-Serna et al. 2014). Studies of wheat and corn inoculated with *F. graminearum* on field conditions suggest that wheat can have elevated amounts of DON, but frequently little to none ZEN, whereas corn can be contaminated with both mycotoxins (CAST 2003). Trichothecenes are toxic sesquiterpenoid metabolites with a rigid tetracyclic ring (Loi et al. 2017), they are produced mainly by fungi of the genera *Fusarium*, *Trichoderma*, *Myrothecium* and *Phomopsis*. Hundreds of trichothecenes have been identified, however only few of them represent a risk as contaminants of food and feed, namely, DON, NIV and T-2. Trichothecene biosynthesis starts with the formation of the sesquiterpene trichodiene, which is then oxygenated, esterified, and cyclized (CAST 2003). DON (Fig. 13) is the most common trichothecene, it is produced mainly by *Fusarium graminearum* and *F. culmorum*. DON producing strains can generate different acetylated variants such as 3-acetyl-DON (3-ADON) and 15-acetyl-DON (15-ADON) (Haile et al. 2019). Optimal and minimal temperature for DON production in corn grains is 29-30°C and 11°C, respectively (Mannaa and Kim 2017b). If grains are stored under appropriate conditions, no further accumulation of DON is observed (Richard 2007).

Trichothecenes biosynthesis precursors start on the mevalonate pathway, where the mevalonate pathway enzyme HMG-CoA reductase (Hmr1) catalyzes the synthesis of mevalonate, leading to farnesyl pyrophosphate (FPP), which is also used by essential metabolic pathways (e.g. biosynthesis of ubiquinone, steroids and terpenes). Then FPP is cyclized, catalyzed by the enzyme trichodiene synthase Tri5. Posterior oxygenation of the trichodiene product by Tri4 creates the toxic epoxide moiety in isotrichodermol and subsequent pathway intermediates. Further reactions catalyzed by Tri101, Tri11, Tri3, Tri1, and Tri8, generate deoxynivalenol (DON) and 15-acetyldeoxynivalenol (15-ADON) (Fig. 14) (Boenisch et al. 2017). The reason why Tri14 is required for DON synthesis *in planta* remains unknown (Brown et al. 2020) but its deletion reduced DON production and *F. graminearum*

pathogenicity (Dyer et al. 2005). During this work, studies will concentrate on DON and 15-ADON production.

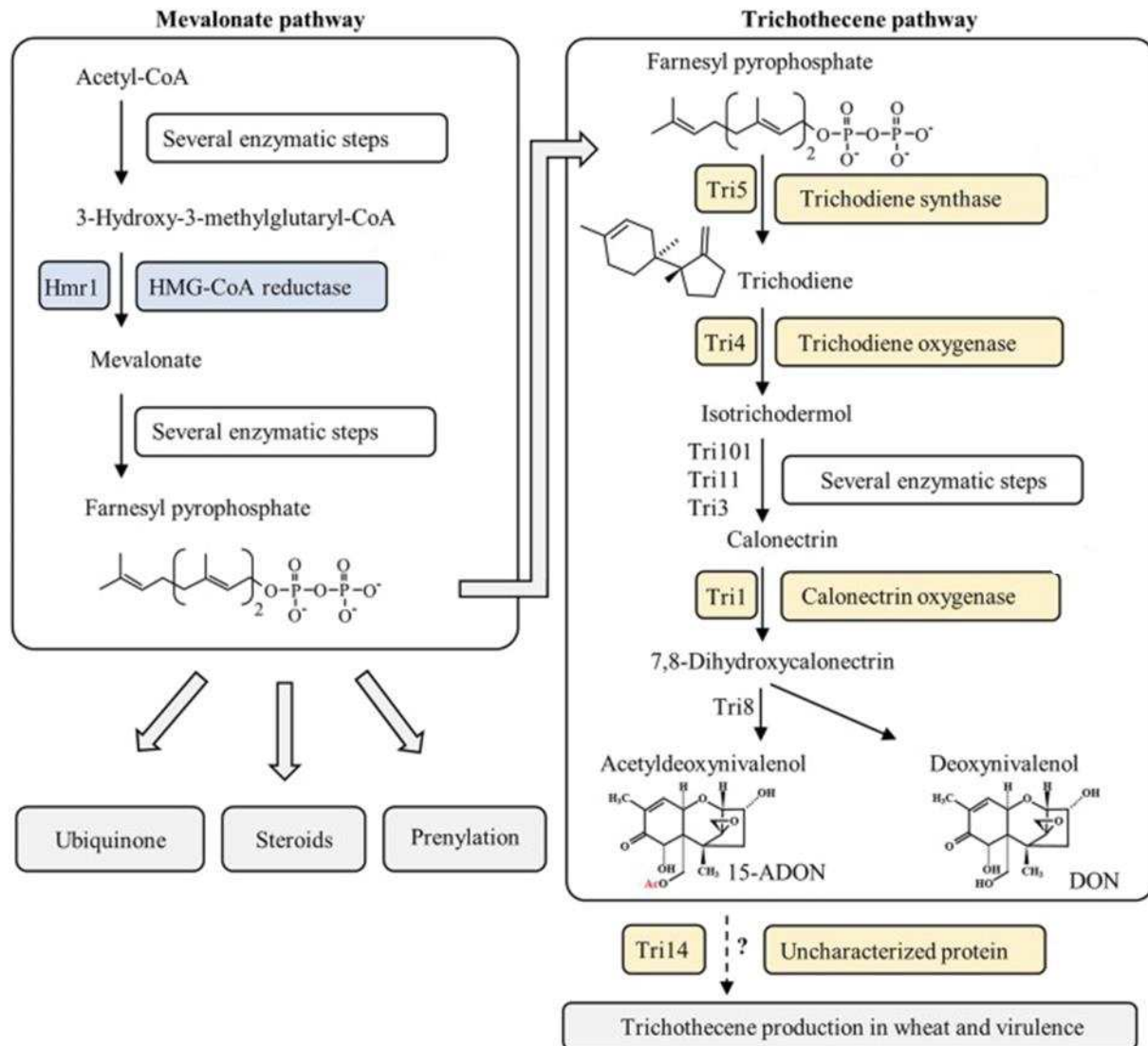


Figure 14. Primary and secondary metabolism pathways in *Fusarium graminearum* (Boenisch et al. 2017).

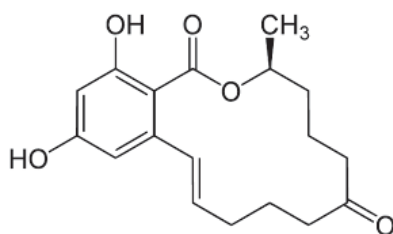


Figure 15. Chemical structure of zearalenone (ZEN).

ZEN is an estrogenic mycotoxin produced primarily by *Fusarium graminearum* and *F. culmorum* when they develop in substrates with high humidity and low temperatures. ZEN is a phenolic resorcylic acid lactone (Fig. 15) that resembles the natural estrogen 17 $\beta$ -estradiol, which confers it the capacity to bind to estrogen receptors, hence its estrogenic potential (Loi et al. 2017). It has been detected frequently in different cereals, such as wheat, barley, maize, sorghum, rye, and rice (Gil-Serna et al. 2014). It has also been detected in



processed products like bread, chocolate, flour, malt and milk (Mahato et al. 2021). Optimal production of ZEN has been observed at 25°C, 16% moisture and neutral pH. ZEN is known to be heat stable up to 150°C and to resist food and feed processing (Rai, Das, and Tripathi 2020).

### 2.1.3.2.1.2 Toxicity of DON and ZEN

The ingestion of trichothecenes in high doses can cause immune response inhibition, nausea and vomiting (Mannaa and Kim 2017b), as well as reduced growth, anorexia and changes in brain neurochemicals (Ferrigo et al. 2016). At the molecular level, DON disturbs standard cell function by obstructing protein synthesis and activating protein degradation in the proteasome, leading to translation inhibition (Smith et al. 2018).

ZEN is of major interest because despite its low acute toxicity, it has proven to be hepatotoxic, immunotoxic and carcinogenic to several mammalian species (Ferrigo et al. 2016). Effects of ZEN involve hormonal changes, as it binds to estrogen receptors. However, mortality is not a concern due to this mycotoxin (Richard 2007). In humans, ZEN occasionally causes hypoestrogenic syndromes and its suspected to provoke precocious puberty development in girls (Smith et al. 2018). Additionally, ZEN causes altered progesterone level, decreased sperm count and serum testosterone level, and infertility. Its involvement in the occurrence of esophageal cancer and breast cancer has been reported around the world (Rai et al. 2020). ZEN has been categorized as a Group 3 carcinogen by the International Agency for Research on Cancer (IARC) due to the lack of evidence on its carcinogenicity to humans (Mahato et al. 2021).

### 2.1.3.2.1.3 Regulation of deoxynivalenol and zearalenone

DON limits for unprocessed durum wheat, oats and maize is 1750 µg/kg. For cereals intended for human consumption, cereal flour and pasta the limit was established at 750 µg/kg. Bread, pastries, biscuits, cereal snacks and breakfast cereals have a limit of 500 µg/kg. Lastly, DON maximal limit for foods destined to babies, infants and young children is 200 µg/kg.

ZEN limit for unprocessed cereals is of 100 µg/kg, and of 75 µg/kg for cereals intended for direct human consumption and cereal flour. The limit for unprocessed maize, as well as maize intended for direct human consumption and derived products is 200 µg/kg. Bread, pastries, biscuits, snacks, breakfast cereals derived from cereals and maize must not exceed 50 µg/kg of ZEN. Cereal-based and maize-based foods for babies, infants and young children must remain under 20 µg/kg (European Commission 2006).

#### 2.1.3.2.2 *Fusarium verticillioides*

##### **Generalities**

*Fusarium* ear rot (FER) in maize is caused mainly by *Fusarium verticillioides* and *Fusarium proliferatum*. *F. verticillioides* is mainly a maize pathogen, however it has been found on wheat (Cendoya et al. 2018) such as in its stalks (Palazzini et al. 2013) and husks (Kuzdraliński et al. 2017). *Fusarium verticillioides* (previously *F. moniliforme*) can grow between 4 and 37°C with an optimal temperature of 30°C. Regarding humidity, this pathogen requires a minimal  $a_w$  of 0.90 (Mannaa and Kim 2017b). Fungal contamination by *F. verticillioides* occurs in the field, seemingly under drought stress followed by warm, wet weather (Richard 2007). It can infect the roots, stalk, ears and kernels. In addition to causing plant diseases, *F. verticillioides* is capable of producing fumonisins. To date, 28 fumonisin analogues have been identified. However, only 3 occur abundantly and have elevated toxicity, namely, fumonisin B<sub>1</sub> (FB<sub>1</sub>), B<sub>2</sub> and B<sub>3</sub> (Deepa and Sreenivasa 2017).

##### **Life cycle and pathogenesis**

*F. verticillioides* colonizes residues in the soil, it can be found in corn seeds and can infect the seedlings. However, the most common infection pathway is the entrance of airborne micronidia by the silks or through existent wounds (CAST 2003). Figure 16 describes the disease cycle of *F. verticillioides* around the life cycle of maize: **A**, seeds that germinate in contaminated soils could develop rot and seedling blight. **B**, endophytic colonization starts as asymptomatic. **C**, Stalk wounds from mechanical or insect damage become fungal entrances and may result in stalk rot. **D**, During the silk stage, fungus can colonize kernels via the stylar canal, generating the “starburst” pattern on kernels. **E**, Insects feeding on the kernels generate an entrance site for *F. verticillioides* stalk and ear rots. **F**. After harvest, the fungal pathogen is

capable of surviving and sporulating on crop residue, generating inoculum for subsequent infections (Blacutt et al. 2018).

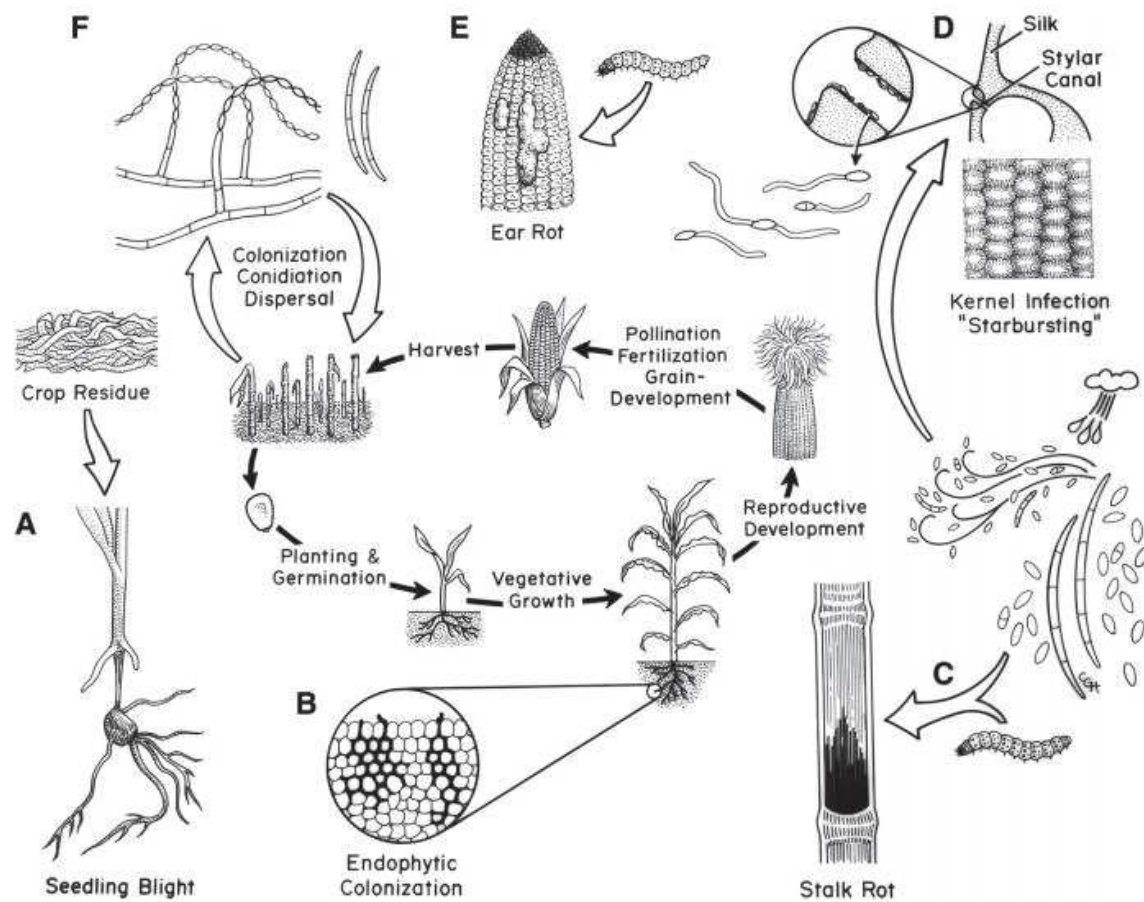


Figure 16. *Fusarium verticillioides* disease cycle, centered on the life of maize (Blacutt et al. 2018).

#### 2.1.3.2.2.1 Synthesis of fumonisins

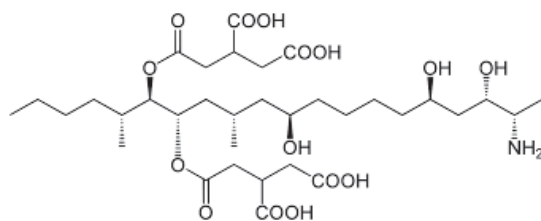


Figure 17. Chemical structure of fumonisin B<sub>1</sub>.

Fumonisins are non-fluorescent mycotoxins primarily produced by *F. verticillioides* and *F. proliferatum*. Their structure was first elucidated by Bezuidenhout et al. in 1988 (Bezuidenhout et al. 1988). They consist in diesters of propane-1,2,3-tricarboxylic acid and

long-chain aminopolyol backbones (Fig. 17). Their structure is similar to those of the sphingoid bases sphinganine (SA) and sphingosine (SO) (Loi et al. 2017). The principal fumonisins are FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> and they are mainly found on maize. Although their occurrence has been documented also in barley, wheat, sorghum and rice (Gil-Serna et al. 2014). Fungal

proliferation can continue after harvest, under poor storage conditions, which provokes mycotoxin accumulation on stored moist grains (Mannaa and Kim 2017b). An  $\alpha_w$  of 0.93 is needed for fumonisin B<sub>1</sub> production. Temperature must remain between 10 and 37°C, with an optimal range between 15 and 30°C.

Fumonisin is synthesized through the condensation of the amino acid alanine to an acetate derived precursor (Sweeney and Dobson 1998). At the end of their biosynthesis, fumonisins' constitution is as follows: the 18-carbon backbone from C-3 to C-20 is derived from acetate, and C-1 and C-2, as well as the C-2 amino, are derived from alanine. The two methyl groups at C-12 and C-16 are derived from methionine. The hydroxyl at C-3 is derived from acetate, while those at C-5, C-10, C-14, and C-15 are derived from molecular oxygen. The two tricarballic esters at C-14 and C-15 are likely derived from the citric acid cycle. These transformations consisting on several hydroxylations and esterifications are regulated by a series of enzymes such as reductases (Fum13p), oxygenases (Fum6p) and transferases (Fum14p) (Fig. 18) (Du et al. 2008).

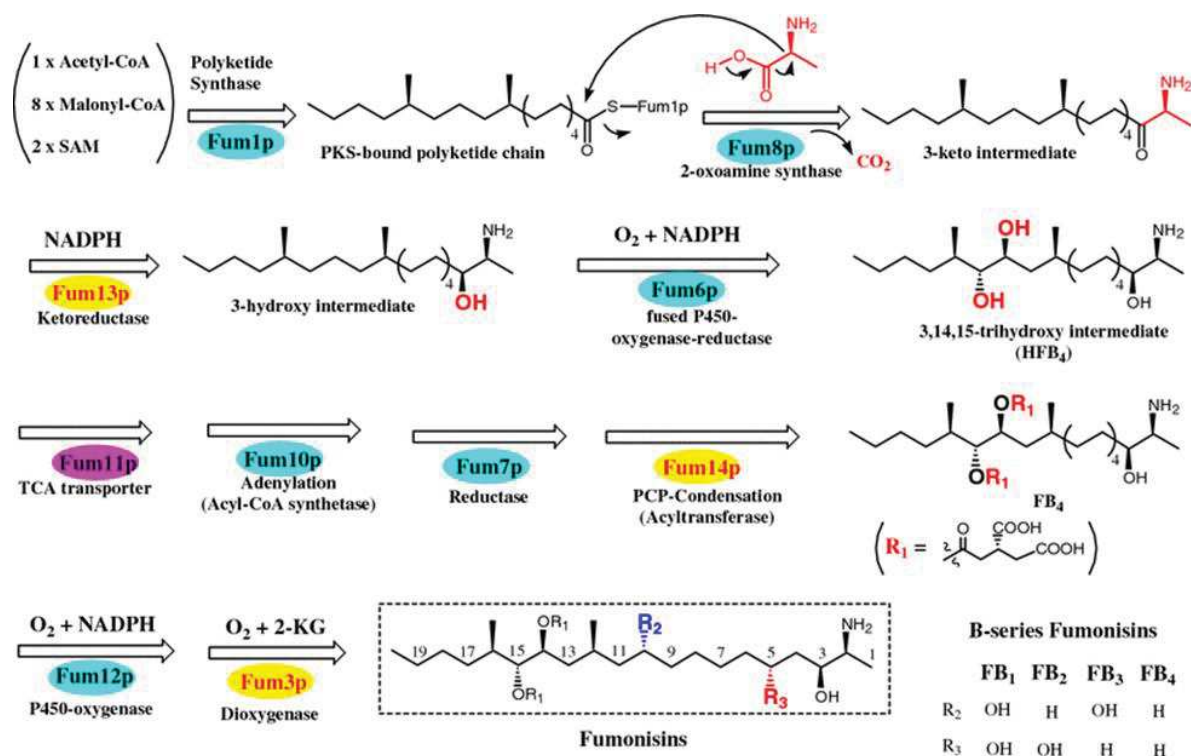


Figure 18. A proposed pathway for fumonisins biosynthesis (Du et al. 2008).

#### 2.1.3.2.2.2 Toxicity of fumonisins

Fumonisin are competitive inhibitors of ceramide synthase. They obstruct the biosynthesis of complex sphingolipids and promote the accumulation of sphinganine, which is cytotoxic at high concentrations. This provokes an alteration of the cell morphology and its interactions with other cells, the conduct of cell-surface proteins, the activity of protein kinases, the metabolism of other lipids, and cell growth and viability (Merrill, Liotta, and Riley 1996). Fumonisin cause leukoencephalomalacia (softening of the white matter in the brain) in horses and lung edema in swine (Richard 2007). In rodents, FB<sub>1</sub> is hepatocarcinogenic and nephrotoxic (Gil-Serna et al. 2014). Fumonisin are classified by IARC as a class 2B possible human carcinogens (IARC 1993). There is evidence linking the presence of *Fusarium verticillioides* in corn to the high prevalence of human esophageal cancer in South Africa and China (CAST 2003). Recent studies suggest that exposure to fumonisins could be related to stunting in children (Cendoya et al. 2018). The addition of β-chloroalanine, which inhibits sphinganine formation (by inhibiting serine palmitoyl transferase), prevented partially the effects of fumonisin on cell growth and cell death (Yoo et al. 1996).

#### 2.1.3.2.2.3 Regulation of fumonisins

The presence of fumonisins is only regulated for maize products despite the fact that they can be found on wheat. The limit for fumonisins (sum of B<sub>1</sub> and B<sub>2</sub>) in unprocessed maize is 2000 µg/kg. The limit decreases to 1000 µg/kg for flour, meal, grits, germ and refined oil. Foods for direct human consumption must remain under 400 µg/kg. Lastly, the maximum limit for processed maize-based foods for babies, infants and young children is 200 µg/kg (European Commission 2006).

Some pathogenic fungi found in the field are capable of producing mycotoxins. These fungal toxic metabolites have functions that seem to go from the competition against other microorganisms, through fungal protection, to abiotic stresses, and until plant infection factors. Several parameters affect mycotoxin production such as temperature, humidity and pH. Due to their toxic effects, the presence of main mycotoxins on food and feed is regulated in most countries, especially for cereals.

### 2.1.3.3 Grain storage fungi: *Penicillium* and *Aspergillus* genera

Fungal contamination from pre-harvest stages leads to post-harvest grain deterioration during storage, such alterations include discoloration, musty odors, dry matter loss, tissue disintegration, nutritional and processing quality decrease, and mycotoxin accumulation. Losses due to this deterioration around the world are estimated at 10%, going up to 50% in tropical regions (Mannaa and Kim 2017b). Storage fungi are predominantly species of *Aspergillus* and *Penicillium*, which are able to develop at the low humidity of seed storage. They provoke the appearance of several toxins such as aflatoxins and ochratoxin A. To avoid their development on the seeds, good storage conditions have to be implemented, such as reducing the humidity content and the temperature (Bothast 1978). Factors affecting fungal growth and mycotoxin production during storage include: water activity, temperature, pH, light and oxygen and carbon dioxide amount, among others (Atanda S. A 2011). Optimizing storage conditions is fundamental, otherwise, after extensive periods of time, initial fungal inoculum and mycotoxin amount could increment considerably and make the product unfit for consumption. Crop losses of 20% to 50% were recorded in developing countries due to inadequate storage practices (Agriopoulou, Stamatelopoulou, and Varzakas 2020).

#### 2.1.3.3.1 *Penicillium verrucosum*

Fungi of *Penicillium* genus can develop over a wide range of temperatures, but they are more abundant in temperate to cold climates. They are mostly associated with storage rather than preharvest contamination (CAST 2003). *Penicillium verrucosum* (Fig. 19) is the main ochratoxin producing fungus present on stored grain in Northern Europe, it can also produce citrinin under certain conditions, such as oxidative stress (Schmidt-Heydt et al. 2015). As a saprophytic fungus, *P. verrucosum* develops mostly during food and feed storage. It develops at a wide range of temperatures (0-35°C) and at an optimal  $a_w$  of 0.95, the minimal being 0.80 (Mannaa and Kim 2017b). It also grows over a pH range going from 2.1 to 10, with an optimal between 6 and 7 (Pitt et al. 2000).

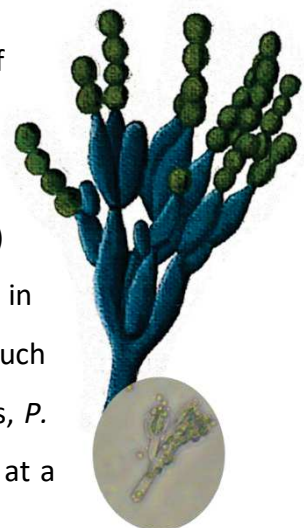


Figure 19. Author's illustration of microscopical observation of *P. verrucosum* conidiophores.

## 2.1.3.3.1.1 Synthesis of ochratoxin A and citrinin

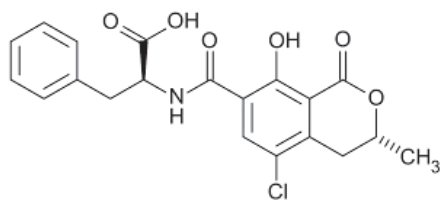


Figure 20. Chemical structure of ochratoxin A.

Ochratoxin A (OTA) was first described by van der Merwe et al. in 1965 as produced by the fungus *Aspergillus ochraceus* (Van Der Merwe et al. 1965). OTA is composed by a phenylalanine molecule and a dihydroisocoumarine moiety (Fig. 20) (Loi et al. 2017). It

is a fluorescent compound produced by several *Aspergillus* and *Penicillium* fungi. Main OTA producers include *A. steynii*, *A. westerdijkiae* and *A. ochraceus* in warm and tropical regions, while *P. verrucosum* is mainly found in temperate to cold regions (Gil-Serna et al. 2011; Schlösser and Prange 2019). OTA is an ubiquitous mycotoxin, as it can be found in several food products including cereals, coffee, nuts, grapes, chocolate, meat and spices, as well as in some beverages such as wines, beers and juices (Eskola et al. 2020; Geisen et al. 2018; Petzinger and Weidenbach 2002). Humidity, pH and temperature are crucial factors affecting OTA production (Mannaa and Kim 2017b). OTA optimal production by *P. verrucosum* occurs at 25°C but it can also be produced between 5 and 10°C at its optimal  $a_w$  (0.90-0.95). A minimal  $a_w$  of 0.83-0.85 is necessary for OTA production.

OTA biosynthesis originates from acetate and malonate by the formation of a polyketide dihydroisocoumarin moiety by a polyketide synthase (PKS), that then is linked via amide bond to the amino acid phenylalanine synthesized via the shikimic acid pathway. A non-ribosomal peptide synthase (NRPS) is essential for this step, and the resulting molecule is ochratoxin B (OTB). Finally, a halogenation step is required to add the chlorine atom to the molecule via a halogenase (HAL), which yields ochratoxin A (OTA) (Fig. 22). The process is likely to involve other enzymes such as oxidases, esterases, transporters and transcription factors (Gallo et al. 2017).

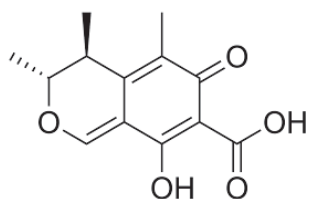


Figure 21. Chemical structure of citrinin.

Citrinin (Fig. 21) is a yellow mycotoxin produced by some species of *Penicillium* and *Aspergillus*, including ochratoxin producer *Penicillium verrucosum* which has the ability to switch between OTA and citrinin under light induced stress conditions (Stoll et al. 2014). The occurrence of this mycotoxin has been documented in rice, fruits, cheese and dried fruits (Gil-Serna et al. 2014).

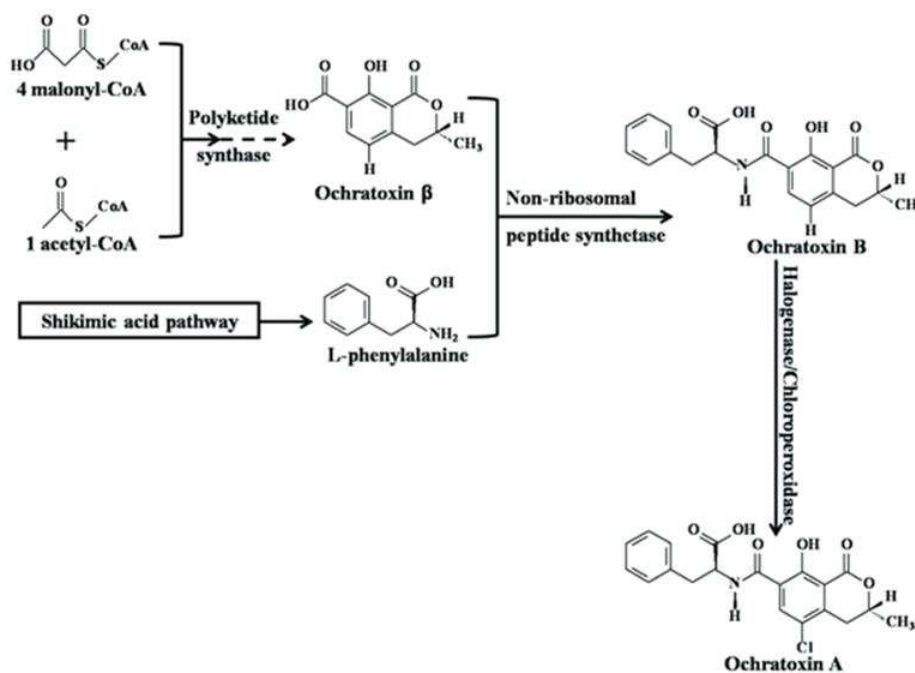


Figure 22. Critical steps of ochratoxin A biosynthesis (Chen et al. 2018).

#### 2.1.3.3.1.2 Toxicity of OTA and citrinin

OTA is suspected to be the causative agent of the Balkan Endemic Nephropathy (BEN) originally described in 1956 as a pathology in Balkan areas where the presence of OTA-contaminated food was frequently reported (Duarte, Pena, and Lino 2010). Because of its analogy to the structure of the amino acid Phenylalanine, OTA is a competitive inhibitor of tRNA phenylalanine synthetases which causes a cease in protein synthesis. Furthermore, OTA provokes the occurrence of DNA adducts and indirect oxidative DNA damage (Loi et al. 2017).

OTA was classified as genotoxic and possible human carcinogen, as well as hepatotoxic and nephrotoxic (it affects mainly the kidney) and also teratogenic (Pfohl-Leskowicz and Manderville 2007). Besides, it has recently been classified as immunosuppressant (Petzinger and Weidenbach 2002). In the last two decades, European countries most affected by OTA contamination have been Germany, Italy, Switzerland, Sweden, the UK and Czech Republic. OTA presence can be detected in blood, urine and milk samples. Its rapid transportation through the body and bio-accumulation is due to its ability to bind to proteins, such as albumin (Heussner and Bingle 2015; Pfohl-Leskowicz and Manderville 2007), which provokes an elevated elimination half-life in humans (35.5 days) (Abrunhosa, Paterson, and Venâncio 2010).



Ochratoxin has three main forms A, B and C. OTB being a non-chlorinated form of OTA and OTC being an ethyl ester of OTA. The toxicity of OTA is given by its isocoumarin moiety. OTB has been proved to be less toxic thanks to the rapidity of its elimination, since it has less affinity for plasma proteins. However, OTC remains almost as toxic as OTA, as it becomes available in the bloodstream (Abrunhosa et al. 2010). Another known metabolite is OT $\alpha$ , which results from the hydrolysis of the amide bond, which also releases phenylalanine. OT $\alpha$  has been found to be about 100 times less toxic than OTA, with a 10-times shorter elimination half-life, and even if the isocoumarin is present, it seems to need the presence of phenylalanine to have a toxic effect (Heussner and Bingle 2015).

As OTA, citrinin causes kidney damage in laboratory animals and its interaction with OTA allegedly causes a synergic effect (CAST 2003). The cellular mechanism of toxicity caused by citrinin is associated to a modification in mitochondrial functions by an alteration of enzymes and the redox chain, which induces oxidative stress (Gil-Serna et al. 2014).

#### 2.1.3.3.1.3 Regulation of ochratoxin A and citrinin

The maximal residual level for OTA in cereals has been settled at 5  $\mu\text{g}/\text{kg}$  by the European Legislation (European Commission 2006). However, many countries lack of ochratoxin limits regulation, including the USA, Australia, New Zealand, Japan, Mexico and South Africa (Heussner and Bingle 2015).

EU limits for OTA were established at 0.5  $\mu\text{g}/\text{kg}$  for processed cereal-based foods and baby foods; 2  $\mu\text{g}/\text{kg}$  for wine, grape juice, grape nectar and grape must intended for direct human consumption; 3  $\mu\text{g}/\text{kg}$  for products derived from unprocessed cereals; 5  $\mu\text{g}/\text{kg}$  for unprocessed cereal, roasted coffee beans and ground roasted coffee beans and ground roasted coffee; 10  $\mu\text{g}/\text{kg}$  for dried vine fruit and soluble coffee; 15  $\mu\text{g}/\text{kg}$  for certain spices; 20  $\mu\text{g}/\text{kg}$  for liquorice root for herbal infusion and 80  $\mu\text{g}/\text{kg}$  for liquorice extract. Regarding animal feed, OTA maximal limits are 50  $\mu\text{g}/\text{kg}$  for feed materials, 50  $\mu\text{g}/\text{kg}$  for complementary and complete feeding stuffs for pigs and 100  $\mu\text{g}/\text{kg}$  for poultry. The USA Food Drug Administration (FDA) does not settle limits for this toxin (European Commission 2006; Smith et al. 2016).

Until recently, there was no legislation regarding citrinin presence in foodstuffs. The opinion of EFSA experts published in March 2012 did not recommend the regulation of citrinin

levels yet, among other aspects, because there was not sufficient data on toxicity or citrinin occurrence in food and feed in Europe in order to determine an accurate risk assessment (Gil-Serna et al. 2014). However, since 2019, the limit for citrinin amounts on products derived from the red rice yeast has been settled at 100 µg/kg (European Commission 2019)

#### 2.1.3.3.2 *Aspergillus flavus*

Fungi of the genus *Aspergillus* are distributed worldwide, notably in subtropical and warm temperate climates. They are saprophytes that can grow at high temperatures (up to 42-43°C) and low water activity; which allows them to proliferate on a variety of grain and nut crops (CAST 2003). *Aspergillus flavus* (Fig. 23) is present mainly during seed storage, however, it can also be found in the field, on senescent or stressed plants (Agriopoulou et al. 2020). Furthermore, its occurrence in the field is increasing with global warming (Battilani et al. 2016). The optimal temperature for the development of *A. flavus* is 35°C and it requires a minimum  $a_w$  between 0.78 and 0.84, 0.95 being the optimal  $a_w$  (Mannaa and Kim 2017b). It grows over a pH range going from 2 to 10 (Pitt et al. 2000). The infection of *A. flavus* in the fields is due to conidia development in the ear and kernel surfaces, which leads to the invasion of the seeds and cobs, and the entry by eventual wounds caused by insects. This finally leads to grain contamination, which increases during storage. Elevated temperatures and drought stress increase *A. flavus* initial infection (CAST 2003).

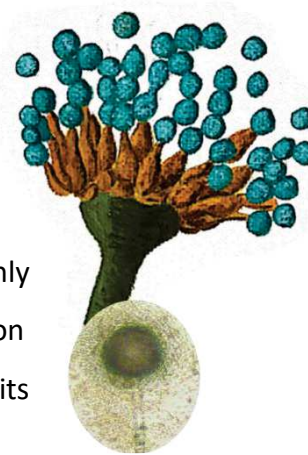


Figure 23. Author's illustration of microscopical observation of *A. flavus* conidiophores.

##### 2.1.3.3.2.1 Production of aflatoxins

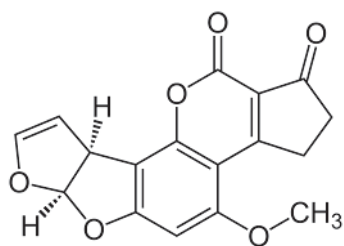


Figure 24. Chemical structure of aflatoxin B<sub>1</sub>.

Aflatoxins are polyketides derived from acetate, their structure comprises two furan rings, linked together to a coumarin moiety (Fig. 24). Furofuran and coumarin rings are disposed in a planar configuration, which leads to their typical fluorescence (Loi et al. 2017). Aflatoxins are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. There are principally four major aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> (B=blue and G=green fluorescence), plus two

additional metabolic products, M<sub>1</sub> and M<sub>2</sub>. *A. flavus* produces only AFB<sub>1</sub> and AFB<sub>2</sub>, while *A. parasiticus* can produce also AFG<sub>1</sub> and AFG<sub>2</sub> (Verheecke, Liboz, and Mathieu 2016). The M aflatoxins were isolated for the first time from the milk of lactating animals fed aflatoxin preparations; hence, the M designation (CAST 2003). Aflatoxins contaminate various agriculture and food products. The crops usually affected by aflatoxins are wheat, corn, groundnuts and tree nuts, as well as on soya, rice, coffee and cacao (Gil-Serna et al. 2014). They can also be found on spices such as paprika, cumin and pepper (El Mahgubi et al. 2013). The optimal temperature for aflatoxin production by *Aspergillus flavus* is 28°C, minimal and optimal *a<sub>w</sub>* are 0.84 and 0.99, respectively (Mannaa and Kim 2017b).

The aflatoxin biosynthesis pathway of *Aspergillus parasiticus* has been elucidated (Yu, Bhatnagar, and Cleveland 2004) and it is highly homologous with that of *A. flavus*, as 25 genes within the cluster are the same (Al-Saad et al. 2016). Aflatoxins biosynthesis is coded by an 80 kb long DNA sequence containing 30 putative genes. Figure 25 by Verheecke et al. 2015 presents a synthesis of the aflatoxin biosynthesis pathway focusing on structural genes denominated *afID* (early), *afIM* (medium) and *afIP* (late). The gene *afID* encodes a reductase enzyme implicated in the transformation of norsolorinic acid to averantin; *afIM* is needed for the conversion of versicolorin A to demethylsterigmatocystin; and *afIP* encodes a methyltransferase converting sterigmatocystin to O-methylsterigmatocystin. Two cluster specific regulators are also known: *afIR*, which encodes a transcription activator that binds a consensus sequence in the promoter regions of aflatoxin structural genes, and AfIS is a potential co-activator of AfIR. In addition to AfIS and AfIR, the clustered genes are also regulated by specific transcriptional regulators such as LaeA or Ap-1 (Verheecke, Liboz, Anson, Diaz, et al. 2015).

#### 2.1.3.3.2.2 Toxicity of aflatoxins

Aflatoxins are the most significant and dangerous mycotoxins due to their genotoxic, mutagenic and carcinogenic effects (Mannaa and Kim 2017b). Aflatoxin B<sub>1</sub> is classified as a class I carcinogen by the IARC (IARC 1993). In addition, aflatoxins have oncogenic and immunosuppressive properties (Edite Bezerra da Rocha et al. 2014). The furofuran ring is mainly responsible for the toxic and carcinogenic activity upon activation of the C<sub>8</sub>-C<sub>9</sub> double bond to 8-9 epoxide by the cytochrome P450 in animals (Guengerich et al. 1998). However,

the lactone ring seems to also contribute to the toxicity (Lee et al. 1981). Initially, AFB<sub>1</sub> is converted by P450 cytochromes to the highly reactive aflatoxin B<sub>1</sub>-8,9-epoxide (Pitt et al. 2000). The epoxidation of AFB<sub>1</sub> allows its binding to N7-guanine and, followed by Guanine to Tyrosine transversions, to the DNA molecule. This mechanism is behind the carcinogenic effect. Also, epoxidized aflatoxins form Schiff bases with cellular and microsomal proteins, leading to acute toxicity (Loi et al. 2017). Acute intoxication with aflatoxins provoke hemorrhagic necrosis and fatty acid infiltration in liver cells, and in some cases death (Gil-Serna et al. 2014). AFB<sub>1</sub> is easily transformed in aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) in mammals, which then is excreted through the milk. Moreover, AFM<sub>1</sub> was found to be highly stable to pasteurization temperature. AFM<sub>1</sub> is less hepatotoxic and immunotoxic than AFB<sub>1</sub>, however, its classified as a possible human carcinogen (group 2B) by the IARC (Gil-Serna et al. 2014).

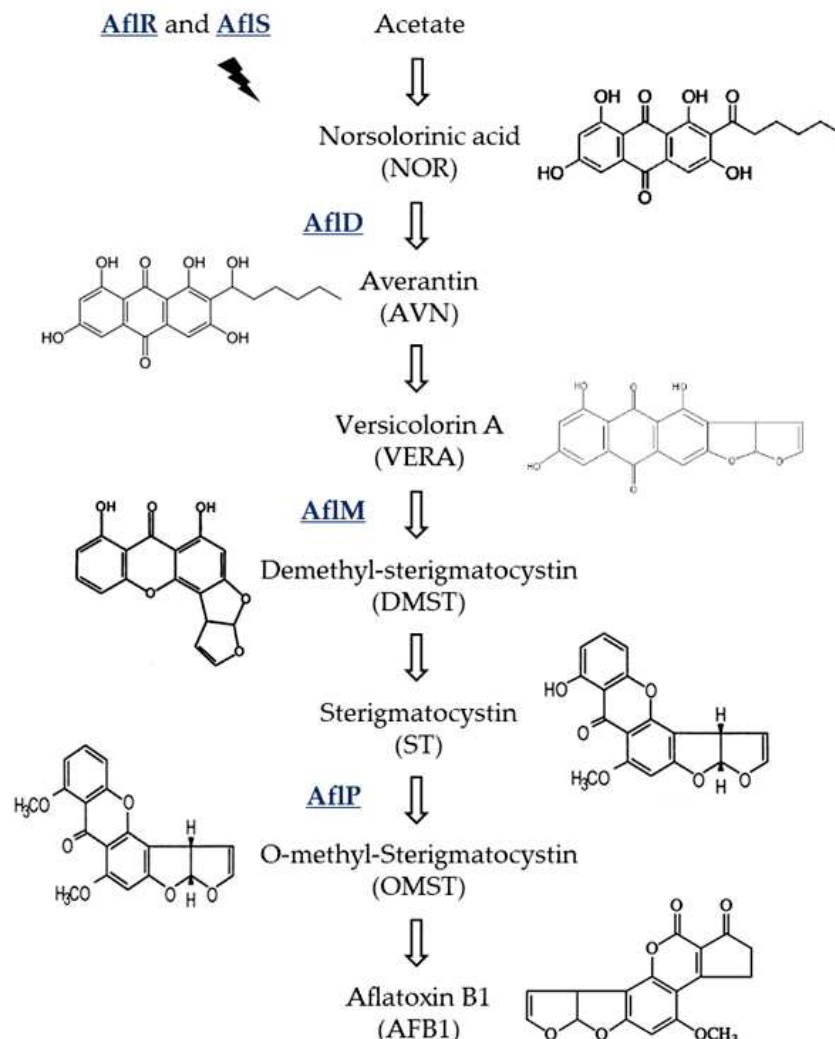


Figure 25. Aflatoxin biosynthesis pathway with a focus on structural enzymes: early (AfID), medium (AfIM) and late (AfIP) and two cluster-specific regulators (AfIR and AfIS) in *Aspergillus parasiticus* (Verheecke et al. 2015).

## 2.1.3.3.2.3 Regulation of aflatoxins

Established limits for the sum of aflatoxins in the EU are 4 µg/kg for groundnuts (2 µg/kg for AFB<sub>1</sub> alone), dried fruits and cereals intended for human consumption; 10 µg/kg (5 µg/kg for AFB<sub>1</sub> alone) for tree nuts, dried fruits, maize and rice subjected to sorting or any other physical process before human consumption, as well as spices, almonds, dried figs, apricot kernels, hazelnuts, and brazil nuts intended for human consumption; 15 µg/kg (8 µg/kg for AFB<sub>1</sub> alone) for peanuts, almonds, pistachios, apricot kernels, hazelnuts and Brazil nuts subjected to sorting or other physical treatment before human consumption (European Commission 2006). The FDA limit for the sum of aflatoxins is 20 µg/kg for the foodstuff described before (Smith et al. 2016).

Regarding animal feed, EU established a limit for AFB<sub>1</sub> of 20 µg/kg for feed materials and 10 µg/kg for complementary and complete feed, the limit for compound feed for young animals was settled at 5µg/kg. The FDA in comparison, established an aflatoxins limit of 20 µg/kg for corn, peanut products and other feeds for immature and dairy animals; 100 µg/kg for corn and peanut products for breeding cattle, breeding swine and immature poultry; 200 µg/kg for finishing swine and 300 µg/kg for finishing beef cattle, as well as cottonseed meal for beef, cattle, swine or poultry (European Commission 2006; Smith et al. 2016).

For milk and milk-based products, only AFM<sub>1</sub> is considered, as AFB<sub>1</sub> gets metabolized to AFM<sub>1</sub> by dairy cattle and then excreted in the milk. The EU limit was established at 0.05 µg/kg, while the FDA established the limit at 0.5 µg/kg (Smith et al. 2016).

Fungal species from the *Penicillium* and *Aspergillus* genus are capable of developing under storage conditions and to provoke the accumulation of harmful mycotoxins in grains. Aflatoxins are considered the most hazardous mycotoxins found in stored cereals, followed by ochratoxin A. Their occurrence increases the health risk of cereals. Thus, maximal limits for their levels in foodstuff have been established.

## 2.2 Control of molds

Wheat disease management nowadays is primarily based on the use of chemical fungicides and resistant wheat cultivars (Syed Ab Rahman et al. 2018). In spite of the protection measures currently implemented in farmer's fields, yield losses from wheat diseases in North Western Europe are estimated at approximately 25% (Willocquet et al. 2021). The implementation of good agricultural practices such as crop rotation and tillage help to reinforce the fungal control approaches mentioned before. Moreover, the use of biological control agents is an important strategy being developed to mitigate mold occurrence (Syed Ab Rahman et al. 2018). Several factors affect fungal growth during farming, harvesting and storage, such as the presence of spores, plant damage, the presence of insects, drought stress, nutrients availability, pH, oxygen and CO<sub>2</sub> levels. Knowledge and management of all the factors influencing fungal development allow to prevent pathogenic proliferation and mycotoxin occurrence (Amézqueta et al. 2009).

### 2.2.1 Pre and post-harvest agricultural practices/management

The first line of defense against mold contamination starts by optimizing crop management, in order to reduce the initial appearance of pathogenic fungi. However, despite the actions taken at pre-harvest stages, fungal growth can occur during the storage of grains. If contamination persists, it should be addressed during post-harvest stages, in order to prevent further pathogenic fungi development and its consequences, that go from grain deterioration to mycotoxin accumulation.

#### 2.2.1.1 Pre-harvest prevention of fungal contamination

Damaged plants tend to be an easy target for fungal contamination, which is why crops should be protected against mechanical and insect damage since pre-harvest stages (Mannaa and Kim 2017a). Timely harvest, crop rotation, tillage, appropriate irrigation, weed control and fertilization are some of the actions that could help prevent the apparition of molds since the earliest stages. If possible, products must be collected when they are mature (Zhu et al. 2017). Products overripen and damaged must be discarded, as they could represent a source of

contamination during storage. Additionally, inoculum sources, dirty farming materials and agricultural residues must be eliminated (Amézqueta et al. 2009).

#### 2.2.1.2 Post-harvest management of fungal development

Management of storage conditions constitute the principal post-harvest prevention method. When possible, after product collection, it should be fastly and homogeneously dried prior to storage, to prevent fungal development. Ideally, products should be turned over to facilitate their aeration, particularly under tropical conditions (Amézqueta et al. 2009). During storage, reducing insect infestation is also essential, as besides causing grain damage, they produce CO<sub>2</sub> and water, that increment seed respiration, humidity and temperature, which altogether favors fungal development. In addition, insect motion contributes to the dissemination of fungal spores (Birck, Lorini, and Scussel 2006). Modification of the storage atmosphere by enriching the amount of CO<sub>2</sub> and reducing that of O<sub>2</sub> is a method of food preservation largely studied. It preserves the quality of foodstuffs and increases their storage life by reducing the respiration rate, which subsequently inhibits fungal activity. When the gas conditions are regulated during the whole storage period it creates a controlled atmosphere, which requires more technical factors (Mannaa and Kim 2017b). It has been demonstrated that these practices reduce the growth rate of most storage-related fungi, as well as their mycotoxin production (Cairns-Fuller, Aldred, and Magan 2005; Giorni et al. 2008; Samapundo et al. 2007). These preservation effects are increased in combination with reduced  $a_w$  and low storage temperatures (Ellis et al. 1993).

#### 2.2.2 Chemical treatments

##### **On the field**

The implementation of good agricultural practices contributes to the reduction of fungal contamination; however, they do not completely eliminate this risk. For this reason, chemical fungicides constitute one of the main components in the Integrated Pest Management (IPM), due to their efficacy for pests and disease control. Main fungicides against plant pathogens are composed of azoles, strobilurins, also known as quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs) or demethylation inhibitors

(DMIs), or a mixture of several of them. In addition to a direct antifungal effect, QoIs inhibit mitochondrial respiration, which increases flag leaf lifespan and consequently impedes fungal sporulation, as fungus requires senescent tissue to develop. However, resistance to this kind of fungicides by some wheat pathogens has become frequent (Solomon et al. 2006).

Wheat rusts are controlled by triazoles (e.g. Tilt, Folicur, Proline, Popimax) and QoIs (e.g. Quadris, Headline). Some commercialized products against rust include both of them (e.g. Stratego, Quilt) (Marsalis and Goldberg 2016). STB is controlled by mixtures of azole (e.g. triticonazole) and SDHIs (e.g. chlorothalonil) (Torriani et al. 2015), as well as by DMIs (Tiley et al. 2018). QoIs are now ineffective against STB due to a widespread resistance (Ponomarenko et al. 2011; Tiley et al. 2018). Foliar fungicide sprays are effective against SNB, the most generally recommended are DMIs (e.g. metaconazole and prothioconazole), QoIs (e.g. pyraclostrobin, azoxystrobin, and picoxystrobin) and combinations of both (e.g. trifloxystrobin + prothioconazole) (Mehra et al. 2019). Some protectant fungicides such as mancozeb and copper can prevent infection by SNB if they are applied at the right time (Li et al. 2008). WB can, under some conditions, be controlled at the heading stage by fungicides combining triazols with QoIs (e.g. carbandazim and tebuconazole) (Debnath et al. 2019). Another approach for the management of WB is the treatment of seeds by active ingredients such as iprodione, fluazinam, difeconazol, carboxin and thiram (Maciel 2019). Several other substances have also been tested against WB, namely, jasmonic acid, deacetylated chitosan, potassium silicate and potassium phosphate, the last being effective to decrease mycelium growth (Kumar et al. 2020).

Main fungicidal treatments applied against FHB consist on DMIs (e.g. metconazole, propiconazole, prothioconazole and tebuconazole) (Ferrigo et al. 2016). A novel fungicide, phenamacril shows not only a suppression of FHB but also a decrease on DON production (Chen et al. 2019). As Fusarium ear rot (FER) in wheat caused by *F. verticillioides* has only been described in the recent years (Gagkaeva and Yli-Mattila 2020), there is a lack of studies indicating the effectivity on chemical fungicides to control this disease on wheat. Triazole groups of fungicides used for FHB management were evaluated for their efficacy against *F. proliferatum* and they were found to reduce fungal growth. However, some fungicide sub-lethal doses stimulated fumonisin production (Cendoya et al. 2020).



**During storage**

Various organic acids have been applied for the control of fungi during grain storage, namely, propionic acid, formic acid and butyric acid. Potassium sorbate and potassium benzoate also showed an inhibitory effect on germination and growth of *A. flavus* and *A. parasiticus* (Mannaa and Kim 2017a). Synthetic food grade antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trihydroxybutyrophenone (THB), and propyl paraben (PP) are allowed for use as antimicrobial agents during storage of foodstuffs (Nesci et al. 2016). Ozone is a powerful fungicide and insecticide, which makes it an alternative technology for the disinfection of wheat. One of the main advantages of ozonation is the absence of residues, as ozone rapidly decomposes to diatomic oxygen. Mycotoxin decontamination by ozone has also been documented (Wu, Doan, and Cuenca 2006). The fumigant methyl bromide, used for many years to control fungal pathogens during storage of grains, was discontinued in 2015 as a part of the “Montreal protocol”, due to its ozone depletion. This led to its replacement by gaseous phosphine (PH<sub>3</sub>) (Hocking and Banks 1991), which has been found useful to control the growth of *A. flavus* on maize (Birck et al. 2006). Other alternatives such as chlorine dioxide (ClO<sub>2</sub>) have been recently researched (Deok Han et al. 2018).

Despite the fact that chemical fungicides have helped reach indispensable crop yields, their systematic use has provoked resistance phenomena among the pathogens, which then leads to higher dosages and higher costs every year (Syed Ab Rahman et al. 2018). Furthermore, the use of chemical pesticides causes several undesirable side effects, such as acute and chronic toxicity in humans, food contamination, environmental pollution and the destruction of beneficial organisms, like pollinator species. To these effects is added the reduction of the diversity of beneficial microbial species in the soil, that can repress the competition against pathogens and facilitate their development (Newitt et al. 2019). Besides, when used to manage mycotoxinogenic fungi, some chemical fungicides were found to stimulate mycotoxin production, even when fungal development was inhibited (Medina et al. 2007). Moreover, evidences indicate that pathogenic fungal exposure to sub-lethal fungicide concentrations can motivate mycotoxin biosynthesis, for example by triggering oxidative stress (Ferrigo et al. 2016). The previous reasons have motivated the search for more

environmentally friendly pathogen management strategies, among which biological control has demonstrated to be a promising alternative.

### 2.2.3 Biological control

Biological control or biocontrol is defined as the association of factors aiming to decrease the undesirable effects of detrimental organisms and promote the growth of beneficial organisms. It refers both to the macro and microorganisms themselves and to the natural substances obtained from them (Syed Ab Rahman et al. 2018). The organisms used in biocontrol are called biocontrol agents (BCAs) while their derived genes, enzymes and metabolites, are defined as “biopesticides” or “biofertilizers”, depending on their application (Pal and McSpadden Gardener 2006). The use of fungi and bacteria against plant pathogens offers a more ecological alternative to chemical pesticides, as they are naturally associated with plants, which implies a minor impact on native microbial equilibrium (Knudsen and Dandurand 2014). Besides, they are able to inhibit fungal development and, in some cases, improve plant defense and resistance against fungal diseases (Pal and McSpadden Gardener 2006). Good BCAs often exhibit more than one antagonistic mechanism (Palaniyandi et al. 2013).

Most biocontrol research has focused on a limited number of bacterial (*Bacillus*, *Burkholderia*, *Lysobacter*, *Panotea*, *Pseudomonas*, *Rhizobium* and *Streptomyces*) and fungal (*Ampelomyces*, *Coniothyrium*, *Dactylella*, *Gliocadium*, *Paecilomyces*, and *Trichoderma*) genera, due to the simplicity of their culture (Pal and McSpadden Gardener 2006). The genera *Pseudomonas* spp., *Bacillus*, *Rhizobium* and *Trichoderma* in particular have been mostly used for the control of soil-borne plant pathogenic fungi (Chet and Inbar 1994). *Pseudomonas* have been largely studied for their production of antibiotics. *Bacillus* are especially attractive for their practical use, as they produce stable endospores which can survive the heat and desiccation processes that can take place during the formulation of BCAs (Legein et al. 2020). *Trichoderma* are able to parasite plant pathogens in addition to their production of antimicrobial compounds (Handelsman' and Stabb 1996). Actinobacteria, especially *Streptomyces* genus, represent promising biocontrol agents as they combine several of the

advantages previously described for the most studied BCAs. Detailed information will be presented in the last chapter of this bibliographical synthesis.

Several strategies are implemented during pre and post-harvest stages to avoid the presence of pathogenic fungi. Pre-harvest preventive approaches include crop rotation, tillage and proper irrigation. Post-harvest measures are based on the optimization of storage conditions. Chemical fungicides are intensively employed in the field to control the fungal contamination, which has led to a pathogenic resistance phenomenon. Biocontrol with active microorganisms exhibiting a variety of antagonistic mechanisms and plant beneficial traits, presents a more ecological alternative to decrease fungal development.

## 2.3 Decontamination of mycotoxins

Several approaches are applied to monitor and avoid mycotoxins presence in the production chain, comprising field, storage, transportation and processing. Unfortunately, mycotoxin occurrence cannot always be prevented. For this reason, detoxification methods are sometimes applied to food and feed in order to reduce mycotoxin risk to the lowest possible amount, and possibly salvage contaminated batches, as long as there is no alteration in the expected quality (Vanhoutte, Audenaert, and De Gelder 2016). Some physical methods such as separation, milling and dehulling allow mycotoxin decrease during industrial processes. Moreover, the use of adsorption agents has become popular since the EU authorized their use as feed additives (Zhu et al. 2017). The application of chemical treatments to food, exclusively with mycotoxin decontamination purposes is not authorized. However, some food preparation processes include the addition of chemical products that incidentally help to decrease mycotoxin risk. Lastly, biological detoxification has been achieved by several microorganisms either naturally present on food matrices or added for technological purposes (Gil-Serna et al. 2014).

### 2.3.1 Modified mycotoxins

*In vivo* decontamination of mycotoxins occurs when plants, animals and fungi use detoxifying mechanisms to protect themselves from mycotoxins as a part of their defense against xenobiotics. These mechanisms can lead to chemical alterations in the structure of mycotoxins, resulting in modified chromatographic profiles, epitope conformation or polarity (Smith et al. 2016), which hinders their correct chromatographic identification. These modified forms may be produced via conjugation with sugars or other biological components, escaping in this manner conventional analytical methods, thus called “modified mycotoxins”. They may also evolve into non-extractable forms on account of a strong interaction with the matrix, becoming “hidden mycotoxins”. In both cases, they represent a latent risk as even when their conjugated form is partially less toxic, like for the FB<sub>1</sub>-glucose reaction products (Fernández-Surumay et al. 2004), they can turn bioavailable again during digestion (Berthiller et al. 2013). Some of the metabolism derivatives known include zearalenone-14-glucoside (ZEA14G), zearalenone-14-sulphate (ZEA14S),  $\alpha$ -zearalenol ( $\alpha$ -ZEL),  $\beta$ -zearalenol ( $\beta$ -ZEL),

deoxynivalenol-3-glucoside (DON3G), 15-acetyldeoxynivalenol (15-ADON) and 3-acetyldeoxynivalenol (3-ADON) (Cirlini, Dall'Asta, and Galaverna 2012).

As stated before, decrease on the mycotoxin amount not necessarily means detoxification, as mycotoxins could be present in masked or modified forms that are no longer detectable but could still be toxic or revert to its toxic form during digestion. Besides, industrial processes could also release modified mycotoxins and make them bioavailable (Karlovsky et al. 2016). In some other cases, mycotoxin degradation by-products remain toxic. For example, yeasts biotransform ZEN to  $\alpha$ -ZEL which is more estrogenic (Böswald et al. 1995). This fact highlights the importance of the study of degradation mechanisms and the characterization of degradation by-products. Designation of mycotoxin transformations as detoxification must be first corroborated (Berthiller et al. 2013). Therefore, there are two ways of validating an effective detoxification of matrices and foodstuffs: either less toxic degradation by products are detected and identified or the reduction of toxicity is assessed by one or more toxicity bioassays (Vanhoutte et al. 2016).

For the evaluation of mycotoxin decrease and the search of degradation by products, chromatographic techniques are most commonly applied, such as HPLC (High Performance Liquid Chromatography), LC-MS (Liquid Chromatography-Mass Spectrometry), GC-MS (Gas Chromatography-Mass Spectrometry), TLC (Thin Layer Chromatography), and NMR (Nuclear Magnetic Resonance Spectroscopy). As well as specific mycotoxin detection by Enzyme-Linked Immunosorbent assay (ELISA) (Zhu et al. 2017).

### 2.3.2 Evaluation of residual toxicity

If a decontamination protocol is applied to food matrices, it is essential to control their residual toxicity. Various methodologies allow the identification of toxicity *in vitro* and *in vivo*. The *in vitro* procedures determine cell viability, apoptosis/necrosis, DNA damage and oxidative damage using cell models corresponding to major organs targeted by mycotoxins (Smith et al. 2016). Toxic effects of trichothecenes, aflatoxins and zearalenone are evaluated using various cell lines which include human intestinal cells (Caco-2), human leukaemia monocytes (THP-1), human hepatic cells (HepaRG), intestinal porcine epithelial cells (IPEC-1), chinese hamster ovary cells (CHO-K1) and monkey kidney epithelial cells (Vero) (Smith et al.

2018; Zhu et al. 2017). One of the most extensively used *in vitro* tests for measuring mutagenicity is the Ames test, which measures reverse mutations from histidine auxotrophy to prototrophy in several especially constructed mutants (Quillardet and Hofnung 1993). A complementary *in vitro* tool for the determination of genotoxicity of compounds, such as aflatoxins, is the SOS-chromotest, a colorimetric assay that measures the *sfiA* expression during the SOS repairing system in a mutant strain of *E. coli*, by assaying the  $\beta$ -galactosidase activity. The fusion of two operons, places *lacZ* (structural gene of  $\beta$ -galactosidase) under the control of the *sfiA* gene which ensures that the  $\beta$ -galactosidase activity is strictly dependent on *sfiA* expression. In addition, the strain was made genetically more susceptible to genotoxic agents and has a mutation that renders the cell envelope more permeable (Krifaton et al. 2011; Quillardet and Hofnung 1993). The toxicity of other mycotoxins such as zearalenone and ochratoxin A can be assessed by using the bioluminescent bacterium *Aliivibrio fischeri* as its light emission decreases in the presence of toxic compounds (Krifaton et al. 2010). The nematode *Caenorhabditis elegans* is an *in vivo* approach used to evaluate the effect of diverse mycotoxins (e.g. AFB<sub>1</sub>, DON, FB<sub>1</sub>, ZEN) on growth, reproduction, lethality and lifespan (Yang et al. 2015). Regarding the teratogenicity of OTA *in vivo*, the zebrafish embryo toxicity assay (ZETA) using the zebrafish *Danio rerio* can be employed to assess the lack of toxicity of the degradation by-products (Haq et al. 2016).

### 2.3.3 Physical methods for mycotoxin decontamination

Physical approaches to decrease mycotoxin content on foodstuff include manual and mechanical sorting, density segregation, milling, steeping and extrusion, as well as binding matrices, thermal inactivation and irradiation (CAST 2003; Karlovsky et al. 2016). Technologies such as pulsed light exhibited high degrading percentages of zearalenone (84.5%), deoxynivalenol (72.5%), AFB<sub>1</sub> (92.7%) and OTA (98.1%) in solution. But were only able to eliminate the toxicity for aflatoxin B<sub>1</sub> (Moreau et al. 2013).

Separation of damaged kernels based on weight, size, color and visible fungal growth has proven to be useful to reduce aflatoxin contamination, as broken and damaged cereal kernels often carry the highest mycotoxin amounts. Flotation separation is achieved by using concentrated NaCl or sucrose solutions that cause the damaged kernels to float. For instance,

reductions of fumonisins, aflatoxins, DON and ZEN by around 70-80% on maize, wheat and barley are achieved by these processes (He and Zhou 2010; Karlovsky et al. 2016). However, sorting techniques are unable to decrease fumonisins contamination as *Fusarium verticillioides* infected kernels are frequently exempt of symptoms (Karlovsky et al. 2016).

Milling and dehulling allow the reduction of mycotoxins present in the outer layer of the grain. Indeed, the distribution of the mycotoxins in the grain influences the efficacy of the detoxification by milling, as elucidated by Schwake-Anduschus et al., who revealed that ZEN is mainly concentrated on the fiber-rich parts of the wheat grain, whereas DON is distributed equally between all the grain fractions. Water soluble mycotoxins (e.g. fumonisins) can be partially eliminated by subjecting the grains to a washing or steeping process (Schwake-Anduschus et al. 2015). Sodium carbonate solutions are often used to improve the effectiveness of washing protocols for mycotoxins with less water solubility (Karlovsky et al. 2016).

Heat treatment remains one of the most useful processes to reduce mycotoxin amounts on the final product. However, the several mycotoxins are chemically and thermally stable. Heating process include cooking, frying, baking, roasting, extrusion and canning (Bullerman and Bianchini 2007). The addition of an alkaline treatment usually increases the efficacy of extrusion and heating. Interestingly, bread baking does not eliminate OTA, while biscuits baking decreased it by about two thirds. Industrial processes such as malting, extrusion and roasting can also contribute to reduce OTA amounts (Abrunhosa et al. 2010). As fumonisin B<sub>1</sub> is relatively heat stable, boiling does not eliminate it, and processes involving higher temperatures are needed, such as canning, extrusion or roasting. The last two treatments have been reported to reduce fumonisins by 34-95% in maize grits. DON is eliminated from bread, biscuits and cookies at variable percentages (24-71%) by baking (Bullerman and Bianchini 2007).

Non-ionizing radiation such as solar, UV light and microwaves, as well as ionizing gamma radiation, can reduce fungal contamination in food and also partially decrease mycotoxin amount (Agriopoulou et al. 2020). Furthermore, aflatoxins in cereals can be decreased up to 75% by photodegradation after 30 hours of direct sunlight. OTA and fumonisins can also be reduced by gamma radiation. Microwaves can partially degrade DON (around 40%) (Karlovsky et al. 2016). Cold plasma has strong antimicrobial effects. Hence, it

allows the disinfection of fragile or temperature-sensitive surfaces. However, there is a lack of studies about the potential formation of toxic compounds in food matrices by this sterilization technique (Agriopoulou et al. 2020). Nevertheless, its efficacy for destroying about half of the amount of aflatoxins, fumonisins, OTA, DON and ZEN has been documented (Karlovsky et al. 2016).

Another physical method for removing mycotoxins from foodstuffs is the use of binding matrices that impede the passage of toxic molecules into the bloodstream (Agriopoulou et al. 2020). Some examples include activated charcoal, aluminosilicates and bentonite, which can be added to animal feed as granules, powders and pellets. The use of resins for the elimination of mycotoxins in liquid products was also studied (He and Zhou 2010; Karlovsky et al. 2016). Such techniques are currently being researched for their use as food additives for reduce the exposure to mycotoxins in highly affected regions (Afriyie-Gyawu et al. 2008; Wang et al. 2005, 2008).

#### 2.3.4 Chemical methods for mycotoxin decontamination

During food processing, a number of steps may include chemical treatments, such as ammoniation and ozonation (CAST 2003), which transform the nutritional quality, texture and flavor of the matrix. It has been found that some chemicals involved in industrial processes are able to transform mycotoxins. Regardless, chemical treatments with the objective of decontamination or detoxification of mycotoxins are not authorized in the EU (Karlovsky et al. 2016).

The majority of mycotoxins are resistant to weak acids. However, strong acids such as HCl are able to detoxify aflatoxins, but their application to food is not allowed. Furthermore, food-grade acids, such as acetic acid, citric acid and lactic acid were evaluated during cooking process, revealing that lactic acid was the most effective to detoxify AFB<sub>1</sub> by transforming it into AFB<sub>2</sub> and AFB<sub>2a</sub> (hemiacetal form) (Karlovsky et al. 2016).

The efficacy of bases involved in food processing as a degradation technique varies according to the temperature, pressure, moisture, duration and substrate. For instance, aflatoxins are unstable to alkaline treatments, as their lactone ring is opened under these



conditions, however this reaction is reversible. Moreover, the use of ammonia has proven useful for the partial degradation of aflatoxins, resulting in aflatoxin D<sub>1</sub> and D<sub>2</sub>, which are less toxic. Other alkaline agents able to detoxify aflatoxins include NaOH, Ca(OH)<sub>2</sub>, Na<sub>2</sub>CO<sub>3</sub>, Na<sub>3</sub>PO<sub>4</sub>, methylamine, ethylene-diamine and ethanolamine (Karlovsky et al. 2016). Manufacturing of tortillas by alkaline cooking and steeping of the corn (nixtamalization) and further processing into tortilla chips and corn chips led to a reduction of aflatoxins by 52% in the tortillas, 85% in the tortilla chips and 79% in the corn chips (Torres, Guzmán-Ortiz, and Ramírez-Wong 2001). OTA was also decomposed by ammoniation in maize, wheat and barley (Chelkowski et al. 1981). FB<sub>1</sub> was reduced by 79% in wheat by ammoniation, but the procedure was ineffective in maize. Besides, toxicity of the products resulting of FB<sub>1</sub> alkaline hydrolysis was variable, indicating the need of supplementary research in order to use this procedure for detoxifying food matrices (Karlovsky et al. 2016).

The addition of ozone (O<sub>3</sub>) as an oxidizing agent has given positive results for the degradation of aflatoxins, the putative mechanism involves the opening of the lactone ring, followed by the rearrangement of the aflatoxin molecule into derivatives such as ketones, aldehydes and organic acids. Indeed, the presence of a terminal double bond in the dihydrofuran ring of AFB<sub>1</sub>, AFG<sub>1</sub> and AFM<sub>1</sub> makes them more susceptible to the attack of oxidizing agents than AFB<sub>2</sub>, AFG<sub>2</sub> and AFM<sub>2</sub> (Karlovsky et al. 2016). Furthermore, OTA, FB<sub>1</sub> and ZEN were also degraded after being in contact with 10% of O<sub>3</sub> for 15s. However, degradation of FB<sub>1</sub> yielded a 3-keto derivative that remained toxic during two bioassays (McKenzie et al. 1997). Despite its promising results, ozone application for the decontamination of foods is not yet allowed in several countries, including the EU. Ozone application on food as an antimicrobial agent and for decontamination purposes has been approved by the FDA in United States since 1997 (Suman 2021). Furthermore, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) has proven effective to detoxify aflatoxins and to decrease ZEN, which is also destroyed by oxidation with ammonium persulfate. Contrastingly, OTA was resistant to oxidation by H<sub>2</sub>O<sub>2</sub> (Karlovsky et al. 2016). Aflatoxins B<sub>1</sub>, G<sub>1</sub> and M<sub>1</sub>, as well as DON can be destroyed by the addition of sodium bisulfite (NaHSO<sub>3</sub>) in maize and dried fruits (Karlovsky et al. 2016).

Figure 26, modified from Karlovsky et al. 2016, presents a summary of the main physical and chemical treatments used in food processing being able to mitigate mycotoxin risk.

Selected food commodities	Relevant mycotoxin(s)	Physical processes											Chemical processes				
		Sorting	Sieving	Flotation	Washing	Dehulling	Steeping	Milling	Heating	UV radiation	γ radiation	Plasma	Acid	Alkaline	Oxidation	Reduction	Ammoniation
Cereals	Trichothecenes	Green	Green	Green	Yellow	Green	Yellow	Green	Green	Red	Yellow	Yellow	Yellow	Green	Yellow	Yellow	Yellow
	Ergot alkaloids	Green	Green	Yellow	White	White	Yellow	White	White	Yellow	Yellow	Yellow	White	White	White	White	White
	Ochratoxin A	Yellow	Yellow	White	White	White	White	Green	Green	Yellow	Yellow	Yellow	Yellow	Red	Green	Yellow	Yellow
	Zearalenone	Yellow	Yellow	White	Yellow	Green	Yellow	Green	Green	Red*	Yellow	Yellow	Yellow	Yellow	Red	Green	Yellow
Maize	Trichothecenes	Green	Yellow	Green	Yellow	Green	Green	Green	Green	Red	Yellow	Yellow	Yellow	Green	Yellow	Yellow	Yellow
	Ochratoxin A	Yellow	Yellow	White	White	Green	White	Green	Green	Yellow	Yellow	Yellow	Yellow	Red	Green	Yellow	Yellow
	Aflatoxins	Green	Yellow	Green	Yellow	Green	Green	Green	Green	Yellow	Yellow	Yellow	Yellow	Green	Green	Green	Green
	Fumonisin	Green	Yellow	Green	White	White	Green	Green	Green	Red	Green	Yellow	Yellow	Red	Red	Red	Red
	Zearalenone	Yellow	Yellow	White	Yellow	Green	Green	Green	Green	Red*	Yellow	Yellow	Yellow	Yellow	Green	Yellow	Yellow
Apples	Patulin	Green	Red	White	Yellow	Red	Red	Red	Red	**	Green	White	Red	Red	Red	Red	Red
Grapes	Ochratoxin A	Green	Red	White	Red	Red	Red	Red	White	Yellow	Yellow	Yellow	Yellow	Red	Red	Red	Red
Cocoa beans	Ochratoxin A	Yellow	White	White	White	Yellow	Red	Red	Green	White	Yellow	White	White	Yellow	Yellow	Yellow	Yellow
	Aflatoxins	Yellow	White	White	White	Yellow	Red	Red	Green	White	Yellow	White	White	Yellow	Yellow	Yellow	Yellow
Coffee beans	Ochratoxin A	Yellow	White	White	White	Yellow	Red	Red	Green	White	Yellow	White	White	Yellow	Yellow	Yellow	Yellow
	Aflatoxins	Yellow	White	White	White	Red	Red	Red	Green	White	Yellow	White	White	Yellow	Yellow	Yellow	Yellow

\* Conversion to a more estrogenic *cis*-form

\*\* Experimentally demonstrated on apple juice

	Experimentally demonstrated
	Partly successful or scientifically conceivable
	Not possible or incompatible with the commodity
	Not enough information or not applicable

Figure 26. Summary of physical and chemical processes applied to food commodities being able to decrease mycotoxin amount. Modified from Karlovsky et al. 2016.

### 2.3.1 Biological and enzymatical methods of mycotoxin decontamination

Biological mycotoxin decontamination methods have proven to be more specialized and more environmentally friendly than physical and chemical methods, which tend to be time-consuming and have limited efficacy. Additionally, they may cause nutrient loss and require specialized equipment (Agriopoulou et al. 2020). Prokaryotic and eukaryotic microorganisms from various sources, as soil, plants and seeds are capable of transforming mycotoxins, sometimes by using them as carbon sources (He and Zhou 2010).

Decontamination could be provoked either by an adsorption on the microorganism cell wall surface (Haskard et al. 2001; Pierides et al. 2000) or an irreversible enzymatic transformation, such as acetylation, glucosylation, ring cleavage, hydrolysis, deamination and decarboxylation (McCormick 2013). Determining which of the two alternatives is behind an observed mycotoxin decrease is of the utmost importance, as bound mycotoxins could be released during the consumption of the final product. However, in many studies, the mechanisms involved during detoxification remain sometimes unexplored.

Certain microorganisms involved on fermentation processes are capable of detoxifying mycotoxins, yet, no strain addition has been authorized with the sole objective of degrading mycotoxins. Despite this, selecting starter cultures able to detoxify mycotoxins, without modifying the technology, could improve the safety of these aliments (Karlovsky et al. 2016).

Some enzymes naturally present in the food matrices can detoxify them by degrading the mycotoxins during fermentation processes, however, better results are obtained by the addition of purified and/or molecular engineered enzymes. Unfortunately, not many enzymes have been identified, purified and characterized for this activity (Loi et al. 2017; Lyagin and Efremenko 2019). It should be noted that in the EU no enzyme has been authorized so far for the reduction of mycotoxin contamination in food. Nevertheless, few commercial feed additives are available, namely, Mycofix<sup>®</sup>, FUMzyme<sup>®</sup>, Biomin<sup>®</sup> BBSH 797 and Biomin<sup>®</sup> MTV (Loi et al. 2017; Lyagin and Efremenko 2019). A defining property of enzymes is their specificity, nevertheless, enzymes such as laccases and peroxidases modify a large number of substrates, making them able not only to destroy mycotoxins, but also important food components. Besides, some of them could cause an allergic reaction, so their allergenic potential has to be investigated before approval. So far, no allergic reaction has been reported towards currently used food enzymes. The most important limitation for the application of mycotoxin degrading enzymes in real matrices is the occurrence of matrix effects that reduce their effectiveness as moisture, acidity and texture have a great influence in enzymatic performance (Zhu et al. 2017). Nevertheless, enzymes potential is vast as they can be expressed by several industrial microorganisms (e.g. yeasts, lactic acid bacteria) and their versatility allows for them to be used free or immobilized, which present several advantages (Loi et al. 2017).

## 2.3.2 Mechanisms of mycotoxins detoxification

### 2.3.2.1 Detoxification of DON and ZEN

The toxicity of trichothecenes comes essentially from the 12,13-epoxide ring. However, the degree of toxicity is dependent on the presence of substituents (acetyl, acyl) on C-4 and C-15. For the non-acetylated trichothecenes (e.g. DON, NIV), de-epoxidation is the first step towards detoxification, while for the monoacetyl trichothecenes (e.g. 3-ADON, 15-ADON) de-acylation is the predominant route. After de-epoxidation, DON is degraded by oxidation and isomerization. The transformation of DON to de-epoxy-DON (DOM-1) has been documented for microorganisms such as *Eubacterium* and *Citrobacter freundii*. Its subsequent oxidation to 3-keto DON was reported for a strain belonging to the *Agrobacterium-Rhizobium* group. Lastly, the transformation to 3-epi DON was achieved by the soil bacterium *Devosia mutants* and by *Nocardioides* sp., the resulting product being at least 50 times less toxic than DON (He et al. 2016; Vanhoutte et al. 2016). In a recent study, Carere et al. isolated the enzymes DepA and DepB produced by *D. mutants*, which catalyze the conversion of DON to 3-epi-DON (Carere et al. 2018a, 2018b). An actinobacteria strain capable of degrading DON is *Marmoricola* sp., which was reported to degrade DON from wheat kernels after applying a bacterial cell suspension (Ito et al. 2012). Rumen or intestinal bacteria's ability to transform DON into DOM-1 was also reported. Same mechanism was exhibited by enterobacteria such as *Serratia*, *Clostridium*, *Citrobacter* and *Enterococcus*. In fact, DOM-1 is 55 times less toxic than DON (McCormick 2013). In human liver, DON can be conjugated to glucuronides producing a non-toxic by-product, glucuronide conjugated-DON (Smith et al. 2018).

Enzymes such as amylases, glucanases and proteases are commonly used during beer brewing and in bakery, where DON contamination is usually observed. Thus, the addition of detoxifying enzymes could be compatible with these processes (Karlovsy et al. 2016). Unfortunately, food suitable enzymes able to irreversibly detoxify DON are not available yet, although some promising candidates have been identified (He et al. 2016; Ito et al. 2013; Zhang et al. 2020).

ZEN toxicity derives from its lactone ring and the free C-4 hydroxyl group, which allows the binding to the estrogen receptor (Vanhoutte et al. 2016). Reported fungal transformations

of ZEN include reduction of carbonyl ketones, reduction of double bonds, hydroxylation, methylation, sulfation, glycosylation, and ring cleavage (McCormick 2013). The main detoxification method remains the lactone ring cleavage, which is catalyzed by esterases. The resulting hydroxyketones spontaneously decarboxylate, making the degradation irreversible. *In vivo*, ZEN gets reduced to  $\alpha$ - and  $\beta$ -zearalenol ( $\alpha$ -ZEL and  $\beta$ -ZEL), both by-products are estrogenic and  $\alpha$ -ZEL is more estrogenic than ZEN. A subsequent reduction leads to the formation of  $\alpha$ - and  $\beta$ -zearalanol ( $\alpha$ -ZAL and  $\beta$ -ZAL), which are less estrogenic than ZEN (Böswald et al. 1995; Loi et al. 2017).

ZEN degradation by modification of the C-4 hydroxyl group has been observed for *Cunninghamella bainieri*, *Thamnidium elegans*, and *Rhizopus* sp., among others (McCormick 2013). *C. bainieri* converted ZEN into 2,4-dimethoxyzearalenone, while *T. elegans* and *Mucor bainieri* transformed it into ZEN-4-O- $\beta$ -glucoside. However, this mechanism cannot be considered a true detoxification as the conjugates may be hydrolyzed during digestion. Several *Pseudomonas* species have also been documented to degrade ZEN (Ji, Fan, and Zhao 2016). The yeast *Trichosporon mycotoxinivorans* was able to convert zearalenone into ZOM-1, which is no longer estrogenic, by opening the ring structure of the ketone group at C-6 (Vekiru et al. 2010). Fungal species such as *Gliocladium roseum* and *Trichoderma aggressivum* were reported capable of degrading ZEN by a ring cleavage. Cell cultures of *Bacillus natto* and *B. subtilis* also degraded ZEN by a similar mechanism. A cleavage of the benzene ring may be involved in degradation observed by *Aspergillus niger* and *Acinetobacter* sp. (Vanhoutte et al. 2016). The estrogenic activity of ZEN was decreased by several *Rhodococcus* strains, namely, *R. erythropolis*, *R. ruber* and *R. pyridinivorans*, which had ZEN degradation efficiencies between 14% and 70% (Cserháti et al. 2013). Furthermore, *Streptomyces rimosus* converted ZEN to 8'-hydroxyzearalenone, which is no longer estrogenic (Harkai et al. 2016; Ji et al. 2016; Vekiru et al. 2010). A lactonohydrolase produced by the fungus *Clonostachys rosea* was able to degrade ZEN. A laccase-mediator system, patented by Novozyme, degrades ZEN by means of a laccase from *Streptomyces coelicolor*. Finally, ZEN is also degraded by an oxidoreductase, Prx (2-Cys peroxiredoxin) (Loi et al. 2017).

### 2.3.2.2 Detoxification of fumonisins

Toxicity of fumonisins is given by two structural groups on its structure, the free primary amino group in C-2 and the two tricarballic acids (Vanhoutte et al. 2016). Fumonisin's detoxification mechanism consists in a deesterification by a carboxylesterase, which releases the two tricarballic acid moieties, resulting in hydrolysed FB<sub>1</sub> (HFB<sub>1</sub>), also known as aminopentol 1 (AP1). Next step consists on an oxidative deamination by an aminotransferase that produces 2-keto HFB<sub>1</sub> (Loi et al. 2017), which can be further converted to hemiketal derivatives by cyclization of the hydroxyl at C-5 and the C-2 (McCormick 2013). The N-acetylation of the amino group can also decrease FB<sub>1</sub> toxicity (Vanhoutte et al. 2016). HFB<sub>1</sub> is also produced by the addition of lime during nixtamalization (Palencia et al. 2003). However, biochemical studies showed that hydrolyzed fumonisins could be transformed to highly toxic derivatives *in vivo* (Humpf et al. 1998). Fumonisin detoxification has been observed by microorganisms such as *Sphingomonas* sp., *Sphingopyxis* sp., *Exophiala* sp, *Bacillus* and yeasts (Vanhoutte et al. 2016). Enzymes involved in the degradation by these microorganisms consist mainly on carboxylesterases, aminotransferases and, when the amino group is targeted, deaminases (Loi et al. 2017). The carboxylesterase produced by *Sphingopyxis* sp. is commercialized as a feed additive (FUMzyme®) by Biomin (McCormick 2013).

### 2.3.2.3 Detoxification of ochratoxin A and citrinin

The toxicity of OTA is mainly attributed to its isocoumarin moiety, due to its Cl group, than to the phenylalanine moiety (Vanhoutte et al. 2016). Two pathways could be involved during microbial degradation of OTA. The first consists in the hydrolysis of the amide bond between the phenylalanine and the isocoumarin residue, which results in the formation of Phenylalanine and OT $\alpha$  (Loi et al. 2017). The second, more hypothetical, involves the hydrolysis of the lactone ring, which results in an opened lactone form of OTA (OP-OTA) (Abrunhosa et al. 2010). Soil bacteria *Phenyllobacterium immobile* putative degradation mechanism consisted in (i) a dioxygenase attack on the phenylalanine moiety followed by (ii) a dehydrogenation to a catechol derivative that suffers a meta-ring cleavage and finally (iii) a hydroxylation step that releases OT $\alpha$  (Wegst and Lingens 1983).

Several protozoal, fungal, yeast and bacterial species were found able to degrade, transform or bind OTA (Chen et al. 2018). Rumen protozoa of ruminants, are able to degrade OTA by hydrolysis of the amide bond, which makes them resistant to OTA toxic effects. The main mechanism involved in the decontamination by lactic acid bacteria and yeasts is the absorption of OTA to the cell walls, however some catabolism may be involved, as some authors reported reduced toxicity. OTA decrease by yeasts such as *Saccharomyces cerevisiae* has been documented during brewing, but the phenomenon was reversible, indicating a binding mechanism (Petruzzi et al. 2014). Other yeasts such as *Rhodotorula*, *Cryptococcus* and *Trichosporon mycotoxinivorans* can hydrolyze the amide bond of OTA and produce OT $\alpha$  and phenylalanine (McCormick 2013). Several *Aspergillus* and *Rhizopus* species are able to detoxify OTA, as well as *Botrytis cinerea* and *Pleurotus ostreatus* (Abrunhosa et al. 2010).

The presence of an amide bond in the OTA molecule allows the hydrolytic action of enzymes such as carboxypeptidases, chymotrypsins, amidases, proteases and lipases (Loi et al. 2017). Two classes of carboxypeptidases can be involved in OTA degradation, namely Carboxypeptidase A (CPA) and Y (CPY) (Vanhoutte et al. 2016). Many proteases, such as Protease A from *Aspergillus niger*, were able to hydrolyze OTA to OT $\alpha$  (Loi et al. 2017). Lipases, like the one produced by *A. niger* are also able to hydrolyze OTA and to produce the less toxic OT $\alpha$  (Stander et al. 2000). Carboxypeptidase Y from *Saccharomyces cerevisiae* was found to be able to hydrolyze OTA, in addition to the yeast adsorption phenomenon (Abrunhosa et al. 2010).

Citrinin degradation was achieved by a solvent-tolerant bacteria *Moraxella* sp., which converted citrinin to decarboxycitrinin, which also results from a heat treatment of the mycotoxin (Zhu et al. 2017). By a mechanism of oxido-reduction, citrinin transforms into dihydrocitrinone in urin (Li et al. 2020). Moreover, degradation of this mycotoxin has been reported by *Klebsiella pneumoniae* and *Rhizobium borbori* (Ben Taheur et al. 2019).

#### 2.3.2.4 Detoxification of aflatoxins

The toxicity of AFB<sub>1</sub> originates mainly from the lactone ring. Its cleavage leads to a non-fluorescent compound with 450 times reduced mutagenicity and 18 times reduced toxicity. The difuran ring moiety, especially the double bond in the terminal furan ring, also contributes to the toxicity. Generally, two main detoxification pathways are observed: modification of the difuran ring or modification of the coumarin structure (Vanhoutte et al. 2016).

Several, not food related, microorganisms have been screened for their ability to degrade aflatoxins, the first report published in 1966 by Ciegler and co-workers (Ciegler et al. 1966). Several fungal species have been found to be able to transform AFB<sub>1</sub> into less toxic metabolites. These species include *Pleurotus ostreatus*, *Trametes versicolor*, *Rhizopus* sp., *Mucor* sp.; some yeasts such as *Trichosporon mycotoxinivorans*, *Saccharomyces cerevisiae*, *Trichoderma* and *Armillariella tabescens*. Degradation by the latter originated a product that remained fluorescent, which suggests that the modification took place in the difuran rather than in the lactone fraction of the molecule (McCormick 2013). Bacterial ability to decrease AFB<sub>1</sub> was also documented, several decontamination studies were performed on Lactic Acid Bacteria (LAB), however, as mentioned before, this reduction was mainly due to cell wall binding of the mycotoxin, which seems to be reversible (Guan et al. 2008). On the other hand, bacteria able to effectively degrade AFB<sub>1</sub> include *Rhodococcus erythropolis* (Alberts et al. 2006; Teniola et al. 2005), *Mycobacterium fluoranthenorans* (Teniola et al. 2005) and *Nocardia corynebacterioides* (previously *Flavobacterium aurantiacum*) (Smiley and Draughon 2000). As well as *Streptomyces lividans* and *Streptomyces aureofaciens* (Eshelli et al. 2015). The mechanism of degradation of AFB<sub>1</sub> and AFG<sub>1</sub> was described by the actinobacteria *Mycobacterium smegmatis*. First, a reduction of the  $\alpha,\beta$ -unsaturated double bond between C-2 and C-6 by a F<sub>420</sub>-H<sub>2</sub> dependent reductase takes place, followed by a spontaneous breakdown of the product. The F<sub>420</sub> cofactor does not occur in microorganisms applied in food processing but seem to be widespread among the *Actinomycetales* family (Lapalikar et al. 2012; Taylor et al. 2010).

Several enzymes have been studied for their capacity to degrade aflatoxins, such as the laccase enzyme produced by *Trametes versicolor* which degraded AFB<sub>1</sub> by 87% (Alberts et al. 2009). Degradation of AFB<sub>1</sub> into AFB<sub>1</sub>-8,9-dihydrodiol was observed from the white rot fungi



*Phanerochaete sordida* by a manganese peroxidase, as well as from the “aflatoxin-detoxifzyme” (ADTZ) produced by the fungus *Armillariella tabescens*, which was confirmed to be an oxidase and renamed aflatoxin oxidase (AFO). The mechanism proposed for the degradation by this enzyme consists in a AFB<sub>1</sub> transformation into AFB<sub>1</sub>-8,9-epoxide, followed by an hydrolysis into a dihydrodiol-derivative (Loi et al. 2017). Another metabolite, produced by a peroxidase from the rot fungus *Pleurotus ostreatus*, was dihydrohydroxyaflatoxin B<sub>1</sub> (AFB<sub>2a</sub>), which has reduced mutagenicity (Vanhoutte et al. 2016). By-products of the modification of lactone ring in the coumarin moiety include aflatoxins D<sub>1</sub>, D<sub>2</sub> and D<sub>3</sub>. This mechanism was observed for *Pseudomonas putida* (Samuel, Sivaramakrishna, and Mehta 2014). Aflatoxicol is another degradation product that results of the reduction of the C-3 keto on the cyclopentanone ring and was produced by *Rhizopus* sp. *Hypomyces rosellus* and *Cornybacterium rubrum* (McCormick 2013). An extracellular enzyme extracted from bacterium *Myxococcus fulvus* was designated as myxobacteria aflatoxin degradation enzyme (MADE) (Zhao et al. 2011). In several studies, degradation by-products were not identified but the reduction of toxicity was evaluated.

A summary of biological detoxification of mycotoxins is presented in Table 1.

Mycotoxin decontamination can be achieved by some physical and chemical methods, as well as by biological degradation. Some mycotoxin transformations lead to the modification of the original structure creating a “modified form” that can be difficult to detect with standard analytical methods. Certain physical steps involved in cereal processing, such as sorting, dehulling and heating, facilitate mycotoxin decrease. Additional mycotoxin decontamination is achieved by some chemical treatments like nixtamalization and ozonisation. Microorganisms and their enzymes have proven to be a more rapid, effective and ecological approach for mycotoxin degradation. Furthermore, the majority of the known biological degradation by-products, resulting mostly from hydrolysis and oxidation, have proven reduced toxicity. A combination of a mycotoxin decrease measurement, the identification of degradation by-products and the evaluation of residual toxicity is essential to guarantee that an effective detoxification has occurred.

Table 1. Main mycotoxins found in cereals and their biological detoxification mechanisms, resulting products and detoxifying microorganisms.

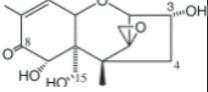
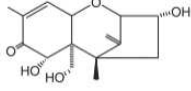
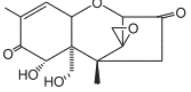
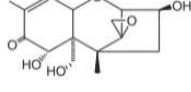
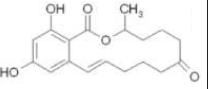
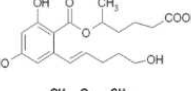
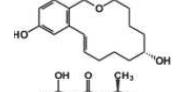
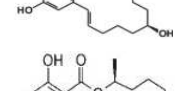
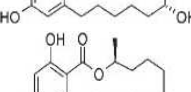
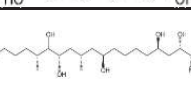
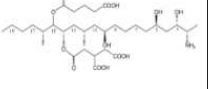
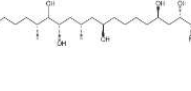
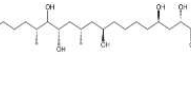
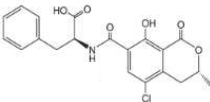
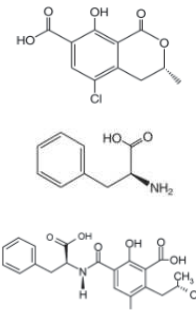
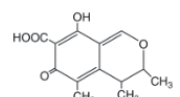
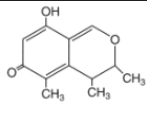
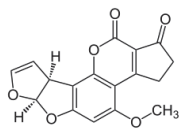
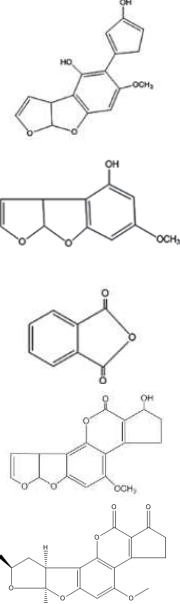
Mycotoxin	Fungi	Source of toxicity	Degradation mechanism	By-products	Structure	Examples of degrading microorganisms	References
	<i>F. graminearum</i> <i>F. culmorum</i>	12,13-epoxide ring Substituents on C-4, C-15, C-7 and C-8	De-epoxidation  Oxidation  Isomerization	DOM-1  3-keto DON  3-epi DON	    	<i>Eubacterium</i> sp. <i>Citrobacter freundii</i> <i>Serratia</i> <i>Clostridium</i> <i>Citrobacter</i> <i>Enterococcus</i> <i>Agrobacterium-Rhizobium bacteria</i>  <i>Devosia mutants</i> <i>Nocardioides</i> sp.	(Ito et al. 2012) (McCormick 2013) (He et al. 2016) (Vanhoutte et al. 2016) (Smith et al. 2018) (Zhang et al. 2020)
	<i>F. graminearum</i> <i>F. culmorum</i>	Lactone ring C-4 hydroxyl group	Reduction of carbonyl ketones Reduction of double bonds Hydroxylation Methylation Sulfation Glycosylation Ring cleavage	ZOM-1  $\alpha$ -ZEL  $\beta$ -ZEL  $\alpha$ -ZAL  $\beta$ -ZAL	        	<i>Trichosporon mycotoxinivorans</i> <i>Cunninghamella bainieri</i> <i>Thamnidium elegans</i> <i>Rhizopus</i> sp. <i>Gliocladium roseum</i> <i>Trichoderma aggressivum</i> <i>Aspergillus niger</i> <i>Acinetobacter</i> sp. <i>Bacillus natto</i> <i>Bacillus subtilis</i> <i>Rhodococcus</i> sp.	(Böswald et al. 1995) (Vekiru et al. 2010) (McCormick 2013) (Cserháti et al. 2013) (Vanhoutte et al. 2016) (Ji, Fan and Zhao 2016) (Loi et al. 2017) (Li et al. 2020)
	<i>F. verticillioides</i> <i>F. proliferatum</i>	Two tricarballic acids Free amino group in C-2	Deesterification  Oxydative deamination	HFB1  2-keto HFB1	  	<i>Sphingomonas</i> sp. <i>Sphingopyxis</i> sp. <i>Exophiala</i> sp. <i>Bacillus</i> Yeasts	(Humpf et al. 1998) (Palencia et al. 2003) (McCormick 2013) (Vanhoutte et al. 2016) (Loi et al. 2017)

Table 1. Continuation.

Mycotoxin	Fungi	Source of toxicity	Degradation mechanism	By-products	Structure	Examples of degrading microorganisms	References
OTA 	<i>P. verrucosum</i> <i>A. ochraceus</i> <i>A. carbonarius</i>	Isocoumarin moiety	Hydrolysis amide bond  Hydrolysis lactone ring	Ota  Phenylalanine  OP-OTA		<i>Rhodotorula</i> sp. <i>Cryptococcus</i> sp. <i>Trichosporon mycotoxinivorans</i> <i>Brevibacterium</i> sp. <i>Aspergillus niger</i> <i>Rhizopus</i> sp. <i>Botrytis cinerea</i> <i>Pleurotus ostreatus</i> <i>Phenylbacterium immobile</i>	(Stander et al. 2000) (Abrunhosa et al. 2010) (McCormick 2013) (Petruzzi et al. 2014) (Vanhoutte et al. 2016) (Loi et al. 2017) (Chen et al. 2018)
Citrinin 	<i>P. verrucosum</i> <i>P. expansum</i>		Decarboxylation	Decarboxycitrinin		<i>Moraxella</i> sp.	(McCormick 2013)
AFB1 	<i>A. flavus</i> <i>A. parasiticus</i>	Lactone ring	Modification of the coumarin	AFD1  AFD2  AFD3  Aflatoxicol  AFB2a		<i>Trametes versicolor</i> <i>Mucor</i> sp. <i>Trichosporon mycotoxinivorans</i> <i>Saccharomyces cerevisiae</i> <i>Trichoderma</i> sp. <i>Rhodococcus erythropolis</i> <i>Mycobacterium fluoranthenivorans</i> <i>Nocardia corynebacterioides</i> <i>Streptomyces lividans</i> <i>Streptomyces aureofaciens</i> <i>Mycobacterium smegmatis</i> <i>Pseudomonas putida</i>  <i>Rhizopus</i> sp. <i>Hypomyces rosellus</i> <i>Corynebacterium rubrum</i>  <i>Pleurotus ostreatus</i> <i>Armillariella tabescens</i>	(Giegler et al. 1966) (Smiley and Draughon 2000) (Teniola et al. 2005) (McCormick 2013) (Samuel, Sivaramakrishna, and Mehta 2014) (Eshelli et al. 2015) (Vanhoutte et al. 2016) (Loi et al. 2017)

## 2.4 *Streptomyces* genus as BCA and mycotoxin detoxifying agents

### 2.4.1 Actinobacteria

*Actinobacteria* are Gram-positive bacteria with high G+C content in their DNA. They exhibit a wide variety of morphologies, from coccoid (*Micrococcus*) or rod-coccoid (e.g. *Arthrobacter*) to fragmenting hyphal forms (e.g. *Nocardia*) or permanent and highly differentiated branched mycelium (e.g. *Streptomyces*) (Ventura et al. 2007). *Actinobacteria* are ubiquitously distributed in both aquatic and terrestrial ecosystems. In soil they develop particularly in the rhizosphere or inside plant roots (Rey and Dumas 2017). Most *Actinobacteria* are saprophytic and contribute to organic matter decomposition into humus (Dhanasekaran and Jiang 2016). Filamentous actinobacteria develop by tip extension and branching of hyphae, constituting a vegetative mycelium. Then, in response to nutrient depletion, among other signals, they may form aerial mycelium carrying spores (Colombo et al. 2020). Due to their extensive metabolism, these bacteria are of major importance for biotechnology, medicine, and agriculture. The genus *Streptomyces* accounts for over 95% of the *Actinomycetales* strains isolated from soil (Barka et al. 2016). Figure 27 depicts the phylogenetic tree of *Actinobacteria*.

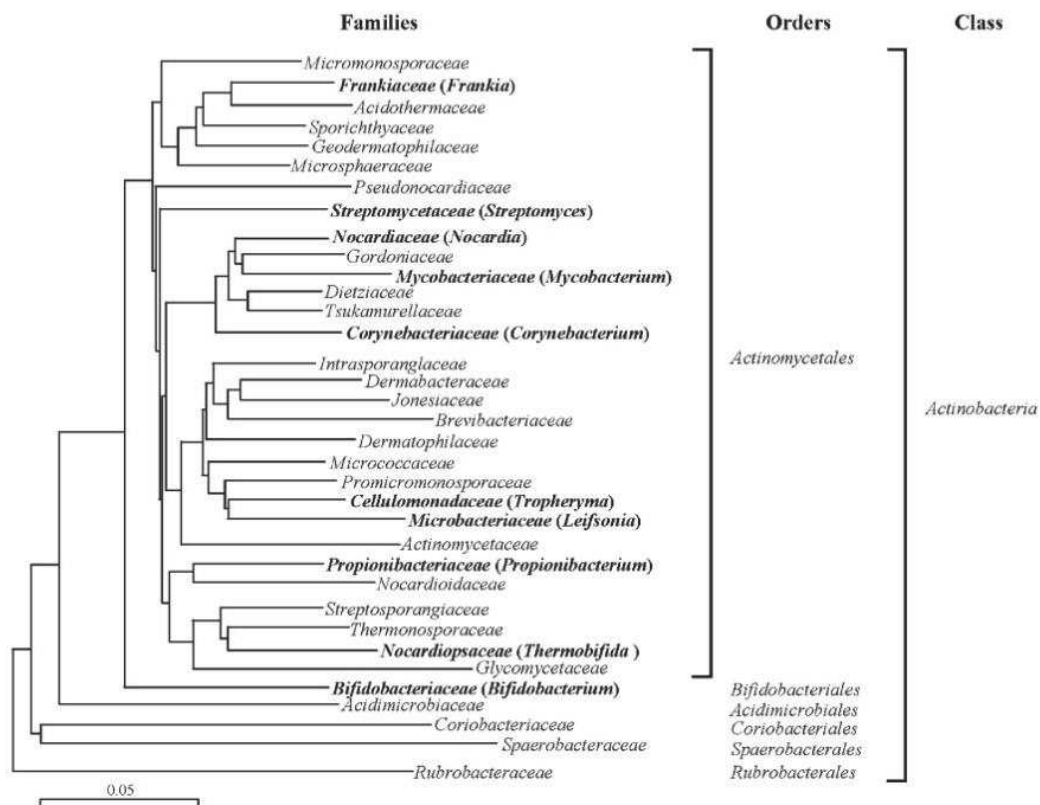


Figure 27. Phylogenetic tree of Actinobacteria based on 1,500 nucleotides of 16S rRNA. Scale bar, 5 nucleotides (Ventura et al. 2007).

### 2.4.2 *Streptomyces* genus

Streptomycetes are aerobic members of the order *Actinomycetales* within the classis *Actinobacteria* (Anderson and Wellington 2001). The term *Streptomyces* was conferred by Selman Waksman in 1942, who also discovered their ability to produce antibiotics, for which he received a Nobel Prize (Chater 2006). Nowadays, *Streptomyces* (Fig. 28) comprise more than 570 different species, of which only 10 are pathogenic, causing mainly the common scab of potatoes, e.g. *Streptomyces scabies* and *S. europaeiscabiei* (Chater 2016). The rest are saprophytes and include many endophytes (Newitt et al. 2019; Vurukonda, Giovanardi, and Stefani 2018). *Streptomyces* are the most important source of antibiotics for medical, veterinary and agricultural applications (Chater 2006). Approximately 60% of agricultural compounds of importance reported in the literature originated from *Streptomyces* species (Sharma and Salwan 2018; Tanaka and Omura 1993), as well as over 80% of all clinical antibiotics of actinobacterial origin (Barka et al. 2016). In fact, the collateral risks associated with antibiotic production is one of the main obstacles to their utilization (Doubou et al. 2001). Several studies have documented the discovery of about 20 biosynthetic gene clusters in the genome of *Streptomyces* which could account for the production of secondary metabolites (Law et al. 2018). The reduction of pathogen damage of plants by *Streptomyces* species has been demonstrated in several studies (Caceres et al. 2018; Colombo, Kunova, Pizzatti, et al. 2019; Danial et al. 2020; Law et al. 2017; Li et al. 2011; Sharkawy, Tohamey, and Khalil 2015; Zarandi 2009), which places them as a promising strategy to manage fungal contamination. The efficacy of *Streptomyces* as BCAs is achieved thanks to a series of mechanisms, such as the production of metabolites and enzymes, including antifungal and antimicrobial compounds, as well as their rhizosphere colonization capacities, and the resulting plant growth promotion (Vurukonda et al. 2018). Moreover, the capacity of *Streptomyces* to form spores confers them resistance to formulation processes and increases their stability during conservation (Newitt et al. 2019).

Figure 28. Left: solid culture of four *Streptomyces* strains showing their typical powdery morphology. Right: microscopical observation of *Streptomyces* mycelium.



2.4.2.1 *Streptomyces* strains development and secondary metabolites production

The intricate life cycle of *Streptomyces* species involves two distinct filamentous cell forms: the growing (or vegetative) hyphae and the reproductive (or aerial) hyphae, which differentiate into long chains of spores. Vegetative mycelia are formed by branching hyphae that prowl for nutrients, grow and proliferate. Hyphae develop by tip extension generating a ramifying mycelial network of filamentous cells delimited by occasional cross-walls (Anderson and Wellington 2001; Bush et al. 2015). During sporulation, hyphal branches differentiate into specialized sporogenic structures called aerial hyphae. Aerial growth is accompanied by a change in the surface properties of the hyphae, which confers them a rodlet hydrophobic layer (Claessen et al. 2006). Following their erection, chromosome replication, segregation, cell division, etc., they give place to aerial hyphae divided into a long chain of spores that, once mature, are liberated and dispersed by the wind or animals. Spores allow extended survival of *Streptomyces* species in the soil in an active form as they are resistant to water and nutrient scarcity (Law et al. 2018). Spores then form new vegetative mycelium under favorable conditions (Fig. 29) (Bush et al. 2015).

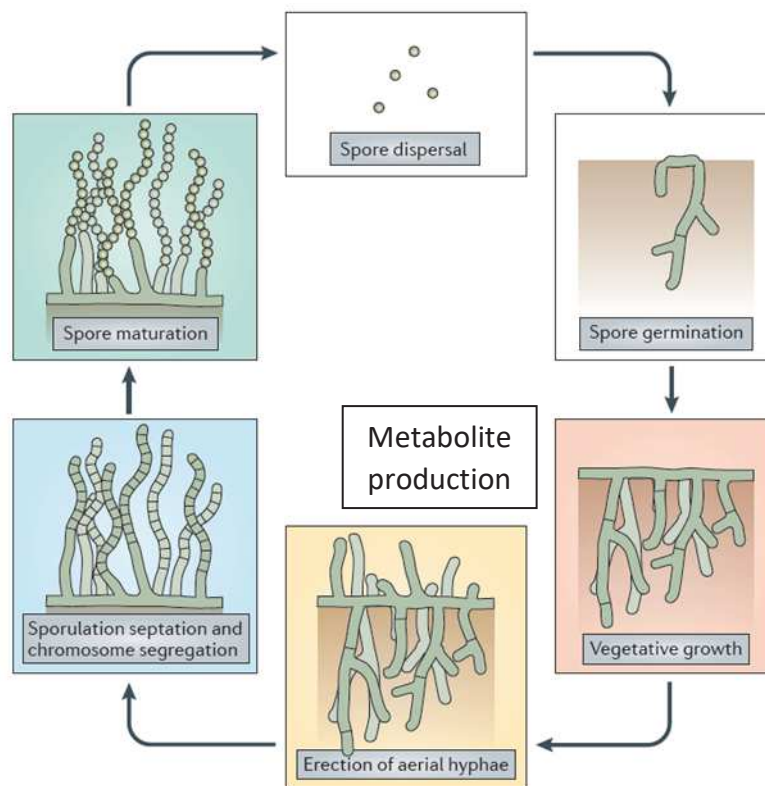


Figure 29. The life cycle of *Streptomyces* (Bush et al. 2015)

*Streptomyces* differentiation between surface and submerged cultures entails a complex modification on their metabolism, which can have an impact on their production of bioactive compounds (Manteca et al. 2008; Manteca and Yagüe 2018). Spore formation, which occurs mainly on solid culture, is closely related to the production of secondary metabolites (Bobek, Šmídová, and Čihák 2017; Law et al. 2018). Variations on culture conditions such as temperature and pH, can also diversify the molecules produced by *Streptomyces* during their active growth (Yu et al. 2008). Secondary metabolites production does not usually occur until one or more nutrients become scarce. Carbon sources, nitrogen sources, and phosphate limitation have been described as important elicitors of secondary metabolism (Běhal 2000).

#### 2.4.2.2 Antagonistic mechanisms deployed by *Streptomyces* as BCAs

As mentioned before, *Streptomyces* species are able to employ a series of direct and indirect mechanisms that allow them to combat pathogenic development, such as the production of antifungal molecules and the competition for the available nutrients. Direct antagonisms are linked to the physical contact of both microorganisms, while indirect antagonisms do not require sensing or targeting the pathogen by the BCA (Pal and McSpadden Gardener 2006). The interaction with the plant host constitutes another mode of action of *Streptomyces* species, as it usually leads to an improvement of plant resistance and adaptation to biotic and abiotic factors. Generally, *Streptomyces* species with potential as BCAs employ more than one antagonistic mechanism (Legein et al. 2020) (Fig. 30).

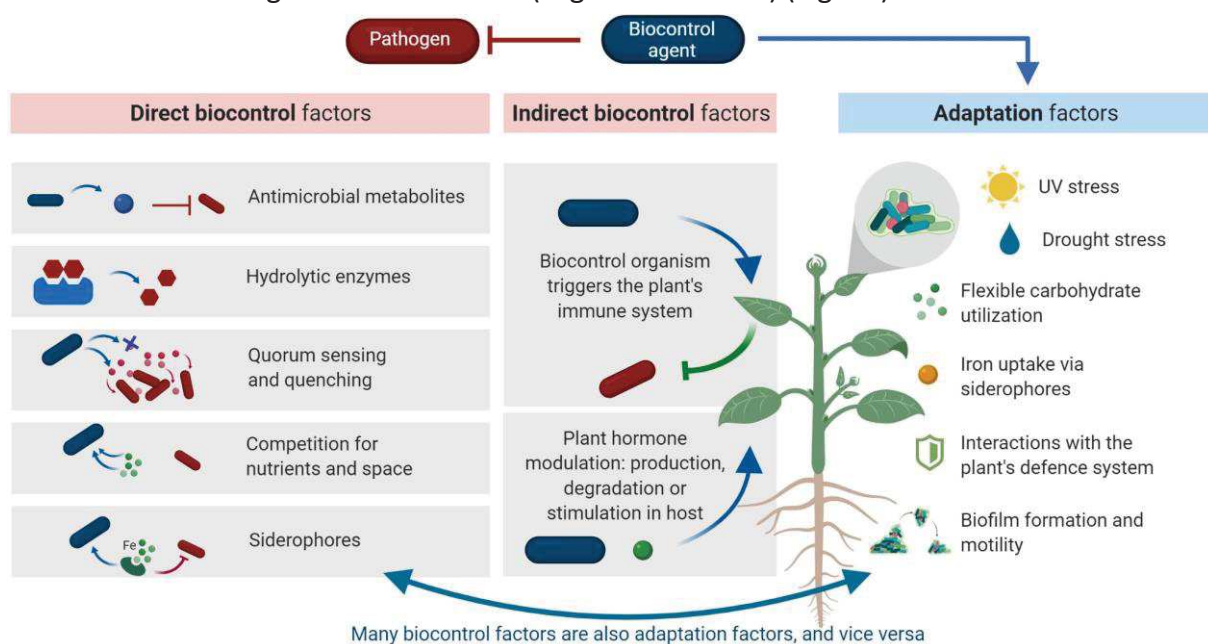


Figure 30. Antagonistic mechanisms exhibited by biocontrol agents (Legein et al. 2020).

#### 2.4.2.2.1 Parasitism

Parasitism or hyperparasitism is the most direct form of antagonism and it occurs when the BCA uses the pathogen for its own benefit, usually as a source of nutrients. Between filamentous microorganisms, usually the antagonistic microorganism wraps around the pathogenic fungi hyphae (coiling), restraining its propagation, however internal parasitism and perforation (lysis) have also been reported (Handelsman' and Stabb 1996). Numerous studies have documented the ability of several *Streptomyces* spp., such as *S. griseous* and *S. griseoviridis* to parasite fungal pathogens resulting on growth decrease and malformation or lysis of the fungal mycelium (Palaniyandi et al. 2013). Ursan et al. observed evidence of hyperparasitism by *Streptomyces* on *Rhizoctonia solani*, when evaluating their efficacy as biocontrol agents against fungal pathogens (Ursan et al. 2018).

#### 2.4.2.2.2 Antibiosis

*Streptomyces* bacteria are largely studied because of their ability to produce a wide variety of enzymes and metabolites of medical and agricultural interest (Sharma and Salwan 2018). These compounds interfere with the pathogen growth and/or activities, for example by disrupting its cell membranes (Pellan et al. 2020). Some enzymes include chitinases, cellulases, glucanases, proteases and phospholipases that, in addition to degrading pathogenic cell walls, are able to inhibit spore adhesion and germination (Palaniyandi et al. 2013). Furthermore, *Streptomyces* strains are able to produce amylases, keratinases, peroxidases, pectinases, phytases and xylanases that allow them to convert complex nutrients into simple mineral forms (Vurukonda et al. 2018). Another protein produced by *Streptomyces* species is an alkaline protease inhibitor (API), which has the ability to inhibit the fungal serine alkaline protease, indispensable for fungal growth (Doubou et al. 2001).

Among the variety of metabolites produced by *Streptomyces* bacteria, several have antifungal properties, such as Polyoxin B and D (*S. cacaoi*) that interfere with the formation of fungal cell wall by inhibiting chitin synthase. As well as Validamycin A, a trehalase inhibitor, which affects only microbes as vertebrates do not depend on the hydrolysis of this disaccharide for their metabolism. Furthermore, Kasugamycin, produced by *S. kasugaensis*, interferes with protein biosynthesis on microorganisms. Another example is mildiomycin, an



inhibitor of fungal protein biosynthesis (Doubou et al. 2001; Vurukonda et al. 2018). *Streptomyces* bacteria can also produce polyketides that provoke the inhibition of fungal growth (Sharma and Salwan 2018). Hydrogen cyanide (HCN), a microbial product, can also contribute to pathogen suppression by blocking the cytochrome oxidase pathway (Pal and McSpadden Gardener 2006). The importance of antibiotic production as an antagonistic mechanism for biocontrol has been assessed by producing mutant strains unable to generate antibiotics, which were then incapable of suppressing the pathogen (Singh 2014). However, as mentioned before, antibiotic production can hinder the use of *Streptomyces* strains, so further research on antibiotic repression could open new perspectives, as long as the targeted strains possess alternative antagonistic mechanisms. In addition to antimicrobials, *Streptomyces* strains are able to produce anthelmintic compounds, such as avermectin, produced by *Streptomyces avermitilis*, which is lethal to nematodes (Newitt et al. 2019).

Several volatile organic compounds (VOCs) are also produced by *Streptomyces* genus, such as isoprene, acetone, 1-butanol, cyclopentanone, dimethyl disulphide and geosmin (Schöller et al. 2002). The latter confers them their characteristic “earthy” odour and has shown the ability to promote selective growth of geosmin-utilizing bacteria (Vurukonda et al. 2018). VOCs are reported to have antifungal activity by suppressing fungal growth, causing morphological abnormalities and inhibiting spore germination in fungi. They could present an alternative to chemical fumigants such as methyl bromide or chloropicrin (Palaniyandi et al. 2013), however their sometimes unpleasant smell could obstruct their utilization.

#### 2.4.2.2.3 Competition

Antagonistic *Streptomyces* strains can compete against the pathogen for nutritive resources such as carbon, nitrogen, iron, water and air; as well as for space in the ecological niche (Nguyen et al. 2017). Competition for nutrients is often achieved by the production of antibiotics or hydrolyzing enzymes, thus, competition and antibiosis are closely linked (Palaniyandi et al. 2013). Iron is an essential element for the development of microorganisms, and its availability in the soil and plant surfaces is limited, which creates a fierce competition (Singh 2014). Thus, the production of siderophores (iron-binding ligands) by certain microorganisms is an effective antagonistic mechanism, as it allows them to compete for the iron in the environment (Pal and McSpadden Gardener 2006). *Streptomyces* can produce

siderophores such as coelichelin and albomycin (Vurukonda et al. 2018). Siderophores produced by *S. albovinaceous*, *S. griseous* and *S. virginiae* may be involved in their antagonism against *Moniliophthora perniciosa* (Macagnan et al. 2008). Moreover, siderophore production by some actinobacteria leads to the enrichment of other actinobacteria in the plant rhizosphere (Palaniyandi et al. 2013), which may lead to the creation of disease suppressive soils for the long-term management of plant pathogens (Kinkel et al. 2012).

#### 2.4.2.2.4 Plant colonization

*Streptomyces* strains as endophytes have been reported to protect plants against pathogens, aid in nutrient uptake, modify plant development and stimulate rhizobial associations (Kinkel et al. 2012). The ability of *Streptomyces* strains to colonize and proliferate in the plant roots, leaves, fruits, etc., confers them an additional advantage as it contributes to a selective adaptation. Also, it provides a supplementary protection from the pathogen attack on the infection site. However, it has been shown that an initial competitive leverage has to be granted, such as high initial cell numbers. Once the beneficial organism is installed, the antagonistic mechanism could involve competition and antibiosis, as well as the stimulation of the plant self-defenses (Singh 2014). *Streptomyces* bacteria might enter plant tissues by natural openings such as stomatal apertures, lenticels, hydathodes, wounds, broken trichomes, and root hair cracks formed by lateral root emergence zones (Sousa and Olivares 2016). In a study performed by Vergnes et al., *Streptomyces* spp. rapidly colonized *Arabidopsis* leaf surface, including the trichomes and stomata (Vergnes et al. 2020). Moreover, *Streptomyces griseoviridis* from the commercial biofungicide Mycostop® has been documented as a good root colonizer (Doubou et al. 2001).

Several *Streptomyces* bacteria are well known Plant Growth-Promoting Rhizobacteria (PGPR), as they develop into the plant rhizosphere and have a beneficial impact on plant development and tolerance against diverse stimuli. Certain *Streptomyces* strains have the capacity to boost plant immune response, as well as to increase the seedling and root length (Sharma and Salwan 2018). The plant growth-promoting effects related to *Streptomyces*-plant interactions can be divided into biofertilization, biostimulation and bioprotection. Biofertilization improves the nutrient acquisition, biostimulation is associated with the production of metabolic compounds with phytohormonal activity and bioprotection consists

in the minimization of the impact of pathogenic fungi (Sousa and Olivares 2016). Actinobacteria can influence soil fertility by rendering nutrients available to the plant (Vurukonda et al. 2018). For example, by fixing the nitrogen from the atmosphere to the soil in the form of ammonium and nitrates, by solubilizing phosphorus into soluble forms, by fixing and reducing iron and by producing/stimulating the production of phytohormones such as Indole Acetic Acid (IAA), which regulates root growth (Doubou et al. 2001; Nguyen et al. 2017). Indeed, *Streptomyces* have been documented to produce phytohormones such as cytokinins, gibberellic acid and indole-3-acetic acid (IAA) (Palaniyandi et al. 2013), which have beneficial impacts on plant growth and development by induction of cell elongation and division (Vurukonda et al. 2018). Biotic and abiotic stresses trigger ethylene production by plants, which decreases plant growth. *Streptomyces* are able to alleviate this stress as they produce an enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase that transforms ACC, the precursor of ethylene, into ammonia and  $\alpha$ -ketobutyrate, enhancing plant growth (Palaniyandi et al. 2013).

#### 2.4.2.2.5 Induction of host resistance

The induction of host resistance is the enhancement of the plant self-defense capacity as a result of its interaction with a microorganism (Nguyen et al. 2017). Plant active defenses involve changes in membrane function, oxidative bursts, cell wall reinforcement and hypersensitive response (HR), which results in programmed cell death (PCD) (Syed Ab Rahman et al. 2018). There are two main pathways of induced resistance. The first, named systemic acquired resistance (SAR), is mediated by salicylic acid (SA), which is often produced by the plant under pathogenic infection; it usually leads to the expression of pathogenesis-related (PR) proteins. These PR proteins consist in a variety of enzymes, that may act directly to lyse invading cells, reinforce cell wall boundaries or induce localized cell death. The second pathway, triggered by BCAs, is referred as induced systemic resistance (ISR) and is mediated by jasmonic acid (JA) and ethylene (ET) (Pal and McSpadden Gardener 2006). ISR results in the thickening of plant cell walls and alteration of the plant metabolic responses, which leads to an increase on plant defense chemicals (Singh 2014). The induction of plant resistance by *Streptomyces* bacteria differs from traditional ISR produced by *Pseudomonas* and *Bacillus* bacteria, which do not induce the transcription of PR proteins (Pal and McSpadden Gardener

2006). Indeed, *Streptomyces*-mediated ISR involves features of both ISR and SAR, as well as the cross-talk between a variety of phytohormone signaling pathways (Newitt et al. 2019).

#### 2.4.2.3 Screening for *Streptomyces* strains as biocontrol agents

*In vitro* evaluation of BCAs is commonly employed during potential antagonistic screenings. However, the protective effect observed *in vitro* is not always correlated with the *in planta* efficacy (Pliego et al. 2011). Thus, complementing *in vitro* assays with *in planta* validation is essential to identify promising biocontrol candidates. Moreover, to improve the first stages of selection, media used for dual culture assays should simulate at best the possible conditions of the targeted environment (Colombo, Pizzatti, et al. 2019). The authors suggested a comprehensive workflow for the selection of *Streptomyces* strains as biocontrol agents. Their proposed integrated approach, which steps are described below, facilitates the effective selection of BCAs whose effectivity persists to the field (Colombo, Kunova, Cortesi, et al. 2019):

- *Streptomyces* species identification. Bacterial species identification and characterization are essential in order to guarantee the safety of their application.
- *In vitro* antagonistic assays. This first step of interaction between *Streptomyces* strains and the pathogen allows to identify antifungal properties and certain mechanisms of action.
- Effect of culture media. Media composition has an impact on the production of secondary metabolites. Thus, ideally, *in vitro* bioassays should be performed on different media to allow the broadening of the number of interesting strains identified.
- Use of fermentation extracts. The use of crude extracts of culture allows the identification of the metabolites and enzymes involved on the antagonistic effects. Further steps of purification can lead to the obtention of bioactive compounds.
- Evaluation of the antagonistic mechanism. This step is essential to better address the impact of BCAs and avoid the risk of dispersing dangerous metabolites in the environment.
- Plant colonization assessment. A verification of the colonization of the intended plant niche is preferable to predict a consistent protection.

- Influence of complex environmental conditions. Subjecting a potential BCA to the conditions expected in the field is convenient as the complexity of environmental factors often impacts the biocontrol efficacy of BCAs.
- Determining plant growth promotion properties. The interaction of the BCA with the plant can lead to a series of positive and/or negative reactions that impact plant development and fitness against the disease. Indeed, some *Streptomyces* strains can be pathogenic, such as *Streptomyces scabies* that causes potato scab.
- Application method. The delivery system must be selected carefully according to the targeted pathogen. Optimized formulation of a BCA is fundamental for its endurance and efficacy.
- Mycotoxin quantification. As discussed before, there is a lack of correlation between fungal development and mycotoxin production. An inhibition of fungal growth does not guarantee a detoxification result, in some cases a stimulation of mycotoxin production was observed. Moreover, some secondary metabolites can limit toxin accumulation without impacting fungal proliferation, which is an important factor often neglected during the selection of BCAs against mycotoxinogenic fungi.

#### 2.4.2.4 Modes of application of *Streptomyces* as biocontrol agents

The differences commonly observed between *in vitro* and *in planta* efficacy of BCAs can be attributed to several factors encountered in the field, such as organic matter, pH, nutrients availability, and humidity of the soil. Which is why the application of a BCA has to focus on facilitating its installation and proliferation under environmental conditions (Law et al. 2017). The application of a biocontrol agent has to be adapted to its nature (living microbes or microbial products), the site or application and the targeted mode of action (Palaniyandi et al. 2013). Products such as Actinovate® (*Streptomyces lydicus* strain WYEC108), sold in Canada and USA, and Mycostop® (*Streptomyces griseoviridis* strain K61), which is also sold in Canada and USA and the EU (Lahdenperä, Simon, and Uoti 1991; Newitt et al. 2019; Rey and Dumas 2017) are sold as dried formulations containing spores and mycelia, which can be either suspended in liquid and sprayed onto crops (foliar spraying), folded into the soil prior to sowing (soil inoculation), or be used as a seed coating. Foliar spray is intended to control pathogens causing foliar diseases (recommended for Actinovate®) while soil inoculation is

designed to manage soil or seed-borne pathogens (recommended for Actinovate® and Mycostop®), or when the BCA strain is particularly vulnerable to desiccation. Another way of application is directly onto the plants root by fluid drill inoculation or root transplant dip. However, these methods are labor-intensive. For seed coating application, seeds are usually immersed in a BCA suspension and dried before germination (Vurukonda et al. 2018). The main advantage of this technique is that it delivers the beneficial organism to the immediate surroundings of the seed and rhizosphere, some disadvantages include shelf-life and storage conditions (Newitt et al. 2019). Figure 31 illustrates some BCA application techniques.

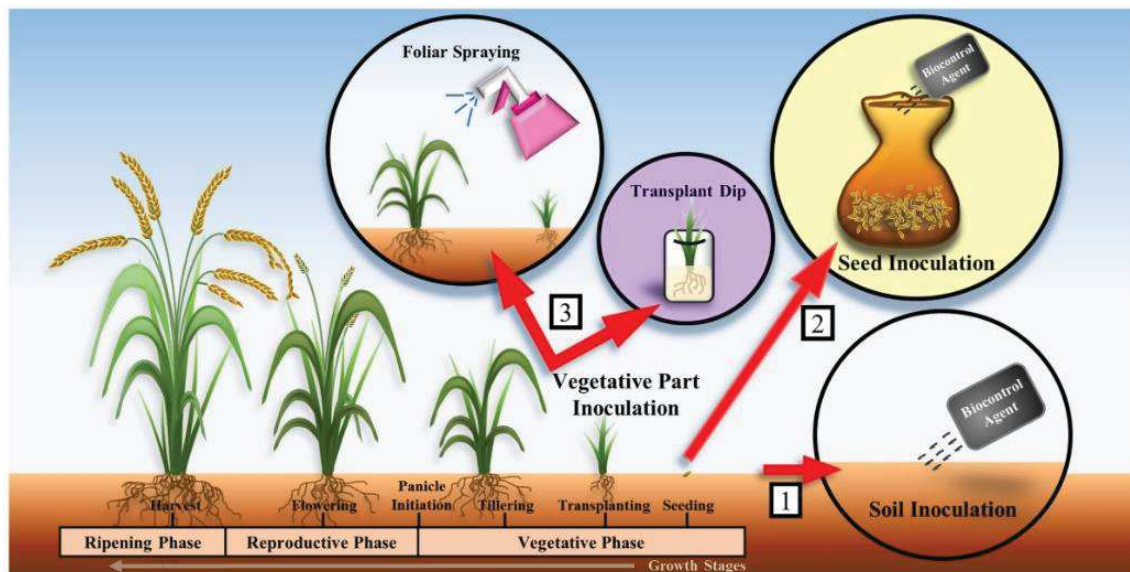


Figure 31. Methods of application of biocontrol agents (Law et al. 2017).

Other than the mode of application, BCA's efficacy, consistency and stability rely on a great manner on its formulation. This process consist in mixing the active ingredient (i.e. bacterial spores, mycelium, secondary metabolites, enzymes) with inert materials such as diluents and surfactants, sometimes amended with nutritional supplements, in order to make it easy to handle and to increase its stability during storage and field application (Junaid et al. 2013; Palaniyandi et al. 2013). Indeed, when introduced to an ecosystem, a BCA encounters abiotic stresses such as desiccation, osmotic stress, UV radiation and extreme temperatures; as well as biotic stresses (Legrand et al. 2017). Some authors have experimented with different formulations to protect and deliver efficient *Streptomyces* BCAs, such as talcum powder, alginate beads, clay pellets and chitosan polyphosphate beads (Jobin et al. 2005; Sabaratnam and Traquair 2002; Tamreihao et al. 2016; Zamoum et al. 2017). It seems to be a consensus about talcum being an effective and stable carrier.

#### 2.4.2.5 *Streptomyces* against phytopathogenic fungi

The use of *Streptomyces* strains as an alternative to the management of phytopathogenic fungi has recently been explored. For this purpose, the application of *Streptomyces* isolates, of culture broths and of bacterial metabolites have been evaluated.

El-Sharkawy et al. established the efficacy of *Streptomyces viridosporus* to control leaf rust under greenhouse and field conditions, with disease reductions going up to 50% and a general increase on yield parameters (spike, grain and kernel weight). They also found that *S. viridosporus* efficacy increased in combination with *Trichoderma harzianum* (Sharkawy et al. 2015). As wheat blast is an emerging disease, most biological control assays have focused on rice blast (Law et al. 2017). However, results obtained could give some leads on the efficacy of *Streptomyces* as a managing tool for *M. oryzae* pv *Triticum*. For instance, greenhouse assays using spore suspension of *Streptomyces sideneusis* strain 263 on rice seedling-leaves, resulted in a strong inhibition of *M. oryzae* and a suppression of leaf symptoms (Zarandi 2009).

Yi et al. determined that fermentation broth of a *Streptomyces* spp. strain was able to decrease leaf rust by 80% (Yi et al. 2004). Furthermore, culture filtrates of *Streptomyces globisporus* JK-1 were found to inhibit *M. oryzae* development and conidial germination, their efficacy was even superior of that of tricyclazole, a commonly used chemical fungicide for the control of this fungus (Li et al. 2011). Additionally, Fermentation broth of *Streptomyces* strain BG2-53, with 96% homology to *S. lipmanii*, according to 16S rDNA sequence analysis, showed potent antifungal activity against *M. oryzae* on rice (Lee et al. 2002).

Li et al. 2008 determined that malayamycin, an antifungal compound produced by *Streptomyces malaysiensis*, inhibited *Parastagonospora nodorum* sporulation *in vitro*, as well as lesion size and pycnidiation in wheat seedlings (Li et al. 2008). In addition, Phenamide, produced by a member of the *Streptomyces albospinus* cluster was also active against *P. nodorum* on assays conducted by Makkar et al. (Makkar et al. 1994). Another compound, Fencicoxamid (Intrateq™ active), a derivative of the natural compound UK-2A, originally isolated from *Streptomyces* fermentation broths, is a fungicide with strong inhibitory capacity against *Zymoseptoria tritici* and *Puccinia triticina* growth on *in vitro* and greenhouse experiments, with effects equivalent to those of the commercial fungicide fluxapyroxad (Owen et al. 2017) (Fig. 32). Also, xiamycins, originated from *Streptomyces* species,

demonstrated complete inhibition of *Z. tritici* growth on assays performed by Pfaffenbach et al. (Pfaffenbach et al. 2019). Antibiotics produced by *Streptomyces* spp. such as Blasticidin-S (*S. griseochromogenes*) and Kasugamycin (*S. kasugaensis*) have been used for the control of rice blast and further Studies on oligomycin A produced by *S. diastatochromogenes* demonstrated its ability to control this disease under greenhouse conditions (Law et al. 2017).

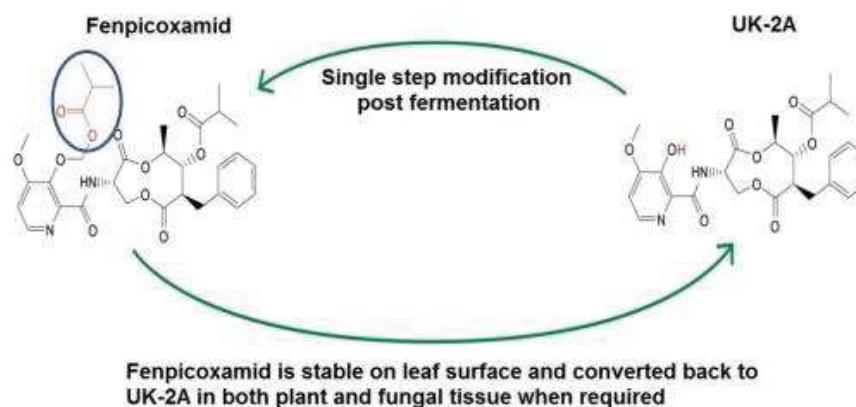


Figure 32. Modification of UK-2A to fepicoxamid and reconversion to UK-2A in plants and fungi (Leader et al. 2018).

#### 2.4.2.6 *Streptomyces* against mycotoxinogenic fungi

When evaluating actinobacteria as biocontrol agents of mycotoxin producing fungi, is of the utmost importance to take into consideration not only the bacteria's capacity to reduce fungal germination, growth and colonization, but also their ability to decrease mycotoxin amount, either by degrading it or by obstructing its production. Indeed, is well known that some pathogens can increase their mycotoxin production under stressful conditions as a defense mechanism (Medina et al. 2017), thus inhibition of fungal growth is not always correlated with a decrease on mycotoxin amount.

According to Jung et al., spores of *Streptomyces* sp. BN1 sprayed onto wheat heads helped to decrease FHB severity during greenhouse experiments (Jung et al. 2013). Moreover, on field experiments performed by Palazzini et al., the application of *Streptomyces albidoflavus* 87B during wheat anthesis decreased FHB severity between 29 and 39%, as well as DON amount between 69 and 85% (Palazzini et al. 2017). In a later study, the same team confirmed that *S. albidoflavus* 87B reduced on 25% FHB incidence, on 30% its severity and up to 51% the DON accumulation in durum wheat (Palazzini et al. 2018). Additional research



conducted by Ursan et al., who evaluated the antagonistic capacity of 60 strains of *Streptomyces* sp. against *Fusarium culmorum* and *F. graminearum* *in vitro*, showed that at least 30% of the evaluated strains were able to inhibit fungal growth. Some of the mechanisms applied by the bacterial strains were the production of hydrolytic enzymes, such as cellulase and chitinase, as well as mycoparasitism and lysis of fungal mycelium. Additionally, two of the evaluated strains decreased ZEN amount (Ursan et al. 2018). A recent *in vitro* study by Colombo et al. demonstrated that four *Streptomyces* strains were capable of inhibiting *F. graminearum* growth up to 71% and DON up to 99% when inoculated on detached wheat grains three days prior to fungal contamination. The evaluated *Streptomyces* strains were ineffective when inoculated simultaneously with the pathogen, which suggest that their antagonistic potential is preventive rather than curative (Colombo et al. 2020).

On *in vitro* experiments performed by Samsudin et al., a *Streptomyces* sp. was able to decrease by almost a half the growth of *F. verticillioides* by the diffusion of antifungal metabolites, and a slight decrease on FB<sub>1</sub> production was also observed (Samsudin and Magan 2016). An *in vitro* study of fourteen *Streptomyces* strains against *F. verticillioides* showed fungal growth decreases going up to 77% by bacterial cells and up to 73% by bacterial CFEs. Furthermore, a considerable reduction on FB<sub>1</sub> and FB<sub>2</sub> production (up to 88% and 98%, respectively) was also reported (Nguyen et al. 2018). *Streptomyces* strain AS1 was able to inhibit *in vitro* growth of *P. verrucosum* and *F. verticillioides* by 90% and 51%, respectively, by hindering germination. Its antifungal activity was hypothesized to be due to the production of metabolites such as valinomycin and brevianamide F. The strain was also able to decrease OTA and fumonisins FB<sub>1</sub> and FB<sub>2</sub> production by both pathogens respectively (Danial et al. 2020). In another study by Campos-Avelar et al., the growth of *P. verrucosum* was strongly decreased by *Streptomyces* isolates during *in vitro* assays, but this inhibition caused sometimes an overaccumulation of OTA. Additionally, bacterial isolates degraded the mycotoxin, mainly during their growth in liquid medium (Campos-Avelar et al. 2020).

An interesting approach presented by Verheecke et al. when evaluating the interaction between *Streptomyces* and *Aspergillus flavus*, was to assess the bacterial impact on mycotoxin biosynthesis gene cluster. Indeed, for aflatoxins, genes such as *afID*, *afIM*, *afIP* (structural) and *afIR/afIS* (regulatory) can be interfered by actinobacteria, thus decreasing aflatoxin production. In addition, they suggested that *afIM* expression could be an aflatoxin indicator

during *Streptomyces* interactions (Verheecke et al. 2015). However, in some studies, the addition of bacterial antagonists inhibited *afID* and still increased AFB<sub>1</sub> production (Al-Saad et al. 2016). *Streptomyces* metabolites such as aflastatins, blasticidins A (Fig 33) and S, and dioctatin A were found to inhibit aflatoxin production by interruption of norsolorinic acid and sterigmatocystin production, both intermediates of aflatoxin. The reduction of the mRNA levels of genes encoding for enzymes needed during aflatoxin biosynthesis and the regulation of the AflR protein were also reported. Concerning dioctatin A, fungal conidiation inhibition was observed on *Aspergillus parasiticus*, as well as a significant increase on its kojic acid production (Sakuda 2010).

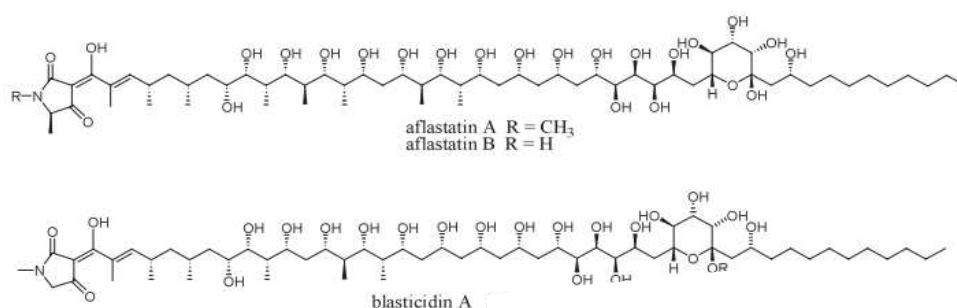


Figure 33. Chemical structures of aflastatins and Blasticidin A. produced by *Streptomyces* sp. (Sakuda 2010).

#### 2.4.2.7 Use of *Streptomyces* in biocontrol, risks and benefits

There are several compounds derived from *Streptomyces* bacteria with biological properties. For example, the first antibiotics employed for the control of pathogenic fungus and bacteria are cycloheximide and streptomycin, both produced by *Streptomyces griseus*. The *Streptomyces* strains capable of producing geldanamycin are also viewed as promising BCAs. Furthermore, the ability of generate several antibiotic compounds increases the BCA's importance, as is the case for *Streptomyces violaceusniger* YCED9, which has been reported to produce nigericin, guanidylfungin A-like compound, and geldanamycin (Palaniyandi et al. 2013). The soil application of azalomycin produced by *Streptomyces malaysiensis* MJM1968 showed antifungal activity against *Fusarium oxysporum*, *Rhizoctonia solani*, *Cladosporium cladosporioides*, *Fusarium chlamydosporum*, *Colletotrichum gloeosporioides*, *Alternaria mali*, and *Pestalotia* spp (Cheng et al. 2010). A thiopeptide produced by the strain *Streptomyces* S4-7 was purified and tested against *Fusarium*, showing a potent inhibitory activity against fungal

cell wall biogenesis (Newitt et al. 2019). Additionally, antibiotics produced by *Streptomyces* are also commercialized as bactericides and fungicides, namely Polyoxin D, Streptomycin, Kasugamycin, Blastidicin-S, Phytomycin, Validamycin, Polyoxorim and Natamycin (Palaniyandi et al. 2013; Vurukonda et al. 2018).

The direct use of bacterial strains has also been proven effective for the management of fungal pathogens. The strain *Streptomyces* BN1 was able to alleviate the reduction in seedling length caused by *Fusarium* when applied as a spore preparation to seeds. This strain also significantly decreased *Fusarium* head blight symptoms when sprayed onto wheat heads (Jung et al. 2013). In addition to the commercial biological fungicides Mycostop® and Actinovate®, some other *Streptomyces*-based products are authorized as “fertilizers”, such as Micosat F® (CCS Aosta srl, Aosta, Italy), containing three different *Streptomyces* spp.; Forge SP® (Blacksmith Bioscience, Spring, TX, USA), containing *S. nigrescens*; and Mykorrhiza soluble 30G (Glückspilze, Innsbruck, Austria), containing *S. griseus* and *S. lydicus* (Vurukonda et al. 2018).

The application of *Streptomyces* as biocontrol agents has various advantages compared to other largely studied microorganisms. For instance, *Pseudomonas* have been investigated as potential biocontrol agents, however, these strains only colonize wheat plants during the early stages of growth before being out-competed, and they are also sensitive to desiccation (Newitt et al. 2019). Thus, the benefit of using *Streptomyces* as BCAs include their saprophytic lifestyle, which allows them to colonize the mature roots of cereal crops (Conn and Franco 2004), as well as their spore-forming capacity, which allows them to survive well under unfavorable conditions (Law et al. 2018).

There are some possible negative impacts of the use of *Streptomyces* strains as biocontrol agents. For instance, as the main antagonistic mechanism consist on antibiosis, pathogens could develop resistance to the antibiotics produced by the actinobacteria. However, *Streptomyces* may also co-evolve with the pathogen and activate numerous putative antimicrobial biosynthetic gene clusters, resulting in the simultaneous production of various antibiotics with different modes of action, which could reduce the rate of evolution of pathogenic resistance (Newitt et al. 2019). Furthermore, these antibiotics may not be specific and affect non-targeted organisms, and they could even be detrimental to human health. Nevertheless, some studies suggest that antibiotics are produced at very low quantities in the

soil and that their role on pathogen inhibition is minor compared to space or resources competition. Also, several gene clusters encoding secondary metabolites may only be activated in the presence of pathogenic species, which gives them a certain selectivity (Newitt et al. 2019). Another possible negative impact is the transfer of genes from pathogenic *Streptomyces* species towards saprophytic species, this has been reported and should be studied carefully (Doubou et al. 2001; Vurukonda et al. 2018).

#### 2.4.2.8 Screening for mycotoxin-biotransforming microorganisms

Mycotoxin-biotransforming microorganisms (MBMs) should be obtained from environmental sources or screened from a previous collection. Next step must consist on evaluating the detoxification abilities and the influencing factors of the selected microorganisms. Later, degradation mechanism and by-products should be investigated. Afterwards, safety assessment of functional strains and biotransformation products should be performed. Finally, potential enzymes should be identified, isolated and/or cloned and expressed in order to be applied for detoxification purposes (Zhu et al. 2017). A workflow of this process is presented in Figure 34.

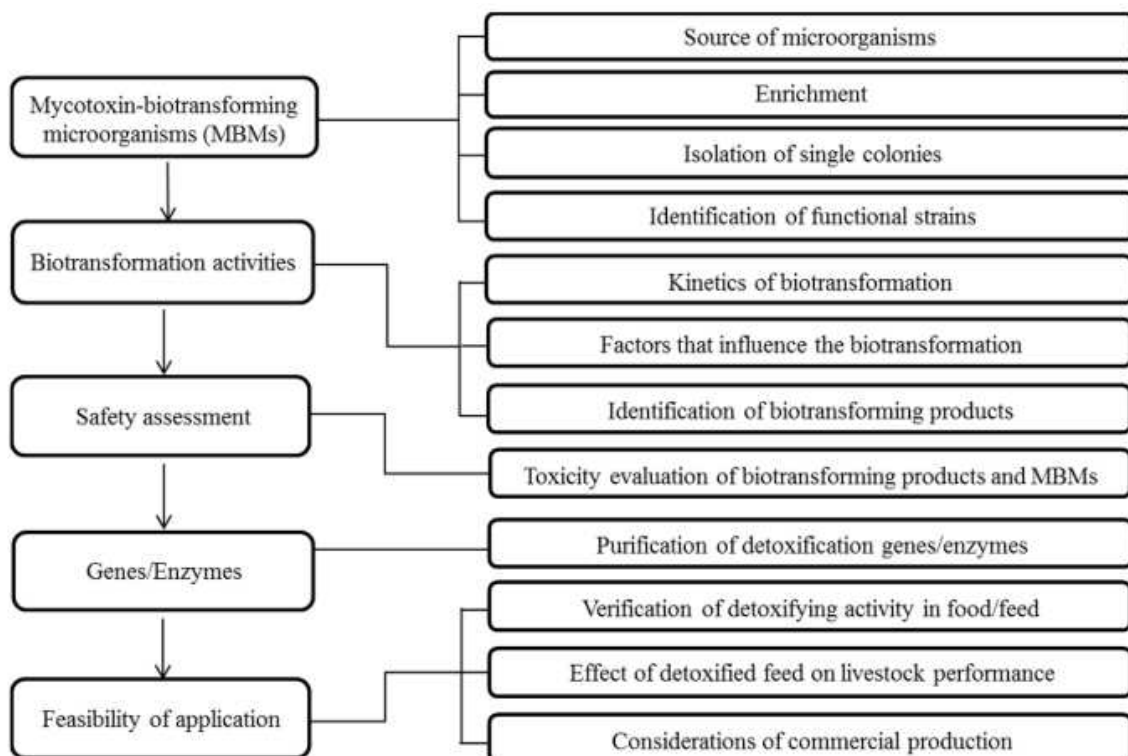


Figure 34. Workflow of research on microbial detoxification of mycotoxins (Zhu et al. 2017).

#### 2.4.2.9 Use of *Streptomyces* as detoxifying tools

Due to the considerable number of secondary metabolites they produce, *Streptomyces* are not considered as GRAS (generally recognized as safe) microorganisms. However, it has already been reported that they are able to produce a variety of metabolites and enzymes with high potential for detoxification, which makes them promising sources for the isolation of compounds that could be later used for detoxification of food and feed.

Several Streptomycete strains studied by Palazzini et al. significantly decreased DON level on wheat grains and spikes in greenhouse experiments (Palazzini et al. 2007). Further work from the same research group, indicated strong inhibition of DON production in wheat spikes on the field by *S. albidoflavus* (Palazzini et al. 2017, 2018). Some *in vitro* studies have demonstrated the ability of streptomycetes to limit fumonisin accumulation. Strains isolated from organic amendments decreased significantly FB<sub>1</sub> and FB<sub>2</sub> production by *F. verticillioides* (Nguyen et al. 2018). FB<sub>1</sub> accumulation was also inhibited by treating milled maize with *Streptomyces* sp. (Samsudin and Magan 2016). A laccase produced by *Streptomyces coelicolor* was patented for its use to decontaminate AFB<sub>1</sub> (Loi et al. 2017). As mentioned before, the compounds aflastatin A, blasticidin A and dioctatin A, produced by *Streptomyces* sp., inhibit aflatoxin production in *A. parasiticus* (Ren et al. 2020; Tian and Chun 2017). In a series of studies by Verheecke et al., they reported that several soil-born *Streptomyces* isolates were capable of degrading AFB<sub>1</sub>, and had a strong bioactivity against AFB<sub>1</sub> and AFB<sub>2</sub> production by *A. flavus* (Verheecke et al. 2014). Moreover, they repressed aflatoxin gene expression on *A. parasiticus* (Verheecke, Liboz, Anson, Zhu, et al. 2015). Eshelli et al. optimized the AFB<sub>1</sub> degradation conditions of *R. erythropolis*, *S. lividans* and *S. aureofaciens*. Subsequently, they identified a series of degradation by-products and proposed a plausible degradation mechanism (Eshelli et al. 2015). Caceres et al. determined that *S. roseolus* was capable of down-regulate the AFB<sub>1</sub> gene cluster in *Aspergillus flavus* (Caceres et al. 2018). *Streptomyces rimosus* degraded zearalenone into 8'(S)-hydroxyzearalenone and 2,4-dimethoxyzearalenone (El-Sharkawy and Abul-Hajj 1988). A laccase, produced by *S. coelicolor* was also capable of degrading ZEN (Loi et al. 2017). Harkai et al. documented the biodegradation of AFB<sub>1</sub> and ZEN by several *Streptomyces* strains, and some of them eliminated the genotoxic and estrogenic effects of the mycotoxins, respectively (Harkai et al. 2016).

The *Streptomyces* genus has a vast potential as BCAs. They benefit from a series of mechanisms that allow them to fight against fungal pathogens but also to facilitate plant development and self defense. The use of *Streptomyces* bacteria as BCAs seems promising due to their technological properties, such as plant colonization and sporulation, which grants them an advantage compared to other potential biocontrol agents and facilitates their formulation and posterior application. Furthermore, *Streptomyces* represent a promising source of metabolites and enzymes with detoxifying properties.

### 3. Objectives of the study

The previous bibliographical synthesis, introduces the context involving the risk of wheat contamination by fungal pathogens and saprophytes, and the potential occurrence of harmful mycotoxins on derived products. The decrease on the use of chemical fungicides in the last decade has urged the search for alternative and most ecological control solutions, such as the use of beneficial microorganisms. This primary goal gave rise to the research project presented in this manuscript, focusing on a specific group of bacteria: actinobacteria. The main purpose started as the screening of an actinobacteria collection for their antagonistic capacities *in vitro* against various phytopathogenic and/or mycotoxinogenic fungi of wheat. Additionally, *in planta* evaluation of the antagonistic capacities of selected actinobacteria were assessed for phytopathogens. For mycotoxinogenic fungi, we included a deeper comprehension of fungal-bacteria interactions and the resulting impact on mycotoxins accumulation.

The experimental approach was based on the following objectives:

1. Assessment of the impact of actinobacteria isolates on fungal growth *in vitro*.
2. Determination of the capacity of actinobacteria to decrease wheat foliar diseases under greenhouse conditions after foliar application.
3. Evaluation of the effect of bacterial isolates on mycotoxin production by mycotoxinogenic fungi.
4. Study of fungal development and mycotoxin accumulation in the presence of bacterial metabolites.
5. Evaluation of the mycotoxin degrading capacity of actinobacteria isolates and their metabolites.

These various assays led to a series of research questions:

- I. Are the inhibitions observed in various fungi-actinobacteria interactions universal among mycotoxinogenic fungi focusing on:
  - a. The growth inhibition
  - b. The reduction of mycotoxin accumulation

- II. What are the mechanisms behind the variation on mycotoxin accumulation when actinobacteria are confronted with fungi?
- III. Does mycotoxin degradation by the actinobacteria isolates lead to an effective detoxification?
- IV. Can actinobacteria antifungal effects on wheat pathogenic fungi *in vitro* be transposed *in planta* on wheat leaves?

The concomitant analysis of the results obtained for the different parameters by Clusterization and Pearson correlation allowed us to compare and regroup the evaluated isolates according to their similarities. The different models studied were analyzed thoroughly to highlight common and contrasting features between them. This allowed the identification of the potential antagonistic mechanisms involved, as well as the selection of promising actinobacteria isolates for further research.

The workflow presented in figure 35 displays the experimental process carried out during this work. The obtained results are presented in six sections presented as scientific articles. One of these articles is already published, the second was recently accepted for publication, and another one has been submitted. Two additional sections include the synthesis and statistical comparison of the results obtained for the different models studied.



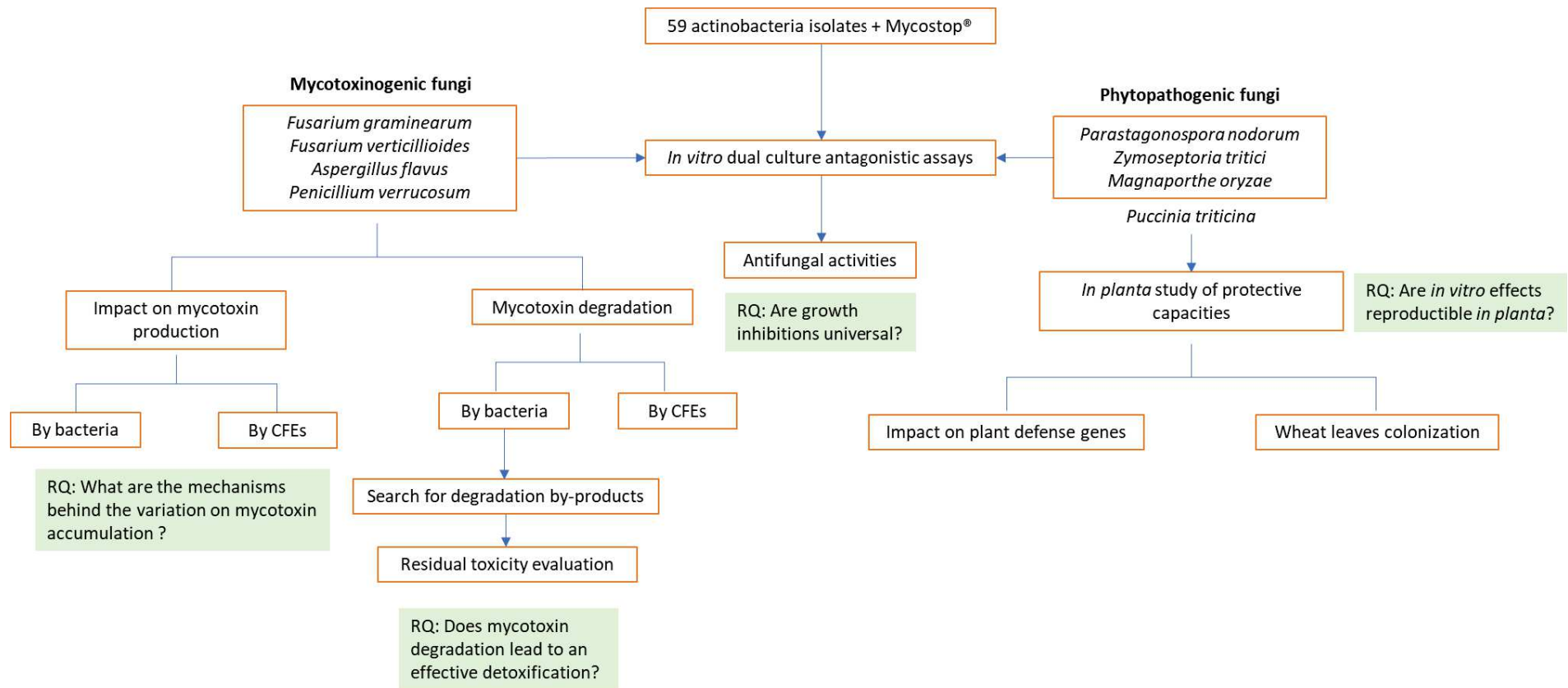


Figure 35. Workflow of the experimental approach followed during this work. Orange boxes represent the implemented experimental protocols and green boxes indicate the main research questions.



## 4. Experimental work

## 4.1 Actinobacteria collection constitution and characterization

### 4.1.1 Introduction

Actinobacteria constitute one of the most abundant microbes in the rhizosphere. They play an important role in protecting plants against harmful pathogens, therefore, they are a potential source for the development of biological control agents (BCAs). Actinobacteria are also associated with promoting plant development and self defense mechanisms. These properties are achieved thanks to the production of a wide variety of secondary metabolites, which confer them plant beneficial and pathogen antagonistic capacities (Sharma and Salwan 2018).

16S rRNA sequencing has led to the recognition of 16 orders, 39 families and 130 genera within the actinobacteria. *Streptomyces* constitute over 95% of the *Actinomycetales* isolated from soil. They form branched vegetative mycelium by tip extension that differentiates into aerial hyphae, which then forms individual and chained spores. During this stage, an active production of enzymes and secondary metabolites takes place, such as antibiotics and melanoid pigments (Barka et al. 2016).

Isolating actinobacteria from a similar environment to which their application is intended may help to maintain an equilibrium of the indigenous microflora and also facilitates their adaptation to the media (Legrand et al. 2017). Several media were specifically created for the isolation and development of *Streptomyces*, and to facilitate the display of their typical morphological traits, such as powdery colonies with greyish colors, the characteristic “earthy” odor and in some cases the production of pigments (Dhanasekaran and Jiang 2016; Shirling and Gottlieb 1966).

Some plant growth promoting traits exhibited by actinobacteria include iron and nitrogen fixation, phytohormone production and phosphate solubilization (Anwar, Ali, and Sajid 2016). They are also able to interfere with plant pathogens by producing degrading enzymes such as chitinases. Actinobacteria possessing these capacities constitute promising alternatives for plant fertilization and particularly for their application as biocontrol agents against pathogenic fungi causing plant diseases (Passari et al. 2015).

Fifty-nine actinobacteria were isolated according to their morphology from soil samples collected at the Hérault region in France and from organic amendments. They were

partially identified as *Streptomyces* by 16S rRNA sequencing. The resulting *Streptomyces* collection belonging to the UMR Qualisud at the University of Montpellier was created after spore production, enumeration and conservation. Afterwards, the *Streptomyces* isolates were evaluated in order to identify their plant growth promoting (PGP) traits and their chitinolytic activities.

#### 4.1.2 Materials and methods

##### Actinobacteria isolation

Microorganisms present in soil samples collected at the Hérault department in France and in domestic organic amendments were cultured on PDA medium (Biokar BK095HA) during previous work (Nguyen et al. 2018). Colonies having actinobacteria morphology were taken from the PDA plates and cultured on three media: ISP2 (4 g/L yeast extract, 10 g/L malt extract, 4 g/L dextrose, 20 g/L bacteriological agar, pH 7.2), ISP4 (10 g/L starch, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 1 g/L MgSO<sub>4</sub>, 2 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/L CaCO<sub>3</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L MgCl<sub>2</sub>, 1 mg/L ZnSO<sub>4</sub>, 18 g/L bacteriological agar, pH 7.2) and CYA (30 g/L sucrose, 5 g/L yeast extract, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.3 g/L NaNO<sub>3</sub>, 0.05 g/L KCl, 0.05 g/L MgSO<sub>4</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L ZnSO<sub>4</sub>, 0.5 mg/L CuSO<sub>4</sub>, 15 g/L agar, pH ~7.4) until the obtention of pure cultures.

##### Actinobacteria spore recovery and conservation assays

Four actinobacteria isolates having different morphological traits (IX06, IX23, IX35, IX43) were selected to evaluate their sporulation and stability during conservation. Isolates were cultured in the dark on ISP4 and CYA medium for 11 days at 28°C. Spores were scraped from the surface of Petri dish plates by adding 5 mL of distilled water + 0.01% Tween 20 and filtered through sterile cotton. Spore amount was determined on an optical microscope (Primo Star, Zeiss, Allemagne) using a Thoma cell counting chamber (Preciss, France). Collected spores were preserved under three modalities: at -80°C with the addition of glycerol (25%), -80°C without glycerol and at 4°C. After 7, 14 and 22 days of conservation, spores were cultured again on ISP4 and CYA media to assess their viability by UFC counting. All tests were done in triplicate.

### Actinobacteria spore production and enumeration

The fifty-nine actinobacteria isolates were cultured on ISP4 medium for 11 days at 28°C. Spores were collected as described above. Spore counting was performed using a Novocyte ACEA flux cytometer. Spore suspensions were aliquoted and conserved at -80°C without the addition of glycerol. Before each test, an aliquot of spores was unfrozen and homogenised at a concentration of 10<sup>7</sup> spores/mL.

### Actinobacteria DNA extraction and 16S rRNA preliminary identification

Actinobacteria were cultured on CYB medium for 5 days at 28°C and 180 rpm. For DNA extraction, 3 mL of culture were centrifugated 5 min at 10000 rpm. Supernatant was eliminated and the pellet was kept at -80°C overnight. Then, samples were lyophilized and kept under liquid nitrogen. Two ceramic beads were added to each tube. Samples were then ground in 3 cycles of 40 seconds using a FastPrep® instrument and kept for 1 minute in liquid nitrogen between each grinding.

Genomic DNA purification was performed using the Gram-positive DNA purification kit MasterPure™ following the manufacturer's instructions. For this, 150 µL of TE buffer were added to each tube, then, samples were vortexed to resuspend the ground pellets. 1 µL of Ready-Lyse Lysozyme were added to each resuspended pellet. Samples were incubated overnight at 37°C. 1 µL of Proteinase K (50 µg/mL) were diluted into 150 µL of Gram-Positive Lysis Solution. 150 µL of the previous solution were added to each sample and mixed thoroughly. Followed by an incubation at 65-70°C for 15 minutes with a brief vortexing every 5 minutes. Samples were cooled to 37°C then placed on ice for 3-5 minutes before DNA precipitation.

DNA precipitation was conducted following manufacturer instructions as well. 175 µL of MPC Protein Precipitation Reagent were added to 300 µL of lysed sample, followed by vigorous vortexing for 10 seconds. Then samples were centrifuged at 4°C for 10 minutes at >10000×g in a microcentrifuge. Supernatant was then transferred to a clean microcentrifuge tube and pellet was discarded. 1 µL of RNase A (5 µg/mL) were added to each sample and mixed thoroughly, followed by an incubation at 37°C for 30 minutes. At the end of the incubation period, 500 µL of isopropanol were added to the supernatant and mixed by inversion for 30-40 minutes. After, tubes were centrifuged at 4°C for 10 minutes at 10000×g

in a microcentrifuge. The supernatant was removed with a micropipette being cautious of not dislodging the DNA pellet. Then, the pellet was rinsed with 70% ethanol and centrifuged if dislodged. Finally, DNA was resuspended in 35  $\mu$ L of ultrapure water.

Genomic DNA was quantified using a spectrophotometer-Nanodrop ND8000 (Thermo Fisher Scientific, Waltham, MA, USA). The 16S rRNA gene was amplified using the universal primers 27F/1492R. Amplification was accomplished by mixing 200  $\mu$ L of AmpliTaq™ Gold Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1  $\mu$ L of each primer at 10  $\mu$ M and 1  $\mu$ L of DNA template (~100 ng). The thermocycling program was started with initial denaturation at 95°C for 10 min and followed by 35 thermal cycles of 95°C for 30s, 52°C for 45s, and 52°C for 2 min. Finally, the final extension was done at 72°C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis and the amplicon (~1.5 kb) was purified. DNA Sanger sequencing was carried out in the ISEM GenSeq platform (University of Montpellier, France). Obtained sequences were trimmed according to the quality scores on the chromatogram sequences. The forward and reverse sequences were aligned using the Geneious Prime (Version 2019.2) software with global alignment with free ends and gaps at 93% of similarity (5.0/-9.026168). The nucleotides obtained were subjected to BLAST analysis using the Geneious database.

### Evaluation of PGPR properties of actinobacteria

In order to evaluate the PGPR capacities of the actinobacteria collection, different media for the identification of PGPR traits were prepared as described below. Actinobacteria were cultured on each specific media under the described conditions.

#### **Chitinase activity**

To determine the ability of actinobacteria isolates to produce chitinase, 10  $\mu$ L of spore suspension at  $10^7$  spores/mL were inoculated on plates of colloidal chitin medium (15 g/L colloidal chitin at 10%, 0.5 g/L yeast extract, 1 g/L  $(\text{NH}_4)_2\text{SO}_4$ , 0.3 g/L  $\text{MgSO}_4$ , 1.36 g/L  $\text{KH}_2\text{PO}_4$ , 15 g/L bacteriological agar) for 5 days at 28°C. Actinobacteria exhibiting a clear halo around the colony were noted as positive.

### Siderophore production

To detect the production of siderophores by the actinobacteria isolates, a CAS (Chrome Azurol Sulphonate) solution was prepared by mixing the three following solutions adapted from (Payne 1994):

- Solution 1: 60 mg of CAS in 50 mL of distilled water
- Solution 2: 2.7 mg of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 10 mL of HCl 10 mM.
- Solution 3: 73 mg of HDTMA (hexadecyltrimethylammonium) in 40 mL of distilled water

Actinobacteria were grown on ISP2 medium for 7 days at 180 rpm. Then, liquid cultures were centrifuged and 0.5 mL of supernatant were mixed with 0.5 mL of CAS solution. After 20 minutes, absorbance was measured at 630 nm. Siderophore production amount was calculated using the following equation:

$$\% \text{ of siderophore units (psu)} = \frac{(Ar - As) \times 100}{Ar}$$

Where:

$Ar$  = Absorbance at 630 nm of reference (uninoculated ISP2 + CAS solution)

$As$  = Absorbance at 630 nm of the sample

### Phosphate solubilization

To determine if actinobacteria were able to solubilize phosphate, 10  $\mu\text{L}$  of a spore suspension at  $10^7$  spores/mL were cultured on PVK (Pikovskayas's) medium (0.5 g/L yeast extract, 10 g/L dextrose, 5 g/L  $\text{Ca}_3(\text{PO}_4)_2$ , 0.5 g/L  $(\text{NH}_4)_2\text{SO}_4$ , 0.2 g/L KCl, 0.1 g/L  $\text{MgSO}_4$ , 0.1 mg/L  $\text{MgSO}_4$ , 0.1 mg/L  $\text{FeSO}_4$ , 15 g/L bacteriological agar) for 7 days at 28°C. A clear halo around the colony indicated a positive result.

### Ammonia production

To verify the production of ammonia by the actinobacteria isolates, 20  $\mu\text{L}$  of a spore suspension at  $10^7$  spores/mL were inoculated on peptone water (10 g/L peptone, 5 g/L sodium chloride) for 15 days at 180 rpm and 28°C. At the end of this period, 0.5 mL of Nessler's

reagent (3.5 g KI; 1.3 g HgI<sub>2</sub>; 70 mL H<sub>2</sub>O, 30 mL NaOH 30%) were added to the culture. A positive result was indicated by the development of a brown to yellow color.

### **Indole-3-acetic acid (IAA) production**

500 µL of actinobacteria spore suspension 10<sup>7</sup> spores/mL were inoculated into flasks containing ISP2 medium, they were cultured at 28°C and 180 rpm for 7 days. 150 µL from the pre-culture were inoculated into flasks containing 100 mL of ISP2 medium with 0.2% L-tryptophan. After 14 days of culture at 28°C and 180 rpm, bacterial liquid cultures were centrifuged at 10000 rpm for 10 minutes. 1 mL of the supernatant was mixed with 2 mL of Salkowski's reagent (2 mL FeCl<sub>3</sub> (0.5M) + 49 mL distilled water + 49 mL perchloric acid 70%) and incubated for 25 minutes in the dark at 28°C. IAA production was observed as the development of a pink-red color in the tubes.

To quantify IAA production by the actinobacteria isolates, a standard curve of IAA was prepared on ISP2 medium. Absorbances were determined at 530 nm.

#### 4.1.3 Results and discussion

##### Actinobacteria spore recollection and conservation assays

After the obtention of pure cultures of actinobacteria, four isolates presenting different morphological characteristics (IX06, IX23, IX35, IX43) were selected to perform sporulation and conservation assays on CYA and ISP4 media. Results indicate that both media allow a good sporulation yield between 1x10<sup>8</sup> and 5x10<sup>9</sup> spores/mL approximatively (Figure 36).

Conservation assays indicated a similar viability for actinobacteria spores regardless of the preservation method, time and medium of origin, with values fluctuating between 1.5x10<sup>7</sup> and 2.5x10<sup>8</sup> UFC/mL (data not shown). Based on these results, we decided selected ISP4 as the medium for spore production, and a conservation at -80°C without glycerol, to avoid the addition of carbon sources to the spore suspensions.



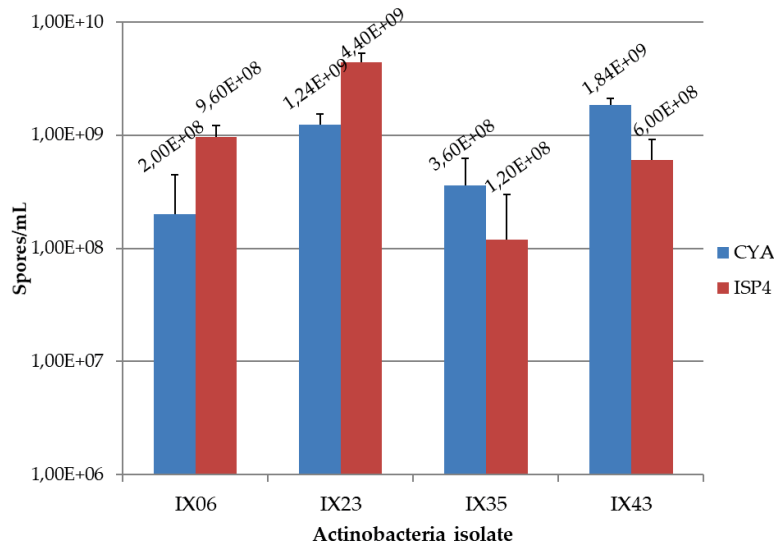


Figure 36. Sporulation rates of four *Streptomyces* strains on CYA and ISP4 medium after 11 days of culture at 28°C.

Actinobacteria spore production and enumeration

Spore suspensions of the 59 actinobacteria isolates were produced on ISP4 medium for 11 days at 28°C and enumerated using a Novocyte ACEA flux cytometer before frozen conservation at -80°C. Flux cytometer distributions of the counted “events” (spores passing through the laser beam) indicate that most of the spores were individually separated, however some of them seemed to be associated in small chains or lots (Fig 37).

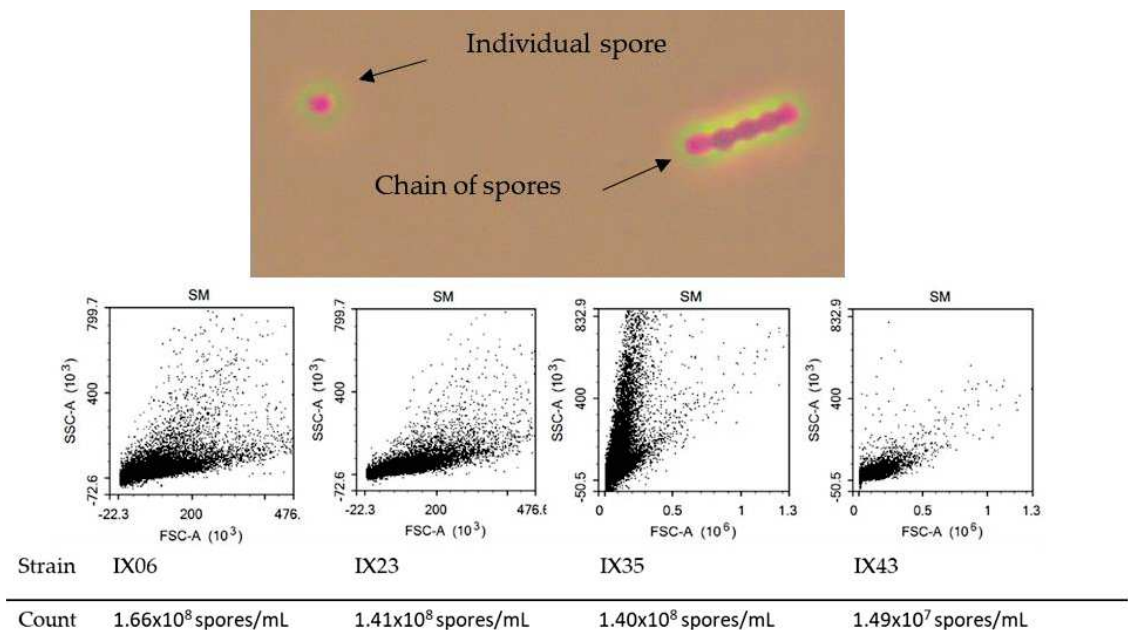


Figure 37. Top: microscopical observation of individual and “chained” actinobacteria spores. Bottom: Examples of plots of “events” (bacterial spores passing through the laser beam) obtained by the flux cytometer for isolates IX06, IX23, IX35 and IX43.

### Actinobacteria DNA extraction and 16S rRNA preliminary identification

The 16S sequencing indicated that the isolates from the collection belonged to the genus *Streptomyces* at 97%. As 16S identification is preliminary, several species can be assigned to the different RNA fragments, resulting in multiple possibilities of identification. For instance, after partial BLAST analysis, isolate IX23 was assimilated to *S. griseolus*, *S. halstedii* and eventually *S. cyaneus*, and isolate IX45 could be *S. zaomycticus* or *S. venezueleae*. However, isolate IX20 had numerous possibilities according to the 16S ribosomal RNA partial sequence, which include *S. cyaneofuscatus*, *S. anulatus*, and *S. praecox*, among 10 others. For this reason, a complete genome sequencing is ongoing. In order to provide a precise identification for further research.

### Evaluation of PGPR properties of actinobacteria

Results indicate that forty-six isolates (78%) are capable of degrading chitin as they form clear halos around their colonies when grown on colloidal chitin medium (Fig. 38). Several studies have determined the chitinolytic capacity of actinobacteria by growing them on colloidal chitin medium, and some of them validated the visual result by amplifying the chitinase gene (Passari et al. 2015). The observation of halos on PVK's medium allowed us to determine that 25 isolates (42%) were able to solubilize phosphate (Fig. 39). Seven isolates (12%) gave positive to ammonia production, as the culture medium turned brown to yellow upon addition of Nessler's reagent. For comparison, around 50% of the actinobacteria isolates screened in the study of Passari et al. were capable of solubilizing phosphate, while all of them produced ammonia (Passari et al. 2015).

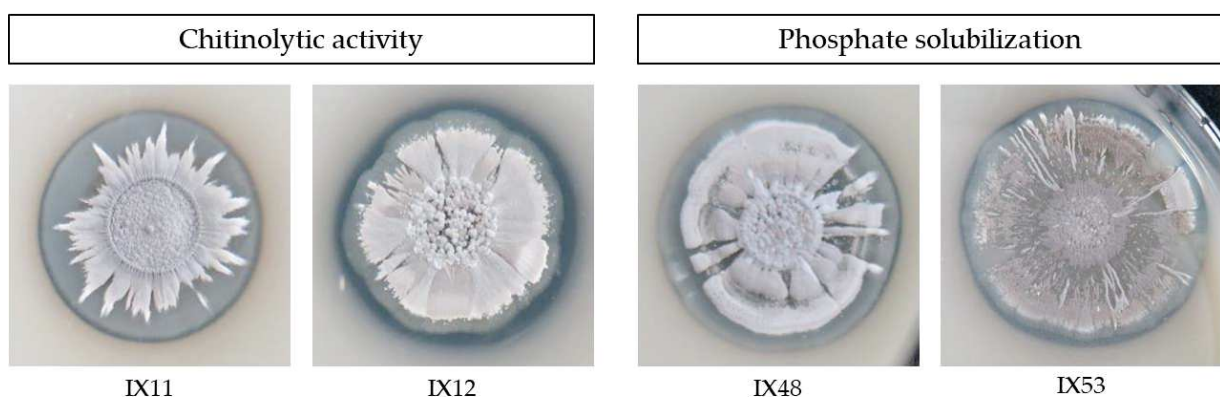


Figure 38. Examples of halo formation by actinobacteria on chitinase degradation and phosphate solubilization assays.

Thirteen isolates (22%) presented positive results to IAA production with phytohormone concentrations going from 58 to 200 µg/mL (Table 2). This is comparable to the results obtained by Anwar et al. when screening rhizospheric actinobacteria. Indeed, their strains produced a maximal amount of IAA between 70 and 80 µg/mL, when cultured with a maximal concentration of 500 µg/mL of L-tryptophan (Anwar et al. 2016), which is 4 times less than the concentration employed in this study. Results obtained by Passari et al. when investigating the PGPR properties of an endophytic actinobacteria collection indicated maximal IAA productions of about 30 µg/mL (Passari et al. 2015). Ranveer et al. estimated a IAA production of about 17 µg/mL by a *Streptomyces* strain (Ranveer and A. 2014). The IAA concentrations produced by *Streptomyces anulatus* and *S. albidoflavus* during a study by Kunova et al. were much lower, with about 4 µg/mL produced by both strains (Kunova et al. 2016).

Our results also indicate that 15 of the tested isolates (25%) were able to produce siderophores with concentrations going from 4 to 82 percentage siderophore units (PSU). Near 60 PSU were obtained by Kumar et al. during the siderophore production estimation of soil isolated bacteria (S. et al. 2017). About 19% of the *Streptomyces* strains tested by Colombo et al. were capable of producing siderophores, as an orange halo was observed around the colonies on Chrome Azurol S agar overlay (O-CAS) medium (Colombo, Kunova, Pizzatti, et al. 2019). All six actinobacteria isolates evaluated by Anwar et al. were capable of producing siderophores (orange halo formation) (Anwar et al. 2016).

Table 2. Quantitative estimation of IAA and siderophore production by the actinobacteria isolates exhibiting positive results. Common isolates between the two tests are indicated in bold.

IAA production		Siderophore production	
Actinobacteria isolate	µg/mL	Actinobacteria isolate	PSU
<b>IX02</b>	87,12	IX01	3,9
IX06	89,58	<b>IX02</b>	5,0
IX07	96,85	IX03	80,5
IX11	82,93	IX04	13,6
IX42	92,17	IX05	14,0
IX43	72,33	IX20	35,0
IX44	57,79	IX25	79,9
IX47	140,47	IX28	7,7
IX51	68,51	IX29	18,1
<b>IX52</b>	199,99	IX35	70,3
IX53	172,64	IX36	57,7
IX54	130,37	IX45	82,2
IX55	84,16	<b>IX52</b>	12,6
		IX58	78,4

Fungal cell walls are conformed of chitin, so bacteria capable to degrading this polymer have an advantage during antagonistic confrontations. In the same way, siderophore production allows them to sequester iron, rendering it unavailable for the fungi. PGPR properties such as phosphate solubilization, ammonia production and the production of the phytohormone IAA, confer them potential as plant development facilitators (Anwar et al. 2016; Colombo, Kunova, Pizzatti, et al. 2019; Kunova et al. 2016; Passari et al. 2015).

Table 3 shows a summary of the actinobacteria isolates exhibiting at least one positive result to the PGPR assays.

**Table 3. Positive of actinobacteria isolates to the PGPR assays. Only isolates having at least one positive result were presented.**

Actinobacteria isolate	Chitinase activity	Siderophore production	Phosphate solubilization	Ammonia production	IAA production
IX01	X	X			
IX02	X	X			X
IX03		X		X	
IX04	X	X	X	X	
IX05		X			
IX06	X				X
IX07	X	X	X		X
IX08	X				
IX09	X		X		
IX10	X		X		
IX11	X				X
IX12	X		X		
IX13	X				
IX14	X				
IX15	X				
IX16	X				
IX17	X		X		
IX18	X				
IX19	X		X		
IX20		X			
IX21	X		X		
IX22	X				
IX23	X				
IX24	X		X		
IX25		X		X	
IX26	X				
IX27	X				
IX28		X	X		
IX29		X	X		
IX30			X		
IX31			X		
IX33	X		X		

IX34	X				
IX35		X	X	X	
IX36		X	X	X	
IX37	X				
IX38	X				
IX39	X		X		
IX40	X				
IX41	X				
IX42	X		X		X
IX43	X		X		X
IX44	X		X		X
IX45	X	X	X		
IX46	X		X		
IX47	X		X		X
IX48	X		X		
IX49	X				
IX50	X				
IX51	X				X
IX52	X	X			X
IX53	X		X		X
IX54				X	X
IX55	X				X
IX56	X				
IX57	X				
IX58		X		X	
IX59	X				

#### 4.1.4 Conclusion and perspectives

The conducted experimental protocols allowed the creation and conservation and preliminary characterization of the Qualisud actinobacteria collection. 16S identification indicates that the collected isolates belong to the *Streptomyces* genus. Additionally, a complete genome sequencing is currently being performed on thirty isolates having showed promising results to the assays in the next chapters of this work. Moreover, the various positive results of the PGPR evaluations show a promising BCA potential for the screened isolates under the perspective of soil treatments. These results constitute the first step towards the comprehension of antagonistic mechanisms established by actinobacteria against wheat fungal pathogens (i.e. chitinase production).

## 4.2 Minimizing Ochratoxin A contamination through the use of actinobacteria and their active molecules

### 4.2.1 Article presentation

During grain storage, temperature and humidity conditions should be controlled carefully, otherwise fungi can develop and lead to a potential risk of mycotoxin accumulation. As the addition to chemical products to stored food and feed is limited due to their undesirable effects, other alternatives for the control of fungal development and mycotoxin decontamination on food matrices have to be explored. The addition of mycotoxin degrading enzymes produced by *Streptomyces* constitutes a promising alternative.

This article presents the screening of a *Streptomyces* collection for their antifungal and antimycotoxinogenic capacities against *Penicillium verrucosum*, a ubiquitous fungus found regularly as a contaminant of cereals, and the main producer of ochratoxin A (OTA) among the *Penicillium* genus. It was found that most of the tested bacterial isolates were capable to inhibit fungal growth. However, fungal growth inhibition was often accompanied by a stimulation of OTA production, probably as a fungal defense mechanism. The evaluation of *Streptomyces*' cell free extracts allowed to determine that they may contain some constituent metabolites and/or active enzymes having an impact on mycotoxin accumulation.

An interesting observation of this work was the impact of the culture conditions on the capacity of bacterial cells to degrade OTA. Indeed, only five isolates were able to degrade the mycotoxin on solid medium, while almost all of them degraded it in liquid culture, some almost completely. This kind of differences were already described in the literature and reveal interesting perspectives in the study of degradation mechanisms related to bacterial metabolism. Despite the evidence of mycotoxin decrease, no degradation by-products were identified after bacterial degradation. *Streptomyces* cell free extracts were unable to degrade OTA, this result led to complementary analysis on the stimulation of bacterial cells during liquid culturing for the production of supernatants, results are presented in the next chapter.

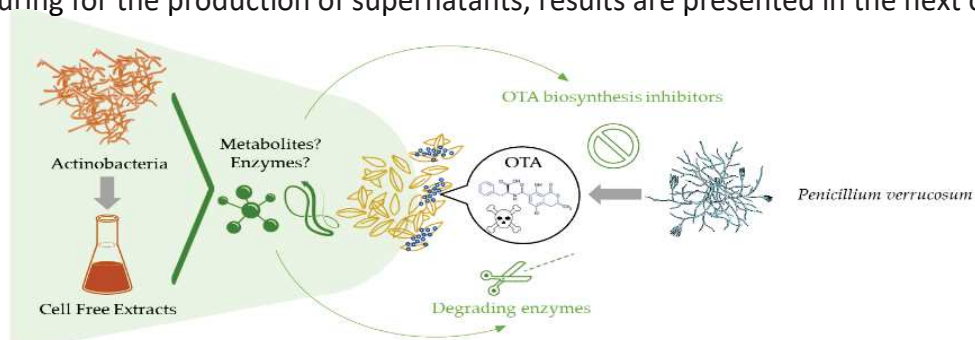



Figure 39. Graphical abstract



Article

# Minimizing Ochratoxin A Contamination through the Use of Actinobacteria and Their Active Molecules

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**Abstract:** Ochratoxin A (OTA) is a secondary metabolite produced by fungal pathogens such as *Penicillium verrucosum*, which develops in food commodities during storage such as cereals, grapes, and coffee. It represents public health concerns due to its genotoxicity, carcinogenicity, and teratogenicity. The objective of this study was to evaluate the ability of actinobacteria and their metabolites to degrade OTA and/or to decrease its production. Sixty strains of actinobacteria were tested for their ability to prevent OTA formation by in vitro dual culture assays or with cell free extracts (CFEs). In dual culture, 17 strains strongly inhibited fungal growth, although it was generally associated with an increase in OTA specific production. Seventeen strains inhibited OTA specific production up to 4% of the control. Eleven actinobacteria CFEs reduced OTA specific production up to 62% of the control, while no substantial growth inhibition was observed except for two strains up to 72% of the control. Thirty-three strains were able to degrade OTA almost completely in liquid medium whereas only five were able to decrease it on solid medium, and two of them reduced OTA to an undetectable amount. Our results suggest that OTA decrease could be related to different strategies of degradation/metabolization by actinobacteria, through enzyme activities and secretion of secondary metabolites interfering with the OTA biosynthetic pathway. CFEs appeared to be ineffective at degrading OTA, raising interesting questions about the detoxification mechanisms. Common degradation by-products (e.g., OT $\alpha$  or L- $\beta$ -phenylalanine) were searched by HPLC-MS/MS, however, none of them were found, which implies a different mechanism of detoxification and/or a subsequent degradation into unknown products.

**Keywords:** *Streptomyces*; fungi; mycotoxins; enzymes; biodegradation; detoxification

**Key Contribution:** Actinobacteria employ different strategies to reduce ochratoxin A through secreting secondary metabolites and by degrading activities. Clustering and Pearson's correlation calculations produced well defined groups regarding actinobacteria specificities and strategies against *P. verrucosum* and ochratoxin A. Several strains inhibiting fungal growth also led to a strong enhancement of OTA specific production.

## 1. Introduction

As climate change progresses, the presence of fungi increases and with them, the occurrence of mycotoxins, which are estimated to contaminate about 25% of global food crops [1]. These secondary

metabolites raise huge concerns due to their harmful effects on human and animal health [2] and to their relative stability to industrial processing [3–5]. *Penicillium verrucosum* is a mycotoxinogenic fungus that develops mostly on stored seeds and is able to produce ochratoxin A (OTA) [5,6]. OTA has carcinogenic, hepatotoxic, immunosuppressive, and genotoxic effects; in addition to potential nephrotoxic and teratogenic incidences [7,8], food authorities have established regulatory measures to limit the use and consumption of contaminated raw materials [9,10].

Facing the millions of tons of grains lost per year due to the presence of mycotoxins, several mitigation techniques are currently under evaluation. At this time, prevailing physical processes include manual sorting, sieving, density segregation, washing, dehulling, steeping, milling, extruding, and heating [11]. Another physical decontamination approach is the use of binding matrices that are able to adsorb toxic molecules such as activated charcoal, bentonite, hydrated aluminum silicates, and zeolites [12]. Emerging techniques such as pulsed light, cold plasma, and gamma irradiation have proven to be efficient to reduce ochratoxin A in nuts and seeds, but the absence of toxic by-products remain to be evaluated before applying these practices during food processing [13,14]. Chemical options consist of acidic and alkaline treatments (e.g., ammoniation) as well as oxidizing (e.g., ozonation) and reducing agents, but these treatments are currently not authorized in the European Union for the decontamination of human food commodities [15].

Aside from physical and chemical treatments, biological solutions include the use of microorganisms and their enzymes [16]. Several microorganisms are able to inhibit mycotoxin biosynthesis and to detoxify mycotoxins once they are produced, either by binding them to their cell wall or by degrading them into less toxic compounds, or a combination of several mechanisms [17–19]. The primary approach is the direct application of detoxifying microorganisms to the contaminated food matrix, but the biological agent must already be recognized as safe for consumption (GRAS) (e.g., lactic bacteria and yeasts) [20]. For microorganisms able to detoxify mycotoxins but not GRAS, their direct utilization in the food matrix is not allowed. Thus, the search for enzymes implied in their degrading capacities can lead to interesting applicative alternatives [21]. Some enzymes known to be involved in ochratoxin A degradation are carboxypeptidases, deoxygenases, lipases, amidases, and proteases [22].

Actinobacteria are filamentous, Gram-positive bacteria that are found in terrestrial and aquatic ecosystems. They are mainly of interest in biotechnology and agriculture due to the huge diversity of enzymes, antibiotics, and antifungal compounds they can produce [23,24]. Along with their antifungal properties, actinobacteria have been repeatedly shown to be able to degrade OTA [16,17] and they can produce enzymes capable of degrading aromatic compounds [25,26]. The main OTA biodegradation mechanism consists of the hydrolysis of the amide bond via hydrolytic enzymes (e.g., carboxypeptidase A), which results in its two primary degradation by-products: OT $\alpha$  and L- $\beta$ -phenylalanine, which are reportedly much less toxic than OTA [15,16]. Another, more hypothetical, mechanism is the cleavage of the lactone ring, giving place to an OTA with an open lactone, however, this process is reversible [27]. To confirm the effectiveness of the detoxification, the identification of the degradation by-products is crucial. HPLC MS and HPLC MS/MS are well suited for the research, identification, and quantification of degradation by-products [28].

Aside from potential degradation mechanisms, some authors have shown that some strains belonging to the *Streptomyces* genus possess the ability to bind mycotoxins onto their cell walls [17]. In addition, *Streptomyces* strains were found to reduce the expression of the *acpks*, *acOTApks*, *acOTAnrps*, and *veA* genes, which modulate the biosynthesis of OTA in *Aspergillus carbonarius* [17]. These remarkable abilities make actinobacteria promising tools for biodegradation, and although their direct application in food matrices is not authorized, they can be the source of various enzymes and metabolites of interest for the reduction of mycotoxins' associated risk.

The aim of our work was to study the interactions between 60 *Streptomyces* strains isolated from compost and amended soil with *P. verrucosum* and its toxin. In a first step, *Streptomyces* strains were confronted with *P. verrucosum* to evaluate their inhibitory effect on growth and OTA specific production (OTAsp). In a second step, we designed experiments to gain further information on the specific

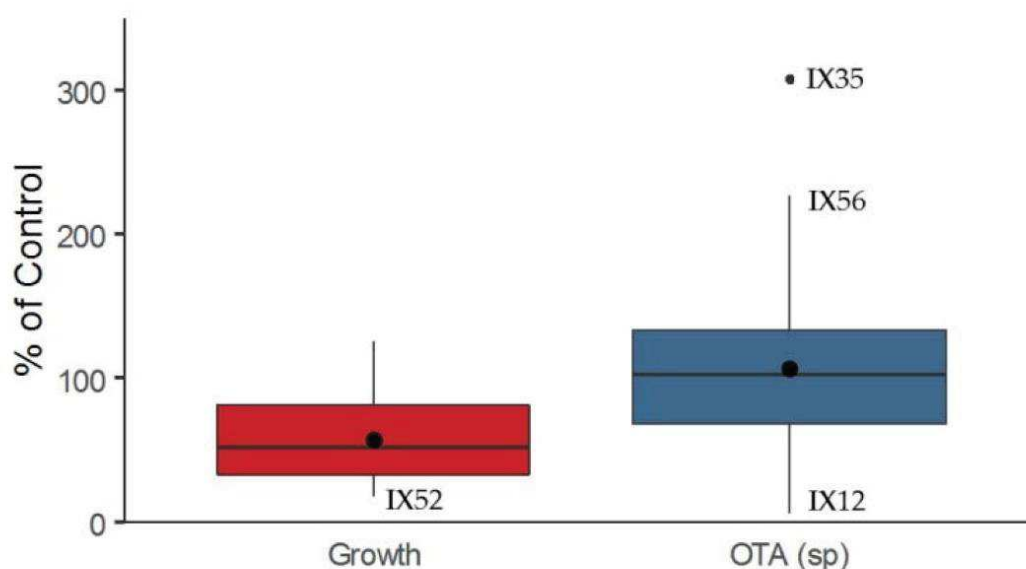


interactions occurring during dual culture assays. The CFEs were added to the growth media of *P. verrucosum* to understand the role of constitutive *Streptomyces* metabolites on inhibiting or promoting fungal growth and/or OTA biosynthetic pathway. Interactions with OTA were studied by adding the toxin to the media during *Streptomyces* growth, and also in their CFEs to understand if OTA reductions could originate from its degradation by secreted enzymes or through its metabolization. All our data were then processed using clustering analysis (heatmap) and Pearson's correlation with the aim of segregating strains with comparable profiles of interest. Finally, OTA degradation was investigated by HPLC-MS/MS in order to draw assumptions about potential degradation by-products typically described in the literature.

## 2. Results

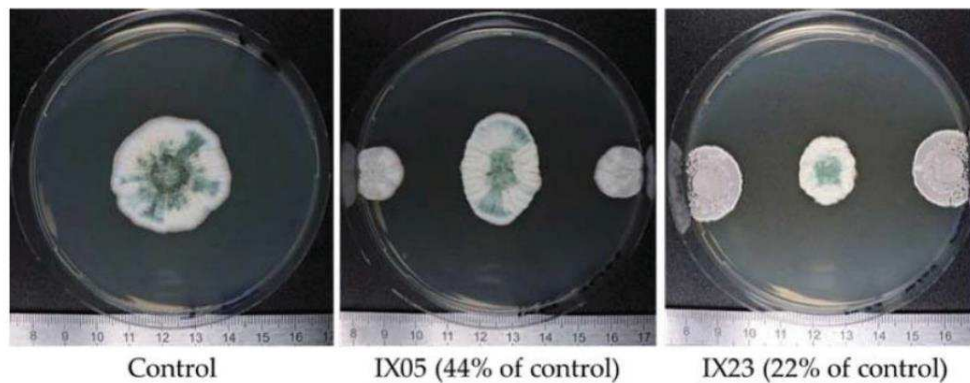
### 2.1. Antagonistic Evaluation of Actinobacteria and Their Cell Free Extracts (CFE)

Sixty strains of actinobacteria were tested by in vitro dual culture assays against *P. verrucosum* on CYA (Czapek Yeast extract Agar) medium at 25 °C for 12 days. Growth was reduced down to 20% of the control with a mean of 58% (median 53%). Ochratoxin A (OTA) specific production decreased to 7% of the control. Nevertheless, 27 strains increased OTA specific production, especially IX56 (227%) and IX35 (307%) (Figure 1). Of note, *Streptomyces* strains with no impact on pathogen growth were more efficient at decreasing OTA specific production, whereas those with strong growth inhibition properties often enhanced mycotoxin production. Strains IX02, IX09, and IX52 were the only strains able to inhibit both *P. verrucosum* growth (38%, 35%, and 19% of the control, respectively) and OTA specific production (51%, 29%, and 46% of the control, respectively).



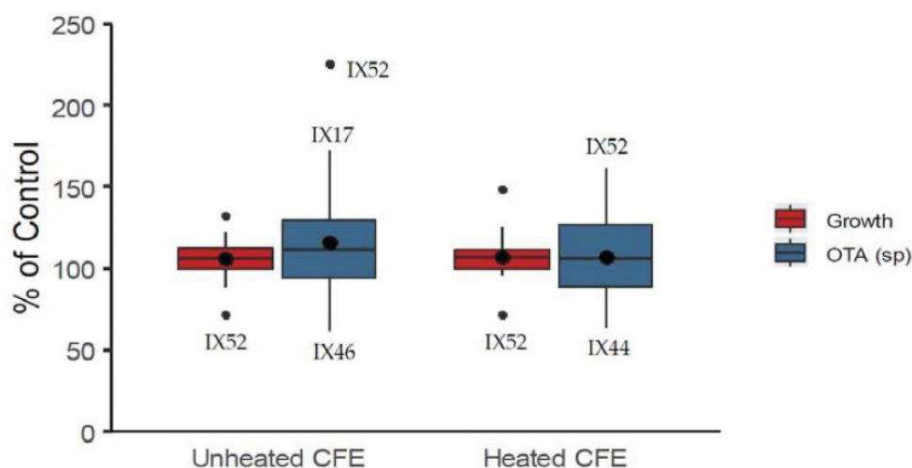
**Figure 1.** Effect of the 60 strains of actinobacteria in dual culture assay with *Penicillium verrucosum* on fungal growth and ochratoxin A (OTA) specific production. Boxplot represents the distribution of the data expressed as % of a control (fungal growth and specific mycotoxin production without actinobacteria).

During the dual culture assays, different inhibition profiles were observed. As shown in Figure 2, the *P. verrucosum* colony had either an oval or round shape, probably reflecting the diffusion and efficacy of antifungal metabolites in the agar.



**Figure 2.** Example of inhibition profiles observed during dual culture assays of actinobacteria strains against *P. verrucosum* on CYA plates at 25 °C for 12 days.

CFEs were produced with CYB (Czapek Yeast extract Broth) liquid culture of actinobacteria grown for five days at 25 °C under agitation (180 rpm) and added at a concentration of 10% in the solid growth medium of *P. verrucosum*. CFEs were much less efficient at reducing both growth and OTA specific production of *P. verrucosum* than that observed in dual culture (Figure 3). Only one CFE (IX52) inhibited *P. verrucosum* growth substantially down to 72% of the control, regardless of whether it underwent a thermal treatment or not (mean = 106%, median = 106% for unheated CFEs and mean = 107%, median = 107% for heated CFEs). OTA specific production was reduced by a few strains up to 62% (IX46) and 64% (IX44) of the control by unheated and heated CFEs, respectively (mean = 116%, median = 112% for unheated CFEs and mean = 107%, median = 106% for heated CFEs). However, half of the CFEs increased OTA specific production during fungal growth. As observed earlier, the most effective CFEs for the reduction of fungal growth were also the ones promoting OTA specific production. For example, CFEs of strain IX52 decreased *P. verrucosum* growth to 72% of the control (with and without thermal treatment), while simultaneously causing an overproduction of OTA with 225% and 161% of the control, for unheated and heated CFEs, respectively.

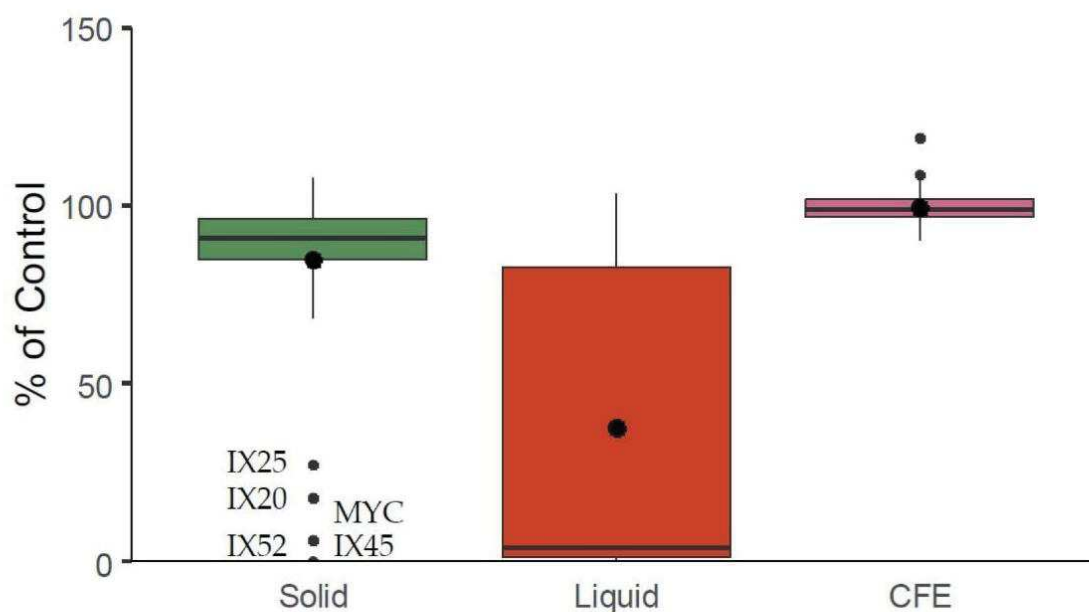


**Figure 3.** Effect of the 60 cell free extracts (CFEs) added at 10% in the culture medium of *Penicillium verrucosum* on growth and OTA specific production. Boxplot represent the distribution of the data expressed as % of a control (fungal growth and specific mycotoxin production without CFEs). Thermal treatment for heated CFEs: 100 °C-10 min.

## 2.2. Mycotoxin Degradation Assay

The ability of the sixty strains of actinobacteria to degrade mycotoxin was evaluated by culturing them in solid CYA and liquid CYB medium supplemented with OTA. A concentration of ~280 ng/mL

was used because it is representative of the maximal toxin concentration typically encountered for *P. verrucosum* alone after 12 days on CYA plates at 25 °C. Furthermore, CFEs were tested for degradation capacities by spiking OTA in CFEs. The control was performed by adding the toxins in CYA medium without bacteria and incubated in the same conditions as the samples. The results are presented in Figure 4. OTA was strongly degraded by only five strains on solid medium (IX25 < IX20 < MYC < IX52 < IX45) from 27% to 0% of the control (mean = 85%, median = 91%), while 33 strains degraded it almost entirely in liquid medium (mean 38%, median 4%). Negligible OTA degradation was noted with a maximum of 90% of the control (mean = 99%, median = 99%) for the unheated CFEs. Thus, heated CFEs were not tested for the degradation assay.



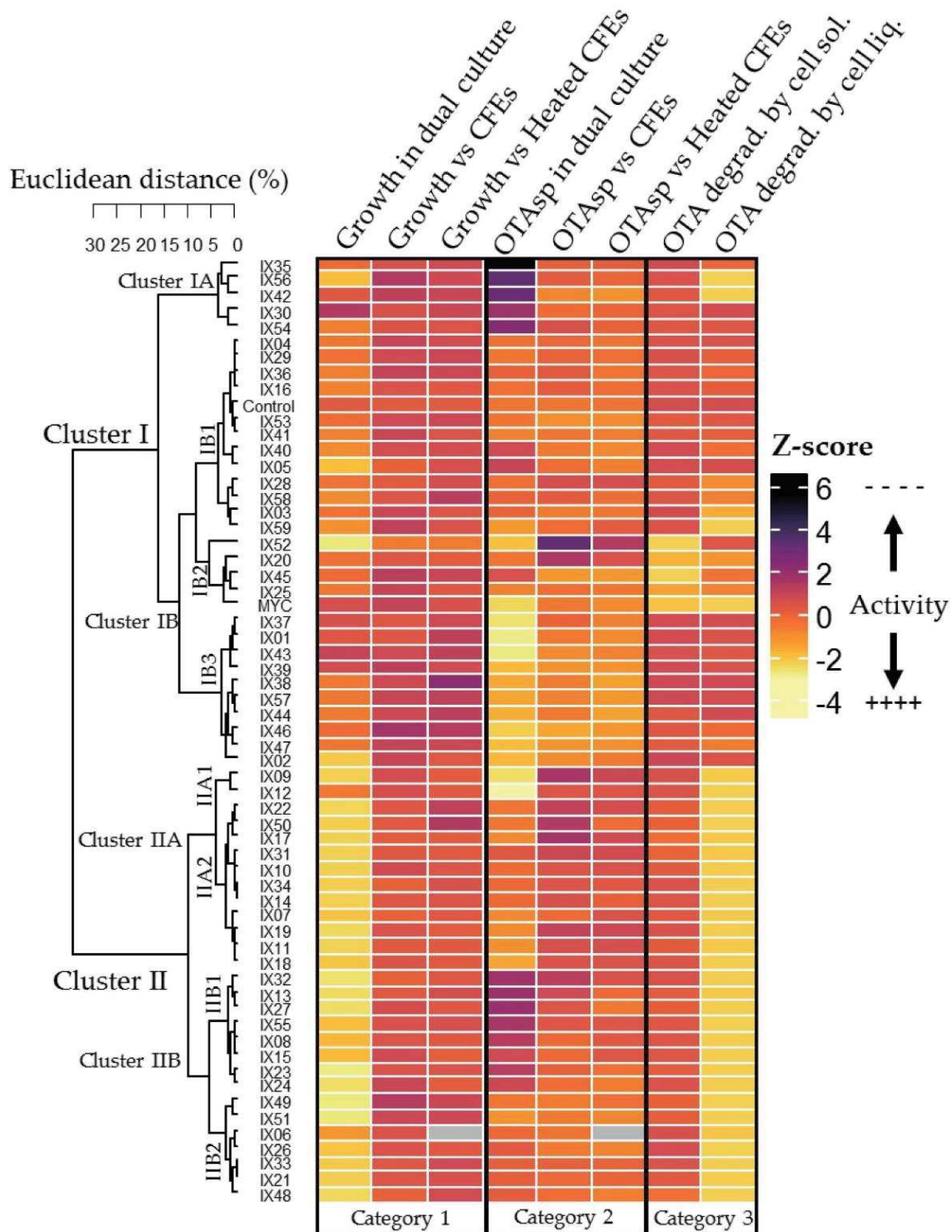
**Figure 4.** Degradation of ochratoxin A by bacterial cells after 10 days of culture at 25 °C in solid and liquid medium, and by unheated CFEs after 48 h at 25 °C under agitation (180 rpm). Boxplots represent the distribution of the degradation efficiency given in % of the control. The control was performed by adding the toxin in CYA or CYB medium without bacteria and incubated in the same conditions as the samples.

### 2.3. Global Analysis of Strains: Heatmap Analysis and Pearson Correlation Index

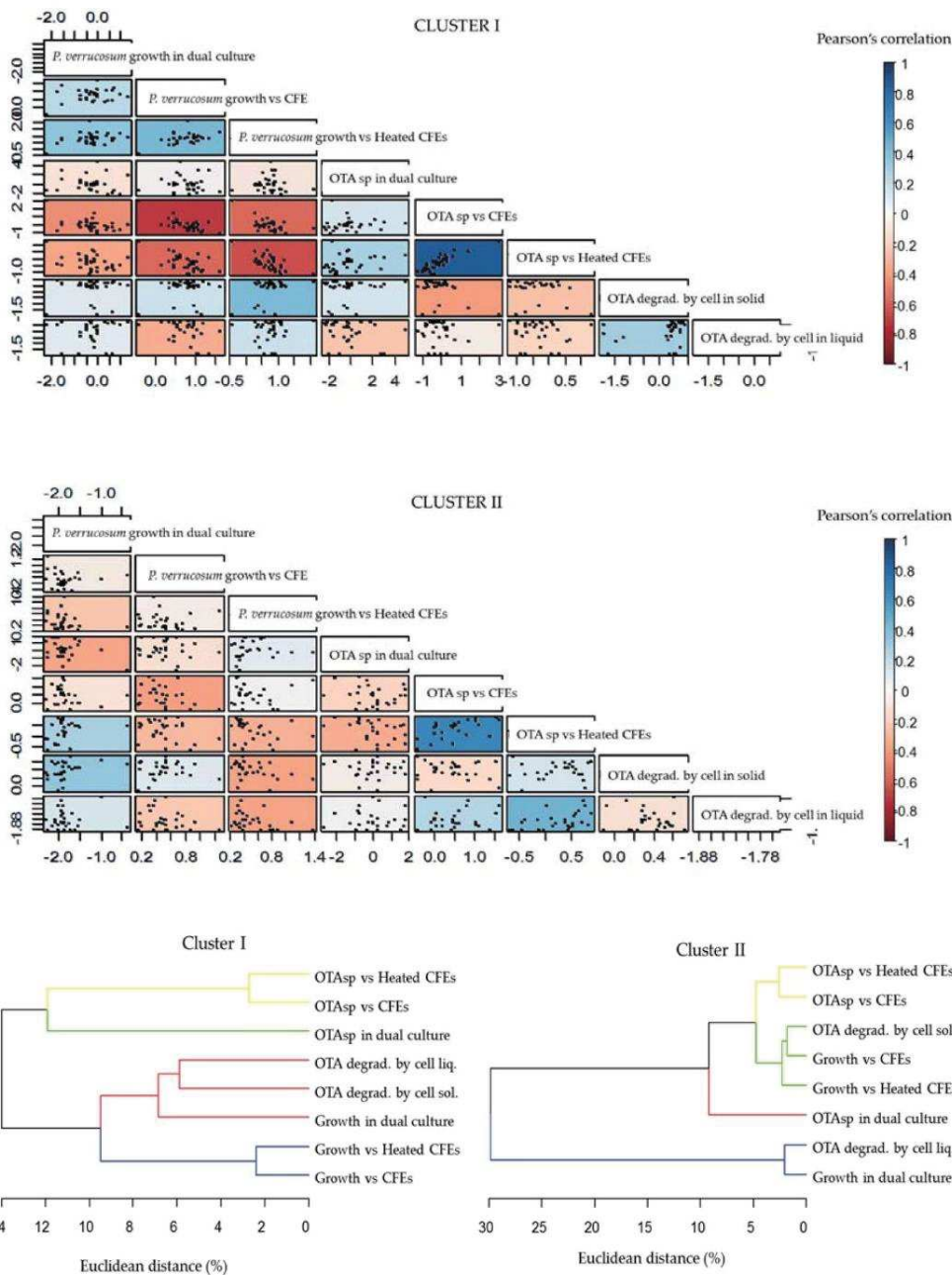
To gain better insight into the specific features of the 60 strains and their potential correlations, all our results were analyzed using a clustering analysis with a heatmap representation (Figure 5) and a correlational analysis through the calculation of the Pearson's correlation index for the two main clusters (Figure 6).

The heatmap depicts the effect of each bacteria regarding three different types of interactions:

- *P. verrucosum* growth in dual culture or with CFE added to the medium (Category 1);
- OTA specific production during *P. verrucosum* growth confronted with bacteria, or with CFE added to the medium (Category 2); and
- OTA degradation by actinobacteria in solid and liquid medium (Category 3).



**Figure 5.** Summary heatmap of the effect of actinobacteria and their CFEs on *Penicillium verrucosum* growth (Category 1) and OTA specific production (Category 2) along with the bacteria’s ability to degrade OTA in solid and liquid medium (Category 3). The results are given in a range of colors according to their Z-score where purple to black indicates a weak activity or an increase compared to the control and yellow indicates the strongest activity. Missing values are indicated in gray.



**Figure 6.** Pearson’s correlation of the effects of actinobacteria and their CFEs in *Penicillium verrucosum* growth and OTA specific production along with the bacteria’s ability to degrade OTA in solid and liquid medium. In the correlation scale, the 1 in blue indicates a strong positive correlation and the –1 in dark red is a strong negative correlation. The correlation was calculated for the strains of each main cluster of the heatmap (Cluster I and Cluster II). Dendrograms in the lower part show the relationship and proximity in % of Euclidean distance of each of the studied parameters for Clusters I and II. Different groups are indicated by different colors.

OTA degradation by CFEs was not included in the analysis because it was found earlier to be ineffective for all our strains. In order to compare different parameters with variable distributions, a standardization was made by converting the original values in % for each test to Z-scores, calculated independently within each of the three previously established categories. The stronger the activity regarding fungal growth, inhibition of OTAsp, or OTA degradation capability, the lower the values of

Z-score. Finally, the Z-score was color coded accordingly: clear yellow represents a strong activity (low Z-score) while dark purple to black represents an enhancement of fungal growth or OTA<sub>sp</sub> compared to the control (high Z-score). Intermediate colors between these extremes represent moderate or no activity. Finally, Euclidean distances were calculated considering the strains' whole profile within each category and sub-category, and further transformed as the percentage of similarity before being clustered accordingly. The main features of each group were identified by plotting boxplots of the different categories for each subcluster (Supplementary Materials Figure S1). Strains were grouped in two main clusters, I and II. Cluster I is characterized by strains with a mitigated effect on *P. verrucosum* growth and contrasting impacts on OTA specific production, either by cells or by CFEs. Strains from Cluster IA provoked a strong increase in OTA specific production while Cluster IB was subdivided in three distinct clusters. Cluster IB1 had no or moderate enhancing effect on OTA specific production, while subcluster IB3 showed a mild to strong ability to decrease it. Five strains (IX20, IX25, IX45, IX52, and MYC) grouped in subcluster IB2 were the only ones able to degrade OTA significantly on a solid medium even if their degradation efficiency was reduced in the liquid medium. Cluster II contained strains with high inhibition rates of *P. verrucosum* and strong capacities to degrade OTA in liquid medium. Furthermore, most of the strains from Cluster IIB caused a moderate (IIB2) to strong (IIB1) increase in OTA specific production, whereas the subcluster IIA showed mild (IIA2) to strong (IIA1) inhibition of the latter. For the sake of clarity, a summary of the main features for each cluster depicted in the heatmap is presented in Table 1.

**Table 1.** Summary of specific features observed during the clustering analysis of the impact of the screened actinobacteria and their cell free extracts (CFEs) on *P. verrucosum* growth and ochratoxin A (OTA) specific production.

Cluster	Specific features	Subcluster	Specific Features
I	Limited effect on <i>P. verrucosum</i> growth (dual culture and CFEs)	A	- Highest increase in OTA specific production (dual culture) Two strains degrading OTA in liquid medium (IX56, IX42)
		B	1 No or moderate increase in OTA specific production
			2 Only cluster with strains able to highly degrade OTA on solid medium Strong degradation capacities in liquid medium
			3 High inhibition of OTA specific production (dual culture and CFEs)
II	Strong effect on <i>P. verrucosum</i> growth (dual culture) High degradation of OTA in liquid medium	A	1 Highest inhibition of OTA specific production (dual culture)
			2 Moderate inhibition of OTA specific production (dual culture)
		B	1 Strong increase in OTA specific production
			2 Moderate increase of OTA specific production (dual culture and CFEs)

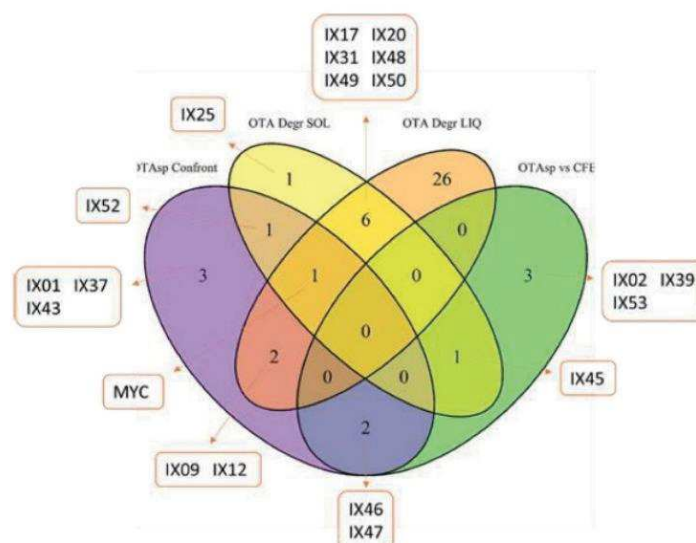
We performed a Pearson correlation test between all the experimental parameters studied, which is illustrated in Figure 6. All correlation values and their significance are presented in the Supplementary Materials (Table S1). The Pearson correlation coefficient of Figure 6 illustrates a strong positive correlation between the effect of heated and unheated CFEs on OTA specific production for both clusters (0.9 for Cluster I,  $p < 0.001$  and 0.7 for Cluster II,  $p < 0.001$ ). However, their low efficiency could explain the strong correlation observed. These results confirm that the degradation through enzymatic activity might not play a significant role in the apparent reduction of OTA specific production by CFEs. Moreover, the absence of the degradation of OTA in CFEs confirms this assumption. There was a strong negative correlation of the effect of CFEs under both treatments on pathogen growth with its OTA specific production in Cluster I (between  $-0.6$ , and  $-0.7$ , all with  $p < 0.001$ ), which indicates that when the CFEs of this cluster were efficient at inhibiting fungal growth, they usually provoked an increase in OTA specific production. In Cluster II, only CFE activity on growth and OTA production were significantly correlated with a negative correlation of  $-0.4$  ( $p = 0.04$ ). Moderate positive correlations were identified between the OTA specific production with heated CFEs and the degradation of OTA by cells in liquid medium in Cluster II ( $0.5$ ,  $p = 0.01$ ). A low but significant positive correlation was observed in Cluster I between the growth of *P. verrucosum* in dual culture and the growth with heated

CFEs (0.4,  $p = 0.04$ ) as well as the effect between heated and non-heated CFEs on pathogen growth (0.4,  $p = 0.01$ ).

The Pearson correlation coefficient between the growth of *P. verrucosum* and its OTA specific production was almost null for Cluster I (−0.1). For Cluster II, moderate negative correlation −0.4 ( $p = 0.054$ ) between the growth inhibition rate and the increase in OTA specific production was noted, showing that stressing conditions might lead to an enhanced OTA production. In fact, a few strains in Cluster II reduced OTA specific production while they inhibited *P. verrucosum* growth (e.g., IX09 and IX51). No significant correlation was observed in Cluster I where subcluster IA (Figure 5) strongly enhanced OTA specific production, even if their effect on fungal growth remained limited.

The dendrograms at the lower part of Figure 6 show how the effect of actinobacteria on the experimental parameters were related within each cluster. Parameters were more closely related in Cluster I than in Cluster II, with a maximum Euclidean distance of 14% and 30%, respectively, which could explain why the correlations were stronger in Cluster I. We can also observe that the parameter association varied slightly between the two clusters. In Cluster I, the specific production of OTA in dual culture was quite distant (12%) from the specific production of OTA with the CFEs, while in Cluster II, they were separated for only 5%. Additionally, degradation of OTA in both mediums were quite close in Cluster I (6%), but the distance increased to 30% in Cluster II. Pathogenic growth either in dual culture or with heated and unheated CFEs was separated by 9% in Cluster I while in Cluster II, there seemed to be a substantial difference between the growth of the pathogen in dual culture and that with CFEs (30%). The relationship between the OTA specific production with heated and unheated CFEs was the only one that remained constant, with about 3% of separation in both clusters.

The Venn diagram of Figure 7 was constructed with strains able to strongly decrease OTA, either by reducing its specific production or by degrading it (at least 50% for dual culture assays and liquid degradation and at least 20% for unheated CFEs and solid degradation). Seven strains were able to degrade OTA both in solid and liquid medium: IX17, IX20, IX31, IX48, IX49, IX50, and Mycostop®. From these, Mycostop® was also able to decrease OTA specific production during dual culture assays. We also noted that the two strains were able to decrease OTA specific production in both dual culture assays and by their CFEs: IX46 and IX47. Two strains that decreased OTA specific production were also able to degrade the mycotoxin in liquid medium: IX09 and IX12. Only IX45 could degrade OTA in a solid medium while being able to decrease OTA specific production with its CFEs.



**Figure 7.** Venn diagram of the effect of actinobacteria and their CFEs in the specific production of OTA during *in vitro* dual culture assays and their ability to degrade pure OTA in solid and liquid medium.

#### 2.4. Search for Degradation by-Products by HPLC

During the last phase of our work, we focused on the search of the main by-products of OTA biodegradation. Three strains with strong degradation abilities in liquid medium were selected from Cluster II (i.e., IX23, IX31, and IX48). They were cultured for three and seven days at 25 °C in CYB liquid medium with ~280 ng/mL of OTA. Main degradation by-products resulting for OTA degradation by microorganisms and already identified in the literature were searched using HPLC MS/MS: OT $\alpha$  and L- $\beta$ -phenylalanine generated by the hydrolysis of the amide bond of OTA [15,25,29], and lactone opened form of OTA (OP-OTA) produced through the hydrolysis of the lactone ring of OTA [28,30,31]. While a strong degradation of OTA at three and seven days was observed as found earlier for these three strains, none of the common by-products were identified (OT $\alpha$ , L- $\beta$ -phenylalanine, OP-OTA). Thus, we also proceeded to research the precursors of the OTA biosynthetic pathway such as OT $\beta$ , which results from the dechlorination of the isocoumarin moiety [16], OT $\beta$ , mellein, viomellein, penicilic acid, and asperlactone [32,33]. However, none of them was identified.

### 3. Discussion

During this work, we evaluated the ability of sixty strains of actinobacteria to inhibit the growth of the mycotoxigenic fungus *Penicillium verrucosum* and to minimize the occurrence of ochratoxin A, either by preventing its production or by degrading it. *Penicillium verrucosum* appears mostly in temperate and colder zones and is one of the main contaminants of stored grains in Europe [34,35], which makes it a main concern for detoxification research. Studies on the antagonistic effects of *Streptomyces* have been mostly conducted on *Aspergillus* sp. [17,36]. Even if the OTA biosynthetic pathway gene cluster is close between *Aspergillus* sp. and *Penicillium* sp., as already suggested in the literature [32,37], an accurate comparison of the regulation of their biosynthetic pathway under similar conditions is scarce. For example, *Penicillium verrucosum* has been shown to switch between OTA and citrinin production under oxidative stress or light [35,38,39] or in response to high osmolarity to adapt to various environments, whereas important *Aspergillus* producers such as *A. carbonarius* do not show such features [40]. Whether *Aspergillus* sp and *Penicillium* sp. OTA producing species behave similarly when exposed to biological stress remains to be elucidated. The wide picture of actinobacteria vs. *Penicillium* sp. interactions presented in this study might represent a good opportunity to select strains with varying effects on *P. verrucosum* growth and OTA production, in order to compare the behavior of *Aspergillus* sp and *Penicillium* sp under similar biological stressing conditions.

Results of the dual culture assays seem to indicate that in some cases, *Streptomyces* metabolites with an effect on *P. verrucosum* growth also led to an enhanced OTA specific production. Correlation analysis confirmed the negative relationship between these two observations for Cluster II with the most representative group being Cluster IIB1. However, the most important increases observed for OTA specific production were related to strains with no effect on growth (Cluster IA). A similar effect was observed for CFEs, regardless of being heated or not, which induced OTA specific production while no substantial effect on growth was observed, even if the thermal treatment somewhat lowered the observed effects. Thus, it seems likely that their effects on OTA specific production were not due to complex molecules such as heat sensitive enzymes, but rather to thermostable antifungal metabolites.

An explanation could be that OTA was overproduced by *P. verrucosum* when the fungus encountered stress conditions such as the presence of inhibitory compounds as a defense mechanism to secure its environmental niche, as suggested earlier by Venkatesh and Keller [41]. It has been demonstrated that several fungi such as *Aspergillus flavus* and *Aspergillus carbonarius* typically overproduce aflatoxins and OTA, respectively, when exposed to low concentrations of fungicides [42]. Thus, special attention must be paid to select strains that do not enhance mycotoxin production when an effect on fungal growth is desired, such as strains from Cluster IIA and especially IIA1 (IX09), which decrease both growth and OTA production. However, the use of specific compounds inhibiting fungal growth raises concern about the potential colonization of the ecological niche by other harmful microorganisms that could also represent a threat for food safety [36]. For future research, strains from



Cluster IB3 that have no impact on *P. verrucosum* growth, but are able to strongly reduce OTA specific production, represent very interesting candidates. The inhibition of OTA specific production with no effect on growth might be explained by an interference between secondary metabolites and OTA biosynthetic pathway, as reported by EL Khoury et al., who showed that *Streptomyces* strains were able to diminish the expression of regulation genes of OTA biosynthesis in *Aspergillus carbonarius*. However, this effect was not attributed to a specific molecule [17,36]. Other similar results were found with other microorganisms such as yeast, which decrease the expression of the *pks* gene and subsequent OTA production [43]. For aflatoxin, identified molecules such as blasticidin and aflastatin A have been described as repressors of the aflatoxin gene cluster, leading to a subexpression of the enzymes responsible for aflatoxin production, without necessarily affecting fungal growth [44]. A similar mechanism acting on the regulation of OTA biosynthetic pathway for *P. verrucosum* seems likely and remains to be verified. Additionally, active secondary metabolites secreted by actinobacteria that have an effect on the OTA biosynthetic pathway could be identified in the future. This complementary research will allow for a refinement of our understanding of mycotoxin biosynthesis inhibition when no effect on growth is observed.

CFEs were less efficient than the bacteria themselves to inhibit *P. verrucosum* growth. In a similar trend, CFEs decreased OTA specific production to a lower degree. However, only one concentration of 10% of CFEs in the media was used during the screening and further studies are required to evaluate the potential of CFEs at higher concentrations. Another assumption could be that secondary metabolites are produced in higher quantity when the bacteria is in contact with the fungi. Many recent studies have highlighted that the co-culture of microorganisms can lead to an overproduction of secondary metabolites [45–48]. Thus, the secretion of OTA or other secondary metabolites by the fungi could enhance the secretion of antifungal compounds or antitoxinogenic compounds by actinobacteria.

Regarding the reduction of OTA during degradation assays, we excluded a binding effect considering that sonication, cellular disruption, and vortexing were performed during mycotoxin extraction. In addition, our extraction solvent was methanol, which is also used in protocols of pellet washing to recover OTA potentially bound by actinobacteria [17,25], making toxin adsorption mechanisms very unlikely in our conditions. A large disparity between the degrading efficacy on solid and liquid media was observed, as OTA was much more effectively degraded in liquid medium. Such differences were also observed by Varga et al. [49] when testing OTA biodegradation by *Aspergillus* species. Better access to the substrate (OTA) in liquid medium could be a simple explanation as the mycotoxin is constantly in contact with the bacteria during agitation. Interestingly, most strains of Cluster II with strong growth inhibition properties were also able to degrade OTA in liquid medium, but none of them could degrade it on solid medium. Indeed, our results raise some interesting questions about the very few strains from Cluster IB2 that were able to degrade OTA with higher efficiency on solid media. Reports of the enhanced production of enzymes on solid media have been reported in the literature [50] and could explain why some strains exhibited higher degradation rates in such conditions. However, this observation was uncommon for the 60 strains of actinobacteria in this study (33 strains with strong degrading ability in liquid medium vs. five strains in solid medium).

The low OTA reduction efficiency in CFEs compared to the degradation by actinobacteria was in accordance with the results of El Khoury et al. [17] and suggests that degradation may be achieved by intracellular enzymes. Such intracellular enzymes with strong OTA degrading ability into OT $\alpha$  have been described for *Alcaligenes faecalis*, a Gram-negative bacteria isolated from soil [51], but not for actinobacteria, as far as we know. Some actinobacteria of other genera such as *Brevibacterium* are able to degrade OTA into OT $\alpha$  and L- $\beta$ -phenylalanine, probably through the action of a carboxypeptidase [25]. Further studies are required in order to identify the active enzymes of the strains that strongly degraded OTA and their intra or extracellular location, since it is well known that actinobacteria are able to produce proteases and peptidases that can be used for biodegradation purposes [25]. Another assumption about the CFEs' inefficiency regarding OTA degradation could be that their production is promoted by the presence of the toxin. To our knowledge, the impact of OTA on *Streptomyces* metabolism and

exocellular enzyme production has so far not been studied. However, it has been demonstrated that chitinase production by *Streptomyces coelicolor* A3(2) is strongly enhanced in soil medium rather than in laboratory media, probably because of the presence of chitin [52]. As actinobacteria and many fungi share the same ecological niches, it would be interesting to study if mycotoxins could act as signaling molecules on *Streptomyces* metabolism, and if they could induce the production of enzymes responsible for their degradation.

No common degradation by-products (i.e., OT $\alpha$ , OT $\beta$ , L- $\beta$ -phenylalanine) were identified by HPLC-MS/MS for the selected strains with strong degrading ability (i.e., IX23, IX31, and IX48), which suggests either a subsequent degradation into unknown products or a different mechanism of detoxification. From one microorganism to another, the OTA's degradation mechanisms differed, as did the degradation by-products. This was confirmed by various studies where the degradation products could not be identified [16,18,30,53]. Shi et al. did not find any common degradation products when evaluating the degradation ability of OTA by *Bacillus subtilis* CW14 [18]. When studying the degradation of OTA by fungi, Bejaoui et al. observed the progressive reduction of OT $\alpha$  and the appearance of an unknown compound after nine days of culture [53] as did Varga et al. after seven days of culture [49]. Thus, in our case, the absence of commonly found by-products could also be due to the length of the incubation period (three and seven days), that possibly allowed a further decomposition into unknown molecules. Further studies could be performed with isotopically labeled OTA in order to elucidate the fate of the degraded mycotoxin [54]. Additionally, residual toxicity assays could be performed on HepG2 cells [55], or by in vivo systems like nematodes or zebrafish [56,57] to ensure the detoxification capacity of the studied actinobacteria strains.

#### 4. Conclusions

In conclusion, several actinobacteria strains were able to minimize the occurrence of OTA either by preventing its formation, degrading it, or both mechanisms. The methodology employed in this study (screening by nine modalities, clustering and correlation analysis) helped easily distinguish the features of each strain and to group them into clusters of comparable abilities. A heatmap representation helped easily select specific strains according to the desired effect. For instance, we could choose strains that strongly inhibit OTA specific production without affecting *P. verrucosum* growth to preserve the ecological niche, or strains that decreased OTA specific production but had low degradation capacities, if we wish to deepen the study of the inhibition of the mycotoxin biosynthetic pathway. Remarkable observations such as the degradation ability of OTA on the solid medium were very scarce among the strains studied, found for only five strains among the sixty. Interesting correlations such as the increase in OTA specific production when growth was strongly reduced have also been observed. Thus, our collection represents a great start for further studies in order to identify active metabolites and enzymes, and to provide a better understanding of the various interactions that can exist between actinobacteria, *P. verrucosum*, and OTA. Among the three strains studied by HPLC MS/MS for OTA degradation, no common by-products were found.

#### 5. Materials and Methods

##### 5.1. Actinobacteria Strains

Fifty-nine actinobacteria strains were isolated from organic amendments and soil samples collected at the Hérault Department in the South of France. The strains constitute a collection maintained in the laboratory of the UMR QualiSud at the University of Montpellier. Mycostop<sup>®</sup> (MYC) strain *Streptomyces griseoviridis* K61 was included in the tests as a commercialized biocontrol agent for comparison. A 16S preliminary identification demonstrated that all the strains in the collection belonged at 97% to the genre *Streptomyces* (data not shown). The strains were grown on ISP4 medium (10 g/L starch, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 1 g/L MgSO<sub>4</sub>, 2 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/L CaCO<sub>3</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L MgCl<sub>2</sub>, 1 mg/L ZnSO<sub>4</sub>, 18 g/L bacteriological agar, pH 7.2) for 11 days at 28 °C. Spores were then

collected by scraping the surface of the Petri dish with 5 mL of distilled water +0.01% Tween 20 and filtered through sterile cotton. The spore suspensions were aliquoted, then stored at  $-80\text{ }^{\circ}\text{C}$ .

### 5.2. Actinobacteria Spore Numeration by Flux Cytometer

For actinobacteria spore count, a Novocyte ACEA flux cytometer was employed. After counting, the results were validated by colony forming units on CYA medium after 11 days of culture. Before each test, bacterial spore suspensions were unfrozen and diluted to a concentration of  $10^7$  spores/mL.

### 5.3. Pathogen Strains

A mycotoxigenic strain of *Penicillium verrucosum* NRRL5571, kindly provided by Dr. Olivier Puel from INRAE's UMR Toxalim [54], was employed to carry out this work. Fungus was maintained on PDA (Biokar BK095HA) inclined tubes covered in paraffin oil prior to use. For fungal spore harvesting, pathogens were grown on PDA plates for seven days at  $25\text{ }^{\circ}\text{C}$ , then spores were scraped from the surface by adding 10 mL of distilled sterile water and filtered through sterile cotton. Pathogen spores were harvested and enumerated before each test.

### 5.4. Antagonistic Evaluation

#### 5.4.1. Actinobacteria Cells

Antagonistic assays were performed on Czapek Yeast Extract Agar medium (CYA: 30 g/L sucrose, 5 g/L yeast extract, 1 g/L  $\text{K}_2\text{HPO}_4$ , 0.3 g/L  $\text{NaNO}_3$ , 0.05 g/L KCl, 0.05 g/L  $\text{MgSO}_4$ , 1 mg/L  $\text{FeSO}_4$ , 1 mg/L  $\text{ZnSO}_4$ , 0.5 mg/L  $\text{CuSO}_4$ , 15 g/L agar, pH  $\sim 7.4$ ), which allowed both pathogen and bacteria proper growth and sporulation without favoring one over the other.

Dual culture assays were implemented on Petri dishes by inoculating 10  $\mu\text{L}$  of the actinobacteria spore suspension at  $10^7$  CFU/mL on each side of the plate. After three days at  $25\text{ }^{\circ}\text{C}$ , 10  $\mu\text{L}$  of the pathogen spore suspension at  $10^6$  CFU/mL were inoculated at the center of the plate and left for nine more days at  $25\text{ }^{\circ}\text{C}$  before image analysis and mycotoxin extraction. As a control, the pathogen was inoculated on a CYA plate without actinobacteria. All tests were done in triplicate.

#### 5.4.2. Cell Free Extracts

Actinobacteria strains were cultured on Czapek Yeast Extract Broth (CYB: 30 g/L sucrose, 5 g/L yeast extract, 1 g/L  $\text{K}_2\text{HPO}_4$ , 0.3 g/L  $\text{NaNO}_3$ , 0.05 g/L KCl, 0.05 g/L  $\text{MgSO}_4$ , 1 mg/L  $\text{FeSO}_4$ , 1 mg/L  $\text{ZnSO}_4$ , 0.5 mg/L  $\text{CuSO}_4$ , pH  $\sim 7.4$ ) medium for five days at  $25\text{ }^{\circ}\text{C}$  and 180 rpm. Then, liquid cultures were centrifuged at  $10,000\times g$  for 5 min and the supernatant was filtered through a PES (Polyethersulfone) filter at  $0.22\text{ }\mu\text{m}$  to eliminate bacterial cells. The resulting cell free extracts (CFEs) were used to evaluate the effect of bacterial metabolites on the growth and mycotoxin production of the fungal pathogen by adding 10% of each CFE in the CYA medium for *P. verrucosum*, then 10  $\mu\text{L}$  of the pathogen spore suspension at  $10^6$  CFU/mL was inoculated at the center of the plate and left for 12 days at  $25\text{ }^{\circ}\text{C}$ . In order to identify a potential effect of actinobacteria extracellular enzymes, CFEs were also tested after a heat treatment at  $100\text{ }^{\circ}\text{C}$  for 10 min. As a control, the pathogen was inoculated on a CYA plate without CFEs. All tests were done in triplicate.

#### 5.4.3. Pathogen Surface Growth Measurement and OTA Specific Production Calculation

Surface growth of *P. verrucosum* colonies was measured in ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018). Growth inhibition was established in comparison to the control without actinobacteria, which represented 100% of growth. Results are given in % of control.

$$\text{Growth ratio (\% of control)} = \frac{\text{Assay area (cm}^2\text{)}}{\text{Control area (cm}^2\text{)}} \times 100 \quad (1)$$

OTA specific production was calculated by dividing the mycotoxin amount in the whole sample by the area of the fungal colony.

$$OTA \text{ specific production } (OTA_{sp}) = \frac{\text{Amount of mycotoxins produced (ng)}}{\text{Colony area (cm}^2\text{)}} \quad (2)$$

Then, the percentage of reduction for OTA specific production was calculated in comparison to the control without actinobacteria.

$$OTA_{sp} \text{ (% of control)} = \frac{OTA_{sp} \text{ assay}}{OTA_{sp} \text{ control}} \times 100 \quad (3)$$

### 5.5. Mycotoxin Degradation Assay

#### 5.5.1. Screening of OTA Degradation by Actinobacteria and Their CFEs

Degradation assays were performed using ochratoxin A (Sigma Aldrich, St. Quentin Fallavier, France) suspended in acetonitrile in order to prepare a stock solution of 31  $\mu\text{g/mL}$ . OTA concentration in the control was about  $\sim 280 \text{ ng/mL}$ . Mycotoxin was added to liquid CYB and solid CYA medium, followed by the addition of 20  $\mu\text{L}$  of actinobacteria spore suspension at  $10^7$  spores/mL and incubated at 25 °C with an agitation at 180 rpm for the liquid cultures. After 10 days, mycotoxin was extracted and analyzed as described before. CFEs, produced as described before, were also tested for their ability to degrade OTA after being in contact with the mycotoxin for 48 h at 25 °C and 180 rpm. As a control, mycotoxin was spiked in solid CYA or liquid CYB medium and incubated following the same incubation protocols as the samples.

$$OTA \text{ degradation (% of control)} = \frac{OTA_{\text{ng/mL assay}}}{OTA_{\text{ng/mL control}}} \times 100 \quad (4)$$

#### 5.5.2. Degradation Assays for the Search of Degradation by-Products

A selection of three strains with strong degradation abilities in liquid medium was made (IX23, IX31, and IX48). These were cultured in liquid medium with OTA ( $\sim 280 \text{ ng/mL}$ ) as described in Section 5.5. for three and seven days at 25 °C and 180 rpm. Controls included liquid CYB medium alone and bacteria cultured in liquid CYB without OTA, both incubated under the same conditions to eliminate potential unknown molecules corresponding to bacterial metabolites and/or culture medium. Main degradation by-products of OTA, namely OT $\alpha$ , L- $\beta$ -phenylalanine, and lactone opened OTA (OP-OTA) as well as biosynthesis precursors such as OT $\beta$ , mellein, viomellein, penicilic acid, and asperlactone were further searched using HPLC-MS/MS.

### 5.6. Mycotoxin and Degradation By-Products Analysis

#### 5.6.1. Sample Extraction

For the mycotoxin extraction of the antagonistic and CFE assays, the whole content of the Petri dish, liquid culture or CFE was transferred into a plastic container. Agar medium was cut into small pieces with a scalpel before extraction. After weighing the sample, acidified methanol (3.85% formic acid) was added to the sample with a ratio of 1.5/5, followed by 20 min of agitation at 250 rpm. A total of 500  $\mu\text{L}$  of the mixture were evaporated in a Speed Vac (Eppendorf® AG, Hamburg, Germany) at 60 °C until dryness, and then 2 mL of the mobile phase (methanol/water/acetic acid, 69:30:1) were added. To ensure that mycotoxin was not bonded to the cells, samples underwent 20 min of sonication, followed by 10 min in a cell disruptor, and finally thoroughly mixed in a vortex before filtering through a 0.45  $\mu\text{M}$  PTFE (Polytetrafluoroethylene) filter for the HPLC analysis.

For HPLC MS/MS, sample extraction was performed similarly, except for the last step of resuspension after evaporation, where the added mobile phase was ultra-pure water acidified with acetic acid (0.5%).

The percentages of recovery for OTA were 97% and 95% after the extraction and resuspension step for fluorescence HPLC and HPLC MS/MS, respectively. The by-product recovery rate was not established because the analysis was only qualitative.

### 5.6.2. OTA Quantification Using Fluorescence HPLC

OTA concentration for all experiments was quantified by HPLC using a fluorescence detector (Shimadzu RF 20A, Japan). The operating conditions were as follows: injection volume of 100  $\mu$ L; C18 reverse-phase HPLC column, Uptisphere type, ODS, 5  $\mu$ m particle size, 5 ODB, 250  $\times$  4.6 mm, with identical pre-column, thermostatically controlled at 35  $^{\circ}$ C; isocratic flow rate of 1 mL/min (mobile phase: methanol/water/acetic acid, 69:30:1); excitation wavelength of 333 nm and emission wavelength of 460 nm. The concentrations were calculated from a calibration curve established from an OTA standard (1  $\mu$ g/mL; ref PD 226 R. Biopharm Rhône Ltd., Glasgow, UK). Detection and quantification limits were established at 0.085 and 0.25 ng/mL, respectively.

### 5.6.3. Search for Degradation By-Products by HPLC-MS/MS

A qualitative analysis was performed on HPLC MS/MS for the research of degradation by-products. The OT $\alpha$  standard was purchased from Romerlabs (Getzersdorf, Austria) and OTB from Sigma Aldrich (St. Quentin Fallavier, France). OT $\beta$  was synthesized from OTB as described by Harris and Mantle [58]. Briefly, 800  $\mu$ L of OTB standard solution (5 $\mu$ g/mL) was hydrolyzed by reflux with 50 mL of HCl 6 M for 18 h and extracted with ethyl acetate. Mellein was extracted from a seven-day old plate of *Aspergillus ochraceus*, as described by Zhang et al. [28].

The MS/MS parameters (MRM, EC) for metabolites investigated were taken from the following articles:

- OTA, OT $\alpha$ , OTB, and mellein “Zhang et al.” [28]
- OT $\beta$  “Wang et al.” [32]
- L- $\beta$ -phenylalanine “Patharajan et al.” [30]
- Lactone opened OTA “Bazin et al.” [31]
- Viomellein [33]
- Penicilic acid [33]
- Asperlactone [33]

Detection of mycotoxin and their by-products were achieved using Ultra High Performance Liquid Chromatography (UHPLC, Shimadzu, Tokyo, Japan) coupled with a mass spectrometer (8040, Shimadzu, Tokyo, Japan). LC separation was performed using a Phenomenex Kinetex XB Column C18 (50 mm  $\times$  2 mm; 2.6  $\mu$ m particles) at 50  $^{\circ}$ C with an injection volume of 50  $\mu$ L. Mobile phase composition was (A) 0.5% acetic acid in ultra-pure water and (B) 0.5% acetic acid in isopropanol (HPLC MS grade, Sigma, St Louis, MO, USA). A mobile phase gradient program was started at 90% A (0.01 min), 45% A at 1.5 min, 15% A at 3.5 min, 20% A at 4 min, 98% A at 4.01 min, and finally 98% A at 11 min, and the mobile phase flow rate was 0.4 mL min $^{-1}$ . The mass spectrometer was operated in electrospray positive (ESI+) and negative (ESI-) ionization mode, and two multiple reaction monitoring (MRM) transitions for each analyte were monitored for quantification and qualification. OTA detection and quantification limits were established at 0.06 and 0.2 ng/mL, respectively. As only qualitative measurements were performed for the degradation by-products, their limits of detection and quantification were not established. All data were analyzed using LabSolution Software (v5.91/2017, Shimadzu, Tokyo, Japan, 2017).

### 5.7. Data Analysis and Data Visualization

Preprocessing of the data for the heatmap consisted of a conversion of percentages to Z-scores calculated as follows:  $Z\text{-score} = (X - \mu) / \sigma$ , where  $X$  is the measurement,  $\mu$  is the mean value of the effect of all strains in a category (fungal growth, OTA specific production, OTA degradation), and  $\sigma$  is the standard deviation of the value of the effect of all strains in the considered category. This conversion allowed all data results within each category to be normalized in order to compare them, as the results of the different categories evaluated had variable distributions. Therefore, with the standardized data, the default Euclidean method was chosen for measuring the distance between the clusters, which is expressed as a percentage of similarity of the effect of actinobacteria regarding the different parameters. Boxplots and Pearson correlation graphics were developed in Rstudio.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6651/12/5/296/s1>, Figure S1: Boxplot of the effect of actinobacteria and CFEs on *P. verrucosum* growth and OTA specific production. Each boxplot represents a subcluster of the heatmap from Figure 5. GDC = *P. verrucosum* growth in dual culture, GCFE = *P. verrucosum* growth vs. cell free extracts (CFEs), GHCFE = *P. verrucosum* growth vs. Heated CFEs, OTADC = OTA specific production (sp) in dual culture, OTACFE = OTAsp vs. CFEs, OTAHCFE = OTAsp vs. Heated CFEs, OTADS = OTA degradation by cells in solid medium, OTADL = OTA degradation by cells in liquid medium, Table S1: Values of the Pearson correlation between the measured parameters during the screening of the effect of actinobacteria and their CFEs on *P. verrucosum* growth and OTA production.

**Author Contributions:** I.C.-A., A.C.d.I.N., and S.S.-G. conceived, supervised, and designed the experiments. I.C.-A. performed all experiments. I.C.-A. and A.C.d.I.N. analyzed the data. V.M. assisted in the mycotoxin extraction from agar plates and microbiological manipulation. N.D. and B.F. executed the HPLC and HPLC MS/MS analysis as well as the search of degradation by-products. S.S.-G., A.C.d.I.N., A.F., and C.S. were in charge of funding acquisition and project administration. I.C.-A., A.C.d.I.N., and S.S.-G. interpreted the data. The manuscript was written by I.C.-A. and A.C.d.I.N. Revised by A.C.d.I.N., S.S.-G., and C.S. All authors have read and agreed to the published version of the manuscript.

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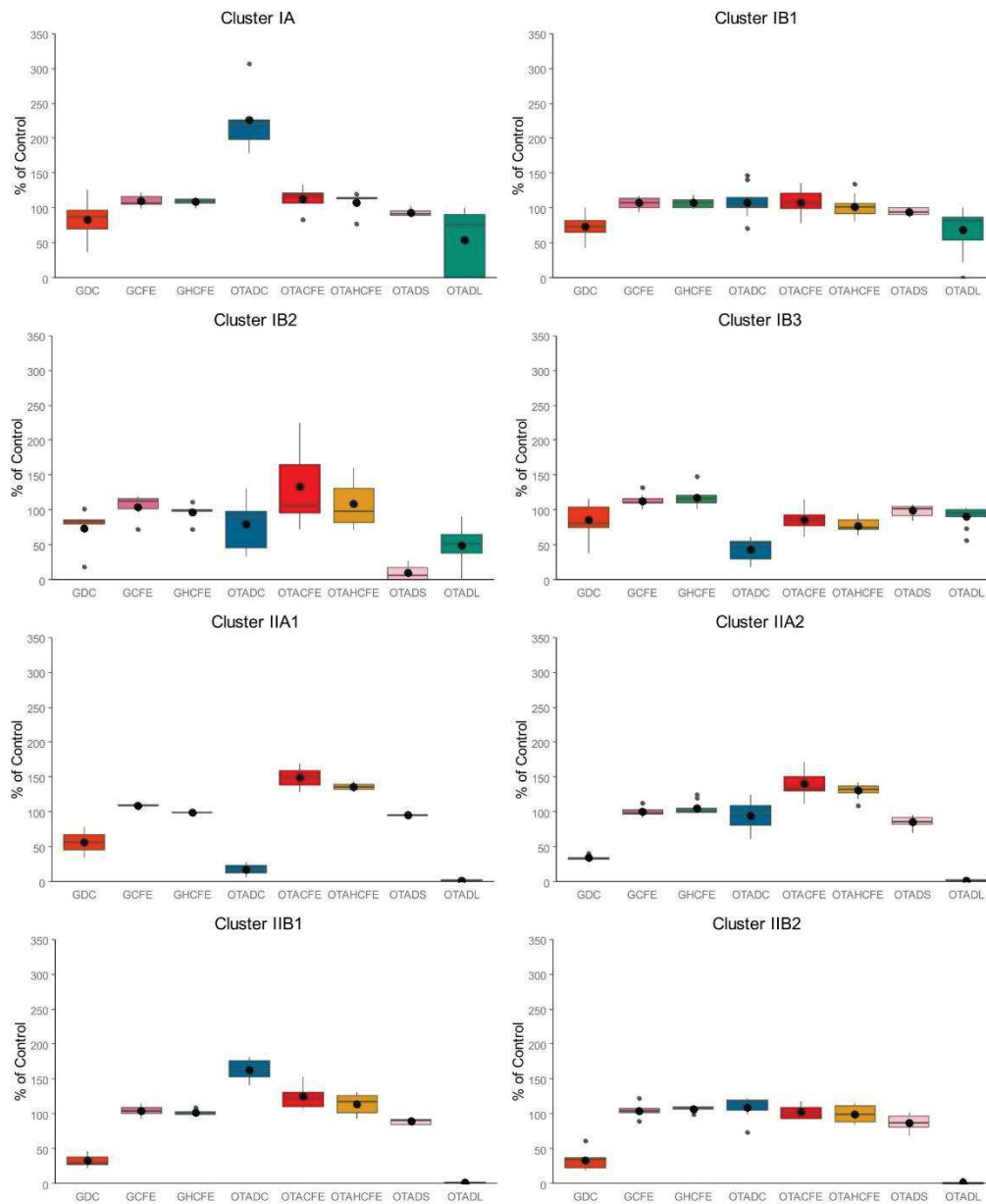
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## Supplementary Materials: Minimizing Ochratoxin A Contamination through the Use of Actinobacteria and Their Active Molecules

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**Figure S1.** Boxplot of the effect of actinobacteria and CFEs on *P. verrucosum* growth and OTA specific production. Each boxplot represents a subcluster of the heatmap from Figure 5. GDC = *P. verrucosum* growth in dual culture, GCFE = *P. verrucosum* growth vs Cell Free Extracts (CFEs), GHCFE = *P. verrucosum* growth vs Heated CFEs, OTADC = OTA specific production (sp) in dual culture, OTACFE = OTAsp vs CFEs, OTAHCFE = OTAsp vs Heated CFEs, OTADS = OTA degradation by cells in solid medium, OTADL = OTA degradation by cells in liquid medium.

**Table 1.** Values of the Pearson correlation between the measured parameters during the screening of the effect of actinobacteria and their CFEs on *P. verrucosum* growth and OTA production.

	GDC	GCFE	GHCFE	OTADC	OTACFE	OTAHCFE	OTADS	OTADL	
Cluster I	1								GDC
	0.2 (0.18)	1							GCFE
	0.4 (0.04)*	0.4 (0.01)*	1						GHCFE
	-0.1 (0.59)	0 (0.1)	-0.1 (0.73)	1					OTADC
	-0.4 (0.01)*	-0.7 (<0.001)***	-0.6 (<0.001)***	0.1 (0.47)	1				OTACFE
	-0.4 (0.04)*	-0.6 (<0.001)***	-0.7 (<0.001)***	0.3 (0.11)	0.9 (<0.001)***	1			OTAHCFE
	0.1 (0.65)	0.2 (0.37)	0.4 (0.01)*	0.1 (0.49)	-0.4 (0.02)*	-0.3 (0.14)	1		OTADS
	0.1 (0.72)	-0.3 (0.053)	0.2 (0.37)	-0.2 (0.17)	0 (0.86)	-0.2 (0.30)	0.3 (0.08)	1	OTADL
Cluster II	1								GDC
	0 (0.80)	1							GCFE
	-0.2 (0.22)	0 (0.86)	1						GHCFE
	-0.4 (0.054)	-0.1 (0.60)	0.1 (0.74)	1					OTADC
	-0.1 (0.64)	-0.4 (0.04)*	0 (0.10)	-0.2 (0.32)	1				OTACFE
	0.3 (0.15)	-0.3 (0.14)	-0.3 (0.10)	-0.3 (0.08)	0.7 (<0.001)***	1			OTAHCFE
	0.4 (0.06)	0.1 (0.68)	-0.4 (0.054)	0 (0.89)	-0.1 (0.47)	0.1 (0.54)	1		OTADS
	0.1 (0.55)	-0.2 (0.22)	-0.4 (0.052)	0 (0.95)	0.3 (0.18)	0.5 (0.01)*	-0.1 (0.60)	1	OTADL

GDC = *P. verrucosum* growth in dual culture, GCFE = *P. verrucosum* growth vs Cell Free Extracts (CFEs), GHCFE = *P. verrucosum* growth vs Heated CFEs, OTADC = OTA specific production (sp) in dual culture, OTACFE = OTAsp vs CFEs, OTAHCFE = OTAsp vs Heated CFEs, OTADS = OTA degradation by cells in solid medium, OTADL = OTA degradation by cells in liquid medium. *P*-values are indicated in the parenthesis. \* *P*<0.05, \*\*\* *P*<0.001.

#### 4.2.2 Complementary research on the stimulation of actinobacteria

During the previously published work (Campos-Avelar et al. 2020), it was determined that actinobacteria were able to decrease the amount of ochratoxin A produced by *P. verrucosum* during dual cultures. Moreover, actinobacteria growing cells were able to degrade, at different rates, pure ochratoxin A present in liquid medium and, in rare cases, in solid medium. However, actinobacteria cell free extracts (CFEs) seemed unable to degrade the mycotoxin. This may indicate that OTA degrading enzymes are not constitutive, and are rather produced when actinobacteria are confronted with the mycotoxin and/or with the pathogen and its metabolites.

Indeed, results from several studies conducted on actinobacteria mixed cultures documented that a large fraction of actinobacteria biosynthetic genes, encoding for secondary metabolites, remains silent during mono cultures (Marmann et al. 2014). Furthermore, when actinobacteria are subjected to co-cultivation with other microorganisms or their components, an elicitation of the secondary metabolism occurs (Bertrand et al. 2014). For instance, an enhanced production of antibiotics and enzymes was observed when co-cultivating actinobacteria strains with other actinobacteria but also with other bacterial species and fungi. From an ecological approach, it could be a way for actinobacteria to protect themselves, to compete with other organisms and/or to better adapt to their environment (Abdelmohsen et al. 2015).

Furthermore, it has been reported that actinobacteria differential development in solid and liquid medium has an impact on their production of secondary metabolites (Manteca and Yagüe 2018). This could give clues about the contrasting results on degradation capacity of actinobacteria grown under both conditions.

With this in mind, we decided to perform a series of complementary tests in an attempt to stimulate the secondary metabolism of actinobacteria to obtain active CFEs, capable of degrading OTA. For this, liquid cultures of actinobacteria were either supplemented with OTA alone, or with the full spectrum of fungal metabolites (fungal CFEs), or co-cultured with the fungi. After this, the resulting CFEs were evaluated for their ability to degrade OTA, as well as for their impact on fungal growth and mycotoxin production.

### 4.2.3 Materials and methods

#### Stimulation of actinobacteria during CFEs production

Five actinobacteria isolates having strong ochratoxin A degrading capacities in liquid medium were chosen for the CFEs stimulation assays (IX12, IX20, IX32, IX56 and MYC). 50  $\mu$ L of actinobacteria ( $10^7$  spores/mL) were inoculated into 50 mL of CYB medium (30 g/L sucrose, 5 g/L yeast extract, 1 g/L  $K_2HPO_4$ , 0.3 g/L  $NaNO_3$ , 0.05 g/L KCl, 0.05 g/L  $MgSO_4$ , 1 mg/L  $FeSO_4$ , 1 mg/L  $ZnSO_4$ , 0.5 mg/L  $CuSO_4$ , pH  $\sim$ 7.4) modified following the 6 modalities below:

1. CYB (unmodified)
2. CYB + Acetonitrile (5.64 mL/L)
3. CYB + OTA (260  $\mu$ g/L) in acetonitrile (5.64 mL/L)
4. CYB + CFEs of *P. verrucosum*'s liquid culture (25%)
5. CYB + CFEs of *P. verrucosum*'s solid culture (25%)
6. CYB + fungal inoculum of *P. verrucosum* (50  $\mu$ L at  $10^7$  spores/mL)

Modality 1 corresponded to the standard conditions previously described for the production of bacterial CFEs (Campos-Avelar et al. 2020). Modality 2 contained acetonitrile, in order to evaluate its impact on actinobacteria growth and metabolism, as the standard solution of OTA is diluted in this solvent. Modality 3 contained 260  $\mu$ g/mL of a standard solution of OTA. For modalities 4 and 5, *P. verrucosum* was cultured in liquid and solid medium for 7 days at 25°C (180 rpm for liquid culture). Fungal metabolites for Modality 4 were retrieved after centrifugation of the liquid culture of *P. verrucosum* at  $10000\times g$ , and filtration of the supernatant (PES filter 0.22  $\mu$ m). For Modality 5, an extraction step was included by cutting the *P. verrucosum* agar plate (15 g) into small pieces with a scalpel and putting it in a container with 50 mL of distilled water, under agitation at 250 rpm for 20 minutes. The water extract was finally recovered after separation by centrifugation and filtration as previously described for liquid fungal CFEs. To ensure homogeneous OTA concentration, the mycotoxin amount in fungal CFEs was measured by HPLC as described earlier (Campos-Avelar et al. 2020) and adjusted at the same concentration as modality 3 (260  $\mu$ g/mL). Modality 6 corresponds to a co-culture of bacterial and fungal cells at the same concentration ( $10^7$  spores/mL) on unmodified CYB.

After 11 days at 25°C and 180 rpm, liquid cultures were centrifuged and filtered through a PES filter at 0.22  $\mu$ m to eliminate bacterial cells and obtain the stimulated CFEs.

### Degrading capacities of stimulated bacterial CFEs

OTA was added to 500  $\mu\text{L}$  of the CFEs prepared under the 6 modalities (226 ng/mL final concentration), before incubation at 25°C and 180 rpm. After 72H, OTA was extracted and the residual concentration was determined by fluorescence HPLC as described in previous work (Campos-Avelar et al. 2020).

### Effect of stimulated bacterial CFEs on growth and OTA production by *P. verrucosum*

Stimulated CFEs (25%) were added to CYA medium before pouring the plates. Spores of *P. verrucosum* were inoculated at the center of the Petri dish (10  $\mu\text{L}$  at  $10^6$  spores/mL) and incubated for 12 days at 25°C. Fungal growth was calculated by measuring the area of the fungal colony on ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018). OTA extraction and quantification was carried out as described earlier (Campos-Avelar et al. 2020).

#### 4.2.4 Results

### Stimulation of actinobacteria during CFEs production

Bacterial CFEs were produced in liquid CYB prepared under 6 modalities by the addition of OTA, fungal CFEs, or by co-culture with the fungus. After 11 days, cultures were filtered and the residual amount of OTA in modalities 3, 4, 5 and 6 was measured by HPLC. Visual observations at the end of the culture period indicated that the addition of acetonitrile (Modality 2) or standard OTA solution (Modality 3) at the tested concentrations did not have a blatant impact on actinobacteria growth as compared to the standard culture (Modality 1). Indeed, the resulting broths presented similar light brown color, apparent cell density and mycelia consistency. Contrasting results were observed for all actinobacteria isolates under modalities 4, 5 and 6, where the addition of colorless fungal metabolites and the bacteria-fungus co-culture generated a dark red or brown broth (Fig. 40).

Figure 41 presents the residual OTA amount in the bacterial supernatants produced with the toxins or in co-culture with the fungi. Results indicate that actinobacteria were unable to degrade the OTA initially present in the liquid medium under modality 3 (CYB + OTA), which differs from the results obtained in our previous work (Campos-Avelar et al. 2020). The unique

difference between our first experiments and the one presented here is the use of glass flasks in this study instead of plastic containers in the precedent.

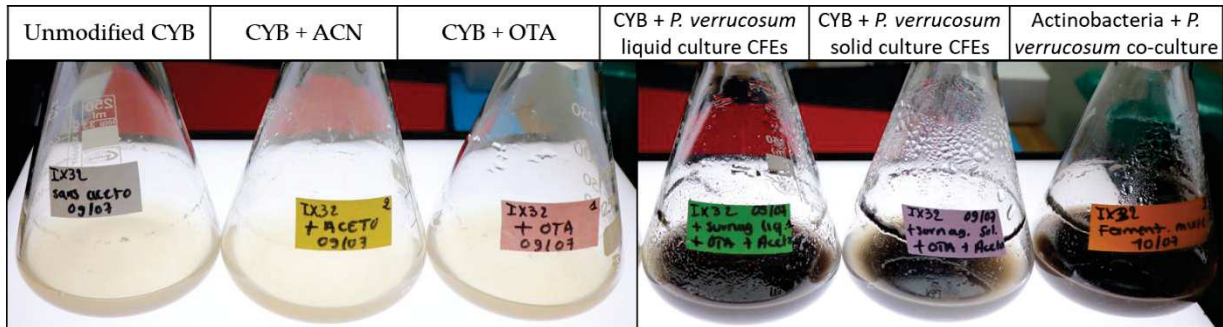


Figure 41. Example of liquid culture of actinobacteria (IX32) under 6 modalities of "stimulation". 1: CYB (unmodified), 2: CYB + Acetonitrile (5.64 mL/L), 3: CYB + OTA (260 µg/L) in acetonitrile (5.64 mL/L), 4: CYB + CFEs of *P. verrucosum*'s liquid culture (25%), 5: CYB + CFEs of *P. verrucosum*'s solid culture (25%), 6: CYB + fungal inoculum of *P. verrucosum* (50 µL at 10<sup>7</sup> spores/mL).

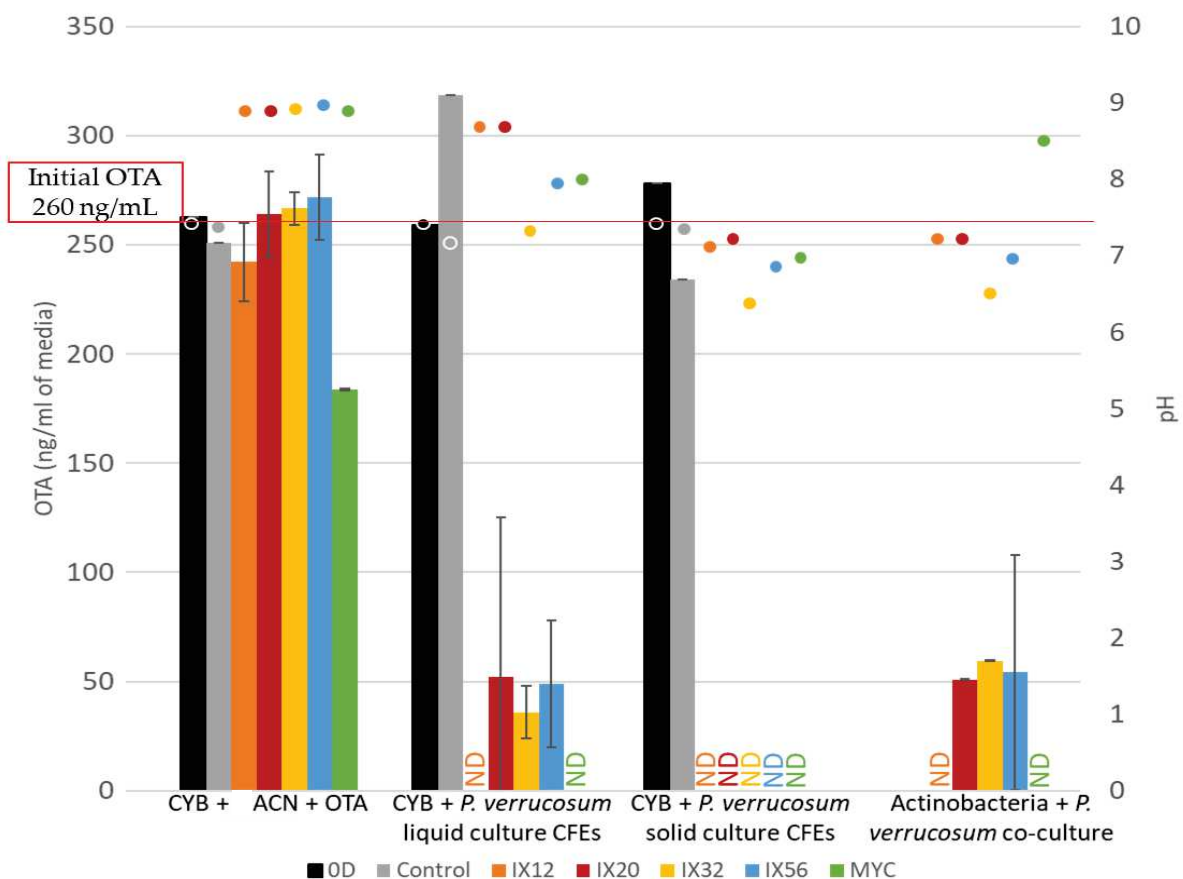


Figure 40. Residual OTA after culturing actinobacteria under stimulating modalities: CYB + OTA (260 µg/L) in acetonitrile (5.64 mL/L), CYB + CFEs of *P. verrucosum*'s liquid culture (25%), CYB + CFEs of *P. verrucosum*'s solid culture (25%), CYB + fungal inoculum of *P. verrucosum* (50 µL at 10<sup>7</sup> spores/mL). OD: OTA dosage at day 0. Control: OTA cultured under the same conditions without actinobacteria. ND: Not detected.

The pH measure revealed that both kind of containers led to very different pH at the end of the culture, from approximatively neutral (pH~7, plastic containers) to more basic (pH~8.9, glass flasks). In order to verify this observation, an additional test was performed by comparing the two protocols with three isolates (IX12, IX56 and MYC). Once again, actinobacteria samples cultured in plastic containers had neutral pH (~7.4) and degraded almost completely the OTA, while actinobacteria cultured in glass flasks sealed with cotton all reached basic pH values (~9.1) and were unable to degrade the mycotoxin (Fig. 42). During the production of standard actinobacteria CFEs, cultures were carried out on glass flasks and the pH at the end of the culture was usually basic (data not shown). This suggests that the use of plastic containers modified somehow the bacterial development by acting on the culture parameters, such as the rate of aeration.

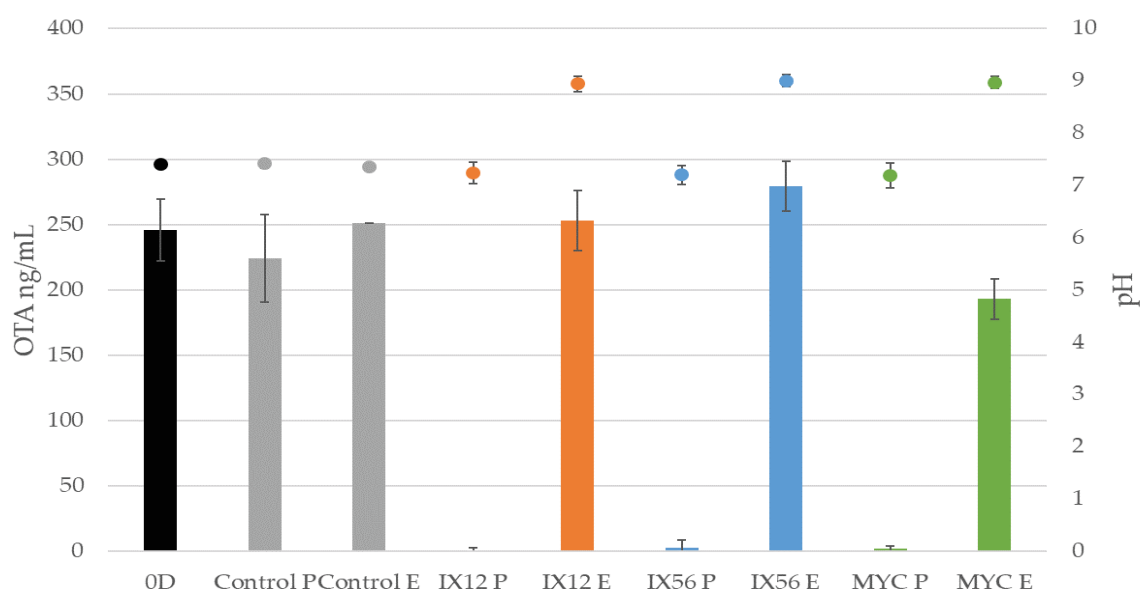


Figure 42. Bar plot of the OTA degradation by three actinobacteria isolates during their development in liquid CYB, and dot plot of the resulting pH values. OD: OTA dosage at day 0. Control: CYB medium + OTA cultured under both conditions: P = plastic container, E = Erlenmeyer flask.

Regarding the remaining modalities in Figure 41, actinobacteria grown under modalities 4 and 5, containing *P. verrucosum* metabolites, were able to degrade the initial OTA added to the medium (260 ng/mL) whether the resulting pH was more neutral (Modality 5) or basic (Modality 4). However, the pH range was always lower than for Modality 3 where no degradation was observed. During the mixed fermentation in Modality 6, microscopical observations showed that fungal growth was scarce, while actinobacteria mycelia was abundant, as exemplified by the Figure 43 displaying the co-culture of *P. verrucosum* (red arrows) and actinobacteria isolate IX20.



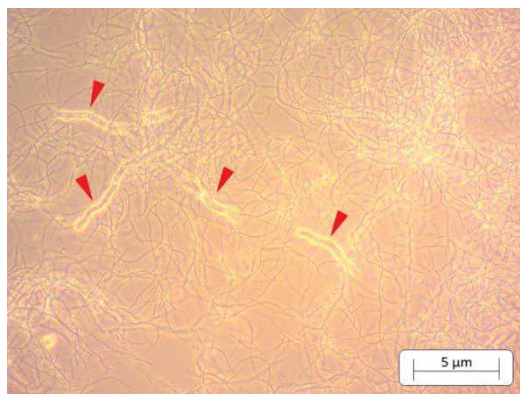


Figure 43. Microscopical observation of mixed culture of IX20 and *P. verrucosum* on CYB medium after 11 days at 25°C and 180 rpm. Fungal mycelium is indicated by the red arrows.

### Degrading capacities of stimulated bacterial CFEs

The resulting bacterial CFEs were evaluated for their OTA degrading capacities by incubating them with OTA (~226 ng/mL) for 72H. As a control, OTA was added to liquid CYB and cultured under the same conditions.

As shown by OTA dosage in Figure 44, the mycotoxin amount was not decreased after its culture with the CFEs under all modalities. Even the CFEs produced under modalities 4, 5 and 6 (where bacteria were able to degrade OTA during cell growth) were unable to degrade the mycotoxin afterwards, despite the pH of some of the samples being neutral.

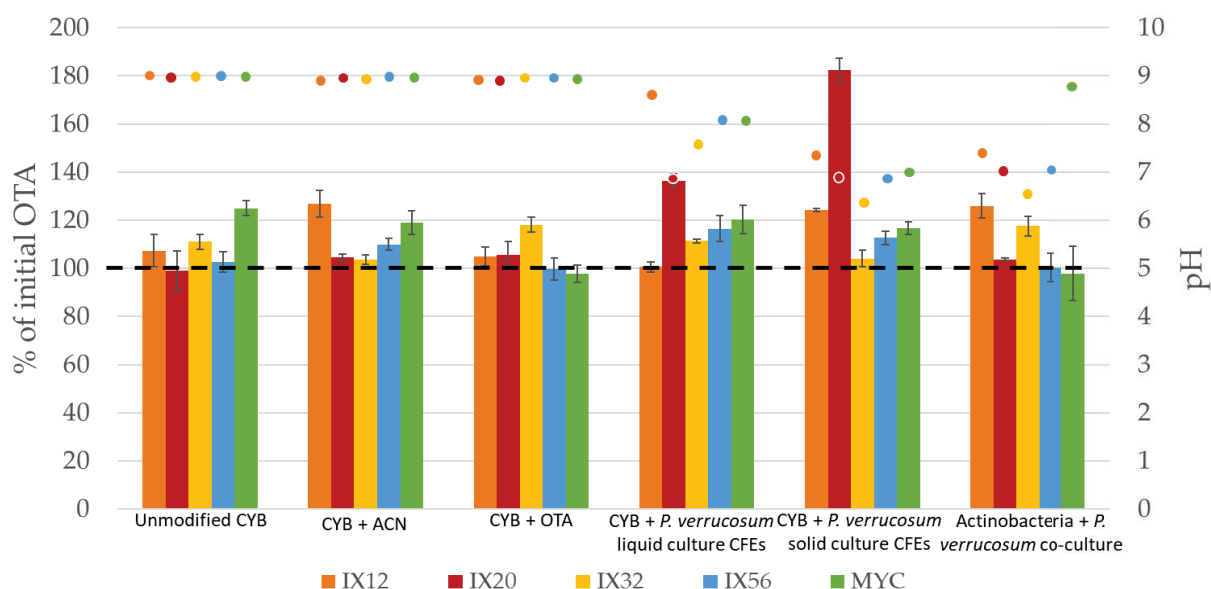


Figure 44. OTA degradation by stimulated actinobacteria's CFEs. Initial OTA = 226 ng/mL. Culture: 72 hours at 25°C and 180 rpm.

**Effect of stimulated bacterial CFEs on growth and OTA production by *P. verrucosum***

The “stimulated” bacterial CFEs were added at 25% to the growth medium of *P. verrucosum* in order to evaluate their effect on fungal development and OTA production.

Results on fungal growth are presented in Figure 45. Globally, CFEs under modalities 1,2 and 3 did not have a significant impact on fungal growth. However, CFEs produced under modalities 4, 5 and 6 seemed to have stimulated the antifungal properties of all the isolates at different extents, except for IX12. CFEs of isolates IX32, IX56 and MYC were particularly active, a complete inhibition of fungal growth was caused by CFEs of isolates IX32 and IX56 under Modality 4 of stimulation.

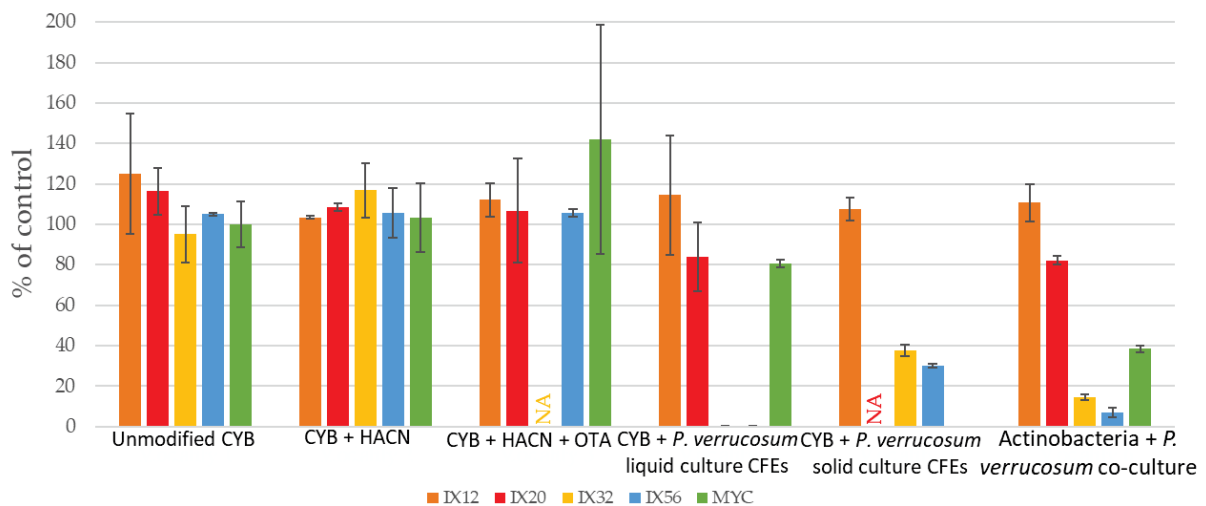


Figure 45. Impact of 25% of actinobacteria "stimulated" CFEs on *P. verrucosum* growth on solid CYA. Control: CYA medium without bacterial CFEs. Modality 1: CYB (unmodified), 2: CYB + Acetonitrile (5.64 mL/L), 3: CYB + OTA (260 µg/L) in acetonitrile (5.64 mL/L), 4: CYB + CFEs of *P. verrucosum*'s liquid culture (25%), 5: CYB + CFEs of *P. verrucosum*'s solid culture (25%), 6: CYB + fungal inoculum of *P. verrucosum* (50 µL at 10<sup>7</sup> spores/mL). NA: Not available data.

The impact of CFEs on OTA production varied according to the bacterial isolate and the stimulation modality. Isolate IX12 had a slight impact under modalities 1 through 5 but caused the highest overproduction in modality 6. Isolates IX20 and IX32 caused an OTA overproduction in general, except under modality 4, where they reduced mycotoxin amount. However, this decrease seems to follow the strong growth inhibition observed. A similar result was observed for isolate IX56, which caused also a mycotoxin decrease under modality 6. MYC decreased OTA amount under all modalities, except for modality 4.

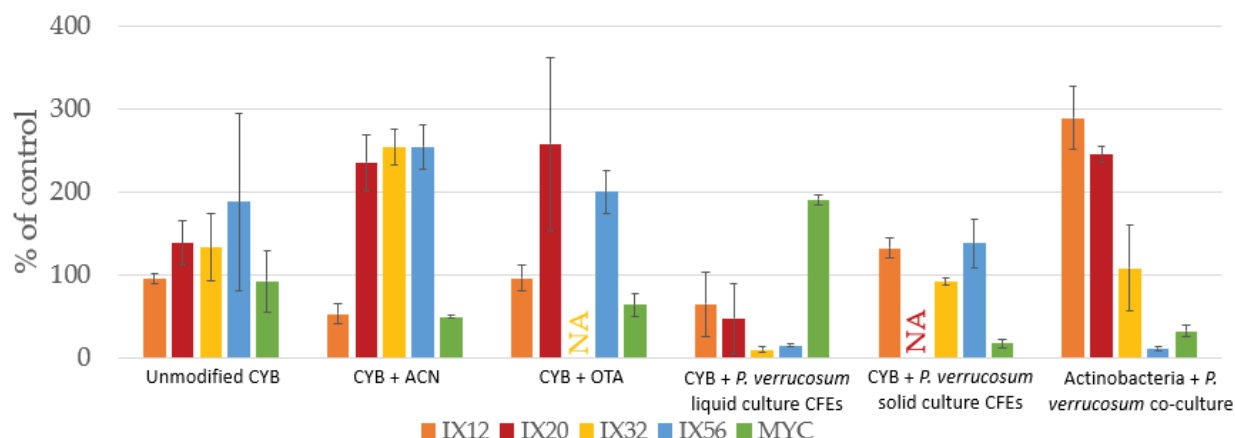


Figure 46. Impact of 25% of actinobacteria "stimulated" CFEs on *P. verrucosum*'s OTA specific production in solid CYA. Control: CYA medium without bacterial CFEs. Modality 1: CYB (unmodified), 2: CYB + Acetonitrile (5.64 mL/L), 3: CYB + OTA (260 µg/L) in acetonitrile (5.64 mL/L), 4: CYB + CFEs of *P. verrucosum*'s liquid culture (25%), 5: CYB + CFEs of *P. verrucosum*'s solid culture (25%), 6: CYB + fungal inoculum of *P. verrucosum* (50 µL at 10<sup>7</sup> spores/mL). NA: Not available data.

#### 4.2.5 Discussion

During this work we attempted to activate the *Streptomyces* secondary metabolism, by the hypothesis that OTA could act as a metabolism elicitor. OTA degrading capacity of actinobacteria' CFEs was particularly aimed in this complementary study, considering that previously published results were highly divergent regarding OTA degradation by bacterial cells and by their CFEs (Campos-Avelar et al. 2020).

As mentioned before, our approach included the addition of OTA during bacterial growth as, like other mycotoxins, OTA production by *P. verrucosum* may facilitate its competition against other microbes for space and nutrients (Pfliegler et al. 2020), and as such, it could act as signaling molecule for other microorganisms (Venkatesh and Keller 2019). We also stipulated that the previously observed OTA degradation by actinobacteria in liquid cultures (Campos-Avelar et al. 2020) was conducted by enzymes, and that the addition of the substrate (OTA) could further stimulate CFEs enzymatic activity. An example of this induction mechanism was observed for *S. coelicolor*, for which the addition of soil containing chitin activated the expression of chitinase genes (Nazari et al. 2011).

In addition to OTA, the impact of supplementing bacterial medium with fungal metabolites, on the bacterial CFEs degrading capacities was also evaluated. Nevertheless, in some cases, the activation of microbial metabolism requires cell to cell interactions, as the

metabolites alone (cell free supernatants, killed cells, extracts) are not always capable of induce compound production (Bertrand et al. 2014). Indeed, co-cultivation or mixed fermentation allows to mimic the conditions encountered by microorganisms in complex ecosystems where the production of secondary metabolites is enhanced by competitive or antagonist relationships (Marmann et al. 2014). To achieve this, a stimulating modality consisting in a co-culture of actinobacteria with *P. verrucosum* was also included in our study. As acetonitrile was used as a solvent to bring OTA into the culture medium, it was included as a control to evaluate its potential influence on the growth and metabolism of actinobacteria. However, some studies indicate that acetonitrile utilization by bacteria was only possible at very high pH (10) and moderate salinity (Sorokin et al. 2007) so its utilization under the conditions of this study seems unlikely.

Visual observation of the resulting bacterial broths indicated that the addition of neither acetonitrile nor OTA had a visible impact on actinobacteria development, while bacterial growth with the addition of fungal metabolites and in co-culture, exhibited an intense brown pigmentation. In fact, the co-cultivation of microorganisms can trigger the production of pigments (Bertrand et al. 2014), and some studies have found that a defense mechanism against degrading enzymes can be behind this effect (Marmann et al. 2014). In a study conducted by Traxler et al., color change in a co-culture of *Streptomyces coelicolor* with several other actinobacteria was caused by the production of pigmented antibiotics (Traxler et al. 2013). Co-cultures of *Streptomyces albospinus* and *Phomopsis* sp. lead to a morphology change by a color switch from clear to dark brown and the production of the antifungal compound amphotericin B (Chagas and Pupo 2018). Thus, we can conclude that OTA does not trigger any pigment production, but that other *P. verrucosum* metabolites clearly impact the metabolism of the actinobacteria isolates in our study.

The dosage of the residual amount of OTA in the resulting broths indicated that actinobacteria were unable to degrade OTA during their growth in unmodified medium (Fig. 41, Modality 1), which differs from the results of previous work (Campos-Avelar et al. 2020). The different nature of the containers between the precedent and the actual study led to a strong variation on pH values. Indeed, the results of this complementary assay seem to indicate that OTA degradation occurs mainly when the pH remains neutral. Two different hypotheses can be drawn from these findings:

1. Actinobacteria produce their degrading enzymes only when the growth is limited by stressful conditions (lack of aeration?) and before they reach basic pH
2. Enzymes are produced all along the actinobacteria growth, but they are not active at high pH

To better understand the discrepancies observed, similar experiments could be performed in buffered medium to eliminate the influence of pH variation during the culture. Moreover, the calibration of all bacterial CFEs at lower pH range might be interesting to verify how the pH range influences the degradation of OTA in the latter.

Regarding the impact of *P. verrucosum* metabolites (Modalities 4 and 5) on the production of CFEs, bacterial isolates were able to degrade the OTA during their growth, regardless of having a more neutral or basic pH (Fig. 41). The variation obtained in the pH range at the end of the culture of actinobacteria with fungal CFEs might originate either from a growth reduction due to fungal metabolites, or to the presence of organic acids produced by *P. verrucosum* (Frisvad, Lund, and Elmholt 2005), acting as buffering agents. This result suggests that a compound, other than OTA, produced by the fungus was able to stimulate bacterial cells so they could degrade the OTA present in the medium. In fact, *P. verrucosum*, is capable of producing a wide variety of secondary metabolites (e.g. verrucine A, verrucolone, anacine), of which about 80% remain unknown (Hautbergue et al. 2017). The screening of the influence of known *P. verrucosum* metabolites on *Streptomyces*' OTA degrading abilities could help in understanding the mechanism behind mechanism behind the observed stimulating effect.

A small amount of OTA was detected in mixed fermentation, which could be explained by the scarce fungal growth observed by microscopy. Nevertheless, mycotoxin inhibition or degradation phenomena cannot be excluded, as they have already been documented during fungal-bacterial interactions (El Khoury et al. 2017, 2018). For instance, Rodriguez et al. found that *Brevibacterium* sp. were able to completely degrade OTA by hydrolyzing it into OT $\alpha$  and phenylalanine during liquid cultures, probably by means of a carboxypeptidase (Rodriguez et al. 2011). The observed degradation of OTA by bacteria, when exposed to fungal CFEs, also provides indications that such phenomenon could be involved.

The bacterial CFEs were unable to degrade OTA under any modality even when the pH values were neutral (Modalities 4, 5 and 6), which indicates that neither OTA, nor the whole spectrum of *P. verrucosum* fungal metabolites, nor the mixed culture granted the capacity to

further degrade the mycotoxin. Our results differ from those of El Khoury and co-workers, as they found that actinobacteria strains were able to degrade OTA from about 20% to 50% while growing in liquid medium, and those strains' CFEs also decreased OTA. However, the observed degradation in CFEs were quite low (14%-22%) (El Khoury et al. 2017). For *B. subtilis*, cell free extracts were able to degrade 97.6% of 6 µg/mL OTA in two hours. However, intracellular supernatant was unable to degrade the mycotoxin (Shi et al. 2014). The strong degradation observed in the presence of the cells for Modalities 4 and 5 along with the total absence of OTA degradation activity of the resulting supernatants points towards an intracellular degradation rather than a secretion of extracellular enzymes, independently of the pH.

Despite their lack of degrading activity, CFEs exhibited an impact on *P. verrucosum* development and OTA production. In particular, CFEs of modalities 4, 5 and 6, as some of them were able to almost completely inhibit fungal growth (IX32 and IX56, modality 4). This suggests that the presence of fungal metabolites, other than OTA, in the last three modalities seemingly stimulated the production of antifungal metabolites. As described by Bertrand et al., the production of antimicrobial compounds is commonly induced during microbial interactions (Bertrand et al. 2014). Furthermore, it was demonstrated that a slightly acidic pH (6) favors the production of antifungal antibiotics by *Streptomyces rimosus* (Yu et al. 2008). In our study, the most active CFEs (IX32 and IX56) were produced under modalities 4, 5 and 6, whose pH was around 6-6.5, which could have promoted the production of antifungal compounds that later inhibited *P. verrucosum* growth. The impact on OTA production was variable, with some isolates causing an overproduction and others strongly decreasing the mycotoxin amount. Moreover, the same isolate exhibited different effects depending on the stimulation modality, which evidences their sensitivity to the parameters evaluated. These results are of the utmost interest as they indicate that different isolates may have different antagonistic mechanisms and that their interactions with the fungi or its metabolites can lead to bioactive compounds for mycotoxin inhibition or stimulation.

#### 4.2.6 Conclusion and perspectives

The different approaches evaluated during this complementary study for the stimulation of actinobacteria CFEs, were ineffective at conferring them OTA degradation capacities. Nevertheless, the presence of fungal metabolites greatly enhanced the degradation capacity by the bacteria itself. Moreover, some antifungal properties of actinobacteria CFEs were also enhanced by the presence of fungal metabolites or by the fungus itself during bacterial growth. Finally, an interesting phenomenon was elucidated concerning the impact of growth conditions on the capacity of actinobacteria to degrade OTA in liquid culture. It appears that peculiar growth conditions could possibly stress actinobacteria, modifying their development and allowing them to degrade/consume the OTA in the medium. Further studies under controlled aeration and pH could allow to validate this hypothesis and/or to elucidate new degradation mechanisms.

### 4.3 Aspergillus flavus growth inhibition and Aflatoxin B<sub>1</sub> decontamination by Streptomyces spp. and their metabolites

#### 4.3.1 Article presentation

The *Streptomyces* collection previously screened against *Penicillium verrucosum* and OTA was this time evaluated against *Aspergillus flavus* and aflatoxins. Both pathogens occur mainly during grain storage and provoke the accumulation of mycotoxins. The purpose of this study was to identify the antifungal and detoxifying capacity of *Streptomyces* isolates, in order to set the basis for the potential identification and isolation of bioactive compounds for the detoxification of food matrices.

The conducted experiments were based on the approach previously described: *in vitro* dual culture assays; impact of CFEs on fungal growth and mycotoxin production; mycotoxin degradation by cells grown on artificially contaminated solid and liquid medium and degradation by bacterial metabolites (CFEs), as well as the search for degradation by-products. An additional step of verification of the residual toxicity complemented this work.

Results showed that *A. flavus* growth was easily inhibited by most *Streptomyces* isolates. The antifungal efficacy of their CFEs at the tested concentration was inferior, indicating either the involvement of a direct mechanism or the need to increase the concentration in order to have a similar impact. Moreover, thermally treated CFEs had a weak antifungal capacity, which evidences an enzymatic activity. Aflatoxin B<sub>1</sub> and B<sub>2</sub> production was highly decreased during dual cultures, suggesting a general detoxifying mechanism. CFEs caused also a mycotoxin general decrease, but to a lesser extent. However, some CFEs caused an overproduction of aflatoxins, which drives attention to the stimulating metabolites present in the bacterial extracts. Degradation assays confirmed the ability of bacterial cells to degrade AFB<sub>1</sub> and also the degrading capacity of their CFEs. Residual toxicity assessment allowed us to validate an effective decrease on genotoxicity. These results lead to a promising research perspective for the production and isolation of aflatoxin degrading enzymes that could be applied in the food industry.

A comparison of the results obtained for both storage pathogens and both main mycotoxins is presented at the end of this section.

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Article

# *Aspergillus flavus* growth inhibition and Aflatoxin B<sub>1</sub> decontamination by *Streptomyces* isolates and their metabolites

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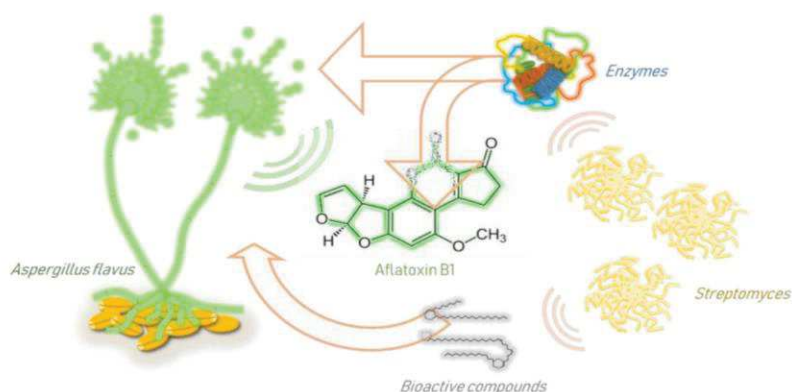
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## Abstract:

Aflatoxin B<sub>1</sub> is a potent carcinogen produced by *Aspergillus flavus*, mainly during grain storage. As pre-harvest methods are insufficient to avoid mycotoxins presence during storage, diverse curative techniques are being investigated for the inhibition of fungal growth and aflatoxins detoxification. *Streptomyces* sp. represent an alternative as they are a promising source of detoxifying enzymes. Fifty-nine *Streptomyces* isolates and *Streptomyces griseoviridis* strain from the commercial product Mycostop<sup>®</sup>, evaluated against *Penicillium verrucosum* and ochratoxin A during previous work, were screened for their ability to inhibit *Aspergillus flavus* growth and decrease aflatoxins amount. The activities of bacterial cells and cell-free extracts (CFEs) from liquid cultures were also evaluated. Fifty-eight isolates were able to inhibit fungal growth during dual culture assays, with a maximal reduction going down to 13% of the control. Aflatoxins specific production was decreased by all isolates to at least 54% of the control. CFEs were less effective in decreasing fungal growth (down to 40% and 55% for unheated and heated CFEs, respectively) and aflatoxins specific production, with few CFEs causing an overproduction of mycotoxins. Nearly all *Streptomyces* isolates were able to degrade AFB<sub>1</sub> when growing in solid and liquid medium. A total degradation of AFB<sub>1</sub> was achieved by Mycostop<sup>®</sup> on solid medium, and an almost complete degradation by IX20 in liquid medium (6% of the control). CFEs maximal degradation went down to 37% of the control by isolate IX09. The search for degradation by-products indicated the presence of few unknown molecules. The evaluation of residual toxicity of tested isolates by SOS chromotest indicates a detoxification of at least 68% of AFB<sub>1</sub>'s genotoxicity.



**Keywords:** Actinobacteria; fungi; mycotoxins; enzymes; biodegradation; detoxification.

**Key contribution:** The impact of the tested *Streptomyces* isolates on *A. flavus* AFB<sub>1</sub> seems to be ruled by a common mechanism, as all the isolates were able to decrease its amount, either during dual cultures or by direct degradation. *Streptomyces* are able to effectively detoxify AFB<sub>1</sub>. Clustering and Pearson's correlation allow to identify groups of microorganisms with particular traits for future research.

## 1. Introduction

During food crop production, aside the yield losses due to fungal infections, the appearance of mycotoxins represents a critical health risk for consumers, even more than the presence of pesticides and synthetic residues [1]. The term mycotoxin was first employed in the 1960s, when investigating the cause of death of several turkeys in England, after consuming a groundnut meal. The toxic molecule was later identified as Aflatoxin B<sub>1</sub> [2]. AFB<sub>1</sub> is carcinogenic, mutagenic and immunosuppressive, and is considered the most harmful mycotoxin [3]–[6]. *Aspergillus flavus* is the main producer of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and aflatoxin B<sub>2</sub> (AFB<sub>2</sub>). As an opportunistic plant pathogen, it can develop on cereals such as wheat and maize, as well as on cotton, nuts and spices [7].

Aflatoxins constitute the second main hazard in France according to the Rapid Alert System for Food and Feeds (RASFF) with 34 alerts notified in 2018 [8]. Moreover, as climate change progresses, aflatoxins occurrence will increase [9]. Due to their potential health risk and their stability during industrial processing, the European Commission has established AFB<sub>1</sub> maximal content according to the type of food commodity. Limits for groundnuts before processing were set at 8 µg/Kg. For nuts, dried fruit, spices and maize before processing the limit is 5 µg/Kg. Finally, for all the listed commodities after processing, plus all cereals and derived products, the limit decreases to 2 µg/Kg [10].

Prevention and curation techniques to avoid aflatoxins presence are being largely studied. Some examples of effective pre-harvest measures are the genetic modification of crops, crop rotation, irrigation, insect prevention and biocontrol by non toxinogenic *Aspergillus*, such as Afla-guard® and Aflasafe®, which are already commercialized. Post-harvest strategies include the precise control of storage conditions (low water activity and temperature) as well as sorting and elimination of contaminated grains [11]. However, as aflatoxin contamination persists, curative techniques must be implemented on foodstuffs, such as adsorption or physical and chemical reduction by degradation or inactivation. Unfortunately, these methods often lead to the decrease of nutritional properties, food quality reduction and increment of production costs. Several studies performed mainly on yeasts, lactic acid bacteria and actinobacteria showed that these microorganisms have the ability to decrease AFB<sub>1</sub> [12], [13]. However, the main reduction mechanism of AFB<sub>1</sub> by lactic bacteria and yeasts, is based on their ability to bind the mycotoxin to their cell walls, which makes this decontamination technique very limited [14], [15]. Actinobacteria presents an interesting natural and cost-effective alternative for the effective biodegradation of mycotoxins [1].

Actinobacteria are filamentous Gram-positive bacteria found in several ecological niches both on the soil and in the water. *Streptomyces* in particular are largely researched for their ability to produce numerous molecules of interest, namely antibiotics, antifungal compounds, and enzymes, such as chitinases, which provide them strong antagonistic capacities against fungal development [16]. The impact of actinobacteria on *Aspergillus flavus* growth has already been assessed. As well as their ability to degrade AFB<sub>1</sub> [14], [17]–[19] and to inhibit its production. Indeed, some *Streptomyces* strains produce aflastatin A, blasticidin A and dioctatin A, three molecules that inhibit aflatoxin biosynthetic pathway [20], [21]. In addition, a *Streptomyces roseolus* strain was found to reduce AFB<sub>1</sub> production by inhibiting aflatoxin gene cluster expression on *A. flavus* [22].

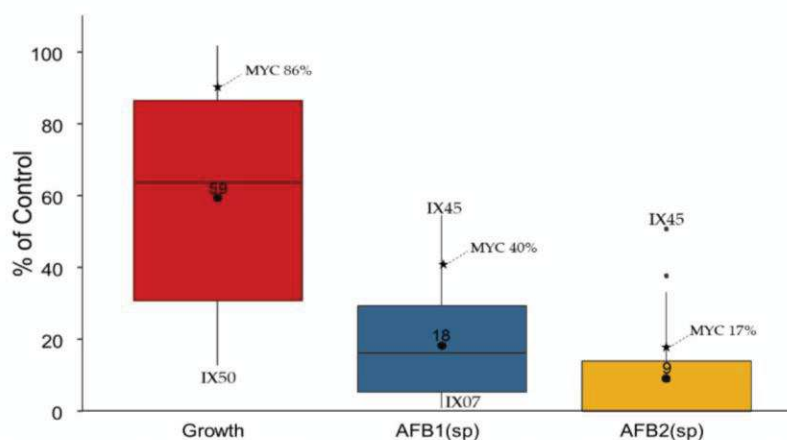
The major mechanisms involved in the degradation of AFB<sub>1</sub> consist of a cleavage of the lactone group or a modification of the difuran ring or of the coumarin structure. Some enzymes known to be involved in AFB<sub>1</sub> degradation are laccases, peroxidases, oxidases and reductases [14], [23], [24]. AFB<sub>1</sub> main degradation metabolites include Aflatoxicol, Aflatoxin B<sub>2a</sub> and Aflatoxin D<sub>1</sub>, which are reportedly less toxic [15]. Moreover, two other modified forms, AFB<sub>1</sub>-8,9-epoxide and AFM<sub>1</sub>-8,9-epoxide, can bind to the DNA and provoke carcinogenic and mutagenic effects [25]. Hence, it is essential to ensure that AFB<sub>1</sub> is effectively detoxified in less harmful molecules. For the evaluation of residual toxicity, *in vitro* tests allow to evaluate AFB<sub>1</sub> genotoxicity and mutagenicity, such as the Ames test and the SOS-chromotest [26]. In the SOS-chromotest, the bacterial gene *sfiA* expression, controlled by the SOS system for DNA damage, is monitored by assaying  $\beta$ -galactosidase activity [27]. It uses a mutant strain of *Escherichia coli* PQ37 which carries a *sfiA::lacZ* fusion and has a deletion of the normal *lac* region, so that  $\beta$ -galactosidase activity is strictly dependent on *sfiA* expression [28].

The aim of this work was to evaluate the *in vitro* interactions of 59 *Streptomyces* isolates, Mycostop®'s strain, and their cell-free extracts (CFEs) with *Aspergillus flavus* and its aflatoxins. First, *Streptomyces* isolates were confronted with *A. flavus* to assess their direct impact on fungal growth and mycotoxin accumulation in the medium. Then, AFB<sub>1</sub> degradation ability was studied during bacterial cells development in solid and liquid medium, as well as by incubating the mycotoxin with their CFEs, to determine if degrading enzymes were produced extracellularly. Degradation by-products were researched by HPLC-MS and their residual toxicity was evaluated by a SOS-Chromotest.

## 2. Results

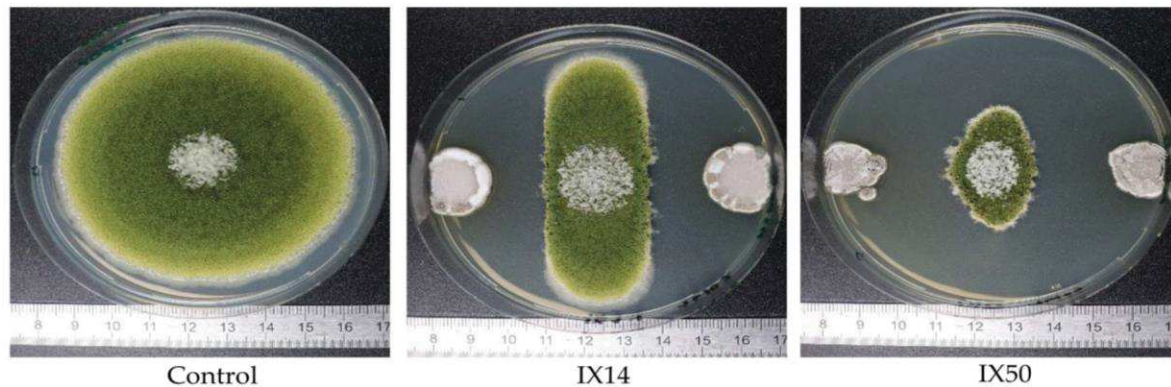
### 2.1. Evaluation of the Antagonistic Activity of *Streptomyces* Isolates and Their Cell Free Extracts (CFEs).

The effect of fifty-nine *Streptomyces* isolates and Mycostop®'s *Streptomyces griseoviridis* strain (further referenced as MYC) on *A. flavus* growth and aflatoxin B<sub>1</sub> and B<sub>2</sub> specific production was evaluated by a dual culture assay (Figure 1). Fungal growth was decreased until 13% of the control (mean = 59%, median = 64%) by isolate IX50. AFB<sub>1</sub> specific production (AFB<sub>1</sub>sp) was strongly reduced by all isolates (mean=18%, median = 16% of the control), with at least 54% of the control for isolate IX45 to a negligible amount for isolate IX07 (0.7% of the control). The effect on AFB<sub>2</sub>sp was stronger and followed the same tendency as for AFB<sub>1</sub>sp, with half of the isolates provoking a high to full reduction of AFB<sub>2</sub>sp (mean = 9%, median = 0.08 %). No isolate led to a specific overproduction of aflatoxins in the medium, meaning that an inhibition of AFB<sub>1</sub>sp occurred and/or that mycotoxins were degraded. Globally, most of the isolates had stronger antifungal and mycotoxin accumulation reduction properties than Mycostop®.



**Figure 1.** Effect of 59 *Streptomyces* isolates and Mycostop® (MYC) on *Aspergillus flavus* growth and aflatoxin B<sub>1</sub> and B<sub>2</sub> specific production (accumulation) during dual culture on CYA medium at 25°C for 8 days. The boxplots represent the distribution of the data expressed as a % of a control (fungal growth and aflatoxin specific production without bacteria).

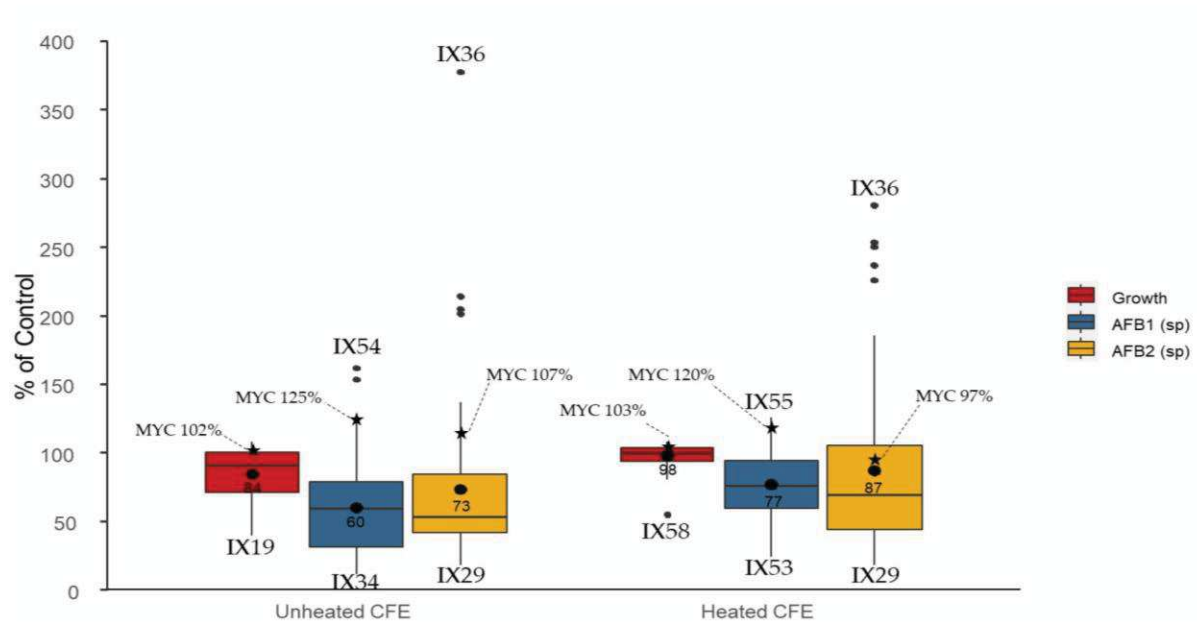
Figure 2 shows the inhibition profiles of *A. flavus* growth by isolates IX14 and IX50 on CYA, where fungal growth was decreased up to 37% and 13% of the control, respectively. As we can observe, the distance between bacterial and fungal colonies is almost the same in both cases, however, isolate IX50 strongly inhibited fungal growth on the edges, which highlights the importance of measuring the area of the whole fungal colony rather than the antagonistic distance.



**Figure 2.** Growth profiles of *Aspergillus flavus* during dual culture assay against *Streptomyces* isolates IX14 and IX50, cultured on CYA for 8 days at 25°C.

CFEs were added in the growth medium (10% v/v) of *Aspergillus flavus* with and without thermal treatment, in order to inactivate enzymes and discriminate for their potential impact on fungal growth and AFB<sub>1</sub>sp. As showed by the boxplots in Figure 3, CFEs at 10% were less efficient at inhibiting fungal growth than bacteria in dual culture, with a maximal decrease going until 40% of the control (mean = 84%, median = 91%) for unheated CFEs (IX19) and until 55% of the control (mean = 98%, median = 100%) for heated CFEs (IX58).

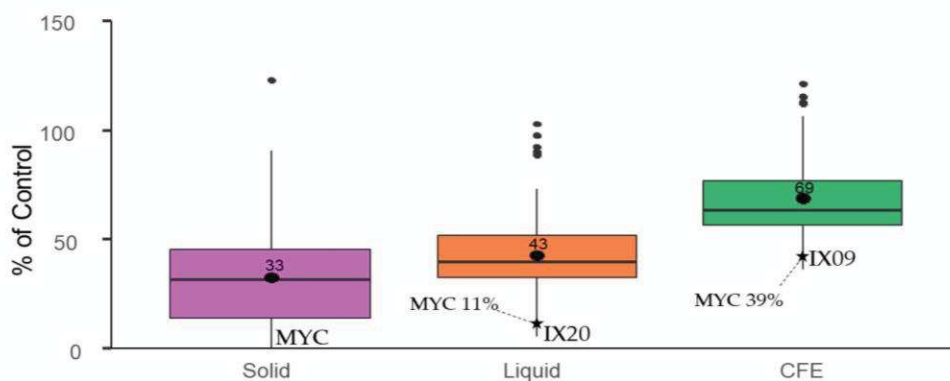
Regarding the effect of CFEs, the results reveal a maximal decrease on AFB<sub>1</sub>sp for unheated CFEs with 11% of the control (mean = 60%, median = 59%) by IX34, and 23% (mean = 77%, median = 76%) for heated CFEs (IX53). Five unheated CFEs provoked an increase of AFB<sub>1</sub>sp going up to 161% of the control (IX54). Heated CFEs increased AFB<sub>1</sub>sp in ten cases, going up to 125% of the control (IX55). CFEs were less effective at decreasing AFB<sub>2</sub>sp with a mean of 73% (median = 53%) and 87% (median = 69%) of the control by unheated and heated CFEs, respectively. The maximal decrease in AFB<sub>2</sub>sp was observed for the CFE of IX29 with a specific production lowered to 18% of the control whether being heated or not. Ten CFEs caused a rise in AFB<sub>2</sub>sp going up to 377% of the control for IX36, while 16 heated CFEs lead to similar effects (up to 280% of the control). CFEs of Mycostop® did not cause a significant change on fungal development nor mycotoxin specific production.



**Figure 3.** Effect of cell free extracts (CFEs) of 59 *Streptomyces* isolates and Mycostop® strain (MYC), added at 10% in the solid culture medium (CYA) of *Aspergillus flavus*, on fungal growth and aflatoxins specific production (accumulation) after 8 days at 25°C. Boxplot represents the distribution of the data expressed as a % of control (fungal growth and specific mycotoxin production without CFEs). Thermal treatment for heated CFEs: 100°C for 10 min.

2.2. Mycotoxin Degradation Assay.

*Streptomyces* isolates, MYC and their CFEs were evaluated regarding their capacity to degrade AFB<sub>1</sub> at a concentration of 2 µg/mL. Boxplots in Figure 4 show that most isolates were able to degrade AFB<sub>1</sub> on solid medium (mean = 33%, median = 32%) after 10 days of culture, down to undetectable residual amounts for MYC. In liquid medium, 5 days of culture allowed bacterial cells to degrade AFB<sub>1</sub> down to 6% of the control for IX20 (mean = 43%, median = 39%). CFEs were less efficient at degrading the mycotoxin, with a maximal decrease down to 37% of the control for IX09 (mean = 69%, median = 64%). Mycostop® exhibited also strong degradation capacities in liquid medium (11% of the control) and by its CFEs (39% of the control).



**Figure 4.** Degradation of aflatoxin B<sub>1</sub> by *Streptomyces* isolates and Mycostop® (MYC) cells in solid (CYA) and liquid (CYB) medium after 10 and 5 days of culture, respectively, and by their unheated CFEs after 48 hours at 25°C and 180 rpm. Boxplots represent the distribution of the mycotoxin decrease as a % of Control. Controls were included by adding AFB<sub>1</sub> to CYA or CYB media, incubated under the same conditions.

2.3. Global Analysis of Strains: Cluster Analysis and Pearson Correlation Index.

In order to better visualize the specific characteristics and similarities of each of the 59 *Streptomyces* isolates and Mycostop® strain, the obtained results were interpreted using clustering analysis with a heatmap representation (Supplementary materials Figure S1) and a correlational analysis by calculating the Pearson’s correlation index for the two main clusters (Supplementary materials Figure S2). The set of correlation coefficients (r) and their significance (p-value, p) are also presented in the Supplementary Materials (Table S1).

The heatmap in S1 shows the effect of each *Streptomyces* isolate regarding three types of interactions with *A. flavus* and its toxins:

*A. flavus* growth in dual culture or with the addition of CFEs to the medium (Category 1);

AFB<sub>1</sub> specific production by *A. flavus* during growth in dual culture or with CFEs added to the medium (Category 2); and

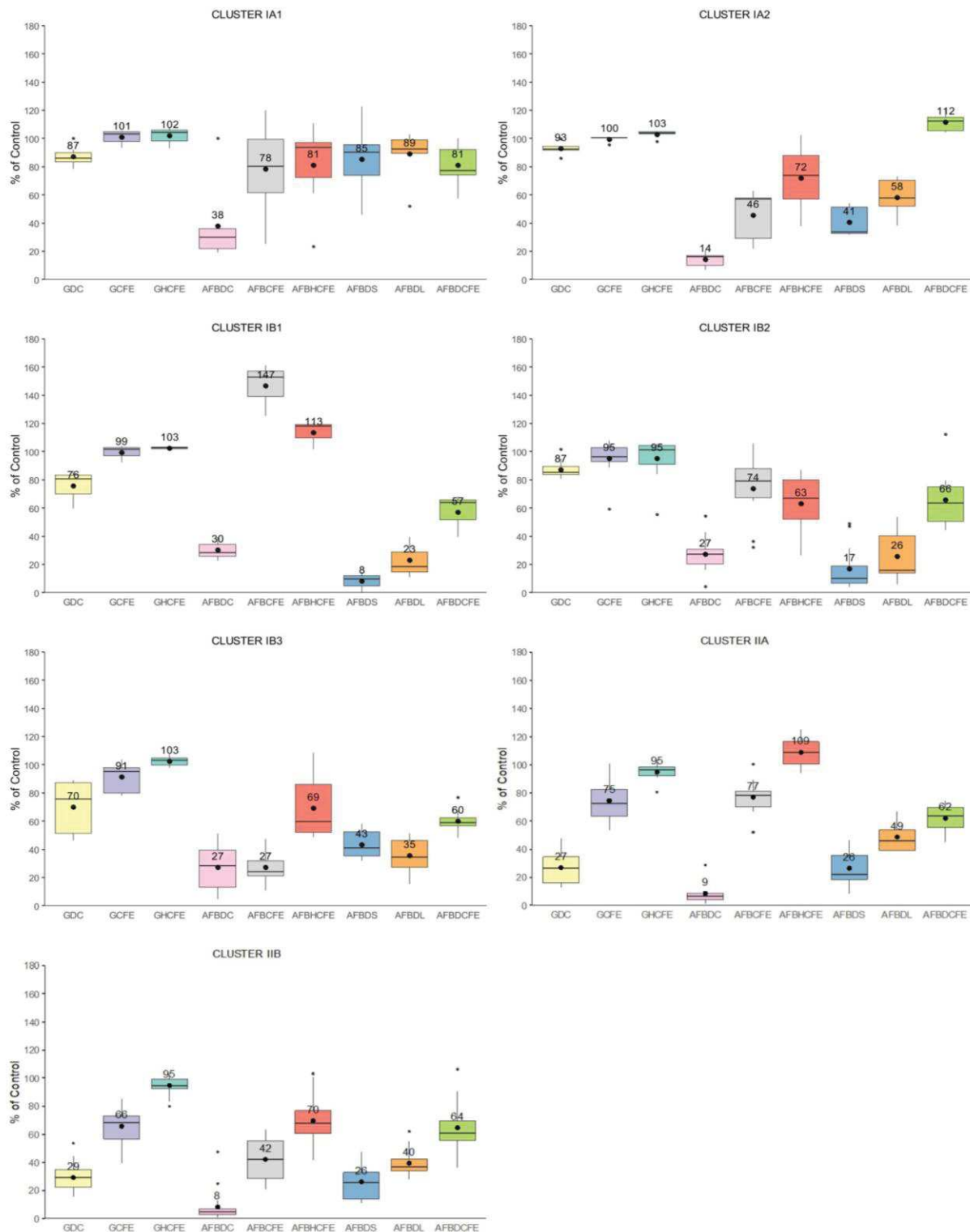
AFB<sub>1</sub> degradation by *Streptomyces* isolates in solid and liquid medium as well as by their CFEs (Category 3).

As the parameters analyzed had different magnitudes, a standardization was performed by transforming each percentage value to a Z-score, calculated by grouping the results of each of the three categories described before. A lower value of Z-score represents a strong activity regarding the inhibition of fungal growth, of AFB<sub>1</sub>sp or its degradation. For the heatmap representation, a color scale was assigned to the Z-scores: clear yellow defines a strong activity (low Z-scores) and dark purple to black represents a lack of activity or an increase as compared to the control for AFB<sub>1</sub>sp (high Z-scores). Intermediate colors represent moderate activity. Finally, Euclidean distances were calculated considering the complete profile of the isolates within each category and sub-category which allowed to clusterize them according to their percentage of similarity.

*Streptomyces* isolates were separated in 2 main clusters and 7 subclusters according to their effects recorded in each assay. Their principal features were evidenced by plotting the distribution of the measured parameters for each subcluster (Figure 5), and their main characteristics are summarized in Table 1.

**Table 1.** Summary of specific features observed during the clustering analysis of the impact of the screened *Streptomyces* isolates and their cell free extracts (CFEs) on *Aspergillus flavus* growth and aflatoxin B<sub>1</sub> specific production (AFB<sub>1</sub>sp), together with their ability to degrade AFB<sub>1</sub>.

Cluster	Specific features	Subcluster	Specific features	Isolates		
I	Moderate effect on <i>A. flavus</i> growth. Contrasted effect on AFB <sub>1</sub> specific production and its degradation.	A	Limited inhibition of <i>A. flavus</i> growth. Moderate decrease of AFB <sub>1</sub> sp in dual culture. Limited degradation capacity.	IX16, IX37, IX38, IX47, IX53, IX57		
			2	Lack of inhibition of <i>A. flavus</i> growth. Strong decrease of AFB <sub>1</sub> sp in dual culture. Moderate capacity of CFEs to decrease of AFB <sub>1</sub> sp. Moderate degradation capacity of bacterial cells.	IX30, IX40, IX41, IX43, IX44	
				B	Moderate decrease of AFB <sub>1</sub> sp in dual culture. Strong increase of AFB <sub>1</sub> sp provoked by CFEs. Strong degradation capacity of bacterial cells.	IX05, IX54, MYC
		2	Moderate decrease of AFB <sub>1</sub> sp. Strong degradation capacity of bacterial cells.		IX01, IX03, IX04, IX12, IX20, IX25, IX28, IX35, IX36, IX39, IX45, IX58	
			3		Strong decrease on AFB <sub>1</sub> sp in dual culture and by unheated CFEs. Moderate degradation capacities.	IX02, IX06, IX08, IX29, IX33, IX34, IX42, IX46
		II	Strong inhibition of <i>A. flavus</i> growth (dual culture). Strong effect on AFB <sub>1</sub> sp. Moderate degradation by bacterial cells and CFEs.	A	Strong inhibition of <i>A. flavus</i> growth in dual culture. Strong decrease of AFB <sub>1</sub> sp in dual culture. Moderate degradation by bacterial cells and by CFEs.	IX14, IX22, IX23, IX48, IX50, IX55, IX56, IX59
					B	Strong inhibition of <i>A. flavus</i> growth in dual culture. Strong decrease of AFB <sub>1</sub> sp in dual culture. Moderate decrease of AFB <sub>1</sub> sp by CFEs. Moderate degradation by bacterial cells and by CFEs.



**Figure 5.** Boxplot of the effect of *Streptomyces* isolates and CFEs on *Aspergillus flavus* growth and AFB<sub>1</sub> specific production (AFB<sub>1</sub>sp) (accumulation). Each boxplot represents a subcluster of the heatmap from Figure S1. GDC = *A. flavus* growth in dual culture, GCFE = *A. flavus* growth vs Cell Free Extracts (CFEs), GHCFE = *A. flavus* growth vs Heated CFEs, AFBDC = AFB<sub>1</sub>sp in dual culture, AFBCFE = AFB<sub>1</sub>sp vs CFEs, AFBHCFE = AFB<sub>1</sub>sp vs Heated CFEs, AFBDS = AFB<sub>1</sub> degradation by cells in solid medium, AFBDL = AFB<sub>1</sub> degradation by cells in liquid medium, AFBDCFE = AFB<sub>1</sub> degradation by CFEs.

The following description of subclusters is based on the average of each evaluated category. Fungal growth inhibition during dual cultures (GDC) was mainly achieved by isolates from cluster II, which were able to decrease fungal development down to 27% of the control (IIA), with the most effective isolates being IX50 and IX23. Isolates from cluster I had a more limited effect, except for IB3 which decreased it down to 70% of the control and to 76% of the control for IB1. CFEs antifungal activity (GCFE) followed the same tendency as the dual culture assay, with an efficiency mostly limited to the cluster II, since fungal inhibition went from 91% for cluster I (IB3) to 68% of the control for cluster II (IIB). Isolates IX19 and IX18 (subcluster IIB) were the most effective CFEs at decreasing fungal growth. The thermal treatment (GHCFE) ruled out any antifungal activity for all CFEs.

Regarding AFB<sub>1</sub>sp, all subclusters strongly affected toxin accumulation when confronted in dual culture assay (AFBDC), with reductions ranging from 38% (IA1) up to 8% (IIB) of the control on average. Isolates IX07 and IX31 (subcluster IIB) had the strongest mycotoxin reduction activity, with an almost complete decrease of AFB<sub>1</sub>sp (0.71% and 0.83% of the control, respectively). The impact of CFEs on AFB<sub>1</sub>sp (AFBCFE) was less pronounced, and variable effects could be noted: from a strong reduction for subcluster IB3 (27%) -particularly isolates IX34 and IX06-, to intermediate reduction for IA2 (46%) and IIB (42%), and limited for subclusters IIA (77%), IB2 (74%) and IA1 (78%). The subcluster IB1 exhibited singular properties with a strong enhancement of AFB<sub>1</sub>sp, going up to 141% of the control on average. The thermal treatment (AFBHCFE) generally decreased the influence of CFEs on AFB<sub>1</sub>sp in all subclusters, except for IB2 (74% vs 63% of the control after a thermal treatment).

AFB<sub>1</sub> degradation was a common trait among all subclusters under both culture conditions, but it was usually higher in solid medium (AFBDS) rather than in liquid medium (AFBDL), except for subcluster IB3. Subcluster IA1 had the weakest degradation abilities in solid medium (85% of the control) whereas IB1 and IB2 exhibited the strongest, with 8 and 17% of the control, respectively. Other subclusters ranged within 26% (IIA and IIB) to 43% of the control (IB3). The strongest degradation was caused by MYC strain (IB1) and by isolates IX28, IX20, IX03 (IB2). Finally, CFEs degradation abilities (AFBDCFE) were usually lower than the degradation by the cells, and comparable between the subclusters, ranging within 57 to 66% of the control, except for subcluster IA1 (81% of the control) where it was equivalent to the degradation by the cells. Most effective CFEs regarding AFB<sub>1</sub> degradation included those produced by IX09 (IIB), MYC (IB1) and IX45 (IB2). Subcluster IA2 was peculiar as it was the only one having substantial degradation properties by the cells (41% in solid medium and 58% in liquid medium) but none effect in the CFEs.

The Pearson correlation analysis in Figure S2 (Supplementary Materials) indicated a positive correlation coefficient in Cluster I between the effect of unheated and heated CFEs on AFB<sub>1</sub>sp ( $r=0.6$ ,  $p<.001$ ), as well as between bacterial cells' ability to degrade pure AFB<sub>1</sub> in solid and liquid media ( $r=0.8$ ,  $p<.001$ ). A moderate positive correlation is presented between the ability of *Streptomyces* isolates to degrade pure AFB<sub>1</sub> in liquid medium and the degrading capacity of their CFEs ( $r=0.5$ ,  $p=.002$ ). Cluster II exhibits a strong positive correlation between unheated and heated CFEs regarding their impact on *A. flavus* growth ( $r=0.6$ ,  $p<.001$ ) and on AFB<sub>1</sub>sp ( $r=0.8$ ,  $p<.001$ ). A moderate positive correlation also exists between the inhibition of fungal growth in dual culture and AFB<sub>1</sub> degradation by bacterial cells in solid ( $r=0.6$ ,  $p=.0012$ ) and liquid medium ( $r=0.4$ ,  $p=.03$ ). A slight positive correlation appears in dual culture between the inhibition of *A. flavus* growth and the reduction of AFB<sub>1</sub>sp ( $r=0.5$ ,  $p=.02$ ). The dendrograms presented at the bottom of Figure S2 illustrate the relationship and proximity between the results of the *Streptomyces* isolates and their CFEs for the different parameters evaluated. Parameters in Cluster I are slightly more closely related (~18% maximum Euclidean distance) than in Cluster II (~21% maximum Euclidean distance). In both clusters, parameters are associated within three main groups, but associations vary between them. AFB<sub>1</sub> degradation by bacterial cells in solid and liquid medium are close in Cluster I, with a distance of 4%, while in Cluster II they are separated by 21%. In Cluster II the



effect of *Streptomyces* isolates on AFB<sub>1</sub> specific production in dual culture is closely related to the degradation in solid medium by bacterial cells (3%).

#### 2.4. Search for Degradation By-products.

Two isolates (IX20, IX45) and MYC strain, with strong degrading capacities in liquid culture (decrease down to 6%, 10% and 11% of the control, respectively) were selected for the search of degradation by-products of AFB<sub>1</sub> (8 µg/mL). Main breakdown products known to be issued of microbial degradation such as aflatoxicol, aflatoxin B<sub>2</sub> and aflatoxin D<sub>1</sub> and D<sub>2</sub> were searched by HPLC-MS. Figure 6 presents the resulting spectra of the Mass Spectrometry analysis with the peaks and molecular formulas corresponding to the found unknown compounds, as well as their *m/z*.

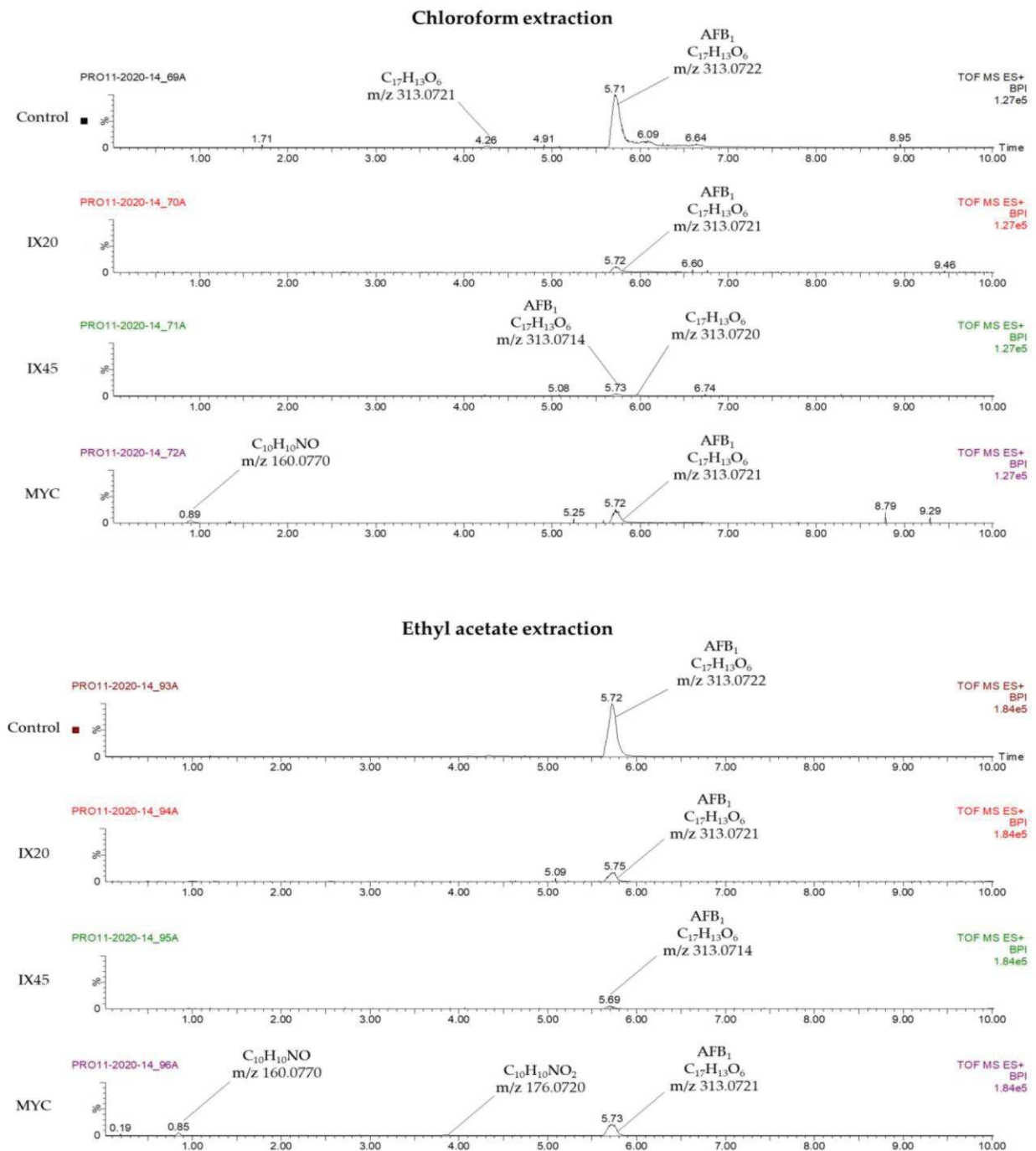
Spectral data were obtained by extracting the supernatant with chloroform or ethyl acetate. Peaks were normalized in order to distinguish the decrease on AFB<sub>1</sub> by the *Streptomyces* isolates, and to compare approximatively the amount of the detected molecules. In both extractions, a clear decrease on AFB<sub>1</sub> peaks is observed for the bacterial cultures, in comparison to the control.

Chloroform extraction spectra exhibits ion peaks at *m/z* 313.0722 and 313.0721 for the control, corresponding to AFB<sub>1</sub> and to what seems to be an AFB<sub>1</sub> isomer, respectively. For IX20 only the ion peak of AFB<sub>1</sub> at *m/z* 313.0722 its observed. For IX45 ion peaks at *m/z* 313.0714 and 313.0720 are present, corresponding to AFB<sub>1</sub> and an AFB<sub>1</sub> isomer, respectively. For MYC ion peaks at *m/z* 313.0721 and 160.0770 are present, corresponding to AFB<sub>1</sub> and to an unknown molecule of formula C<sub>10</sub>H<sub>10</sub>NO, respectively.

Ethyl acetate extraction spectra shows an ion peak at *m/z* 313.0722 for the control corresponding to AFB<sub>1</sub>. For IX20 and IX45 only the ion peak of AFB<sub>1</sub> at *m/z* 313.0721 and 313.0714, respectively, is present. For MYC ion peaks at *m/z* 313.0721, 176.0720 and 160.0770 are present, corresponding to AFB<sub>1</sub>, to a first unknown molecule of formula C<sub>10</sub>H<sub>10</sub>NO<sub>2</sub>, and to a second unknown molecule of formula C<sub>10</sub>H<sub>10</sub>NO, that was also observed for MYC culture extracted with chloroform.

#### 2.5. Evaluation of Residual Toxicity.

To ensure the effective detoxification of AFB<sub>1</sub> by the tested *Streptomyces* isolates and MYC, the liquid cultures resulting from the degradation by-products assay were used to verify the absence of residual toxicity, using the SOS-chromotest method. The control was incubated under the same conditions as the samples, and standard AFB<sub>1</sub> was prepared the same day as the assay. Table 2 presents the residual AFB<sub>1</sub> content of the degraded samples and the SOS Induction Factor (IF) values from the SOS-chromotest assay, as well as their corresponding percentages. IF values of *Streptomyces* cultures indicated that they were able to decrease the genotoxicity of 8 µg/mL of AFB<sub>1</sub> (IF = 7.13) to 32% of the control (IF = 2.26) for IX20, 28% (IF = 1.99) for IX45 and 31% (IF = 2.18) by MYC. Thus, IX45 caused the strongest decrease on genotoxicity. A common interpretation of IF values indicates that a value lower than 1.5 is not genotoxic. In this study, degradation IF values were superior to 1.5. However, a decrease of about a third on genotoxicity is validated in comparison to the control. The concentration on AFB<sub>1</sub> of the control, incubated under the same condition as the samples, and its corresponding IF decreased slightly (~15%) during the incubation period, as compared to the AFB<sub>1</sub> standard. Thus, about 15% of the observed genotoxicity decrease is due to an inherent degradation of AFB<sub>1</sub> during the incubation time.

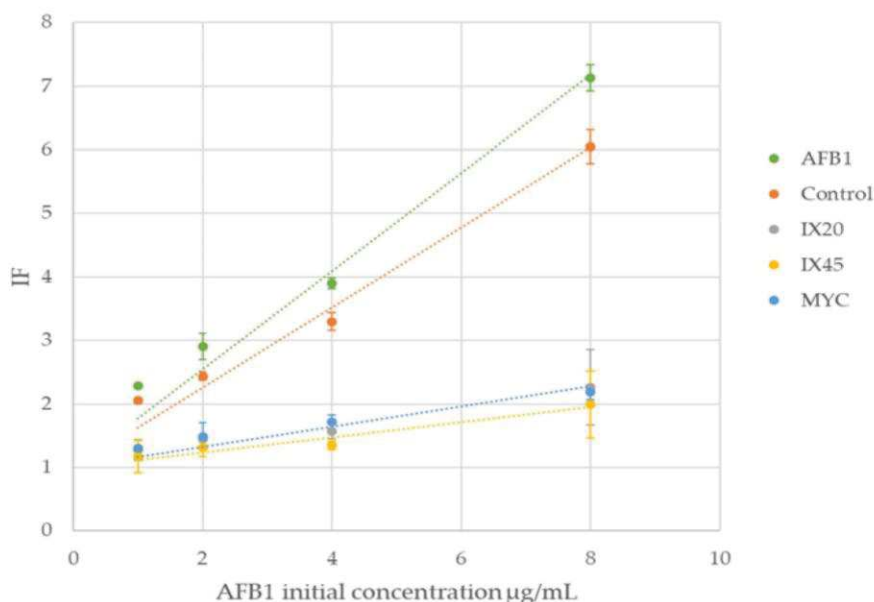


**Figure 6.** Mass Spectrometry spectral data for aflatoxin B<sub>1</sub> remaining after 12 days of incubation at 25°C and 180 rpm with *Streptomyces* isolates IX20, IX45 and MYC strain in CYB medium. Controls consisted of CYB with AFB<sub>1</sub>, incubated without bacteria, under the same conditions. Spectra were obtained for the extraction with two solvents: chloroform (top) and ethyl acetate (bottom). The peaks of the medium and samples without AFB<sub>1</sub> were subtracted as blanks.

**Table 2.** Evaluation of the residual toxicity of CYB supernatants issued of the degradation of 8 µg/mL of AFB<sub>1</sub> by *Streptomyces* isolates IX20, IX45 and MYC strain after 12 days at 25°C and 180 rpm.

Residual AFB <sub>1</sub> (µg/mL)	Standard AFB <sub>1</sub>	Control	<i>Streptomyces</i> isolate/strain Reduction of genotoxicity		
			IX20	IX45	MYC
	8	7.71 ± 0.22	0.98 ± 0.09	0.32 ± 0.04	1.95 ± 1.65
IF	7,13	6.05 ± 0.27	2,26 ± 0,60	1,99 ± 0,53	2,18 ± 0,11
% IF	100	85 ± 4	32 ± 8	28 ± 7	31 ± 2

The IF values for an AFB<sub>1</sub> standard, the control and the samples were plotted as a function of the AFB<sub>1</sub> initial concentration (Figure 7). The intrinsic degradation of the control during the incubation period, compared to the AFB<sub>1</sub> curve, is observed, as well as the stronger decreases on genotoxicity caused by the *Streptomyces* isolates and MYC, compared to the control and to the reference AFB<sub>1</sub> concentration.



**Figure 7.** Plot of the SOS Induction factors (IF) as a function of the initial concentration on AFB<sub>1</sub> during degradation, and residual toxicity assays of *Streptomyces* isolates IX20, IX45 and MYC strain after 12 days at 25°C and 180 rpm. Control consisted on CYB medium without bacteria incubated under the same conditions. Results are compared to the plot of the IF values of AFB<sub>1</sub> standard at the initial concentrations.

### 3. Discussion

In this study, fifty-nine *Streptomyces* isolates and *Streptomyces griseoviridis* strain from the commercial product Mycostop® were confronted *in vitro* to the mycotoxinogenic fungus *Aspergillus flavus*. The effect of *Streptomyces* isolates and their CFEs on fungal growth and aflatoxin specific production was evaluated, as well as their ability to degrade aflatoxin B<sub>1</sub>. A previous study on the effect of the same bacterial collection against the storage fungus *Penicillium verrucosum* and its ochratoxin A was already conducted [29]. As the preceding work brought out isolates with promising features, the collection was evaluated against *A. flavus*, another post-harvest wheat pathogen [11]. A comparison

between the effects observed on *A. flavus* and those previously published on *P. verrucosum* is presented at the Supplementary Material on the form of three heatmaps, one for growth (S3), one for mycotoxin specific production (S4) and the last one for mycotoxin degradation (S5).

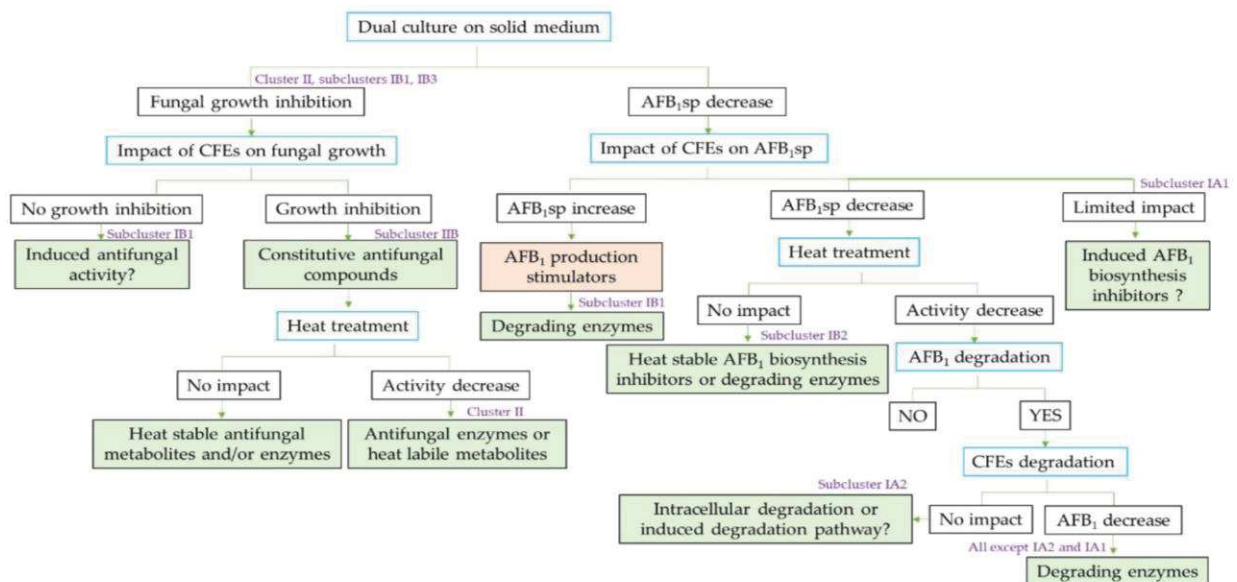
The research approach presented in this study is illustrated by the diagram in Figure 8, which constitutes a workflow towards the identification of the potential mechanisms involved in the interactions observed between the *Streptomyces* isolates and their metabolites, with the fungus and its toxins. Clustering and correlation methods allowed to highlight isolates with promising detoxification capacities, as well as to contrast their mode of action against the two saprophytic pathogens. This visualization, together with the generated workflow, constitutes a guideline for the selection of *Streptomyces* isolates according to the observed effects and the desired application. Furthermore, subsequent experiments, allowing the comprehension of interaction mechanisms between actinobacteria and fungi, will be based on the present general screening, in order to target the particular bioactive isolates identified during this work.

The first observation concerns the inhibition of fungal growth. *A. flavus* development was reduced by most *Streptomyces* isolates from cluster II during dual culture. The most promising strains in cluster II for fungal inhibition include IX50 and IX23. Furthermore, CFEs were significantly less efficient at the tested concentration. However, the results obtained from both procedures are not quantitatively comparable, as only a 10% ratio of CFEs was tested in this assay, and the concentration in active metabolites and enzymes could differ from the dual culture experiment. Nevertheless, various hypothesis can be drawn from the literature. Firstly, the culture environment could be involved, as dual cultures were performed on solid CYA, while CFEs production was conducted in liquid CYB. Indeed, it has been reported that aerial growth in solid media might impact *Streptomyces* metabolism [30]. The attenuated effect of CFEs in comparison to dual culture assays also suggests that bacterial antifungal metabolites and/or enzymes production could be positively induced by the presence of the fungus, as demonstrated by Wakefield et al. during the co-culture of *Aspergillus fumigatus* and *Streptomyces leeuwenhoekii* [31]. Interestingly, bacterial isolates able to decrease *A. flavus* growth in dual culture were also able to reduce *P. verrucosum* development (Figure S3) [29], which implies that growth reduction could also partly originate from a general inhibition mechanism such as hydrolytic enzymes, pH variation or competition. For instance, the production of chitinase is frequently described in the literature for the bacteria from the genus *Streptomyces* [12], [32], [33]. That being said, the heated CFEs efficacy was much lower than their non heated counterpart at inhibiting fungal growth, which comforts the assumption that antifungal enzymes might be implied, produced mainly by strains from cluster II. Furthermore, the remaining antifungal activity of the heated CFEs could be caused by the presence of thermally stable metabolites, as found by several research teams [34]–[36]. The pH increase due to *Streptomyces* metabolism, which in our case goes usually to 8.5 (data not shown), could also be at play in the growth inhibition observed. However, it cannot explain it fully as *A. flavus* growth in the 7–9 pH range is not strongly affected [37]. Finally, direct competition could also influence *A. flavus* growth [38], [39] but the limited repartition and growth of *Streptomyces* isolates on the agar plate during our procedure is unlikely to lead to nutrient exhaustion and subsequent limitation of the fungal development.

In a second step, the impact of *Streptomyces* isolates and their CFE on mycotoxins production and degradation have been evaluated. All isolates were able to decrease AFB<sub>1</sub> specific production to at least 54% of the control during the dual culture assay, particularly IX07 and IX31, which diminish it almost entirely (0.71% and 0.83% of the control, respectively). Moreover, no aflatoxin overproduction was observed when the *Streptomyces* isolates were confronted in dual culture, which contrasts with the previous results obtained with *P. verrucosum* (Figure S4), where strong fungal inhibitions were accompanied by a boost of OTA production [29]. The analysis of CFEs effect on AFB<sub>1</sub>sp, and degradation abilities of the isolates and their CFEs, can help in highlighting the various mechanisms potentially

involved in the observed reduction of AFB<sub>1</sub> during the dual culture assays. Three main hypotheses can be mentioned, which may occur individually or concomitantly: the change in the culture environment (nutrient availability, pH), the presence of inhibitors of AFB<sub>1</sub> pathway, and the degradation of the toxins by actinobacteria through intra or extracellular enzymes. These three hypotheses will be discussed further regarding the results of this work and the available literature.

Some research indicates that sugar (as main carbon source) and yeast extract utilization by aflatoxigenic fungi is linked to their aflatoxin biosynthesis [6], [40]. Thus, if *Streptomyces* isolates limit the nutrients in the medium, making them less available for the fungi, this might provoke the general reduction of aflatoxin production during dual cultures. However, the fact that some CFEs strongly reduced AFB<sub>1</sub>sp (e.g. IX34, IX06), allows to suggest other mechanisms. For instance, *Streptomyces* are well known for their production of active molecules such as Aflastatin A [20] Blastidicin A [41] and Dioctatin A [42], which were found to repress aflatoxin regulation genes, as proposed by Verheecke et al. [18]. Another study by Caceres et al. elucidated that the whole AFB<sub>1</sub> gene cluster of *A. flavus* was down regulated, when exploring the impact of its dual culture with *S. roseolus* [22]. In cluster IA1, where the degradation abilities were weak as compared to the decrease on AFB<sub>1</sub>sp production in the dual culture assay, a repression mechanism seems likely to occur. Furthermore, as mentioned before, most actinobacteria strains raise the pH in the culture medium during growth, which could also inhibit aflatoxin biosynthesis [43], as demonstrated by Keller et al. who found that 10-fold decreases on sterigmatocystin and aflatoxin production by *Aspergillus* spp. were caused at pH 8 [44]. Some CFEs greatly enhanced the AFB<sub>1</sub>sp in cluster IB1 (IX54, IX05 and MYC) showing that some metabolites produced by *Streptomyces* isolates can stimulate the AFB<sub>1</sub> pathway, possibly by triggering a defense mechanism from *A. flavus* [45]–[47]. This implicates that the metabolites produced by these strains must not be applied for biocontrol purposes, as they may increase mycotoxin accumulation. However, they may be interesting for the study of mycotoxin elicitation [48], [49]. A stimulation of aflatoxin biosynthesis by *Aspergillus flavus* due to bacterial biocontrol agents has been previously reported [50].



**Figure 8.** Workflow of the presented study for the preliminary identification of hypothetical antagonistic mechanisms of *Streptomyces* isolates against *Aspergillus flavus* development and AFB<sub>1</sub> accumulation. Blue colored rectangles indicate the different experimental stages, and filled rectangles (green, orange) indicate putative mechanisms. Examples of subclusters exhibiting the described mechanism are indicated in purple.

The majority of the studied *Streptomyces* isolates were able to degrade AFB<sub>1</sub>, either in solid and liquid medium or by their CFEs. This is in agreement with the findings of Eshelli et al. whose degradation assays with three actinobacteria strains proved that all of them were able to significantly degrade AFB<sub>1</sub> [14]. In general, aflatoxins degradation by actinobacteria has been demonstrated by several authors [26], [51], [52]. The observed reduction of aflatoxin by bacterial cells is unlikely to be due to a cell wall binding, as extraction protocol includes methanol and sonication, which are used for pellet washing and mycotoxin detaching [53]. In addition, previous research has showed that aflatoxin cell wall binding by *Streptomyces* is not significant in most cases [51]. Solid medium seems to slightly favorize degradation, with an average reduction down to 33% of the control (43% for liquid medium). However, degradation ability of bacteria cultivated in liquid and solid media remains strongly correlated ( $r=0.8$ ,  $p<.001$ ). This suggests that the differentiated metabolism followed by *Streptomyces* isolates could be influenced by culture environment [30], [54], but is still rather similar in both conditions regarding AFB<sub>1</sub> degradation. By contrast, degradation of OTA by the same *Streptomyces* isolates was scarce on solid medium, whereas it was largely spread in liquid medium (Figure S5) [29], which highlights the differences in the degradation mechanisms involved for both mycotoxins. Of note, *Streptomyces* isolates that were efficient at degrading OTA in liquid medium were also capable of degrading AFB<sub>1</sub> in both liquid and solid environment (Figure S5). The strong AFB<sub>1</sub> degradation capacity of numerous *Streptomyces* isolates such as MYC, IX28, IX20 and IX03 on solid media, is probably one of the main mechanisms regarding the reduction of mycotoxin specific production during dual cultures. The counterintuitive observation in cluster IB1 that CFEs could enhance AFB<sub>1</sub>sp accumulation, while the dual culture assay revealed a decrease of AFB<sub>1</sub>sp, can probably find its explanation in the strong degradation abilities found in this cluster.

A remarkable mechanism was involved in subcluster IA2, where the *Streptomyces* isolates and their CFEs were capable of decreasing AFB<sub>1</sub> amount without affecting *A. flavus* growth, a particularity that can be interesting for ecological niche preservation purposes. Yet, inhibition of fungal development can be desired during storage, as grain quality is strongly affected [55].

The fact that AFB<sub>1</sub> was also degraded in CFEs points towards the presence of constitutive extracellular degrading enzymes, produced during submerged culture, rather than a potential binding to cells. This is comforted by the positive correlation between AFB<sub>1</sub> degradation by *Streptomyces* isolates in liquid CYB and by their CFEs, produced in CYB as well ( $r=0.5$ ,  $p=.002$ ). AFB<sub>1</sub> degradation by extracellular extracts of actinobacteria was already described by Alberts et al. when studying the degradation capacity of *Rhodococcus erythropolis* [56]. As elucidated by Taylor et al., aflatoxin degrading actinobacteria are able to catalyze the reduction of the double bond of the  $\alpha,\beta$ -unsaturated ester moiety of aflatoxins thanks to enzymes using the F<sub>420</sub>H<sub>2</sub> deazaflavin cofactor, leading to spontaneous hydrolysis and detoxification [57]. This mechanism could be widespread among actinobacteria as proposed by Lapalikar et al. [58], and it might apply for the isolates in this study. The capacity of nearly all of the tested CFEs to degrade AFB<sub>1</sub> opens new perspectives for further research to isolate and identify the degrading enzymes involved, and to determine the mechanisms at play. Isolates IX09, MYC and IX45 seem particularly interesting for this purpose.

As already observed for antifungal activity, a thermal treatment of CFEs resulted in weakened detoxification capacities, suggesting the presence of labile mycotoxin degrading enzymes or metabolites. However, the activity of heated CFEs was not completely eliminated, which may indicate the presence of heat resistant compounds and/or enzymes. A complementary method for the confirmation of enzymatic activities in CFEs could be the use of proteinase K, as described by Guan et al. when evaluating the cell free extract of *Stenotrophomonas maltophilia* [59]. This will help at confirming the results obtained with CFEs heat inactivation, as some AFB<sub>1</sub> degrading enzymes have been found to be heat-stable [36], [60]. Finally, a comparison between the activities of intra and extracellular extracts could help to identify the location of the mycotoxin degrading compounds [61]. These two approaches

could be applied on a selection of isolates for an in-depth investigation of the mechanism of degradation and for the subsequent isolation of active compounds, as demonstrated by several research groups [57], [59], [62], [63].

A common bacterial degradation mechanism of AFB<sub>1</sub> consists in the cleavage of the lactone ring, which may lead to the formation of aflatoxin D<sub>1</sub> and D<sub>2</sub> [14], [64]. This cleavage is accompanied by a loss of fluorescence so these compounds cannot be detected by Fluorescence HPLC [65]. As no fluorescent breakdown products were observed in our assay, further search for degradation by-products was conducted by HPLC-MS with two major degrading strains (IX20, IX45) and MYC. During the search for AFB<sub>1</sub> degradation by-products, the peaks of two unknown compounds were observed only for the MYC MS spectra, corresponding to a first compound of formula C<sub>10</sub>H<sub>10</sub>NO (*m/z* 160.0770) and to a second of formula C<sub>10</sub>H<sub>10</sub>NO<sub>2</sub> (*m/z* 176.0720). These molecules are neither present in the mass spectrum of the control nor in the samples grown without AFB<sub>1</sub> addition, which suggests that they were produced during AFB<sub>1</sub> degradation. Additionally, they do not correspond to an already described degradation by-product, and the mechanism of their production was not elucidated. Several unknown breakdown products were identified by Iram et al. when studying the degradation of AFB<sub>1</sub> and AFB<sub>2</sub> by *Corymbia citriodora* [66], by *Ocimum basilicum* and *Cassia fistula* [67], and by *Trachyspermum ammi* [68]. Similarly, some residual products of AFB<sub>1</sub> degradation were described by Eshelli et al., namely, C<sub>17</sub>H<sub>15</sub>O<sub>7</sub> (*m/z* 331.2845), C<sub>16</sub>H<sub>15</sub>O<sub>5</sub> (*m/z* 287.2220) and C<sub>13</sub>H<sub>17</sub>O<sub>4</sub> (*m/z* 237.1123) found after 72 hours of incubation with actinobacteria [14]. The by-products discovered by Iram et al. and Eshelli et al. have a higher molecular weight than the compounds found in this study, this may suggest that after 12 days of culture, known degradation by-products could further be degraded into smaller unknown molecules or the occurrence of a different degradation mechanism. For IX20 and IX45 no degradation by-products were detected in both extraction solvents. This finding is similar to the one of Alberts et al. who were unable to detect degradation by-products by HPLC-MS when studying aflatoxin degradation by *Rhodococcus erythropolis* [56]. Regarding the presence of an AFB<sub>1</sub> isomer –also observed for the control–, no clear explanation was found, other than the theory of a transitional conformation variation during the incubation.

Residual toxicity evaluation by the SOS-chromotest showed that the three isolates tested were able to decrease the genotoxicity of the sample down to about 30% compared to the control. Harkai et al. determined that *Streptomyces cacaoi subsp. asoensis* was able to reduce genotoxicity of AFB<sub>1</sub>, at an initial concentration of 1 µg/mL, as it decreased the SOS induction factor from 2.25 to 1.37 [51]. In the present study, an initial concentration of 4 µg/mL of AFB<sub>1</sub> could be detoxified from an initial IF of 3.9 to a non-genotoxic level (IF<1.5) by the *Streptomyces* isolate IX45, while isolates IX20 and MYC could eliminate the genotoxicity of an initial AFB<sub>1</sub> concentration of ~3 µg/mL (IF=3.32). However, the percentage of decrease on genotoxicity were slightly lower than the percentage of reduction on AFB<sub>1</sub>, which was of 87% for IX20, 96% for IX45 and 75% for MYC. This could indicate that even if the aflatoxin is degraded, some resulting by-products might remain genotoxic to a certain degree, which emphasizes the importance of the verification of the residual toxicity.

#### 4. Conclusions

In conclusion, several *Streptomyces* isolates were able to inhibit *Aspergillus flavus* growth, and most importantly, the entire collection was able to decrease the amount of aflatoxin B<sub>1</sub> accumulated during fungal growth, and also to degrade the mycotoxin, at different rates. Most CFEs also decreased aflatoxin amount, either during fungal growth or by posterior degradation, which is promising for the research and isolation of active enzymes and metabolites. As *Streptomyces* strains are not classified as GRAS (Generally Recognized as Safe) microorganisms, their direct application is not authorized. Nevertheless, they constitute a promising source of mycotoxin degrading enzymes or mycotoxins' metabolic pathway inhibitors for the detoxification of food matrices. A verification of residual toxicity during detoxification assays is imperative to guarantee that the resulting by-products do not represent any risk. The approach

presented in this study allows the evaluation of potential biocontrol agents by their antifungal and detoxifying capacities, as well as their clustering and classification according to common features and the comparison to their effect on different parameters. More importantly, this procedure leads to the identification and selection of promising candidates for subsequent research. The dissimilarities on the effect of *Streptomyces* isolates on AFB<sub>1</sub> and OTA could be related to several factors, such as the different degradation mechanisms involved, distinct enzymes with different optimal conditions, particular functions of each mycotoxin for the fungus, and so on. Further studies on the bioactive isolates identified during this work will allow a better understanding of these mechanisms.

## 5. Materials and Methods

### 5.1. *Streptomyces* Isolates

Fifty-nine *Streptomyces* isolates were retrieved from organic amendments and soil samples collected at the Hérault department in the South of France. A 16S preliminary identification demonstrated that all the isolates in the collection belonged at 97% to the genus *Streptomyces* (data not shown). The isolates constitute a collection maintained in the laboratory of the UMR QualiSud at the University of Montpellier. Mycostop® (MYC) strain *Streptomyces griseoviridis* K61 was included to the tests as a commercialized biocontrol agent for comparison. The isolates were grown on ISP4 medium (10 g/L starch, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 1 g/L MgSO<sub>4</sub>, 2 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/L CaCO<sub>3</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L MgCl<sub>2</sub>, 1 mg/L ZnSO<sub>4</sub>, 18 g/L bacteriological agar) for 11 days at 28°C. Spores were then collected by scrapping the surface of the Petri dish with 5 mL of distilled water + 0.01% Tween 20 and filtered through sterile cotton. The spore suspensions were aliquoted then stored at -80°C.

### 5.2. *Streptomyces* Isolates Spore Numeration by Flux Cytometer

For *Streptomyces* isolates spore count, a Novocyte ACEA flux cytometer was employed. After counting, the results were validated by colony forming units on CYA medium after 11 days of culture. Before each test, bacterial spore suspensions were unfrozen and diluted to a concentration of 10<sup>7</sup> spores/mL.

### 5.3. Pathogen Strains

A mycotoxinogenic strain of *Aspergillus flavus* E73/NRRL62477 kindly provided by Dr. Olivier Puel from INRAE's UMR Toxalim was employed to carry out this work. Fungus was maintained on PDA (Biokar BK095HA) inclined tubes covered in paraffin oil prior to use. For fungal spore harvesting, pathogen was grown on PDA plates for 7 days at 25°C, then spores were scrapped from the surface by adding 10 mL of distilled sterile water and filtered through sterile cotton. Pathogen spores were harvested and enumerated before each test.

### 5.4. Antagonistic Evaluation In Vitro

#### 5.4.1. *Streptomyces* isolates cells in dual cultures on solid medium

Antagonistic assays were performed on Czapek Yeast Agar medium (CYA: 30 g/L sucrose, 5 g/L yeast extract, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.3 g/L NaNO<sub>3</sub>, 0.05 g/L KCl, 0.05 g/L MgSO<sub>4</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L ZnSO<sub>4</sub>, 0.5 mg/L CuSO<sub>4</sub>, 15 g/L agar, pH ~7.4) which allowed both pathogen and bacteria proper growth and sporulation.

Dual culture assays were implemented on Petri dishes by inoculating 10 µL of the bacterial isolate spore suspension at 10<sup>7</sup> CFU/mL on each side of the plate. After three days at 25°C, 10 µL of the pathogen spore suspension at 10<sup>6</sup> CFU/mL were inoculated at the center of the plate and left for 5 more



days at 25°C before image analysis and mycotoxin extraction. As a control, the pathogen was inoculated on a CYA plate without bacteria. All tests were done in triplicate.

#### 5.4.2. Cell Free Extracts of liquid cultures

*Streptomyces* isolates were cultured on Czapek Yeast Extract broth (CYB: 30 g/L sucrose, 5 g/L yeast extract, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.3 g/L NaNO<sub>3</sub>, 0.05 g/L KCl, 0.05 g/L MgSO<sub>4</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L ZnSO<sub>4</sub>, 0.5 mg/L CuSO<sub>4</sub>, pH ~7.4) medium for 5 days at 25°C and 180 rpm. Then liquid cultures were centrifuged at 10,000×g for 5 minutes and the supernatant was filtered through a PES filter at 0,22 µm to eliminate bacterial cells. The resulting cell free extracts (CFE) were used to evaluate the effect of bacterial metabolites on fungal pathogen's growth and mycotoxin production. In order to identify enzymatic activities, CFE were also tested after a heat treatment at 100°C for 10 min.

#### 5.4.3. Pathogen surface growth measurement and AFB<sub>1</sub> Specific Production Calculation

Surface growth of *A. flavus* colonies was measured in ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018). Growth inhibition was established in comparison with the control without bacteria which represented 100% of growth. Results are given in % of control.

$$\text{Growth ratio(\% of control)} = \frac{\text{Assay area (cm}^2\text{)}}{\text{Control area (cm}^2\text{)}} \times 100$$

AFB<sub>1</sub> specific production (AFB<sub>1</sub>sp) was calculated by dividing the mycotoxin amount in the whole sample by the area of the fungal colony. This calculation allows to compare the mycotoxin accumulation in the medium normalized to the fungal surface.

$$\text{AFB}_1 \text{ specific production (AFB}_1\text{sp)} = \frac{\text{Amount of mycotoxin produced (ng)}}{\text{Colony area (cm}^2\text{)}}$$

The percentage of reduction for AFB<sub>1</sub> specific production was calculated in comparison to the control without bacteria.

$$\text{AFB}_{1\text{sp}} \text{ ratio (\% of control)} = \frac{\text{AFB}_{1\text{sp}} \text{ assay}}{\text{AFB}_{1\text{sp}} \text{ control}} \times 100$$

### 5.5. Mycotoxin Degradation Assay

#### 5.5.1. Screening of AFB<sub>1</sub> degradation by *Streptomyces* isolates and their CFEs

Degradation assays were performed using Aflatoxin B<sub>1</sub> (Sigma Aldrich, St. Quentin Fallavier, France and Libios, Vindry sur Turdine, France) suspended in acetonitrile in order to prepare a stock solution of 1 mg/mL. Control concentration was of 2 µg/mL. Mycotoxin was added to solid CYA and liquid CYB medium, followed by the addition of 20 µL of bacterial isolate spore suspension at 10<sup>7</sup> spores/mL and incubated at 25°C with an agitation at 180 rpm for the liquid cultures. After 10 days on solid medium and 5 days in liquid medium, mycotoxin was extracted and analyzed as further described. CFEs, produced as described before, were also tested for their ability to degrade AFB<sub>1</sub> after being in contact with the mycotoxin for 48 hours at 25°C and 180 rpm. As control, mycotoxin was spiked in solid CYA or liquid CYB medium and incubated following the same incubation protocols as the samples.

$$\text{AFB}_1 \text{ degradation (\% of control)} = \frac{\text{AFB}_{1\text{ng/ml}} \text{ assay}}{\text{AFB}_{1\text{ng/ml}} \text{ control}} \times 100$$

#### 5.5.2. Degradation assays for the search of breakdown products

Three isolates with strong degrading abilities on liquid medium (IX20, IX45 and MYC) were selected to perform additional degradation assays. For this, they were cultured for 12 days in 20 mL of CYB medium containing 8 µg/mL of AFB<sub>1</sub> inoculated with 100 µL of a bacterial spore suspension at 1\*10<sup>7</sup> spores/mL and incubated 12 days at 25°C and 180 rpm. The controls consisted on CYB medium with the same concentration of AFB<sub>1</sub> and without the bacteria inoculum, and bacteria cultured in CYB without AFB<sub>1</sub>, both incubated under the same conditions as the main test. These controls allowed to subtract the spectra belonging to bacterial metabolites and culture medium to the spectra obtained during degradation, in order to identify the remaining peaks. All tests were done in triplicate. Several known break up products of AFB<sub>1</sub> degradation such as aflatoxicol, aflatoxin B2a and D aflatoxins were searched on HPLC-MS.

### 5.6. Mycotoxin and Degradation By-products Analysis

#### 5.6.1. Sample extraction

For the mycotoxin extraction of assays with antagonistic strains and CFEs, the whole content of the Petri dish was transferred into a plastic container then cut into small pieces with a scalpel. After weighing the agar, 100 mL of acidified Methanol (3.85% formic acid) were added to the sample followed by 20 min of agitation at 250 rpm. 500 µL of the mixture were evaporated in a Speed Vac (Eppendorf® AG, Hamburg, Germany) at 60°C until dryness and then 2 mL of the mobile phase (water/ methanol 55:45 (v/v) with 119 mg Kbr (0.001 M Kbr) and 350µL of 4M nitric acid) were added. To ensure that mycotoxin was not bind by the cells, samples underwent 20 minutes of sonication, followed by 10 minutes in a cell disruptor and finally thoroughly mixed in a vortex before filtering through a 0.45 µM PTFE filter into a glass vial for HPLC analysis.

For the search of degradation by-products, the resulting supernatants were mixed with equivalent volumes of chloroform or ethyl acetate by agitating vigorously for 20 minutes. Then, the organic phase was retrieved and filtered through a 0.45 µM PTFE filter into a glass vial for HPLC-MS analysis.

#### 5.6.2. AFB<sub>1</sub> quantification by fluorescence HPLC

AFB<sub>1</sub> was quantified by HPLC using a fluorescence detector (Shimadzu RF 20A, Japan) after post-column electrochemical derivatization (Kobra Cell™ R. Biopharm Rhône Ltd, Glasgow, UK). The operating conditions were as follows: injection volume of 100 µL; C18 reverse-phase HPLC column, Uptisphere type 5 ODB, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 40°C; isocratic flow rate of 0.8 mL/min (water/ methanol 55:45 (v/v) with 119 mg Kbr (0.001 M Kbr) and 350µL of 4M nitric acid). Excitation wavelength of 360 nm and emission wavelength of 450 nm. The concentrations were calculated from a calibration curve established from an AFB<sub>1</sub> standard (25 µg/mL; Biopharm Rhône Ltd, Glasgow, UK) [59]. Detection and quantification limits were established at 0.05 and 0.2 ng/mL respectively.

#### 5.6.3. Search for degradation by-products by HPLC-MS

For the search of degradation by-products, the chromatographic apparatus consisted of an Acquity H-Class UPLC (Waters, Milford) equipped with a quaternary pump, solvent degasser, and thermostatted column compartment. A reversed-phase column was used for separation: Kinetex C18 (2,1 x 100 mm 1,7 µm from Phenomenex). Mobile phase A and B consisted of water (0,1% formic acid) and acetonitrile (0,1% formic acid) respectively. The 15 min linear gradient program used was 5–50% B over 8 min, then 50-100%B in 3 minutes, decreasing from 100%–10% B in 0,1 min, followed by a 3,9 min post-run isocratic step at 5% B to re-equilibrate the column. The flow rate was constant at 0.5 mL/min at 25 °C.

Mass Spectrometry detections were carried out using the Waters UPLC system described above coupled to a Synapt G2-S mass spectrometer (Waters, Milford) operating in positive ion mode. Mass spectra were recorded between 50 to 2000 Da. The following parameters were used for all experiments: capillary voltage 3 kV; sampling cone 20V; desolvation temperature 450 °C; source temperature 140 °C.

### 5.7. Evaluation of Residual Toxicity by SOS-Chromotest

The remaining liquid cultures of the two isolates (IX20, IX45) and MYC, selected for the research of degradation by-products were analyzed to determine if the degradation lead to an effective detoxification of the mycotoxin. At the end of the incubation period, a fraction of the samples was extracted as described previously and analyzed by Fluorescence HPLC to determine the decrease in AFB<sub>1</sub> and by HPLC-MS for the search of degradation by-products. Then, the remaining fraction of the liquid culture was filtered through a hydrolyzed PTFE filter to obtain cell free extracts (CFEs), which were evaluated for residual toxicity using an SOS-Chromotest.

The SOS-Chromotest is a colorimetric assay based on the response of a mutant strain of *Escherichia coli* PQ37 to the DNA damage caused by genotoxic compounds. In order to differentiate direct and indirect genotoxic activity, the test uses S9 rat-liver homogenate to identify compounds that require metabolic bioactivation to exhibit a genotoxic reaction. In addition to the measure of β-galactosidase induction, the SOS-Chromotest allows the measure cell survival by means of alkaline phosphatase as a toxicity assay [26]–[28]. The SOS-Chromotest Kit was purchased from Environmental Bio-Detection Products Inc., Canada.

Preliminary tests (not shown) allowed us to determine that the minimal concentration of AFB<sub>1</sub> needed to trigger a genotoxic effect on the *E. coli* test strain was of 2 µg/mL. Thus, AFB<sub>1</sub> concentration for the search of degradation by-products and the posterior evaluation of residual toxicity was increased to 8 µg/mL.

The assay was performed on 96-well flat bottom plates. Using an Enspire Multimode plate reader (Perkin Elmer, Waltham, MA, USA). Positive controls for direct and indirect genotoxicity were respectively 4-nitroquinoline 1-oxide (4-NQO) and 2-amino-anthracene (2-AA) in two-fold dilutions. The background and negative control contained 10% DMSO (dimethyl sulfoxide) in sterile 0.85% saline. Ten microliters of the CFEs obtained as described before were transferred to the corresponding wells to obtain a series of two-fold dilutions. Finally, 100 µL of an overnight culture of the test *E. coli* strain (final OD<sub>600</sub> = 0.5) were added to the control and degradation assay samples and incubated for 2 hours until color development. After the incubation period, absorbances were read at 600 nm to determine β-galactosidase production and at 420 nm to determine viability of the bacteria.

For the analysis of the results the Induction Factor (IF) was calculated as follows:

$$IF = \frac{(OD_{600,i} - OD_{600,B}) / (OD_{600,N} - OD_{600,B})}{(OD_{420,i} - OD_{420,B}) / (OD_{420,N} - OD_{420,B})}$$

Where:

$OD_{600,i}$  = Absorbance at 600 nm for sample wells.

$OD_{600,B}$  = Average absorbance at 600 nm for reagent blank wells.

$OD_{600,N}$  = Absorbance at 600 nm for negative control wells.

$OD_{420,i}$  = Absorbance at 420 nm for sample wells.

$OD_{420,B}$  = Average absorbance at 420 nm for reagent blank wells.

$OD_{420,N}$  = Absorbance at 420 nm for negative control wells.

As a general classification, a SOSIF lower than 1.5 is not genotoxic, between 1.5 and 2 is inconclusive and higher than 2 is genotoxic.

### 5.8. Data Analysis and Data Visualization

Preprocessing of the data for the heatmap consisted of a conversion of percentages to Z-scores calculated as follows:  $Z - score = (X - \mu) / \sigma$ , where  $X$  is the measurement,  $\mu$  is the mean value of the effect of all isolates in a category (growth, mycotoxin specific production, mycotoxin degradation), and  $\sigma$  is the standard deviation of the value of the effect of all isolates in a category. Boxplots and Pearson correlation graphics were developed in R.

### Supplementary Materials:

**Author Contributions:** I.C.-A., A.C.d.I.N., and S.S.-G. conceived, supervised, and designed the experiments. I.C.-A. performed all experiments. I.C.-A. and A.C.d.I.N. analysed the data. V.M. assisted in the mycotoxin extraction from agar plates and microbiological manipulation. N.D. executed the HPLC analysis of mycotoxins. G.C. performed the HPLC MS analysis and the search of degradation by-products. S.S.-G., A.C.d.I.N., A.F., and C.S. were in charge of funding acquisition and project administration. I.C.-A., A.C.d.I.N., and S.S.-G. interpreted the data. The manuscript was written by I.C.-A. Revised by A.C.d.I.N. and S.S.-G. All authors have read and agreed to the published version of the manuscript.

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Supplementary material S1

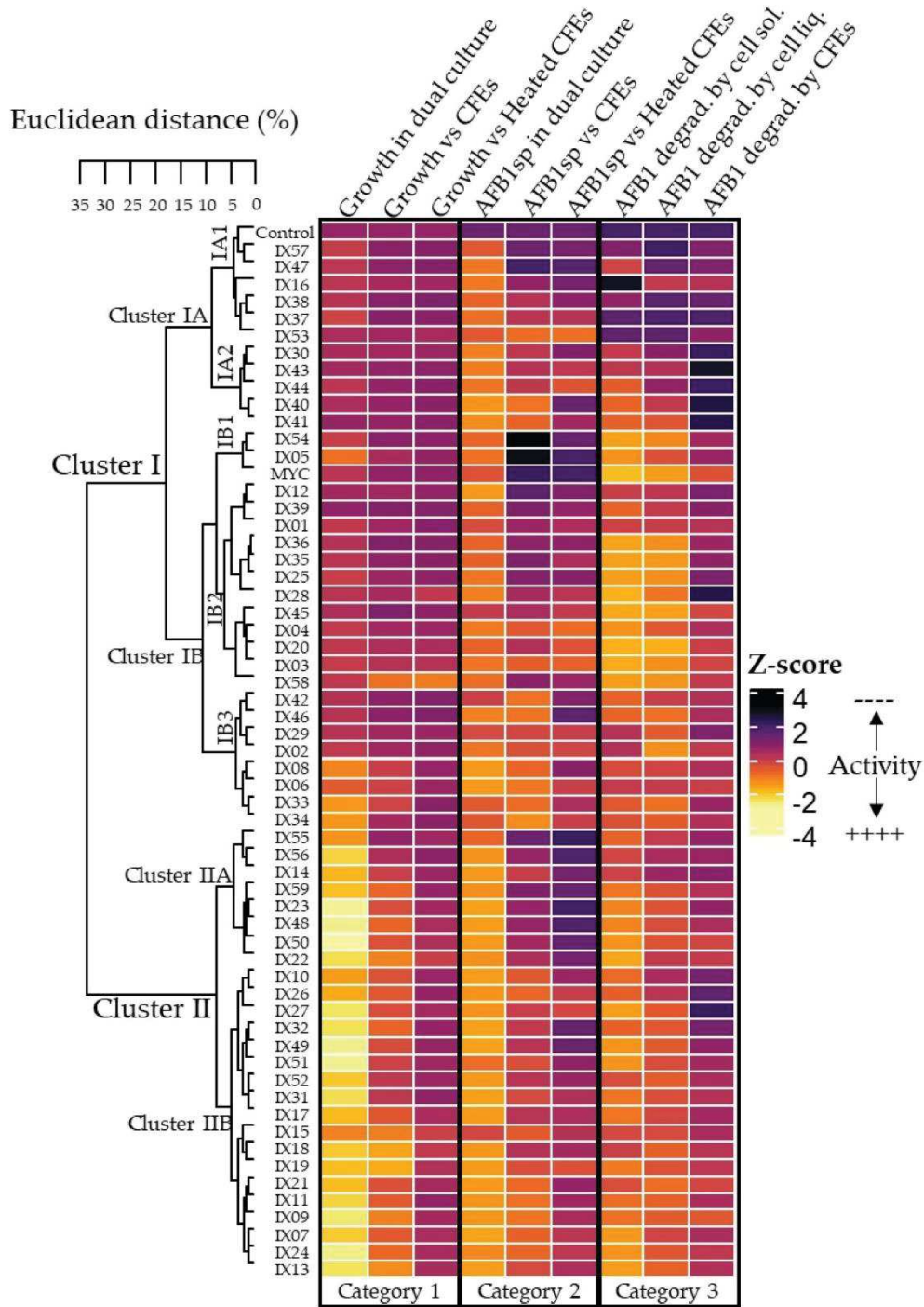


Figure S1. Heatmap of the effect of *Streptomyces* isolates and their CFEs on *Aspergillus flavus* growth (Category 1) and AFB<sub>1</sub> accumulation (Category 2), along with the bacteria's ability to degrade AFB<sub>1</sub> in solid and liquid medium and by their CFEs (Category 3). Results are given in a range of colours according to their Z-score, where purple to black represents a lack of activity or an increase compared to the control, whereas yellow represents the strongest activity.

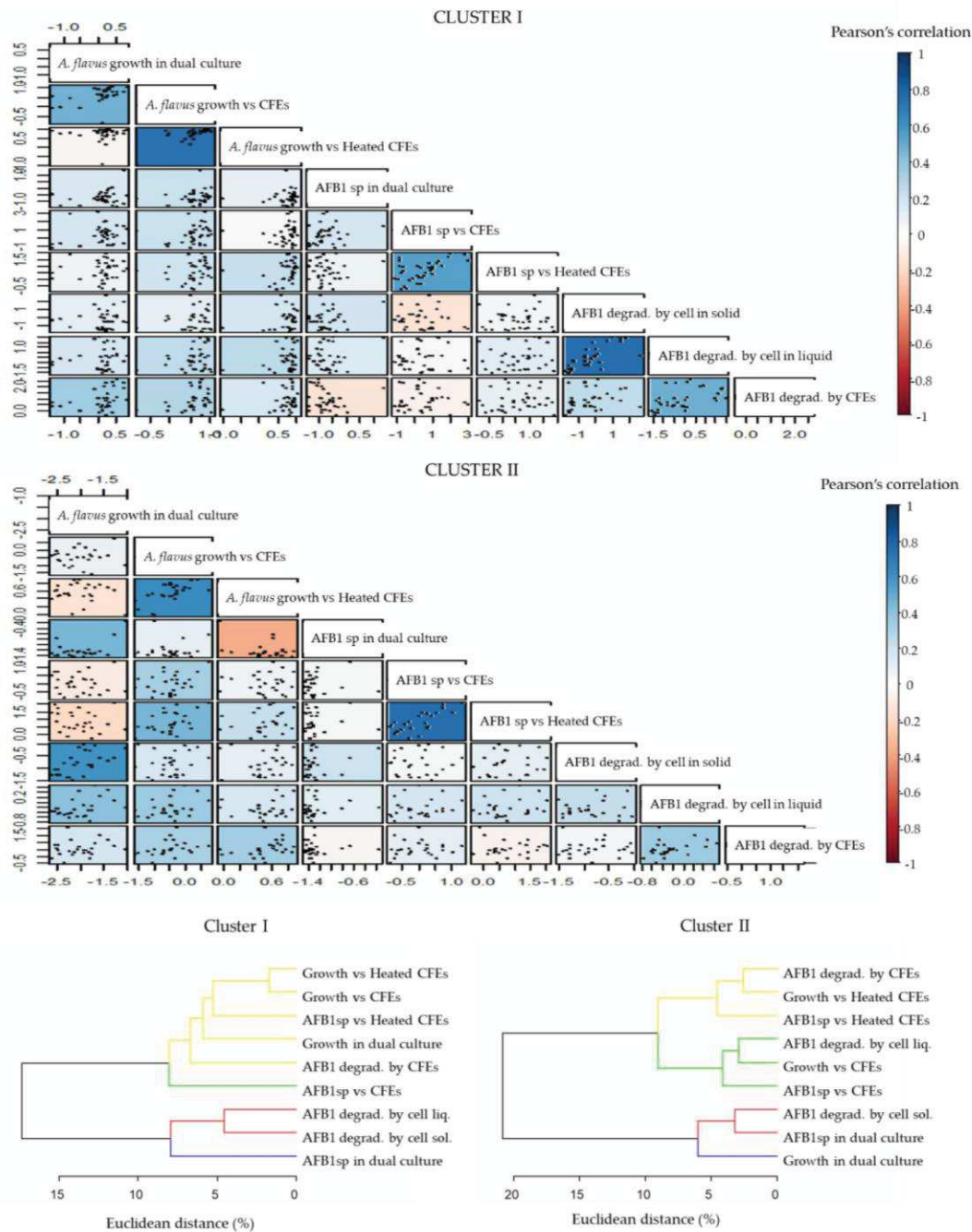


Figure S2. Pearson's correlation of the effects of *Streptomyces* isolates and their CFEs on *Aspergillus flavus* growth and AFB<sub>1</sub> accumulation, along with the degradation by bacteria in solid and liquid medium, and the degradation by their CFEs. The correlation scale goes from 1 in blue which indicates a strong positive correlation, to -1 in dark red that represents a strong negative correlation. The correlations were calculated for the isolates of each main cluster of the heatmap S1 (Cluster I and Cluster II). Values and significance are presented in supplementary Table S1. Dendrograms at the bottom show the relationship and proximity in % of Euclidean distance of each of the studied parameters for Clusters I and II. Different groups are indicated by different colours.

Table S1. Values of the Pearson correlation ( $r$ ) between the measured parameters during the screening of *Streptomyces* isolates and Mycostop® and their CFEs on *A. flavus* growth and AFB1 accumulation.

	GDC	GCFE	GHCFE	AFBDC	AFBCFE	AFBHCFE	AFBDS	AFBDL	AFBDCFE	GDC
	1									
	0.5 (.003)**	1								GDC
	0 (.91)	0.7 (<.001)***	1							GCFE
	0.2 (.37)	0.2 (.18)	0.1 (.64)	1						GHCFE
<b>Cluster I</b>	0.2 (.32)	0.2 (.20)	0 (.99)	0.2 (.32)	1					AFBDC
	0 (.79)	0.2 (.23)	0.2 (.17)	0 (.80)	0.6 (<.001)***	1				AFBCFE
	0.1 (.57)	0.1 (.58)	0.2 (.31)	0.2 (.27)	-0.1 (.43)	0 (.91)	1			AFBHCFE
	0.2 (.32)	0.3 (.14)	0.3 (.13)	0.1 (.39)	0 (.98)	0.1 (.51)	0.8 (<.001)***	1		AFBDS
	0.4 (.04)*	0.3 (.09)	0.2 (.33)	-0.1 (.46)	0 (.85)	0.1 (.67)	0.3 (.14)	0.5 (.002)**	1	AFBDL
										AFBDCFE
	1									GDC
	0.1 (.75)	1								GCFE
	-0.1 (.51)	0.6 (<.001)***	1							GHCFE
	0.5 (.02)*	0.1 (.70)	-0.4 (.07)	1						AFBDC
<b>Cluster II</b>	-0.1 (.71)	0.3 (.09)	0 (.83)	0 (.93)	1					AFBCFE
	-0.2 (.34)	0.5 (.02)*	0.2 (.25)	0 (.95)	0.8 (<.001)***	1				AFBHCFE
	0.6 (.0012)**	0.2 (.41)	0.1 (.63)	0.2 (.29)	0 (.96)	0.1 (.63)	1			AFBDS
	0.4 (0.03)*	0.4 (.07)	0.2 (.43)	0.1 (.60)	0.2 (.40)	0.2 (.28)	0.2 (.22)	1		AFBDL
	0.2 (.38)	0.3 (.10)	0.3 (.08)	0 (.96)	0.1 (.66)	0 (.88)	0 (.92)	0.4 (.07)	1	AFBDCFE

GDC = *A. flavus* growth in dual culture, GCFE = *A. flavus* growth vs Cell Free Extracts (CFEs), GHCFE = *A. flavus* growth vs Heated CFEs, AFBDC = AFB1 specific production (sp) in dual culture, AFBCFE = AFB1sp vs CFEs, AFBHCFE = AFB1sp vs Heated CFEs, AFBDS = AFB1 degradation by cells in solid medium, AFBDL = AFB1 degradation by cells in liquid medium, AFBDCFE = AFB1 degradation by CFEs. P-values are indicated in the parenthesis. \* P<.05, \*\* P<.01, \*\*\* P<.001

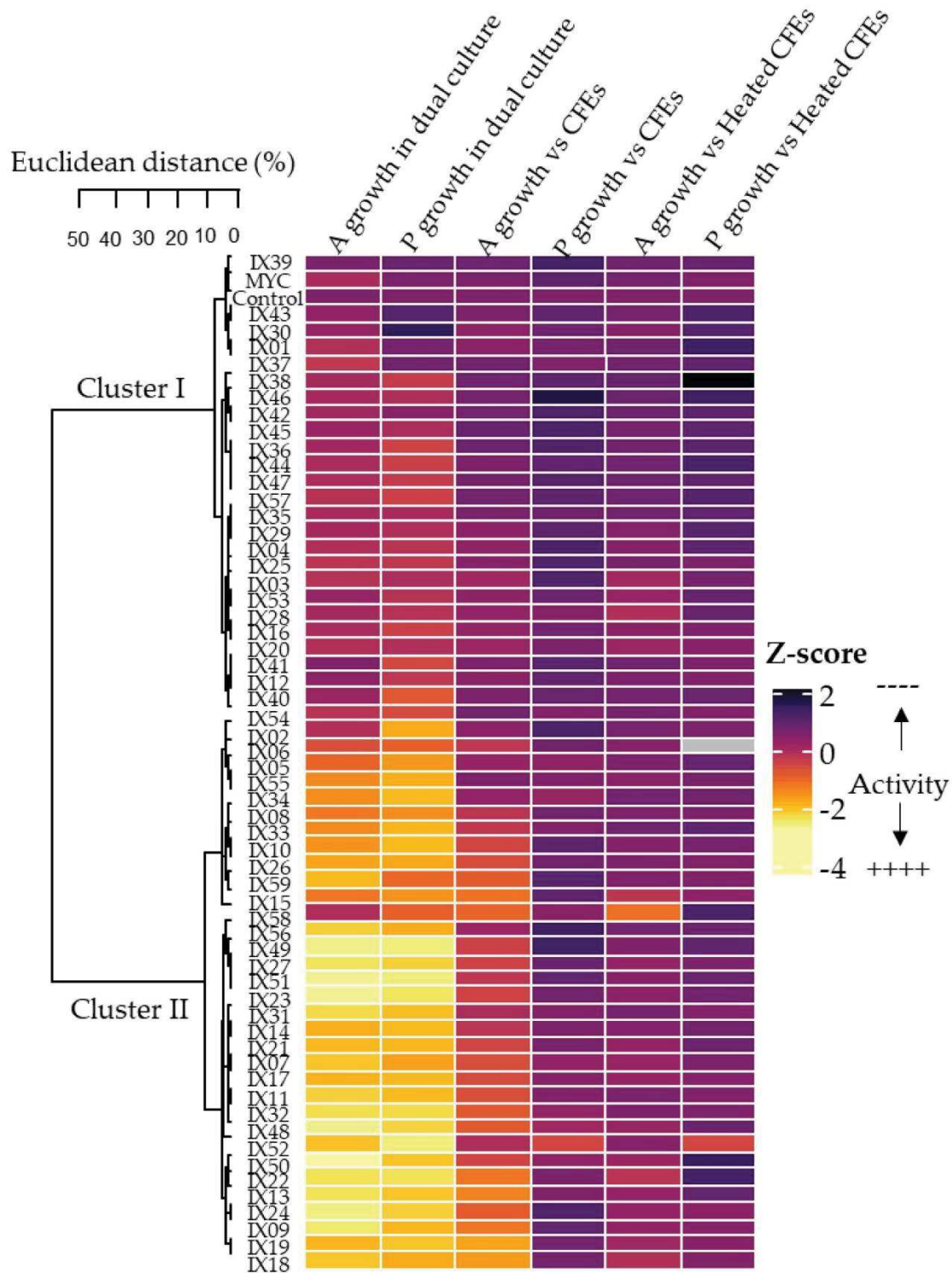


Figure S3. Heatmap of the effect of *Streptomyces* isolates and their CFEs on *Aspergillus flavus* (A) and *Penicillium verrucosum* (P) growth. Results are given in a range of colours according to their Z-score, where purple to black represents a lack of activity or an increase compared to the control, whereas yellow represents the strongest activity. Grey = missing value.

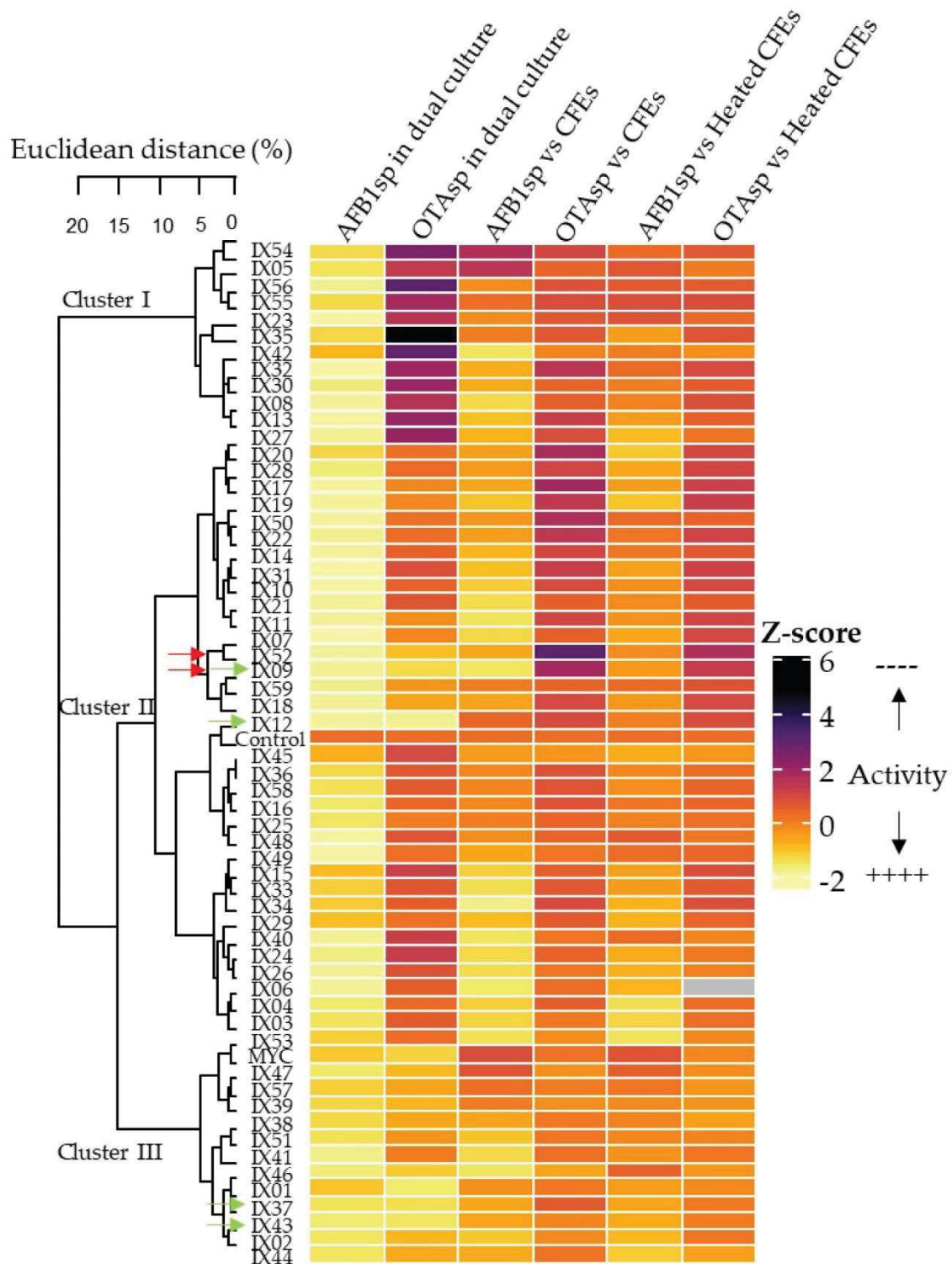


Figure S4. Heatmap of the effect of *Streptomyces* isolates and their CFEs on AFB<sub>1</sub> accumulation by *Aspergillus flavus* and on OTA accumulation by *Penicillium verrucosum*. Results are given in a range of colours according to their Z-score, where purple to black represents a lack of activity or an increase compared to the control, whereas yellow represents the strongest activity. Grey = missing value.

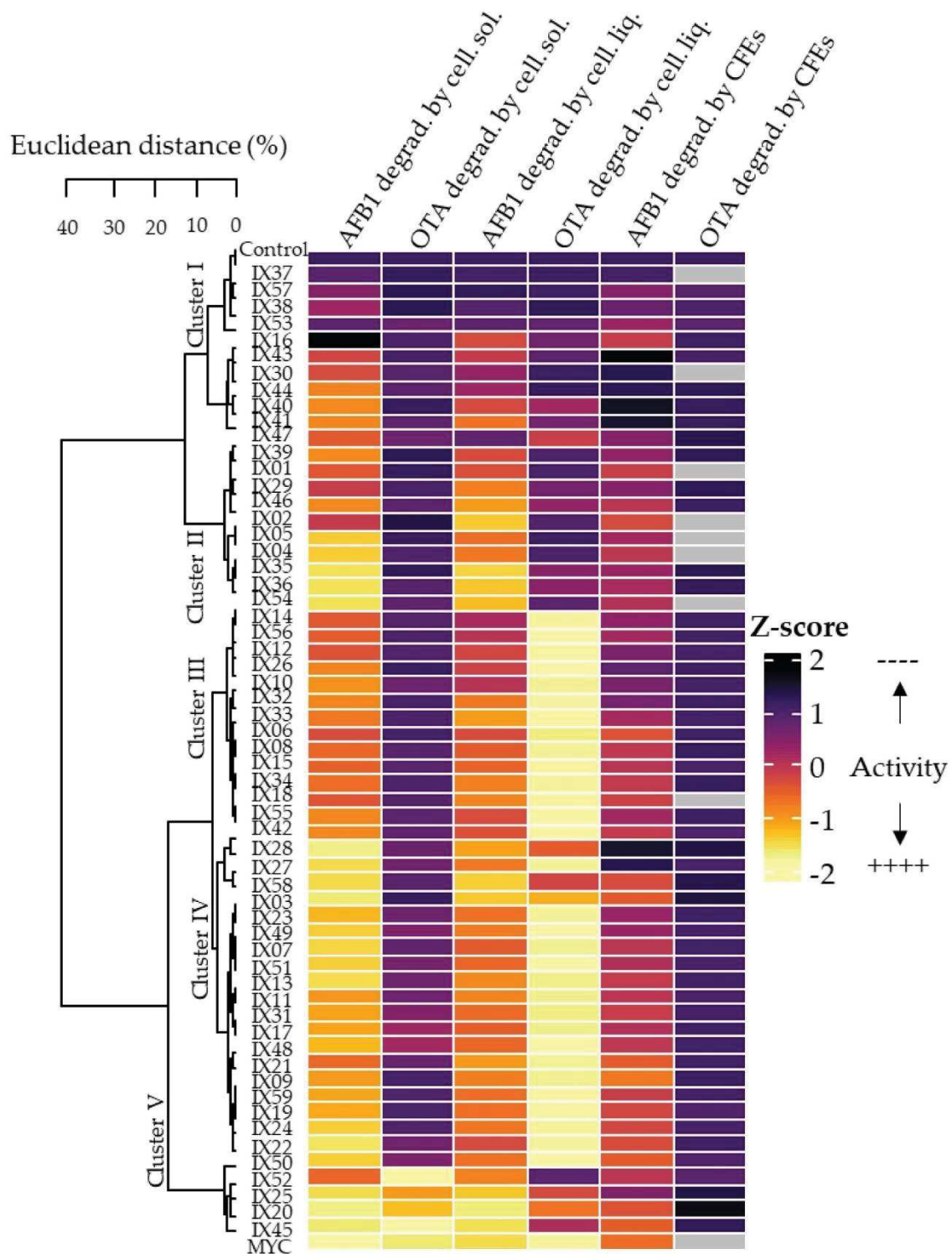


Figure S5. Heatmap of *Streptomyces* isolates and their CFEs ability to degrade AFB<sub>1</sub> and OTA. Results are given in a range of colours according to their Z-score, where purple to black represents a lack of activity or an increase compared to the control, whereas yellow represents the strongest activity. Grey = missing values.

#### 4.4 Comparison of the effect of *Streptomyces* strains on *Penicillium verrucosum* and *Aspergillus flavus* development and their main mycotoxins

As presented in the two previous sections, the impact of *Streptomyces* isolates on both storage pathogens evidenced several marked differences. Such as a general stimulation of OTA production on dual cultures in contrast with a general decrease on aflatoxins amount under the same test conditions. Another important disparity was the fact that OTA degradation was almost only possible during bacterial growth in liquid medium, while degradation of AFB<sub>1</sub> by *Streptomyces* was relatively easy, not only in both culture conditions but also by constitutive bacterial extracellular metabolites. Complementary studies suggested that the tested *Streptomyces* isolates required stressful growth conditions in order to being able to degrade OTA.

The study of the origin of these differences seems interesting, and complementary research should be done to reach a better understanding of mycotoxin degrading mechanisms and its induction by external stimuli. Despite the observed heterogenicity, some isolates exhibited common antagonistic traits against both pathogens. Isolates exhibiting complementary activities represent interesting research prospects, as the presence of multiple mycotoxins is common in contaminated foodstuffs.

The following heatmaps regroup the effect of each *Streptomyces* isolate according to three parameters: fungal growth (Figure 47), mycotoxin specific production (Figure 48), and mycotoxin degradation (Figure 49). Clear yellow indicates a strong activity (antifungal, detoxifying), while dark purple to black indicate a lack of activity or a mycotoxin boost, intermediate colors indicate moderate activities. Isolates are clustered according to the measured activities. For a more detailed explanation about heatmap construction and interpretation, please refer to previously published work (Campos-Avelar et al. 2020).

As observed on the heatmap in Figure 47, *Streptomyces* isolates with the strongest antifungal effect against *A. flavus* (A) were also capable of inhibiting *P. verrucosum* (P) development and therefore are grouped in Cluster II. As mentioned previously, this presents an important advantage considering that during fungal contamination of grains, both pathogens can occur simultaneously. Globally, CFEs were more efficient to control *A. flavus*, as *P. verrucosum* development was hardly affected. A decrease on CFEs antifungal capacities was evidenced for both pathogens.



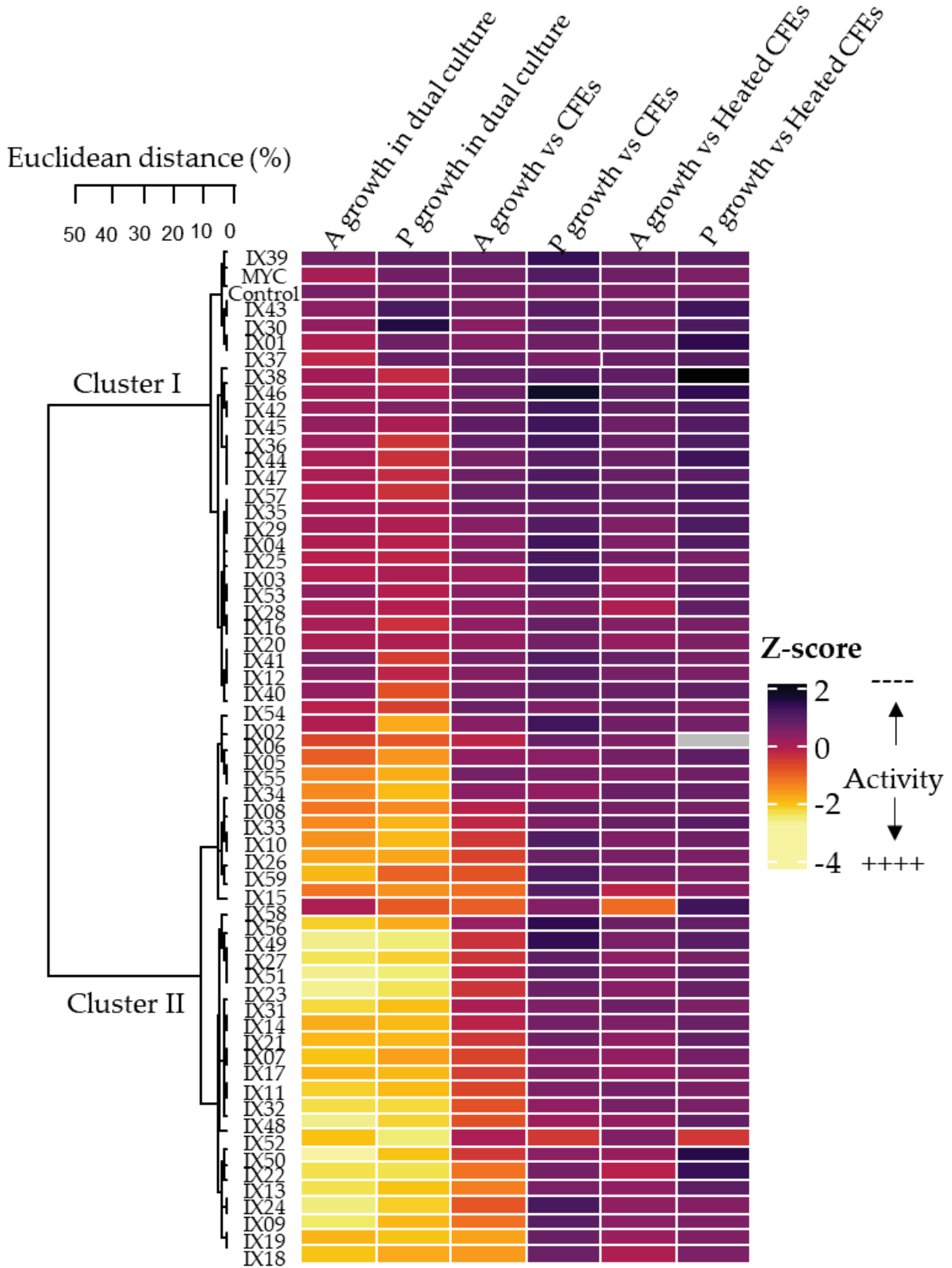


Figure 47. Heatmap of the effect of *Streptomyces* isolates and their CFEs on *Aspergillus flavus* (A) and *Penicillium verrucosum* (P) growth. Results are given in a range of colors according to their Z-score, where purple to black represents a lack of activity or an increase compared to the control, whereas yellow represents the strongest activity. Grey = missing value.

Figure 48 illustrates the effect of the *Streptomyces* collection on mycotoxin specific production by both pathogens. During dual cultures, bacterial isolates were much more efficient at avoiding AFB<sub>1</sub> accumulation than OTA accumulation. No isolate caused an overproduction of aflatoxin by *A. flavus* compared to the control, whereas a whole cluster (II) stimulated OTA production by *P. verrucosum*. Some isolates such as IX09, IX12, IX37 and IX43 (green arrows) were highly efficient at decreasing both pathogens' mycotoxin specific production, which makes them potential candidates for multitoxin inhibition/degradation assays. CFEs were more efficient at controlling AFB<sub>1</sub> amount, compared to that of OTA, as for the latter several of them caused a boost of mycotoxin specific production, especially IX52 and IX09 (red arrows).

Figure 49 presents the capacity of each *Streptomyces* isolate to degrade AFB<sub>1</sub> and OTA, either during bacterial growth on solid and liquid medium or by contact with their CFEs. This heatmap has the most contrasting results. For instance, degradation of OTA during bacterial development on solid medium was only achieved by five isolates (Cluster V), while only 5 isolates were inefficient at degrading AFB<sub>1</sub> under the same conditions (Cluster I). Degradation of AFB<sub>1</sub> by cells on liquid medium was accomplished by all isolates except 4 (first part of cluster I). On the other hand, about a half of bacterial isolates degraded OTA in liquid medium, and the observed degradations were remarkably stronger. Lastly, *Streptomyces* CFEs were unable of degrading OTA, while most of them degraded AFB<sub>1</sub> at different rates. Cluster V contains the most efficient isolates according to their degradation capacities: IX20, IX25, IX45, IX52 and MYC, which were able to degrade both mycotoxins under both culture conditions, additionally, their CFEs were capable of degrading AFB<sub>1</sub>. Future research on these fourth isolates could lead to the identification and isolation of degrading enzymes with a broad spectrum of action. Moreover, pH variations could be tested in order to determine if the lack of OTA degradation by the bacterial CFEs could be due to their rather basic pH which may limit OTA degrading enzymes, while AFB<sub>1</sub> degrading enzymes might not be affected at this pH range.

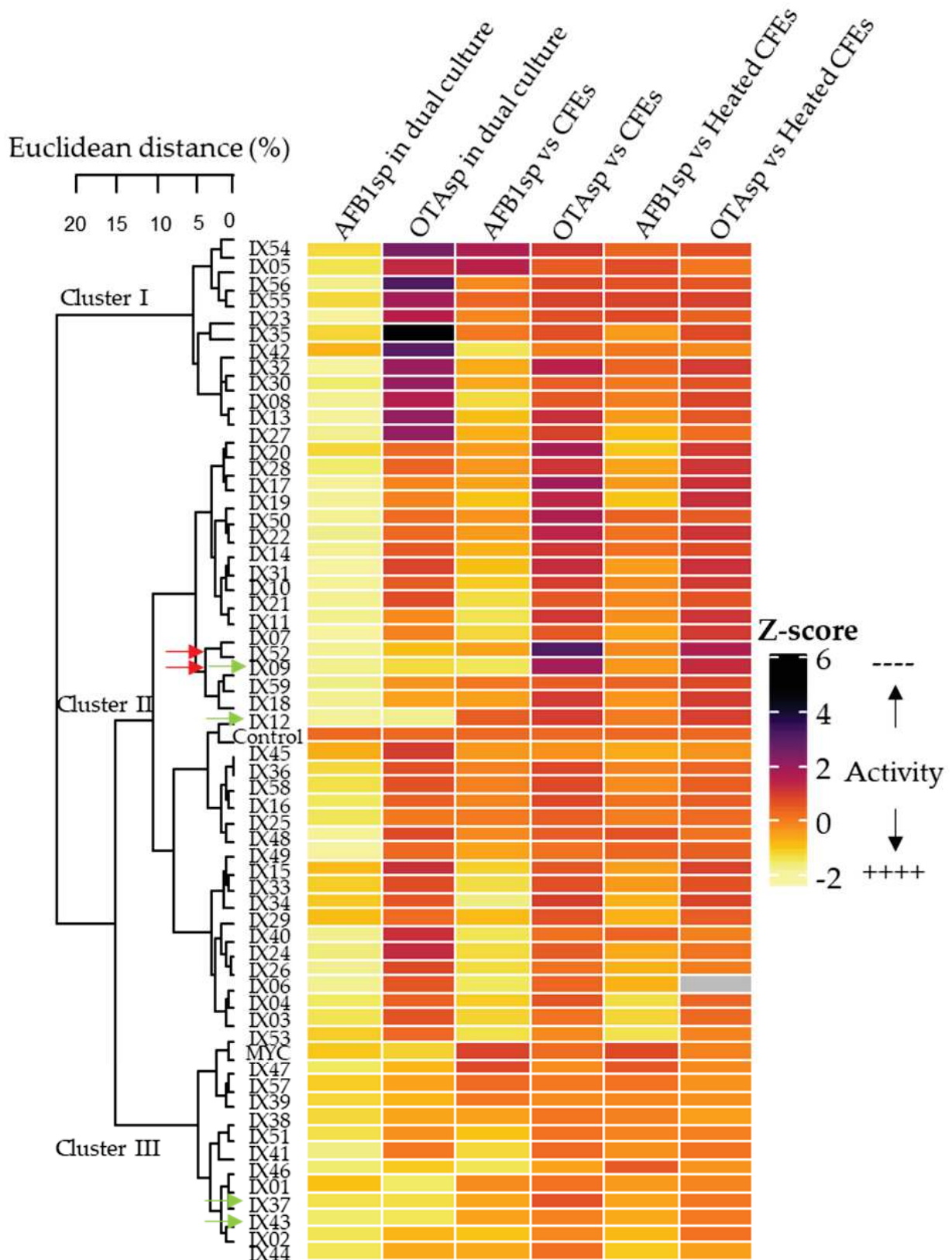


Figure 48. Heatmap of the effect of *Streptomyces* isolates and their CFEs on AFB<sub>1</sub> specific production by *Aspergillus flavus* and on OTA specific production by *Penicillium verrucosum*. Results are given in a range of colors according to their Z-score, where purple to black represents a lack of activity or an increase compared to the control, whereas yellow represents the strongest activity. Grey = missing value.

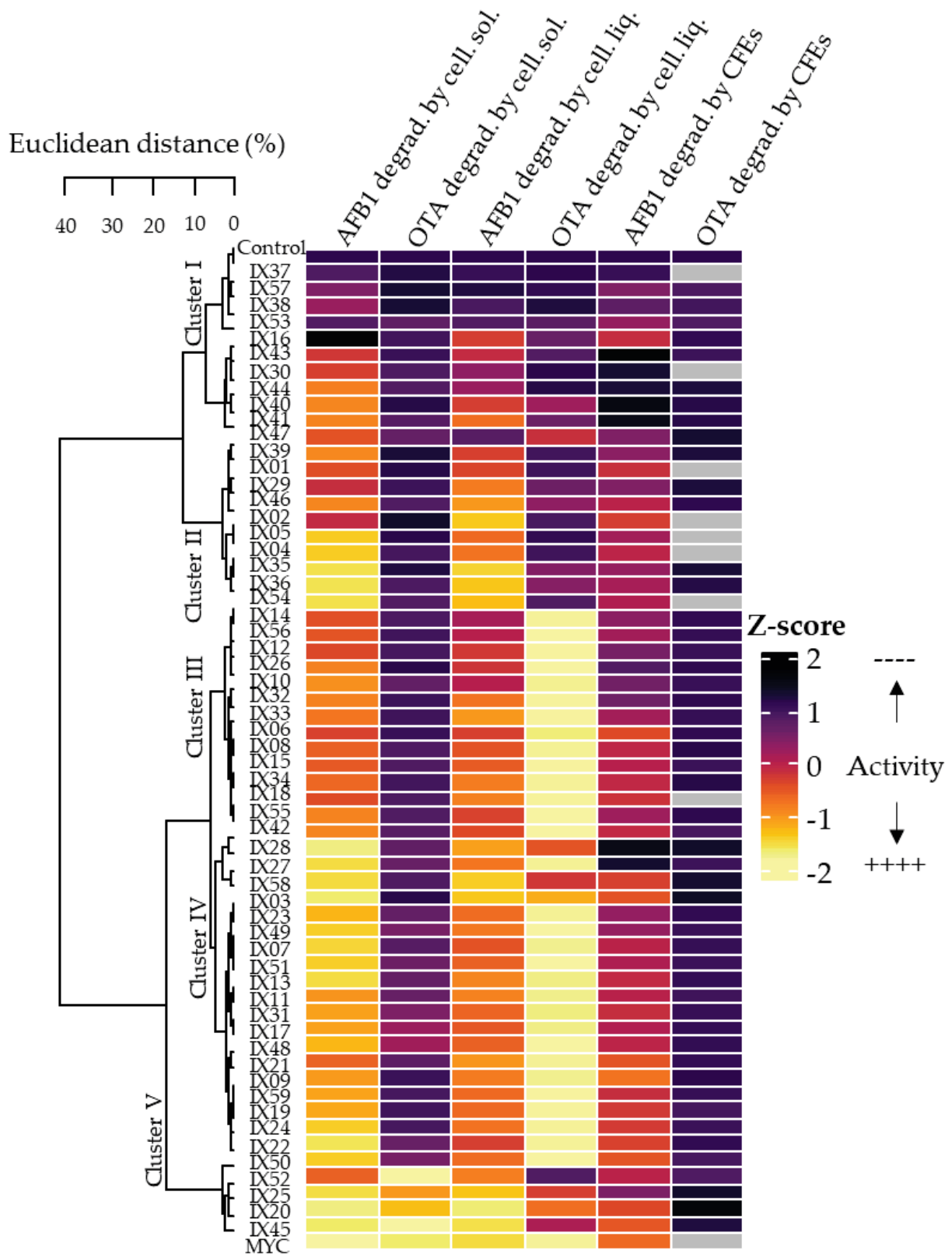


Figure 49. Heatmap of *Streptomyces* isolates and their CFEs ability to degrade AFB<sub>1</sub> and OTA. Results are given in a range of colors according to their Z-score, where purple to black represents a lack of activity or an increase compared to the control, whereas yellow represents the strongest activity. Grey = missing values.

Finally, correlations were calculated between the degrading capacities of *Streptomyces* isolates and their CFEs evaluated on OTA and AFB<sub>1</sub>. Results showed moderate positive correlations between bacterial degradation of the two mycotoxins on both culture conditions (solid and liquid medium) with coefficient values ( $r$ ) between 0.27 and 0.45 ( $p$ -values from 0.03 to 0.001). Isolates IX52, IX25, IX20, IX45 and MYC strain, accounted for the strongest degrading capacities of both mycotoxins. The negative correlation observed between the degrading capacities of OTA by the CFEs and of AFB<sub>1</sub> by the bacterial cells is certainly due to the lack of OTA degradation by CFEs, while bacteria had strong AFB<sub>1</sub> degrading properties.

The capacity of *Streptomyces* bacteria to degrade more than one mycotoxin has already been documented (Hwang and Song 2020). In our case, isolates IX52, IX25, IX20, IX45 and MYC strain, which were able to strongly degrade both OTA and AFB<sub>1</sub>, represent potential candidates for further studies, leading eventually to the isolation of mycotoxin degrading enzymes.

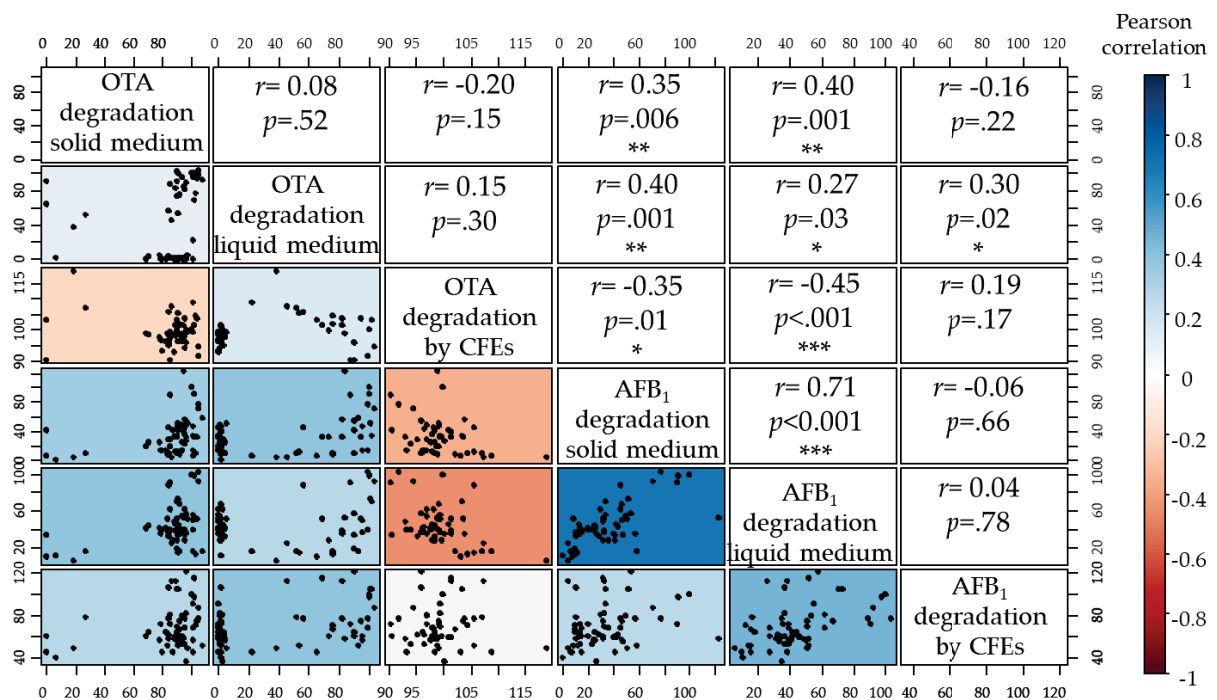


Figure 50. Pearson’s correlation of the OTA and AFB<sub>1</sub> degrading capacities of *Streptomyces* isolates and their CFEs. In the correlation scale, the 1 in blue indicates a strong positive correlation and the -1 in dark red is a strong negative correlation.

## 4.5 Biocontrol of wheat phytopathogens using *Streptomyces* spp.

### 4.5.1 Article presentation

Main phytopathogenic fungi affecting wheat development in the field include rusts, blotchs and blasts, produced by *Puccinia triticina*, *Magnaporthe oryzae*, *Parastagonospora nodorum* and *Zymoseptoria tritici*, respectively. Principal fungal control techniques currently applied include the use of resistant wheat varieties and the application of chemical fungicides. However, both methods have increasing limitations and disadvantages, which urges the exploration of alternative management strategies, such as the biocontrol by beneficial microorganisms.

This section presents at first the observed *in vitro* antifungal activities of a collection of sixty *Streptomyces* isolates against three wheat foliar pathogens: *M. oryzae*, *P. nodorum* and *Z. tritici*. Different inhibition profiles were observed for the various bacterial isolates, in general, *P. nodorum* growth was more easily inhibited, followed by *M. oryzae*. Finally, *Z. tritici* exhibited the higher resistance.

Based on this first screening, twelve isolates were selected for further *in planta* testing against *P. nodorum*. Results showed that only four isolates depicted results significantly different from the control, two of them increasing disease symptoms and the other two decreasing them. These four isolates were later evaluated against *P. triticina* and *Z. tritici*. Results indicated that only MYC had decreased the percentage of lesions caused by *P. triticina*, as well as the percentage of necrosis caused by *Z. tritici*. Unfortunately, in the case of *Z. tritici*, the decrease on plant disease was accompanied by an increased production of pycnidia, which is undesirable, as these structures facilitate fungal proliferation.

The expression of plant defense genes remained practically unchanged, which suggested that the observed protective effects were linked to a direct antagonistic mechanism, such as antibiosis or parasitism. The presence of fungal and bacterial mycelium in the surface of the wheat leaves was assessed by Scanning Electron Microscopy (SEM) and no parasitism was observed between the two microorganisms.

Article submitted to Biological Control on the 7<sup>th</sup> of May 2021



## Biocontrol of wheat phytopathogens using *Streptomyces* isolates

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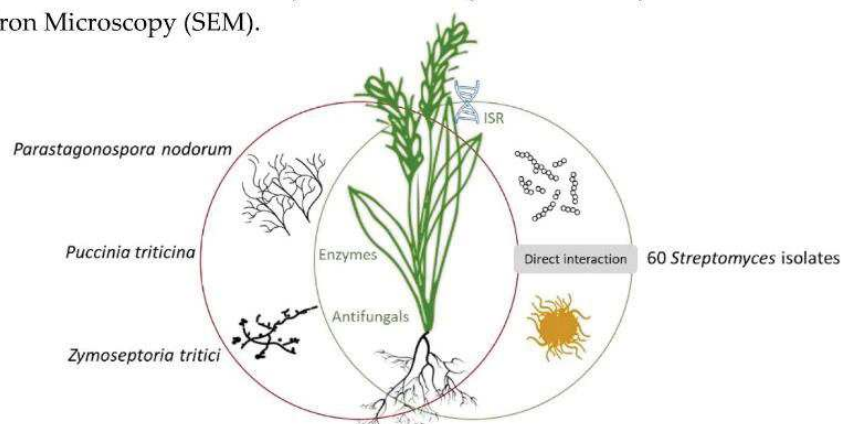
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### Abstract

Wheat is one of the main staple foods consumed worldwide. Reducing the impact of cereal diseases is a main concern for agriculture, as fungal contamination cause about 20% to 40% of yield losses every year. In Europe, the most widespread wheat foliar diseases are caused by *Puccinia* sp., *Zymoseptoria tritici* and *Parastagonospora nodorum*. In addition, the blast fungus *Magnaporthe oryzae* emerges as a new threat in some regions. High production yields on cereal crops have been achieved mainly by the intensive use of chemical fungicides to control diseases, which causes the deterioration of the ecosystem and several adverse effects on human and animal health. Biological control by microorganisms is a promising approach that, together with good agricultural practices and the use of resistant cultivars, could help decreasing and substituting the need for chemical treatments. Fifty-nine *Streptomyces* isolates and strain *Streptomyces griseoviridis* from the commercial product Mycostop<sup>®</sup> were evaluated *in vitro* as antagonistic agents against *Zymoseptoria tritici*, *Parastagonospora nodorum* and *Magnaporthe oryzae*. Results have demonstrated the ability of several isolates to inhibit fungal development, with growth decreases down to 74%, 19% and 10% of the control for *Z. tritici*, *M. oryzae* and *P. nodorum*, respectively. *In planta* greenhouse experiments were also performed against *P. nodorum*, *Z. tritici* and *P. triticina*. Two isolates (IX23 and MYC) significantly decreased the percentage of lesions caused by *P. nodorum*. Furthermore, only MYC was able to decrease the percentage of necrosis caused by *Z. tritici*, but it also provoked an increase on its pycnidia production. Among the *Streptomyces* isolates evaluated against *P. triticina*, only MYC was able to significantly decrease the disease symptoms. The evaluation of plant defense gene levels of expression indicates that the biocontrol efficacy assessed for the tested isolates is not related to an increase on plant defense response, which suggests that a more direct antagonistic mechanism could be involved. Finally, the installation of *Streptomyces* isolates on the surface of wheat leaves was assessed by CFU counting after recovery from the leaf surface and by Scanning Electron Microscopy (SEM).



## Highlights

- Reduction of pathogenic growth *in vitro* by various *Streptomyces* isolates.
- Reduction of foliar disease symptoms *in planta* by isolate IX23 and strain *Streptomyces griseoviridis* (Mycostop®).
- Lack of impact on plant defense genes expression suggesting direct antagonistic mechanisms.
- Surface proliferation and survival of *Streptomyces* isolates on wheat leaves.

**Keywords:** Biocontrol, Actinobacteria, Antagonism, Plant self-defense, SNB, STB, LR, WB

## 1. Introduction

Wheat is the second most important cereal in the world with a production of around 760 million tons in 2020 [1] and the main field crop in France with a production of around 40 million tons in 2019 [2]. As world population grows, wheat demand for food and feed will increase, which will require an increment on production yield and quality. As about 20% to 40% of world crop losses are caused by wheat diseases each year, their management must be improved in order to satisfy the predicted needs. Moreover, consumer expectations have evolved and the agricultural practices must progressively switch from productivity to quality [3]. In order to achieve this, biological, ecological, technological and socio-economic factors have to be considered.

Main wheat diseases include rusts, blotches and head blight/scab, as well as the emerging wheat blast and spot blotch. Rust fungi are obligated biotrophic organisms that depend entirely on the plant nutritional resources. Stem, stripe and leaf rust (LR) are caused by *Puccinia graminis* (*Pgt*), *Puccinia striiformis* (*Pst*) and *Puccinia triticina* (*Pt*), respectively, the last being the most common and extensively distributed [4]. Regions with mild temperature and humidity are the most affected by *P. triticina*, but this pathogen is highly adaptable to a wide range of climates, which increases its occurrence worldwide [5]. The fungi *Zymoseptoria tritici* and *Parastagonospora nodorum* are the causal agents of Septoria tritici blotch (STB) and Septoria nodorum blotch (SNB), respectively. STB is the main disease of wheat in temperate regions, causing huge economic losses in Australia and Europe, especially in Northern France and Germany [6], it often appears concomitantly with SNB. Wheat blast (WB) is caused by the pathogenic fungus *Magnaporthe oryzae* that develops mostly in warm and humid environments. WB is mainly a head disease; the symptoms range from small elliptical lesions to complete bleaching and empty spikes. It causes yield losses from 40% up to 100% [5]. These fungi exhibit different pathogenic mechanisms, which defines their impact on plant health. For instance, *P. triticina* is an obliged parasite, as it requires a living host, and a biotroph, due to its method of extracting nutrients [7]. The colonization of *Z. tritici* is unique as the hyphae grows first for about 14 days without causing any symptoms and inhibits the host defense mechanisms. Then a necrotic phase starts where the pathogen lyses host cells in order to facilitate its sporulation [6]. Contrarily to *Z. tritici*, *P. nodorum* infection develops rapidly thanks to the pathogen's production of proteins facilitating *in planta* growth. *M. oryzae* is an hemibiotroph as it starts as a biotroph and then changes to a necrotrophic lifestyle while host cells are progressively dying [8].

The use of resistant wheat varieties is one of the main approaches for the management of the described diseases. However, the continuous emergence of new virulence profiles impedes the maintenance of resistant cultivars, which leads to the extensive use of fungicides causing pathogen's resistance [8]. The growing inefficacy of chemical fungicides and the environmental threat they represent have encouraged the research for alternative and more sustainable strategies for the management of plant fungal diseases, such as biocontrol with beneficial microorganisms. The mechanisms underlying the efficacy of biocontrol agents consist on direct methods such as antibiosis and lytic enzymes, or indirect approaches by eliciting plant self-defenses, or a mixture of both [9].



Actinobacteria are gram-positive filamentous bacteria highly widespread in the soil. They are beneficial microorganisms that have the ability to colonize plants and induce their systemic resistance (ISR) against pathogens. *Streptomyces* genus is one of the most studied actinobacteria, mainly due to their ability to produce a vast number of metabolites and enzymes and to their plant growth promoting (PGP) properties. *Streptomyces* isolates exhibit several antagonistic mechanisms against pathogenic fungi, such as antibiosis, which is mediated by the production of antifungal molecules and lytic enzymes such as chitinases and glucanases. Competition for resources such as carbon and nitrogen or simply for the available space, as well as parasitism are also some of the mechanisms known to be employed by *Streptomyces* strains [10], [11]. Over the last years *Streptomyces* have been drawing researcher's attention as potential biocontrol agents thanks to their notable antagonistic capacities vis-à-vis a large number of pathogenic fungi *in vitro* and *in vivo* [12]–[21]. Two commercial products based on *Streptomyces* dried spores and mycelia are currently available: Mycostop® (*Streptomyces griseoviridis* K61) and Actinovate® (*Streptomyces lydicus* WYEC108), which are sold as biological fungicides against soil-born fungal pathogens such as *Fusarium*, *Pythium* and *Phytophthora*, among others. These product's preconized method of application is by suspending the formulated powder in water and spraying it onto the leaves, applying it directly to the soil or using it to coat seeds [22].

In this study we evaluated the antagonistic capacity of 59 *Streptomyces* isolates against four phytopathogenic fungi of wheat. Namely, *M. oryzae*, *P. nodorum*, *Z. tritici* and *P. triticina*. The strain *Streptomyces griseoviridis* from the commercial product Mycostop® was included in the experiments for comparison. First, bacterial isolates were confronted on *in vitro* dual culture assays against three pathogens (*M. oryzae*, *P. nodorum* and *Z. tritici*) to measure their inhibitory effect on fungal growth. Then, a selection of active isolates and Mycostop® were evaluated for their protective effect against three foliar wheat diseases: SNB (*P. nodorum*), LR (*P. triticina*) and STB (*Z. tritici*), under greenhouse conditions. To our knowledge, there is no previous report on the antagonistic effect of Mycostop® against foliar wheat pathogens. Furthermore, the impact of *Streptomyces* isolates on various plant defense genes was evaluated, as well as their capacity to install on wheat leaves.

## 2. Material and methods

### 2.1. *Streptomyces* isolates

Fifty-nine actinobacteria isolates were retrieved from organic amendments and soil samples collected in the Hérault department in the South of France. A 16S identification allowed to determine that the isolates belonged to the *Streptomyces* genus (results not shown). The isolates constitute a collection maintained in the laboratory of the UMR QualiSud at the University of Montpellier. Mycostop® (MYC) strain *Streptomyces griseoviridis* K61 was included to the tests as a commercialized biocontrol agent for comparison. The isolates were grown on ISP4 medium (10 g/L starch, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 1 g/L MgSO<sub>4</sub>, 2 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/L CaCO<sub>3</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L MgCl<sub>2</sub>, 1 mg/L ZnSO<sub>4</sub>, 18 g/L bacteriological agar) for 11 days at 28°C. Spores were then collected by scrapping the surface of the Petri dish with 5 ml of distilled water + 0.01% Tween 20 and filtered through sterile cotton. The spore suspensions were aliquoted then stored at -80°C.

### 2.2. Pathogen strains

All the fungal pathogens employed in this study belong to the CIRAD strain collection (CIRAD, Montpellier, France). *Magnaporthe oryzae* BL17, *Parastagonospora nodorum* SE15017 (UMR INRA-AgroParisTech, Thiverval-Grignon, France) and *Zymoseptoria tritici* P1a/M1a were maintained at -80°C, prior to use. For plug collection or spore harvesting, pathogens were pre-cultured for 8 days at 25°C (18°C for *Z. tritici*) before the antagonistic assays. For spore harvesting, spores were scrapped from the

surface by adding 10 mL of distilled sterile water and filtered through sterile cotton. *Puccinia triticina* AD2015 spores were produced on wheat plants by spraying a solution containing: gelatin 0.5%, a drop of Tween 20 per 10 mL and 8 mg of spores per 150 plants. After 10 days, new spores were scraped from the surface of wheat leaves into a sterile tube, then conserved at -80°C prior to use.

### 2.3. Wheat

Durum wheat used for the *in planta* assays was of the spring variety "Pescadou" sold by Florimond Desprez (Cappelle-en-Pevele, France), this variety was selected because of its sensitivity to rust and blotch [23].

### 2.4. *In vitro* antagonistic evaluation of *Streptomyces* against fungal pathogens

Antagonistic assays between the fifty-nine *Streptomyces* isolates plus Mycostop®, and three phytopathogens (*M. oryzae*, *P. nodorum* and *Z. tritici*) were performed on Czapek Yeast Agar medium (CYA: 30 g/L sucrose, 5 g/L yeast extract, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.3 g/L NaNO<sub>3</sub>, 0.05 g/L KCl, 0.05 g/L MgSO<sub>4</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L ZnSO<sub>4</sub>, 0.5 mg/L CuSO<sub>4</sub>, 15 g/L agar, pH ~7.4) which allowed both pathogen and bacteria proper growth and sporulation. As an obliged biotroph, *P. tritici* was not studied by the *in vitro* procedure.

Dual culture assays were implemented on Petri dishes. For the assays against *M. oryzae* and *P. nodorum*, 10 µL of the *Streptomyces* spore suspension at 10<sup>7</sup> CFU/mL were deposited on each side of the plate. After two days at 25°C, a 5 mm plug taken from the border of a week-old colony of the corresponding pathogen was placed in the center of the Petri dish, then cultured at 25°C. For *M. oryzae* the incubation followed photoperiods of 12 hours under light (120 nm) and 12 hours in the dark for 15 days. For *P. nodorum*, plates were incubated under continuous light for 21 days. For the antagonistic assays against *Z. tritici*, whose mycelium does not spread, the pour-plate method was used in order to obtain a homogeneous fungal development on the plate. For this, fungal spores were added to the plates before solidification of the medium (~50°C) at a concentration of 10<sup>4</sup> CFU/mL and homogenized carefully. After solidification of the plates, 10 µL of *Streptomyces* spore suspension at 10<sup>7</sup> CFU/mL were added to the center of the plates, then they were incubated in the dark at 18°C for 15 days. At the end of their incubation period, plates were subjected to image analysis. As a control, each pathogen was inoculated on a CYA plate without bacteria. All tests were done in triplicate.

Surface growth of fungal colonies was measured in ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018). Growth inhibition was established in comparison with the control without *Streptomyces* which represented 100% of growth. Results are given in % of control.

$$\text{Growth ratio}(\% \text{ of control}) = \frac{\text{Assay area (cm}^2\text{)}}{\text{Control area (cm}^2\text{)}} \times 100$$

#### 2.4.1. Heatmap representation of the *in vitro* screening results

Pre-processing of the data for the heatmap representation consisted of a conversion of percentages of fungal inhibition to Z-scores calculated as follows: Z- score = (X- µ)/σ, where X is the measurement, µ is the mean value of the effect of all isolates for each plant pathogen, and σ is the standard deviation of the value of the effect of all isolates for the considered pathogen. This conversion allowed all data results for each antagonistic assay to be normalized in order to compare them, as the effects against each pathogen varied. Therefore, with the standardized data, the default Euclidean method was chosen for measuring the distance between the clusters, which is expressed as a percentage of similarity of the effect of bacteria against the different fungi.

## 2.5. *In planta* evaluation of *Streptomyces* isolates as biocontrol agents under greenhouse conditions

### 2.5.1. Influence of *Streptomyces* spore development stage on its biocontrol efficacy

A comparison of the *in planta* antagonistic efficacy of germinated and non-germinated spores was performed using Mycostop® strain. *P. nodorum* was selected as the fungal pathogen, as it was the most easily inhibited during the *in vitro* assays. For the germinated spore modality, bacterial spores at a concentration of  $10^7$  UFC/mL were cultured on CYB medium (30 g/L sucrose, 5 g/L yeast extract, 1 g/L  $K_2HPO_4$ , 0.3 g/L  $NaNO_3$ , 0.05 g/L KCl, 0.05 g/L  $MgSO_4$ , 1 mg/L  $FeSO_4$ , 1 mg/L  $ZnSO_4$ , 0.5 mg/L  $CuSO_4$ ) for 20 hours at 25°C and 180 rpm. Afterwards, they were washed three times with sterile distilled water. Germinated and non-germinated bacterial spore suspensions were mixed at 1:1 ratio with a 1% gelatin solution. Then, the mixture was applied with a paintbrush on 5 cm of the leaf surface and left to dry. Two days later, fungal spores ( $10^6$  spores/mL) were applied using the same technique. The temperature was set at 24°C during the day and 19°C during the night, the humidity was not regulated. Three pots with four plants each were used for each modality. Disease symptoms were evaluated six days after pathogen infection from a scanned image using the image analysis program LeafTool available on GitHub (<https://github.com/sravel/LeAFtool>) [4].

### 2.5.2. Evaluation of germinated *Streptomyces* spores against *P. nodorum*

Once the influence of the spore development was established, twelve *Streptomyces* isolates from the QualiSud collection, plus Mycostop®, were evaluated against *P. nodorum* by foliar application of germinated bacterial spores, as described previously. Selection of the bacterial isolates was based on the *in vitro* antagonistic assays: isolates with strong antagonistic properties as well as inactive isolates were chosen. Six pots with four plants each were used for each modality. Disease symptoms were evaluated six days after pathogen infection.

### 2.5.3. Evaluation of germinated *Streptomyces* spores against *P. triticina* and *Z. tritici*

Four *Streptomyces* isolates (including Mycostop®) having a significant impact on disease lesions in the previous assay, either by decreasing or increasing the symptoms, were selected to further testing against *Z. tritici* and *P. triticina*. Germinated bacterial spores and *Z. tritici* spores were applied as described before. *P. triticina* spores were inoculated by spraying the spore suspension on the whole plant. Both pathogens were applied two days after bacterial inoculation. Three pots with four plants each were used for each modality. Percentage of leaf surface showing necrosis and pycnidia were evaluated three weeks after infection by *Z. tritici*. The percentage of leaf surface covered by pustules was evaluated 10 days after infection by *P. triticina*.

### 2.5.4. Impact of germinated *Streptomyces* spores on wheat defense genes expression level

A set of wheat plants treated with *Streptomyces* isolates during the previous test were withdrawn before pathogenic infection in order to evaluate the impact of germinated *Streptomyces* spores on the levels of expression of the plant defense genes. For gene expression studies, the protocols were conducted as described by Delteil et al. Briefly, frozen leaf tissues were ground in liquid nitrogen. Then, approximately 500 mL of the resulting powder was treated with 1 mL of TRIZOL (Invitrogen, Carlsbad, CA, USA). Furthermore, RNA samples (5µg) were denatured for 5 min at 65°C with oligo(dT) 18 (3.5 mM) and deoxynucleoside triphosphate (dNTP) (1.5 mM). Samples were later subjected to reverse transcription for 60 min at 37°C with 200 U of reverse transcriptase M-MLV (Promega, Madison, WI, USA) in the appropriate buffer. Two microliters of cDNA (dilution 1:10) were used for RT-QPCR. RT-QPCR mixtures contained PCR buffer, dNTP (0.25 mM),  $MgCl_2$  (2.5 mM), forward and reverse primers (final concentration of 300 or 600 nM), 1 U of HotGoldStar polymerase and SYBR Green PCR mix as per

the manufacturer's recommendations (Eurogentec, Seraing, Belgium). After cDNA amplification, qPCR was performed by an initial denaturation for 5 minutes at 95°C, then 10 seconds at 95°C followed by 40 cycles of 30 seconds at 60°C. Finally, a melting gradient was conducted from 55°C to 95°C at 0.5°C intervals. The list of primers is provided in Table 1. Ratios of gene expression were obtained by dividing them by the level of Ubiquitin expression.

Table 1. Primers used for plant gene expression analysis. PR4: Pathogen-responsive wheat gene. PAL: Phenylalanine ammonia lyase. LOX: Lipoxxygenase. POX: Peroxidase. Chi4: Endochitinase.

Name		Sequence	Final concentration	Efficiency
Chitinase I	F	TGCGCTACTGCAGCATACTC	300 nM	1.836
Chitinase I	R	ACCATACGAATCAACTGATCACA		
PR4	F	ATAAAAAGGCTAGCTAAAAGTAAGTTGA	300 nM	1.883
PR4	R	TTGAATAGATAACCCGTTTGG		
Nodulin	F	GCCGGTTGCACGAGTAAA	300 nM	1.848
Nodulin	R	TTACGAAAAGCGGAAGAGGA		
β-Glucanase II	F	TGCGGCATCCAAGTGTAAC	300 nM	1.896
β-Glucanase II	R	CTGCTTCCATCTCTTTATTCATCA		
PAL	F	CCCCATTGGTGTCCTCAT	600 nM	2.024
PAL	R	ACTGCGCGAACATCAGCTT		
LOX	F	GGGCACCAAGGAGTACAAGGA	600 nM	1.941
LOX	R	GTCCTGATGGTGTGGATGA		
POX	F	GAGATTCCACAGATGCAAACGAG	600 nM	2.067
POX	R	GGAGGCCCTTGTTTCTGAATG		
Chi4	F	TTCTGGTTCTGGATGACCAAC	600 nM	1.815
Chi4	R	ACTGCTTGCAGTACTCCGTG		
Ubiquitin	F	CTGGCGAGGATATGTTCCAT	600 nM	2
Ubiquitin	R	TCGGATGGAAGACCTTTGTC		

#### 2.5.5. Verification of *Streptomyces* isolates persistence on wheat leaves

In order to verify the presence and viability of *Streptomyces* isolates, the wheat leaves were recovered at the end of the assay against *P. nodorum* and swapped with a sterile cotton bud impregnated with tris buffer. The cotton bud was then swirled in a tris solution to resuspend the retrieved material. A separation was made between visually healthier and sicker leaves to determine if there was a relationship between the bacterial amount recovered and the level of disease protection. The obtained tris suspensions were used to inoculate four different media: CYA, AIA (Actinobacteria Isolation Agar), CYA + antibiotics (cycloheximide 50 µg/mL, nystatin 50 µg/mL and nalixidic acid 25 µg/mL) and AIA + antibiotics. CYA is a rich medium while AIA is a selective medium for actinobacteria, for both media, a version with antibiotics was added to suppress fungal and bacterial (other than actinobacteria) growth [24]. Only colonies with actinobacteria morphology were selected. The total amount of CFU numbered was an average of the four media.

#### 2.5.6. Observation of *Streptomyces* installation and interaction with *P. triticina* on wheat leaves by Scanning Electron Microscopy (SEM)

The presence mycelium of *Streptomyces* isolates IX19 and IX23 and of the strain MYC on wheat leaves, retrieved from the *in planta* assay against *P. triticina*, was assessed by Scanning Electron Microscopy (SEM) at the Electronical and Analytical Microscopy Platform at the University of Montpellier. Parameters under Back Scattered Electron Detector or Large Field Detector modes were established as follows: HV of 10 keV and a ×200 magnification, working distance: 9.8 mm, chamber pressure: 4.50<sup>-1</sup> Torr, spot size: 3.5, photo scale: 500 µm.

2.6. Data analysis

Boxplots, Heatmap and statistical analysis were developed in Rstudio (Version 1.3.959, 2009-2020). To compare spore development stage on SNB wheat lesions suppression by MYC a Wilcox's test and an ANOVA were implemented. To evaluate the effect of *Streptomyces* treatments on SNB, STB and LR disease suppression on wheat an ANOVA and either a Dunnett's (SNB, STB) or a Tukey's (LR) test were carried out. For gene expression a Kruskal Wallis' test was performed, followed by a Tuckey's test.  $P < 0.05$  was considered significant.

3. Results

3.1. *In vitro* antagonistic evaluation of *Streptomyces* against fungal pathogens

Fifty-nine *Streptomyces* isolates and the strain *Streptomyces griseoviridis* from the commercial product Mycostop®, were tested against three wheat phytopathogens by *in vitro* dual culture assays on CYA medium. Results showed that globally the *Streptomyces* isolates evaluated were more effective at inhibiting the growth of *P. nodorum*, for which maximal growth inhibition was of 10% of the control when faced to the isolate IX49 (mean=67%, median=64%). *Z. tritici* growth was less decreased with a minimal growth of 48% of the control caused by isolate IX56 (mean=75%, median=72%). Growth inhibition of *M. oryzae* went down to 19% of the control (isolate IX50) (mean=45%, median=47%) (Fig.1).

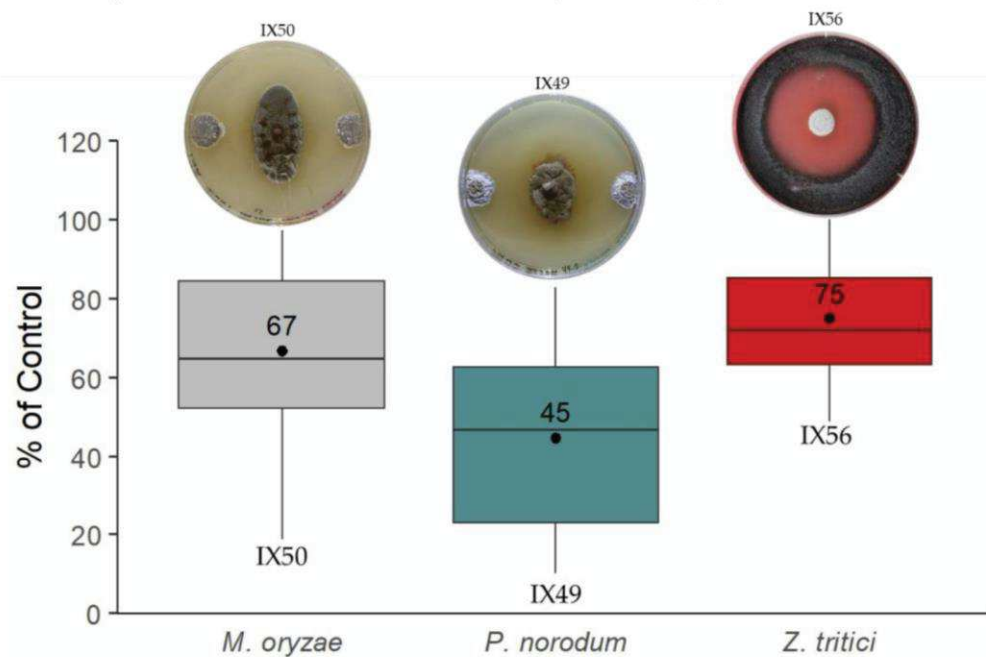


Figure 1. Fungal growth inhibition by 59 *Streptomyces* isolates and Mycostop® in dual culture assays with *Magnaporthe oryzae*, *Parastagonospora nodorum* and *Zymoseptoria tritici* on CYA medium. Boxplot represents the distribution of the data expressed as % of a control (fungal growth without bacteria).

The effect of the evaluated *Streptomyces* isolates on pathogenic fungal growth was assembled into a heatmap (Fig. 2) in order to identify common antifungal properties between them. As the degree of growth inhibition for the three pathogens was different, a standardization was made by converting the original values in % for each test to Z-scores, calculated independently within each of the three pathogens. The stronger was the inhibition of fungal growth, the lower were the values of Z-score. Finally, the Z-score was color coded accordingly: clear yellow represents a strong fungal growth inhibition (low Z-score) while dark purple to black represents a weak or inexistent fungal growth

inhibition compared to the control (high Z-score). Intermediate colors between these extremes represent moderate antagonistic effects. Finally, Euclidean distances were calculated considering the isolates antagonistic properties against each pathogen and further transformed as the percentage of similarity before being clustered accordingly.

Bacterial isolates were grouped in two main Clusters, I and II, they exhibited variable antagonistic activities according to the tested pathogens. Globally, isolates belonging to Cluster II showed a higher inhibitory capacity against the three pathogens. In particular, subcluster IIB, which demonstrated to have a strong antagonistic activity against the three studied pathogens. Isolates from subcluster IA1 were the weakest antagonists. The reference strain MYC exhibited limited inhibitory effect against *M. oryzae* and *P. nodorum* but caused a moderate decrease on *Z. tritici* growth.

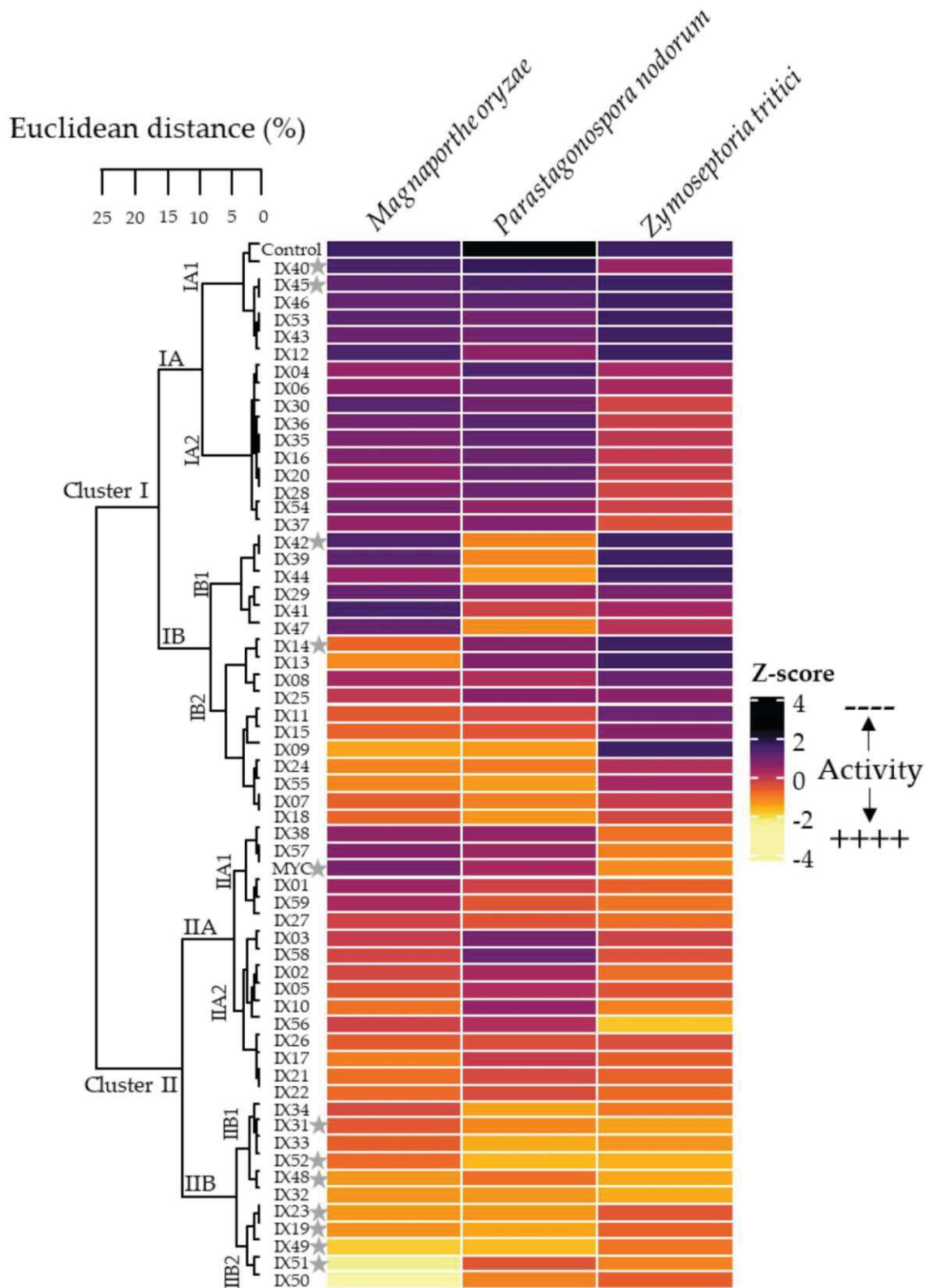


Figure 2. Summary heatmap of the effect of 59 *Streptomyces* isolates and Mycostop® on *Magnaporthe oryzae*, *Parastagonospora nodorum* and *Zymoseptoria tritici* growth on *in vitro* dual assays. The results are given in a range of colors according to their Z-score, where purple to black indicates a weak growth inhibition activity and yellow indicates the strongest growth inhibition activity. Grey stars indicate the isolates selected for *in planta* evaluation.

3.2. *In planta* evaluation of *Streptomyces* isolates as biocontrol agents under greenhouse conditions

3.2.1. Influence of *Streptomyces* spore development stage on its biocontrol efficacy

In order to determine if *Streptomyces* spores should be applied dormant (non-germinated) or in vegetative state (germinated) during *in planta* assays, the biocontrol efficacy of the reference strain Mycostop® (MYC) was assessed under both modalities against *P. nodorum*, as it was the most sensitive pathogen to the *Streptomyces* isolates during *in vitro* assays. Germinated MYC spores reduced significantly the lesions on the wheat leaves ( $p < 0.001$ ) in comparison to non-germinated spores, which were equivalent to the control treatment (water) (Fig. 3). Hence, the following tests were performed with germinated bacterial spores only.

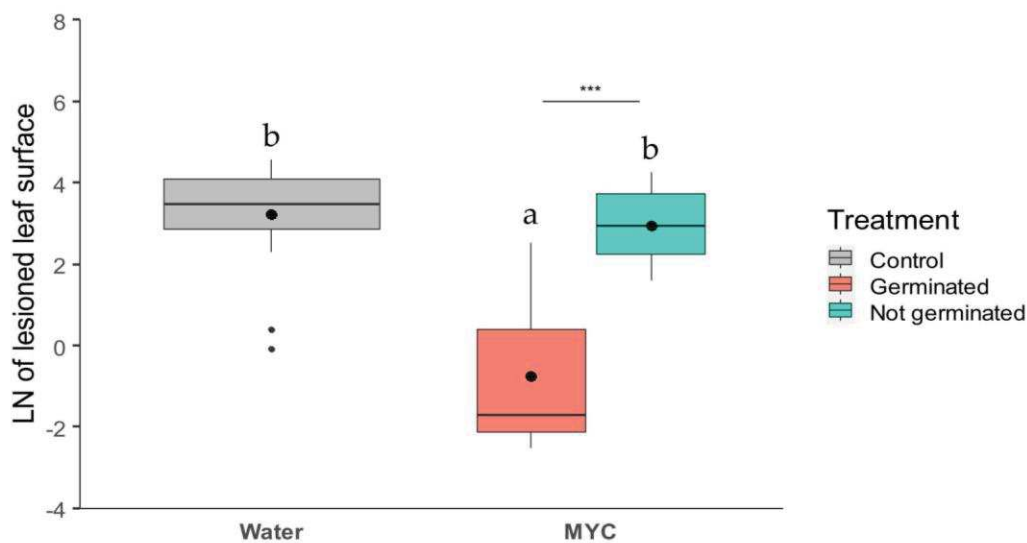


Figure 3. Ability of *Streptomyces griseoviridis* strain from Mycostop® to decrease the lesioned surface caused by *Parastagonospora nodorum* on wheat leaves under greenhouse conditions. Bacterial spores were added either germinated or non-germinated. Boxplots indicate the distribution of the data expressed as LN of the lesioned surface on the wheat leaf. Means were compared by an ANOVA and a Wilcox's test.

3.2.2. Evaluation of germinated *Streptomyces* spores against *P. nodorum*

Twelve *Streptomyces* isolates, including Mycostop®, were evaluated for their biocontrol activity against *P. nodorum in planta*. Wheat leaves were treated with germinated bacterial spores two days before pathogenic spore application. Observations were made after 6 days of incubation with the pathogen. The results show that four bacterial isolates lead to a percentage of foliar lesions significantly different from the control treatment (water). Two of them caused an increase in the percentage of lesions compared to the control (mean=42%, median=38%), namely IX19 (mean=70%, median=76%) and IX51 (mean=66%, median=69%) ( $p=0.0014$  and  $p=0.009$ , respectively). The other two, IX23 (mean=18%, median=12%) and MYC (mean=26%, median=14%) managed to significantly diminish the disease symptoms ( $p < 0.001$  and  $p=0.05$ , respectively) (Fig. 4).



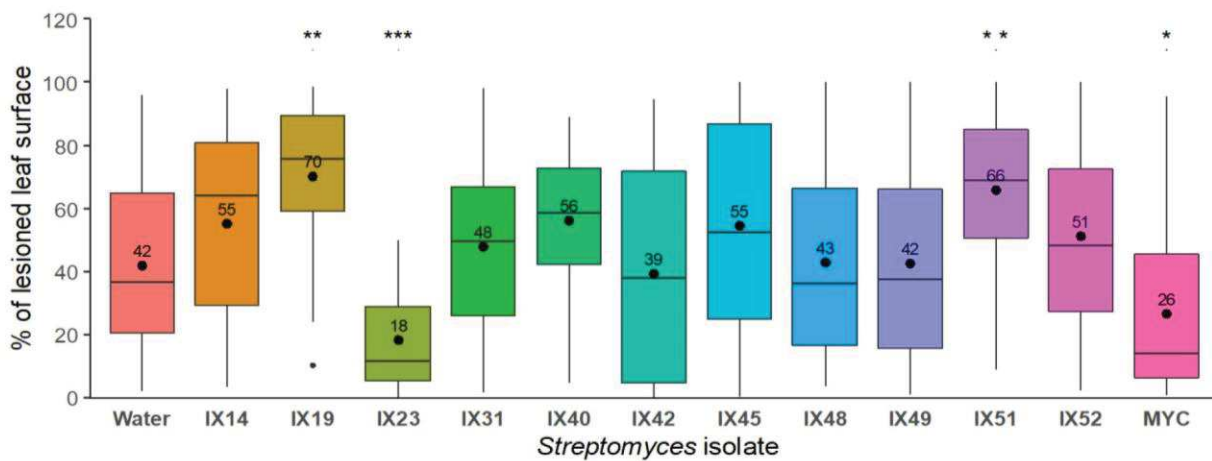


Figure 4. Effect of eleven *Streptomyces* isolates and Mycostop® germinated spores on the percentage of lesioned surface on wheat leaves caused by *P. nodorum* under greenhouse conditions. Boxplots indicate the distribution of the data expressed as % of lesioned leaf surface. The means of the isolates were compared with the mean of the control by an ANOVA and a Dunnett's test.

### 3.2.3. Evaluation of germinated *Streptomyces* spores against *P. triticina* and *Z. tritici*

The three *Streptomyces* isolates (IX19, IX51, IX23) and MYC strain having contrasting effects on *P. nodorum* during the previous test were evaluated against two fungi with diverse pathogenic mechanisms: *Z. tritici*, also a necrotroph, and *P. triticina*, an obliged biotroph.

Results against *P. triticina* indicated that only Mycostop® was capable of significantly decreasing the percentage of lesioned surface on the leaves (mean = 0.13%, median = 0.03%) compared to the control (mean = 0.34%, median = 0.23%) ( $p=0.045$ ). No isolate caused an increase in the percentage of lesions (Fig. 5).

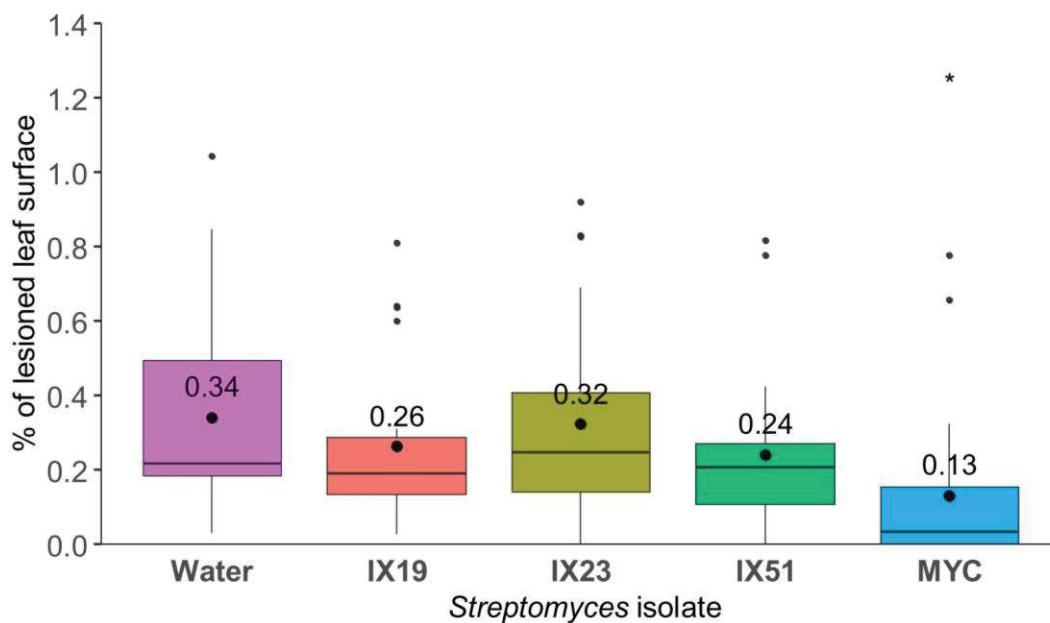


Figure 5. Percentage of wheat leaf's lesioned surface caused by *Puccinia triticina* after a preventive treatment with three *Streptomyces* isolates and Mycostop® under greenhouse conditions. Boxplots indicate the distribution of the data expressed as % of lesioned leaf surface. Means were compared by an ANOVA and a Tukey's test.

Concerning *Z. tritici*, *in planta* evaluation of the *Streptomyces* isolates germinated spores indicates a significant reduction of the percentage of necrosed leaf surface exclusively for MYC (mean = 44%, median = 30%) compared to the control (mean = 73%, median = 92%) ( $p < 0.001$ ). However, a significant increase on the percentage of pycnidia was also observed by this strain (mean = 42%, median = 43%) in comparison to the control (mean = 21%, median = 22%) ( $p = 0.02$ ). IX23, which had previously exhibited a protective effect against *P. nodorum* (mean = 18% of lesioned leaf surface vs 42% for the control) (Fig. 4) was ineffective against *Z. tritici* (mean = 79% of necrosed leaf surface vs 73% for the control) (Fig. 6).

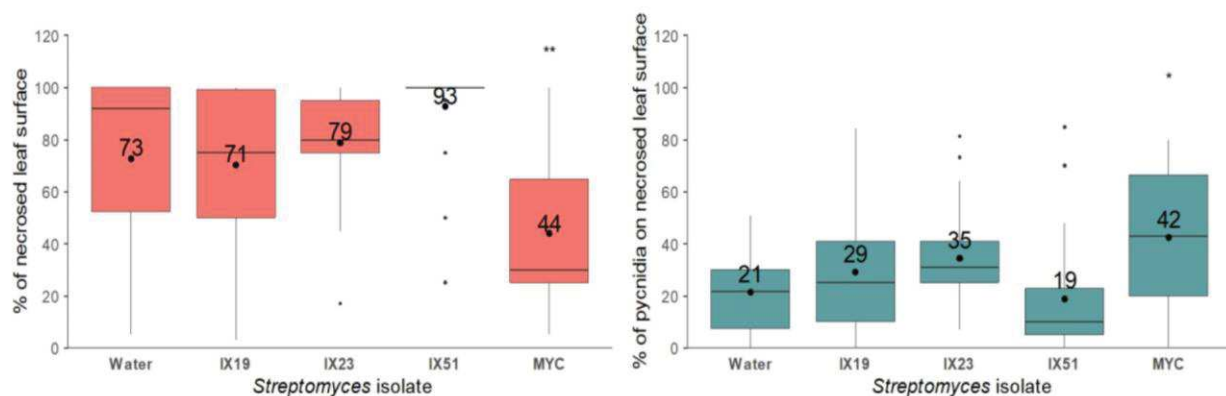


Figure 6. Impact of *Streptomyces* isolates and Mycostop®'s germinated spores on disease suppression and pycnidia production by *Z. tritici* on wheat under greenhouse conditions. Boxplots indicate the distribution of the data expressed as % of leaf surface showing necrosis (left) and % of necrosed surface showing pycnidia (right). Means were compared by an ANOVA and a Dunnett's test.

#### 3.2.4. Impact of germinated *Streptomyces* spores on wheat defense genes expression level

The expression of eight genes related to plant defense was evaluated by retrieving wheat plants treated with *Streptomyces* before fungal infection and performing an RNA extraction and a qPCR analysis. Results indicate that PAL was the only gene affected by the presence of the isolates, with a significant decrease of its expression ratio caused by isolate IX51 ( $p\text{-value}=0.0250$ ) and strain MYC ( $p\text{-value}=0.0285$ ). The expression of the remaining genes was not modified by the *Streptomyces* germinated spores (Fig. 7).

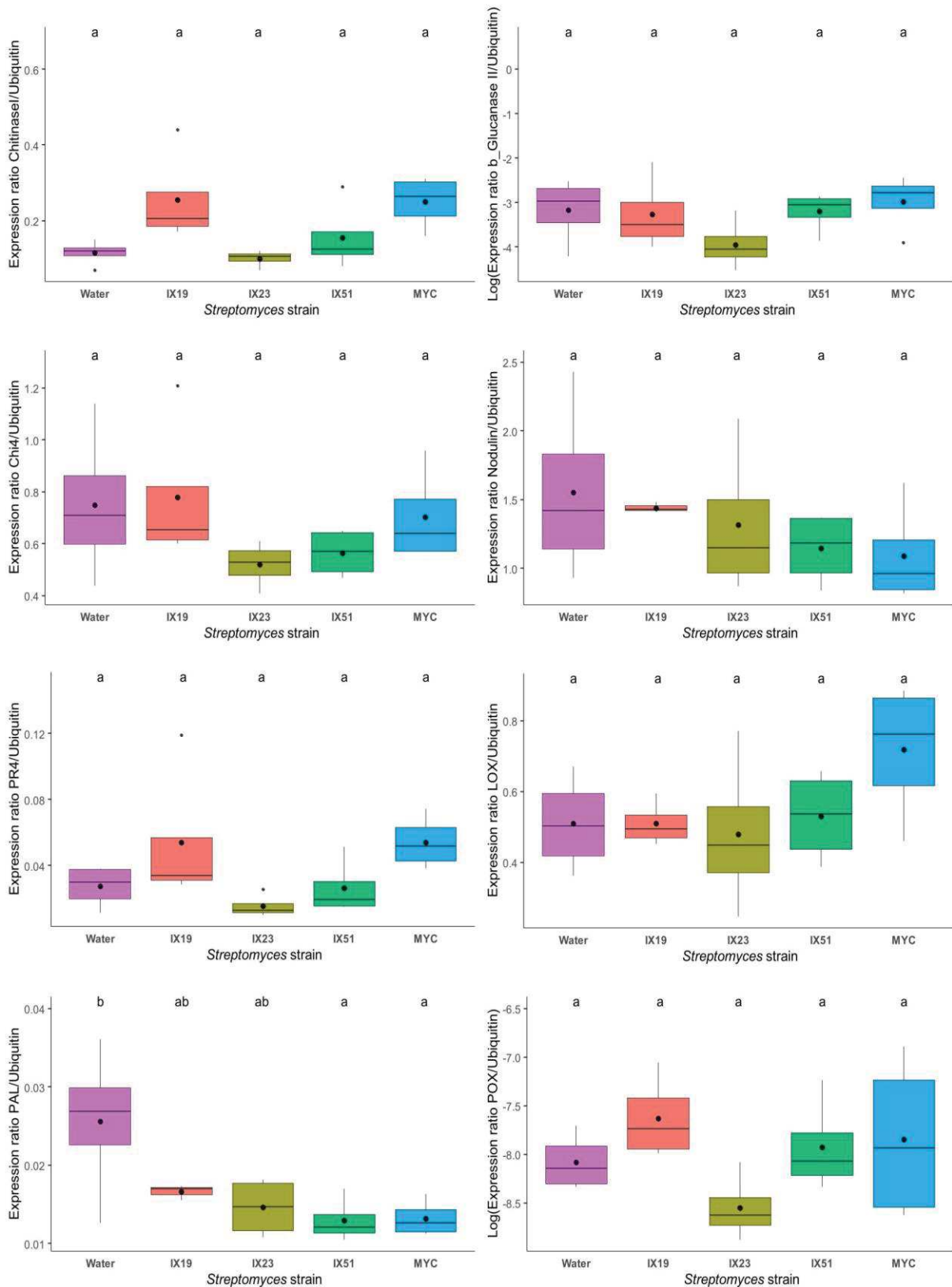


Figure 7. Effect of *Streptomyces* isolates and Mycostop®'s germinated spores on the expression ratio of eight plant defense genes: Chitinase I,  $\beta$ -Glucanase II, Chi4, Nodulin, PR4, LOX, PAL and POX. Ratios of gene expression were obtained after dividing by the level of Ubiquitin expression. Comparison of means was performed by a Kruskal Wallis' test followed by a Tukey's test.

3.2.5. Verification of *Streptomyces* isolates persistence on wheat leaves

At the end of the *in planta* assay against *P. nodorum*, wheat leaves were separated according to their apparent health state (healthy and sick), and swapped with sterile cotton buds in order to verify if *Streptomyces* isolates had settled and remained viable on the leaves surface. The resulting spore suspensions were cultivated on four different media to perform a CFU counting, results include the mean of the UFC counting in the four media. Results reveal that the isolates IX19, IX45 and IX51 had the highest number of CFU/cm<sup>2</sup> of leaf on visually sick leaves ( $3.3 \times 10^3$ ,  $4.6 \times 10^3$  and  $1.9 \times 10^3$  respectively). While only IX23 showed a higher counting on healthy leaves ( $1.4 \times 10^3$  CFU/cm<sup>2</sup>) compared to sick leaves ( $2.7 \times 10^2$  CFU/cm<sup>2</sup>) (Fig. 8). Of note, on the *in planta* assay against *P. nodorum* described previously, IX19 caused an increase on plant lesions, while IX23 had a protective effect.

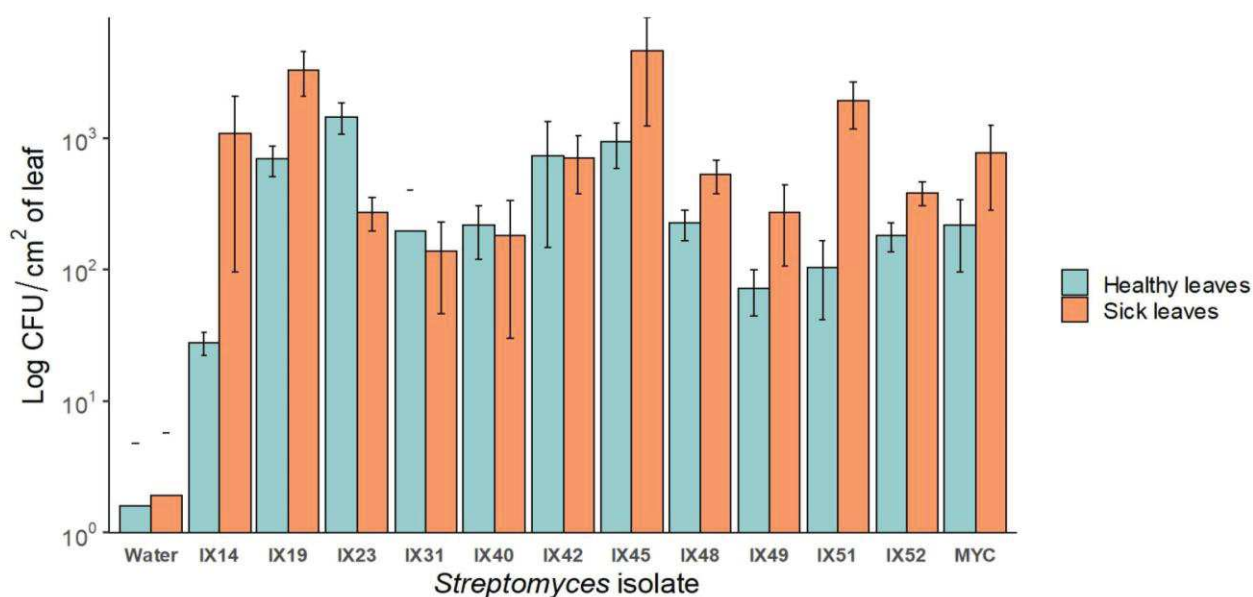


Figure 8. Log of CFU counting of the installed *Streptomyces* isolates on wheat leaves surface during the *in planta* assay against *P. nodorum*.

3.2.6. Observation of *Streptomyces* installation and interaction with *P. triticina* on wheat leaves by Scanning Electron Microscopy (SEM)

Wheat leaves treated with *Streptomyces* germinated spores during *in planta* assay against *P. triticina* were recovered and their surface was observed by Scanning Electron Microscopy (SEM). The observation of bacterial development indicates that the mycelium of all *Streptomyces* isolates tested spreads mostly on the leaf surface and surrounding the leaf trichomes, an example is observed in Figure 9-A for Mycostop®. During the observation of biocontrol assays, the proximity of fungal and bacterial mycelium was assessed in several cases. However, no direct contact nor parasitism by the bacterial isolate were identified for any *Streptomyces* isolate. An example is given in Figure 9-B depicting the mycelium of *P. triticina* (orange arrow) and of *Streptomyces* isolate IX19 (blue arrow).

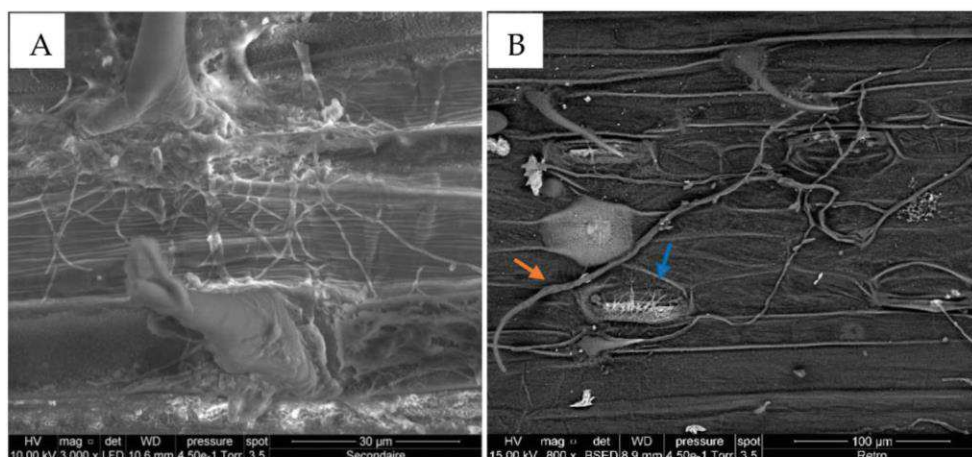


Figure 9. Scanning Electron Microscopy (SEM) of wheat leaves at the end of the *in planta* assay of *Streptomyces* isolates against *P. triticina*. A: Mycostop® *S. griseoviridis* mycelium alone on the leaf surface and surrounding trichomes. B: *P. triticina* mycelium (orange arrow) aside of *Streptomyces* isolate IX19 mycelium (blue arrow).

#### 4. Discussion

*In vitro* dual culture assays demonstrated that several *Streptomyces* isolates from the UMR Qualisud collection exhibit antagonistic traits against the tested wheat phytopathogens. *P. nodorum* was the most easily inhibited, followed by *M. oryzae*, and finally *Z. tritici*, which was less inhibited. However, this is probably due to its simultaneous culture with the *Streptomyces* isolates, while bacteria were inoculated two days before for other fungal models, and also to the confrontation method, which consisted in a single central bacterial spot (two spots for other fungal pathogens). Indeed, *Z. tritici* does not spread on the plate as the other pathogenic fungi in this study, so in order to obtain a development on the entire plate it had to be inoculated by the pour-plate method followed by bacterial inoculation. This implies a disadvantage for the actinobacteria development, as they tend to grow slowly and occupy limited space [25], which is why they are often allowed to install first [22].

The clusterization of *Streptomyces* isolates according to their *in vitro* efficacy allowed to identify isolates with a wide antifungal spectrum, being able to inhibit two or even three pathogens' growth, as well as isolates lacking of fungal inhibiting properties *in vitro*. Both types of isolates were selected for the *in planta* assays, as *in vitro* effects might not correlate with *in planta* results [26]. The differences obtained between *in vitro* and *in planta* testing may be caused by different factors having an impact on the development of the biocontrol agent, such as organic matter, pH, nutrient and moisture level and mostly by the interaction with other microorganisms [27].

The observed *in vitro* antagonistic capacities can be due to a series of mechanisms exhibited by *Streptomyces*, such as the production of degrading enzymes, antifungal compounds and the competition for the available nutrients [9]. Thus, active isolates are potential sources for enzymes and active molecules with antifungal properties. Wheat blast caused by *M. oryzae* is an emerging disease, as this pathogen is mainly found on rice. However, results obtained for its control on rice by *Streptomyces* could accelerate the development of strategies for its management on wheat [27]. Several studies on *M. oryzae* indicate the presence of bioactive compounds produced by *Streptomyces* strains, which supports the results obtained in this study. Indeed, Zarandi et al. observed antagonistic effects on their *in vitro* assays of *Streptomyces sindeneusis* against *M. oryzae* on rice seedling leaves, the active fractions extracted indicated a fungistatic activity [20]. Culture filtrates of *Streptomyces globisporus* JK-1 were able to inhibit *M. oryzae* mycelium growth, conidial germination and appressorial formation [18]. Khalil et al. successfully purified the antifungal compound dihydroxy viridifungin produced by *Streptomyces flavotricini* during its confrontation against *M. oryzae* [28]. Some results have shown that *Streptomyces* isolates and their metabolites have a higher antifungal activity than synthetic fungicides commonly

used to control *M. oryzae*, such as tricyclazole [18], [19]. *Streptomyces sideneusis* strain 263 spore suspensions were able to control *M. oryzae* growth and to suppress leaf symptoms on rice seedling-leaves during greenhouse experiments [20]. Some antibiotics produced by *Streptomyces* are currently used for the control of rice blast, for instance Blasticidin-S and Kasugamycin. Oligomycin A and Rapamycin that showed antifungal activities [27].

Mycostop® *S. griseoviridis* (MYC) biocontrol efficacy against *P. nodorum* *in planta* was assessed by applying dormant spores or vegetative mycelium in order to select the most effective method. Indeed, formulation of biocontrol agents and developmental stage before their application is of paramount importance to guarantee the organism survival and facilitate its proliferation [10]. The observed higher efficacy at decreasing wheat lesions of vegetative mycelium compared to dormant spores may be due to the elimination of the latent phase for spore germination, which facilitates the colonization of the surface of the wheat leaf [29]. Once the application method was selected, eleven *Streptomyces* isolates and Mycostop®'s germinated spores were assessed against the necrotrophic fungus *P. nodorum*. Only 4 isolates exhibited a significant change on the percentage of lesions compared to the control. Isolates IX19 and IX51 increased the percentage of lesions, while IX23 and MYC decreased it. Some studies reported inhibition of *P. nodorum* growth by *Streptomyces* metabolites. For example, Malayamycin, produced by *S. malaysiensis* was capable of delaying fungal growth and sporulation *in vitro*, as well as lesion size and pycnidiation on wheat [14]. Phenamide, produced by *S. albospinus* was also able to inhibit *P. nodorum* *in vitro* [15]. Comparison of the *in vitro* and *in planta* results against *P. nodorum* in this study, confirms a lack of correlation between both modalities for isolates IX19 and IX51, that had exhibited antifungal properties *in vitro*. Isolate IX23 was effective to control the pathogen both *in vitro* and *in planta*, while MYC was more effective *in planta* than *in vitro*.

The germinated spores of IX19, IX23, IX51 and MYC were subsequently evaluated against an obligated biotroph: *Puccinia triticina*, and another necrotrophic fungus: *Zymoseptoria tritici*. Results allowed to determine that a significant ( $p=0.045$ ) plant protection against leaf rust (*P. triticina*) resulted from a preventive treatment with MYC germinated spores only. The efficacy of *Streptomyces* and their metabolites to control leaf rust has already been documented. A study from Yi et al. demonstrated that fermentation broth of a *Streptomyces* spp. strain showed good antifungal activity (80% decrease) against wheat leaf rust caused by *Puccinia triticina* on wheat leaves [12]. Later, El-Sharkawy et al. assessed the efficacy of *Streptomyces viridosporus* to decrease leaf rust by 50% under greenhouse and field conditions. Additionally, a general increase on yield parameters such as spike, grain and kernel weight was observed. They also found that the combination of *S. viridosporus* and *Trichoderma harzianum* augmented the control efficacy [13].

*In planta* assays against *Z. tritici* indicated that, among the four *Streptomyces* isolates evaluated, only Mycostop® *S. griseoviridis* was able to decrease the percentage of necrosis (mean= 44%) on the surface of the wheat leaf compared to the control (treatment with water) (mean= 73%). However, the percentage of pycnidia doubled as compared to the control (42% vs 21%). As *Z. tritici* pycnidiospores are responsible for the spread of the disease by water splash, their increment is undesirable [30]. Nevertheless, further studies on MYC strain could lead to the identification and isolation of antifungal compounds that do not stimulate pycnidia formation. Indeed, bioactive antifungal compounds have been isolated from *Streptomyces* sp., as exemplified by the recently developed fungicide fenpicoxamid (Intrateq™ active), a derivative from the natural antifungal compound UK-2A, isolated from fermentation broths of *Streptomyces*, which was recently found to be active against *Zymoseptoria tritici* and *Puccinia triticina* in greenhouse experiments [16]. Additionally, xiamycins produced by *Streptomyces* spp. caused a complete inhibition of *Z. tritici* growth [17].

The isolate IX23, which was effective against *P. nodorum*, was unable to control *Z. tritici*, which indicates that its efficacy may not be related to the pathogen's trophic mechanism. Furthermore, Mycostop® *Streptomyces griseoviridis* strain seems to have a wide spectrum of action, being able to

control *P. nodorum*, *P. triticina* and *Z. tritici in planta*. These results present interesting perspectives for the application of Mycostop® for the management of wheat foliar diseases, as to date, its efficiency has mainly been reported against soil-borne pathogens, causing wilt/root rot and head blight diseases [22].

The expression of the studied plant defense genes remained unchanged by the inoculation of *Streptomyces* germinated spores, except for PAL, for which IX51 and MYC provoked a significant decrease ( $p=0.250$  and  $p=0.285$ , respectively). This decrease may not be significant, since PAL is only one of the many defense genes regulated upon infection [31]. Additional measures are required to confirm such slight variations on gene expression. As the observed disease suppression by some isolates is not correlated to an increase in the plant defense genes expression, we hypothesized a more direct antagonistic mechanism as antibiosis, competition or parasitism. To validate the presence and viability of *Streptomyces* germinated spores, leaves from the *in planta* assay against *P. nodorum* were used to retrieve bacterial isolates from the surface, and to determine the CFU on different media. CFU counting showed that isolates IX19, IX45 and IX51 were the most viable on visually sicker leaves, with between  $1.9 \times 10^3$  and  $4.6 \times 10^3$  CFU/cm<sup>2</sup> of leaf. Moreover, IX23 was the only isolate for which the CFU counting on healthier leaves was higher ( $1.4 \times 10^3$  CFU/cm<sup>2</sup> vs  $2.7 \times 10^2$  CFU/cm<sup>2</sup>). Interestingly, isolates IX19 and IX51 caused an increase on the percentage of leaf lesions during the *in planta* assay against *P. nodorum*, which could indicate a sensitivity of the plant to the colonization by these *Streptomyces* isolates. On the other hand, a higher colonization of healthier wheat leaves by IX23 could suggest a protective effect of this isolate against SNB.

In order to determine the occurrence of parasitism or lysis of the fungal mycelium of *P. triticina* by the *Streptomyces* isolates IX19 and IX23, and by MYC strain, leaf samples were observed by Scanning Electron Microscopy (SEM). Leaves surface colonization by both fungus and bacteria was identified during the observations. However, no parasitic behavior was detected from the bacterial isolates, which is contrasting to studies that reported parasitic behavior of *Streptomyces*, such as the one of Ursan et al. [32]. Other studies have reported leaf surface colonization by *Streptomyces* on cucumber [33] and *Arabidopsis* [34], accompanied by an activation of plant defense genes and an increased resistance against *Colletotrichum orbiculare* and *Alternaria brassicicola*, respectively. In this study, although leaf colonization by the tested *Streptomyces* isolates was observed, it did not result on plant defense activation nor fungal parasitism, and only MYC was capable of decreasing SNB lesions. This result suggests that rather than plant defense stimulation or parasitism, the production of antifungal compounds (antibiosis) may be behind the antagonistic mechanism displayed by MYC against *P. tritici*, and possibly by the *Streptomyces* isolate IX23 against *P. nodorum*. Therefore, additional research on these particular bacterial CFEs could lead to the identification and isolation of bioactive antifungal compounds. As for isolate IX19, which successfully colonized the leaf surface but was unable to decrease disease symptoms, some hypothesis include that its high colonization rate may have injured the plant or that it was unable to synthesize its bioactive compounds on the leaf.

## 5. Conclusion and perspectives

*In vitro* antagonistic assays have evidenced the antifungal properties of several *Streptomyces* isolates in the collection and Mycostop®. *In planta* validation of the selected *Streptomyces* isolates revealed a lack of correlation between *in vitro* and *in planta* testing. However, promising candidates for the biological control of the studied pathogenic fungi were identified, especially Mycostop®'s strain *Streptomyces griseoviridis*, whose effectiveness to control wheat foliar pathogens had not been previously documented. A direct antagonistic mechanism, such as antibiosis, is suspected, as the plant defense genes were seemingly not stimulated. Furthermore, the presence and viability of *Streptomyces* isolates on the surface of the leaves was validated and no parasitism of the fungal mycelium was observed. As

a successful colonization of aerial plant parts by soil microbes is complicated, other application modes could be evaluated, such as soil treatment and the addition of culture filtrates, as well as the evaluation of the isolates against soil-borne pathogenic fungi. An interesting option could be to isolate active substances and metabolites which can be applied to agricultural processes as described by various research teams. Additionally, the evaluation of the application dose, the protective effect during different periods of time, and the persistence before pathogenic infection will allow to determine the efficacy of the selected biocontrol agents in comparison to commercialized chemical fungicides.

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## 4.6 Control of *Fusarium* and their mycotoxins by *Streptomyces* isolates

### 4.6.1 Introduction

*Fusarium graminearum* causes the disease known as Fusarium head blight (FHB) on wheat, leading to important yield losses in Europe. Besides its pathogenicity, *F. graminearum* can produce mycotoxins such as trichothecenes and zearalenone (ZEN), which can be found from the field to the final products derived from cereals (Duverger et al. 2011). Among the trichothecenes, deoxynivalenol (DON) is the most common and its presence in food and feed represents a major risk, as it causes nausea, vomiting and the inhibition of the immune responses (Mannaa and Kim 2017b). *F. graminearum* can also produce acetylated DON variants such as 3-acetyl-ADON (3-ADON) or 15-acetyl-ADON (15-ADON) (Haile et al. 2019). Different studies have reported that 15-ADON exhibits a higher intestinal toxicity than DON, while 3-ADON is less toxic (Alizadeh et al. 2016; Pinton et al. 2012). Zearalenone is less toxic, but can lead to estrogenic disruptions and hormonal changes (Richard 2007). *Fusarium verticillioides* is mainly a maize pathogen, causing Fusarium ear rot disease (FER). However, it has been recently found to affect wheat (Gagkaeva and Yli-Mattila 2020). In maize, it causes important losses and produces mainly fumonisin B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>). The latter are of major importance, due to their high occurrence and their severe toxic effects. Fumonisins are classified by the IARC as a class 2B possible human carcinogens (IARC 1993). Maximal limits on food and feed have been established for DON, ZEN, and the sum of FB<sub>1</sub> and FB<sub>2</sub>. In the case of foods intended for human consumption, limits were settled at 750 µg/kg, 75 µg/kg and 400 µg/kg, respectively. (European Commission 2006).

The use of chemical fungicides is nowadays the main strategy for the control of both *Fusarium*. However, they lead to fungal resistance and environmental pollution. Thus, new alternatives are currently researched (Syed Ab Rahman et al. 2018). Biocontrol by beneficial microorganisms represents a promising alternative to manage fungal diseases (Knudsen and Dandurand 2014). Actinobacteria are soil microorganisms capable of producing a wide variety of secondary metabolites, including antifungal compounds and degrading enzymes (Barka et al. 2016). The genus *Streptomyces* are the most abundant and studied among actinobacteria. The efficacy of *Streptomyces* as biological control agents to manage *Fusarium* has already been documented (Colombo, Kunova, Cortesi, et al. 2019). Mycostop®, formulated with

spores of *Streptomyces griseoviridis* is already commercialized, and is preconized for the control of *Fusarium* and several other plant pathogens (Lahdenperä et al. 1991).

An aspect often neglected during the evaluation of potential biological agents is the impact on mycotoxin amount, as the reduction of fungal growth is not always accompanied by a decrease on mycotoxins accumulation (Medina et al. 2017).

During our work, we evaluated a collection of 59 *Streptomyces* isolates and the commercial BCA Mycostop® as reference, for their *in vitro* antagonistic ability against *F. graminearum* and *F. verticillioides*. Their impact on fungal growth inhibition and on mycotoxin accumulation (DON, 15-ADON, zearalenone and fumonisins B<sub>1</sub> and B<sub>2</sub>) were evaluated. Additionally, ten bacterial isolates were selected to evaluate the impact of their cell free extracts (CFEs) on fungal growth and mycotoxin production.

### 4.6.2 Material and methods

#### Streptomyces isolates and fungal strains

Fifty-nine *Streptomyces* isolates from Qualisud collection plus a *Streptomyces griseoviridis* strain isolated from the commercial product Mycostop® were employed during this work. Their conservation and spore recollection have already been described in previously published work (Campos-Avelar et al. 2020).

*F. graminearum* isolate BRFM 1967 and *F. verticillioides* isolate BRFM 2251 were purchased from the International Centre of Microbiological Resources in Marseille (CIRM, University of Aix-Marseille, Marseille, France). *Fusarium* strains were maintained at 4°C in inclined PDA (Biokar BK095HA) tubes covered with paraffin oil. Fungal spores were produced by inoculating PDA plates and culturing them at 25°C for 7 days. Then, spores were scrapped from the surface by adding 5 mL of distilled sterile water and filtered through sterile cotton to eliminate fungal mycelium. Fungal spores were cultured, harvested and enumerated before each test.

#### Antagonistic *in vitro* evaluation

Dual culture assays were performed on Czapek Yeast Extract Agar medium (CYA: 30 g/L sucrose, 5 g/L yeast extract, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.3 g/L NaNO<sub>3</sub>, 0.05 g/L KCl, 0.05 g/L MgSO<sub>4</sub>, 1 mg/L FeSO<sub>4</sub>, 1 mg/L ZnSO<sub>4</sub>, 0.5 mg/L CuSO<sub>4</sub>, 15 g/L agar, pH ~7.4). For this, 10 µL of *Streptomyces* isolate at 10<sup>7</sup> spores/mL were inoculated on each side of the plate and allowed

to grow at 25°C. On the third day, 10 µL of a pathogen spore suspension at 10<sup>6</sup> spores/mL were added to the center of the Petri dish. Then, plates were inoculated for 6 and 7 days for *F. graminearum* and *F. verticillioides*, respectively, before image analysis and mycotoxin extraction. As a control, the pathogen was inoculated on a CYA plate without bacteria. All tests were done in triplicate.

### Impact of CFEs on fungal growth and mycotoxin production

Ten *Streptomyces* isolates were selected, according to their dual culture assay results, to produce CFEs in order to evaluate the impact of their metabolites on *F. graminearum* and *F. verticillioides* growth and mycotoxin production. Bacteria were cultured in CYB medium for 5 days at 25°C and 180 rpm. Then, liquid cultures were centrifuged at 10,000×g for 5 min and filtered through a PES (Polyethersulfone) filter at 0.22 µm to eliminate bacterial cells. In order to identify a potential effect of actinobacteria extracellular enzymes, CFEs were also tested after a heat treatment at 100 °C for 10 min. The treated CFEs were added at 10% to CYA medium. Then, 10 µL of the pathogen spore suspension at 10<sup>6</sup> spores/mL were added to the center of the plate and left for 6 or 7 days at 25°C in the dark. As a control, the pathogen was inoculated on a CYA plate without CFEs. All tests were done in triplicate.

### Fungal surface growth measurement and mycotoxin specific production calculation

Surface growth of both *Fusarium* was measured using ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018). Growth inhibition was established in comparison to the control without *Streptomyces* bacteria, which represented 100% of growth. Results are given in % of control.

$$\text{Growth ratio (\% of control)} = \frac{\text{Assay area (cm}^2\text{)}}{\text{Control area (cm}^2\text{)}} \times 100$$

Mycotoxin specific production was calculated by dividing the mycotoxin amount in the whole sample by the area of the fungal colony. This calculation allows to compare the mycotoxin production for the same fungal surface between the control and the different treatments.

$$\text{Mycotoxin specific production (sp)} = \frac{\text{Total amount of mycotoxin produced}}{\text{Colony area (cm}^2\text{)}} \times 100$$

Then, the percentage of reduction for mycotoxin specific production was calculated in comparison to the control without actinobacteria.

$$\text{Mycotoxin}_{(sp)} (\% \text{ of control}) = \frac{\text{Mycotoxin}_{(sp)} \text{ assay}}{\text{Mycotoxin}_{(sp)} \text{ control}} \times 100$$

#### Mycotoxin extraction and quantification

For mycotoxin extraction, the whole content of the Petri dish was transferred into a plastic container and the agar was cut into small pieces with a scalpel. After weighing the sample, 85 mL of solvent (acetonitrile/water/acetic acid, 79:20:10) were added to the container, followed by 20 min of agitation at 250 rpm. For trichothecenes, 500 µL of the mixture were diluted 1:50 with the mobile phase (acidified water, 0.5% acetic acid) before injection. For fumonisins, 500 µL of the mixture were diluted to 1:4 with the mobile phase. After dilution, samples were thoroughly vortexed before filtering through a 0.45 µM CA (cellulose acetate) filter for the HPLC-MS/MS analysis.

#### Mycotoxin analysis

For both pathogens, mycotoxin detection and quantification were achieved using an Ultra High-Performance Liquid Chromatography (UHPLC, Shimadzu, Tokyo, Japan) coupled with a mass spectrometer (8040, Shimadzu, Tokyo, Japan). LC separation was performed using a Phenomenex Kinetex XB Column C18 (50 mm× 2 mm; 2.6 µm particles) at 50 °C, with an injection volume of 50 µL. Mobile phase composition was (A) 0.5% acetic acid in ultra-pure water and (B) 0.5% acetic acid in isopropanol (HPLC MS grade, Sigma, St Louis, MO, USA), and the mobile phase flow rate was 0.4 mL min<sup>-1</sup>. The mass spectrometer was operated in electrospray positive (ESI+) and negative (ESI-) ionization mode, and two multiple reaction monitoring (MRM) transitions for each analyte were monitored for quantification (Q) and qualification (q) (Table 4, Table 5). All data were analyzed using LabSolution Software (v5.91/2017, Shimadzu, Tokyo, Japan, 2017). Limits of detection or quantification (LOD/LOQ in ng/mL, respectively) for each mycotoxin were: DON (4/14), 15-ADON (10/35), FB<sub>1</sub> (0.03/0.1), and FB<sub>2</sub> (0.01/0.05) (Pellan et al. 2020).

Table 4. MS/MS parameters for isotope-labelled internal standard (IS). MRM: Multiple reaction monitoring.

IS	Polarity	MRM	EC
DON C13	-	370.3 > 59.0	35
FB <sub>1</sub> C13	+	756.3 > 356.5-47	

Table 5. MS/MS parameters for mycotoxins. Q: quantification; q: qualification.

Mycotoxin	Polarity	MRM Q	EC MRM Q	MRM q	EC MRM q	R <sup>2</sup> Calibration curve	IS
FB <sub>1</sub>	+	722.35 > 334.5	-43	722.35 > 352.0	-44	0.9982	FB <sub>1</sub> C13
FB <sub>2</sub>	+	706.2 > 318.4	-37	706.2 > 336.4	-44	0.9980	FB <sub>1</sub> C13
DON	-	355.0 > 59.0	35	355.0 > 265.1	35	0.9998	DON C13

### Global analysis of *Streptomyces* isolates: clustering and Pearson correlation index

Clustering and Pearson correlation were performed as detailed in previous published work (Campos-Avelar et al. 2020). Briefly, isolates were grouped according to their effect on fungal growth and mycotoxin accumulation, and illustrated in a color coded heatmap. Clear yellow indicates a strong activity and purple to black indicates a weak activity or an increase on mycotoxin accumulation. Furthermore, the Pearson correlations ( $r$ ) between the different parameters evaluated were calculated. Heatmaps and Pearson correlation graphics were developed in Rstudio.

### 4.6.3 Results

#### Antagonistic *in vitro* evaluation in dual culture

*In vitro* dual culture assays allowed to determine that *F. verticillioides* growth was more easily inhibited by *Streptomyces* isolates than *F. graminearum*, with a decrease going down to 27% of the control by isolate IX48 (mean=63%, median=67%) for *F. verticillioides* and to 66% of the control (mean=83%, median=84%) by isolate IX24 for *F. graminearum* (Fig. 51).

The *in vitro* growth of *F. graminearum* was faster and very invasive, filling the whole surface of the Petri dish in about 6 days, while *F. verticillioides* growth was slightly slower and less voluminous (Annex 1). This slower growth kinetics could contribute to the higher efficiency observed for *F. verticillioides* growth inhibition (Fig. 52).

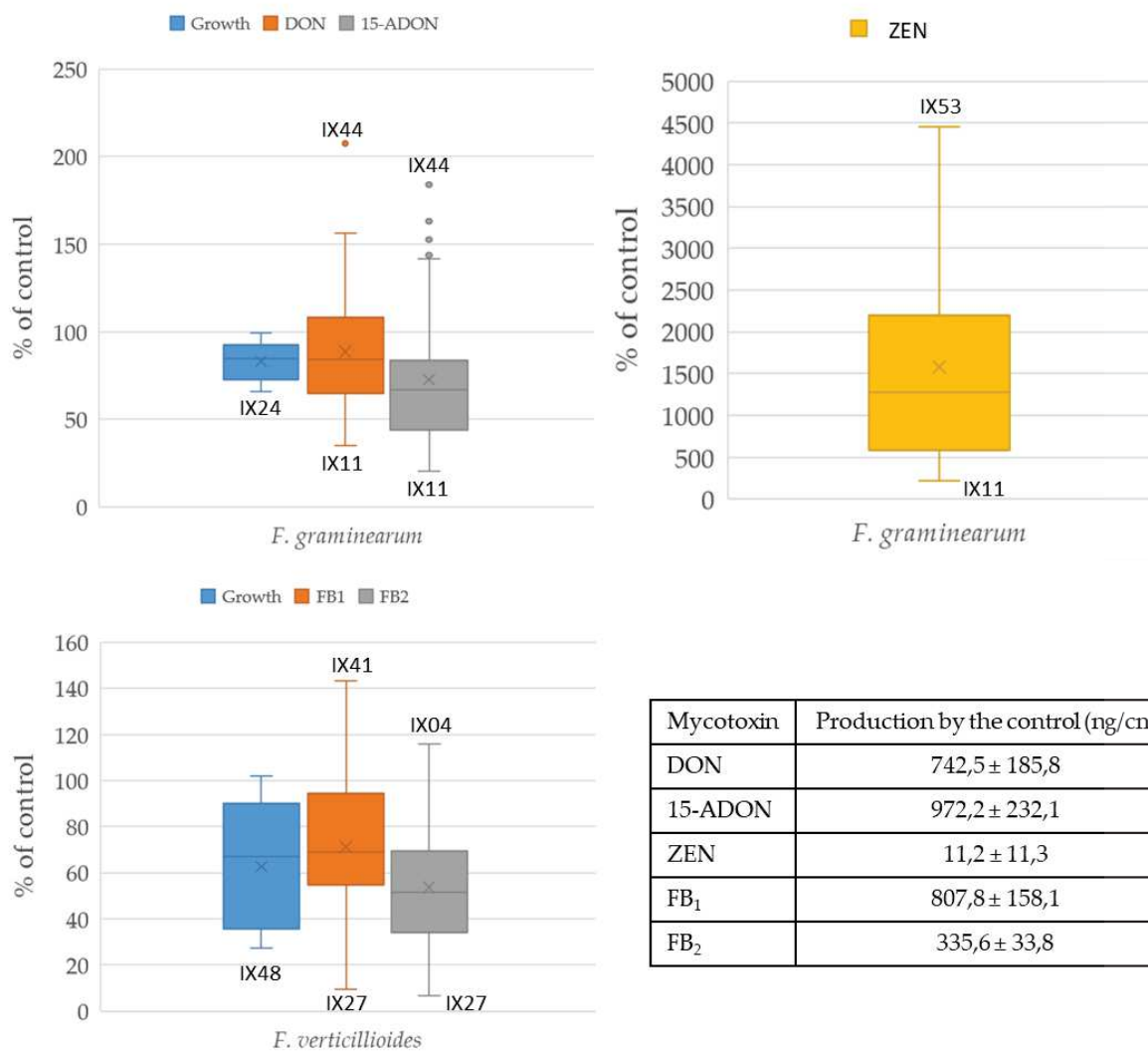


Figure 51. Impact of *Streptomyces* isolates on fungal growth and mycotoxin accumulation by *Fusarium graminearum* (top) and *Fusarium verticillioides* (bottom left) during *in vitro* dual cultures on CYA at 25°C for 6 and 7 days, respectively. Bottom right: Amounts of mycotoxin specific production by the controls.

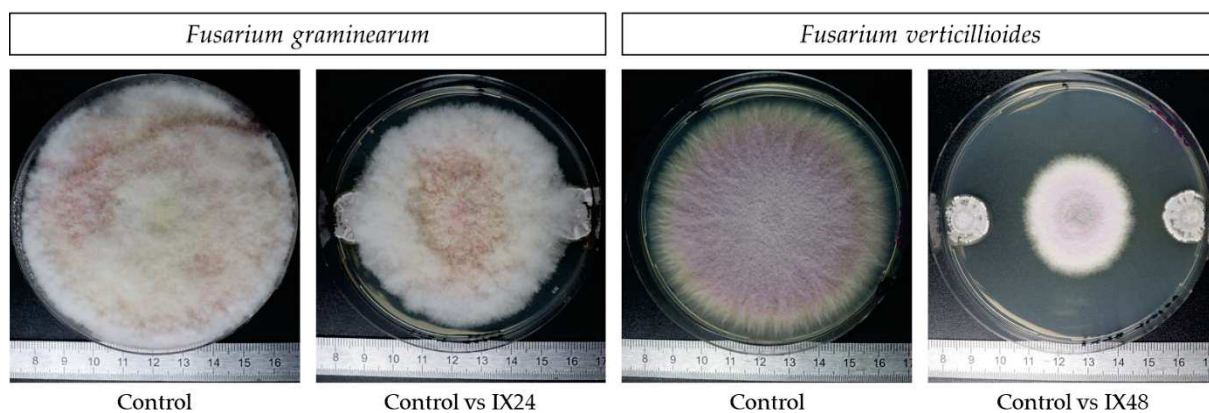


Figure 52. Effect of *Streptomyces* isolates on *Fusarium graminearum* and *Fusarium verticillioides* growth during *in vitro* dual cultures on CYA at 25°C after 6 and 7 days, respectively.



The effect of *Streptomyces* isolates on DON, 15-ADON and ZEN produced by *F. graminearum*, and on FB<sub>1</sub> and FB<sub>2</sub> produced by *F. verticillioides* was also evaluated by comparing them to the mycotoxin accumulation of fungus alone. Mycotoxin accumulation was normalized to the fungus growth area (Fig. 51, bottom right). Results showed that DON specific production was decreased down to 35% of the control (mean=89%, median=84%) by isolate IX11, but IX44 increased DON accumulation twice (208% of the control). A similar behavior was noted for 15-ADON with a reduction down to 20% of the control (mean=73%, median=67%), also by isolate IX11, and an accumulation of 184% caused by IX44 anew. Finally, zearalenone (ZEN) accumulation was highly increased by all bacterial isolates from 222% to 4458% of the control (mean=1580%, median=1277%). Even isolate IX11, that showed the strongest reduction of DON and 15-ADON, caused an overaccumulation of zearalenone, although more limited than the other isolates (222%) (Fig. 51, top right). FB<sub>1</sub> was diminished down to 15% of the control (mean=73%, median=71%) by IX27 while IX41 provoked an FB<sub>1</sub> overproduction of 143% (Fig. 50, bottom left). FB<sub>2</sub>, was strongly diminished down to 9% of the control (mean=55%, median=53%) by IX27 too. A slight increase (116% of the control) was caused by isolate IX04.

The heatmap presented in Figure 53 regroups the observed effects on fungal growth and mycotoxin production for each *Streptomyces* isolate during dual cultures. This representation allows to identify the particular impact of each isolate, as well as to group them according to their characteristics. A standardization was made to allow the comparison between the different parameters (growth, mycotoxin production), by converting the percentage values on z-scores separated in four categories: fungal growth, DON and 15-ADON, zearalenone and fumonisins. The obtained z-scores were then color coded, lower z-scores (clear yellow) correspond to a strong antagonistic activity, while weak antagonistic activities or isolates causing the strongest boost on mycotoxin production were colored dark purple to black. Intermediate colors represent moderate activities. Lastly, the similarity between *Streptomyces* isolates effects was calculated through the Euclidean distance.

As observed in Figure 53, bacterial isolates were separated in two main Clusters, I and II. Cluster I contains isolates with moderate to null effects on fungal growth, and also those isolates causing the highest stimulation of mycotoxin production. Subcluster IA1 provoked an overaccumulation of DON and 15-ADON, while subcluster IB1 caused an overproduction of fumonisins. Finally, subcluster IB2 caused the highest increase on ZEN production.

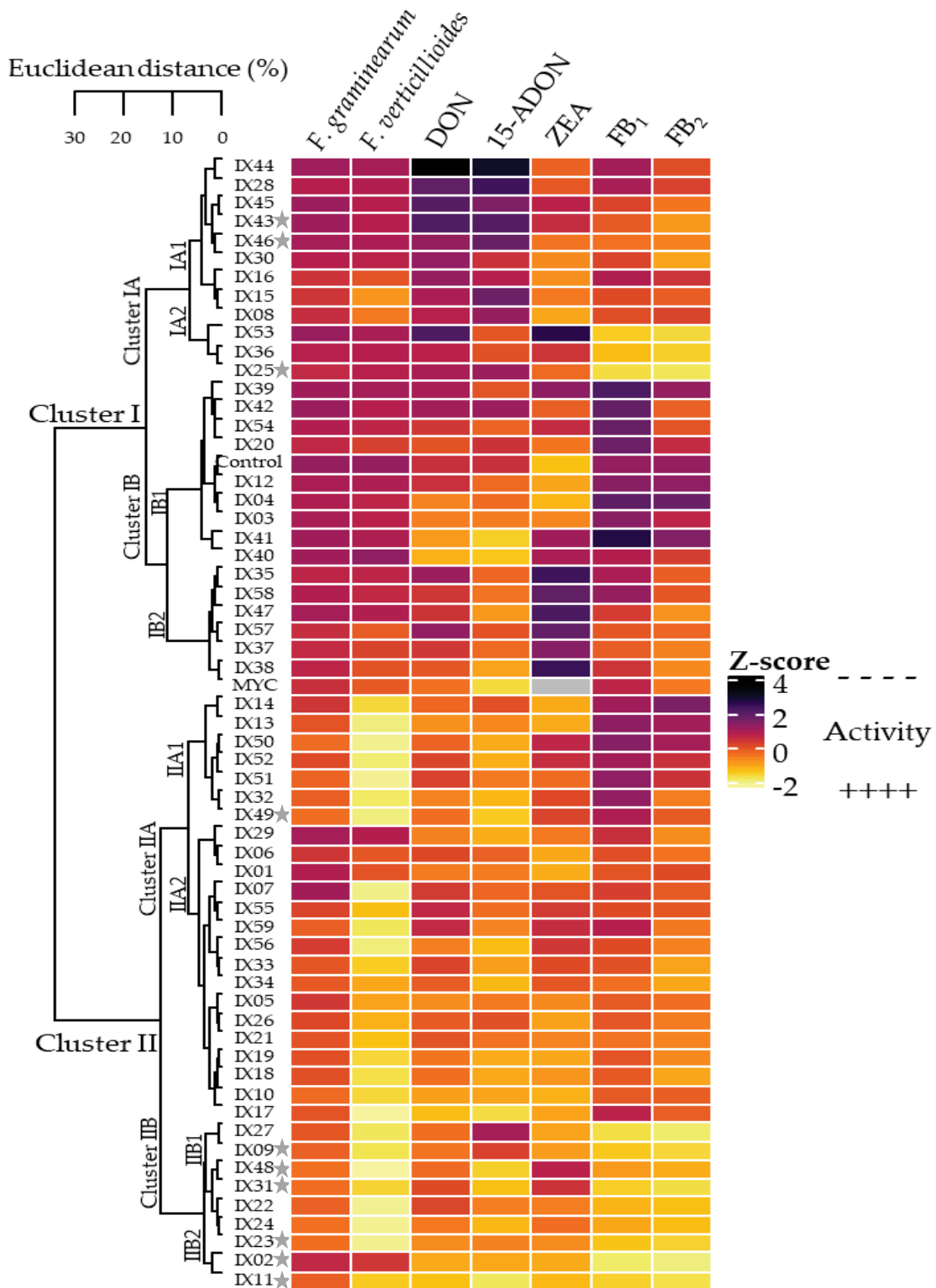


Figure 53. Summary heatmap of the effect of *Streptomyces* isolates on *Fusarium graminearum* and *Fusarium verticillioides* growth and mycotoxin accumulation. The results are given in a range of colors according to their Z-score where purple to black indicated a weak activity or an increase as compared to the control and yellow indicated the strongest activity. Grey stars indicate the isolates selected for CFEs assay. A missing value is indicated in grey.

Cluster II is composed by *Streptomyces* isolates having the strongest growth inhibition capacities, and by the isolates causing the highest decrease on mycotoxins amount. Isolates in subcluster IIB were especially efficient at decreasing both *F. verticillioides* growth and fumonisin accumulation, particularly isolates IX11 and IX23. Most isolates in subcluster IIA also strongly decreased *F. verticillioides* growth, but the reduction of fumonisin accumulation was limited. In fact, isolates in subcluster IIA1 had either no impact on fumonisins or caused very slight overaccumulations.

### Impact of cell free extracts on fungal growth and mycotoxin production

The impact of ten *Streptomyces* isolates cell free extracts (CFEs) on *F. graminearum* and *F. verticillioides* growth and mycotoxin production was also evaluated by adding them to the fungal growth medium (10% v/v).

Results show that fungal growth inhibition by CFEs was limited for *F. graminearum*, with a maximal growth decrease by the unheated CFEs to 86% of the control (mean=95%, median=96%) caused by isolate IX09 and to 80% of the control (mean=91%, median=89%) for the heated CFEs, caused by isolate IX49. Surprisingly, antifungal activity of the CFEs was slightly increased by the thermal treatment (Fig. 54, left). Concerning *F. verticillioides*, unheated CFEs were more effective at decreasing fungal growth down to 37% of the control (mean=61%, median=63%) by isolate IX09 as compared to a decrease down to 49% of the control (mean=71%, median=73%) by the heated CFE of isolate IX49 (Fig. 54, right).

DON and 15-ADON accumulation were decreased to a greater extent by unheated CFEs (DON mean accumulation = 32% of the control, 15-ADON = 26%) than by heated CFEs (DON = 42%, 15-ADON = 48%), with isolates IX09, IX23 and IX49 causing the strongest decreases. No ZEN production was detected on the CFEs assays. In contrast, accumulation of FB<sub>1</sub> and FB<sub>2</sub> was enhanced up to 800% of the control on average for both mycotoxins under both modalities. In particular, unheated CFEs of isolate IX09, provoked one of the highest mycotoxin stimulations (about 13 times more than the control) along with its highest inhibition of fungal growth. The most important increase on fumonisins was caused by IX02 (about 15 times more than the control) (Fig. 54, right).

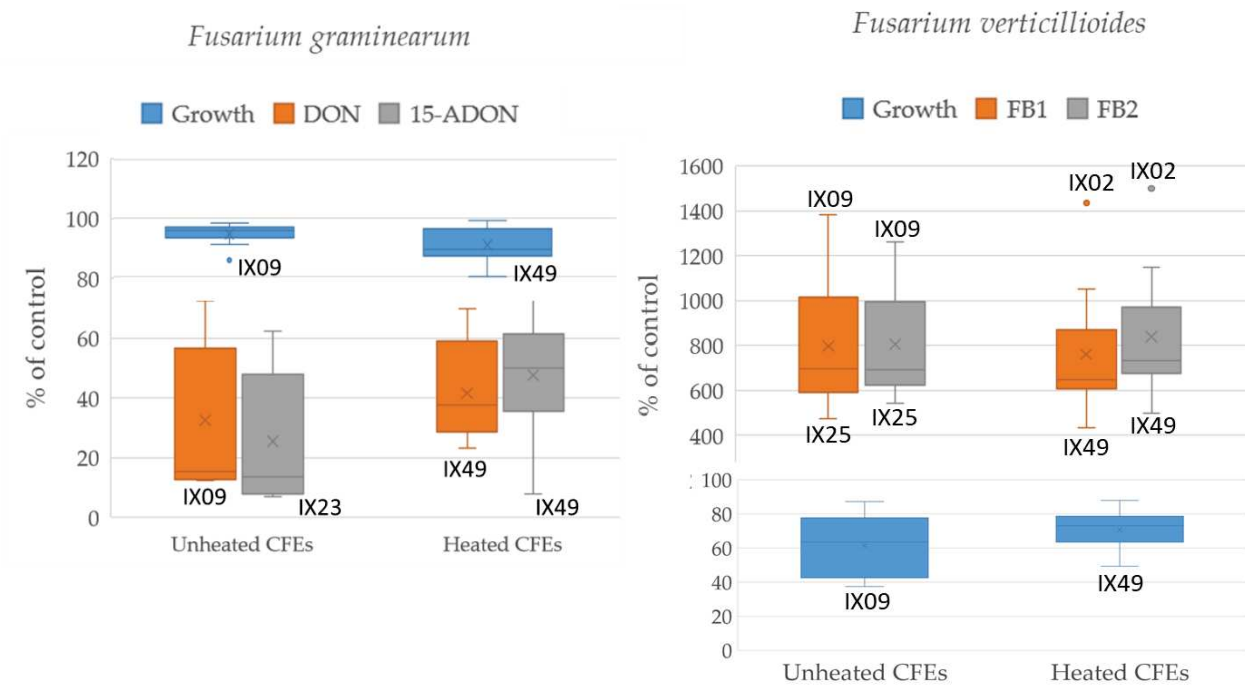


Figure 54. Impact of *Streptomyces* cell free extracts (CFEs) at 10% (v/v) on *Fusarium graminearum* and *Fusarium verticillioides* growth and mycotoxin production on CYA at 25°C.

The Pearson correlation index in Figure 55 shows the correlations between the effect of *Streptomyces* isolates on the accumulation of mycotoxins for both *Fusarium* sp. during dual cultures and on bacterial CFEs assays.

In the case of *F. graminearum*, the effects of *Streptomyces* isolates on DON and 15-ADON accumulation during dual culture are strongly correlated ( $r = 0.79, p < 0.001$ ). Similar observations could be made for CFEs whether being heated ( $r = 0.77, p = .006$ ) or not ( $r = 0.95, p < 0.001$ ). A moderate correlation was also observed between the effect of isolates in dual culture and their unheated CFEs on 15-ADON ( $r = 0.61, p = .045$ ). Finally, unheated and heated CFEs remained correlated regarding their impact on DON ( $r = 0.84, p = .001$ ). A slight correlation was observed between the effect on DON and ZEN on dual cultures ( $r = 0.34, p = .007$ ) (data not shown).

The effect on fumonisins produced by *F. verticillioides* was more variable. For instance, the impact on FB<sub>1</sub> and FB<sub>2</sub> in dual cultures had a positive correlation ( $r = 0.79, p < .001$ ), and a strong positive correlation was also observed between the impact of heated CFEs on both mycotoxins ( $r = 0.995, p < .001$ ). The effects of dual culture and heated CFEs on FB<sub>1</sub> were negatively correlated ( $r = -0.68, p = .02$ ), and the same occurred with FB<sub>2</sub> ( $r = -0.73, p = .01$ ).

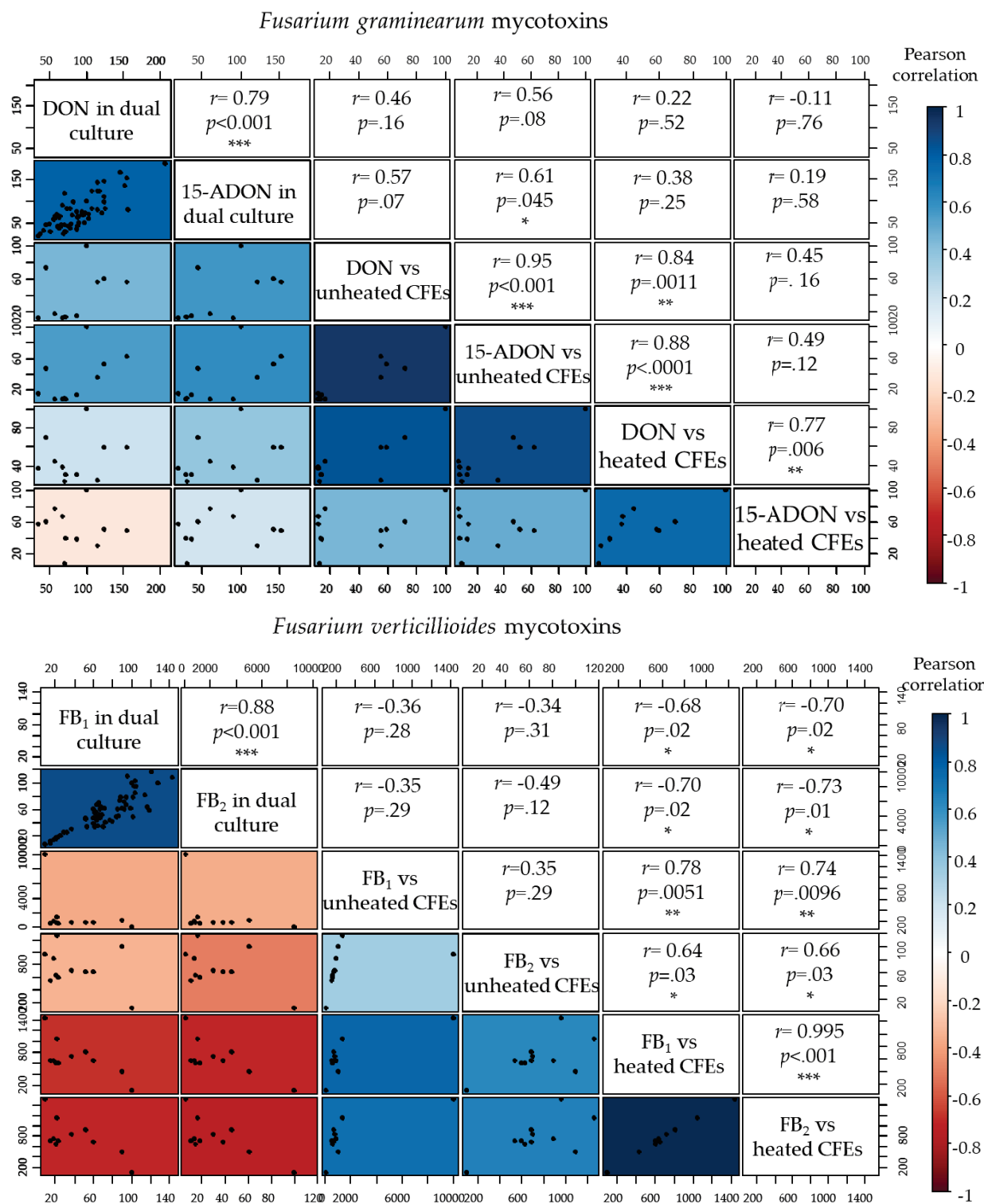


Figure 55. Pearson’s correlation of the effects of *Streptomyces* isolates and their CFEs on DON and 15-ADON produced by *F. graminearum* and on FB<sub>1</sub> and FB<sub>2</sub> produced by *Fusarium verticillioides*. In the correlation scale, the 1 in blue indicates a strong positive correlation and the -1 in dark red is a strong negative correlation.

#### 4.6.4 Discussion

During this study, we evaluated the impact of *Streptomyces* isolates and their CFEs on fungal growth and mycotoxin accumulation by *F. graminearum* and *F. verticillioides*.

Results indicate that fungal development was more easily inhibited for *Fusarium verticillioides* than for *Fusarium graminearum* during the *in vitro* dual culture assays. These results could be explained by the different growth rate of both pathogens. Indeed, microscopical observations by Pellan et al. revealed that *F. graminearum* produced rapidly long, dense and straight hyphae, while *F. verticillioides* developed fine, slightly branched hyphae (Pellan et al. 2020). Moreover, the distant inhibition profiles observed for *F. verticillioides* suggest that this pathogen might be more sensitive to the production of diffusible antifungal compounds by the *Streptomyces* isolates (Goudjal et al. 2016; Samsudin and Magan 2016).

Globally, bacterial CFEs' antifungal activity was similar to that of the bacteria themselves of both fungi. Moreover, thermal treatment did not completely eliminate their antifungal capacity. These results suggest that the tested bacterial isolates represent potential sources of heat-stable enzymes and extracellular antifungal compounds. Indeed, several bioactive molecules, such as allelochemicals, chitinases and volatile organic compounds (VOCs) with antifungal properties, have already been isolated from *Streptomyces* cultures. For this reason, the screening of crude extracts of *Streptomyces* isolates is essential for the subsequent identification and isolation of active compounds, as well as for the understanding of the antagonistic mechanisms (Colombo et al. 2019).

The fungal growth inhibition percentages obtained in our study were inferior to the ones documented in the literature. For instance, Colombo et al. obtained growth inhibitions from 41% to 71% during dual cultures of endophytic *Streptomyces* against *F. graminearum* (Colombo et al. 2019). Another study by this research team reported inhibitions of 60% to 90% of *F. graminearum* and *F. verticillioides* growth, respectively (Colombo, et al. 2019). An evaluation of *Streptomyces* as biocontrol agents was conducted by Ursan et al., who analyzed 60 bacterial strains against *F. culmorum* and *F. graminearum*. They obtained mean growth inhibitions of about 76% for *F. graminearum*. They hypothesized that the observed antifungal properties could be due to the synthesis of hydrolytic enzymes such as proteases and chitinases, produced by their *Streptomyces* strains (Ursan et al. 2018). Nguyen et al. reported

*F. verticillioides* growth inhibitions going up to 77% by bacterial cells and up to 73% by CFEs during an *in vitro* evaluation of *Streptomyces* isolates.

The impact on DON, 15-ADON, amount was moderate during dual cultures, with some bacterial isolates causing strong decreases (e.g. IX11) and some others provoking an overaccumulation (e.g. IX44). In contrast, all the bacterial isolates evaluated caused a huge overproduction of ZEN. The work of Ursan et al. revealed contrasting results, as their *Streptomyces* strains were able to decrease the production of ZEN evaluated by HPTLC (Ursan et al. 2018). An alkalinisation of the media by the *Streptomyces* isolates could explain the overproduction of ZEN observed in our assays, while DON production tends to decrease at higher pH (Wu et al. 2017). However, the impact on DON and ZEN has a slight positive correlation ( $r = 0.34$ ,  $p = .007$ ) so they do not seem to be affected in an opposite way by the bacteria.

The effect of *Streptomyces* isolates on fumonisins led to moderate decreases, with some isolates causing an overaccumulation (e.g. IX41, IX04). Very similar results on fumonisin decrease were obtained by Nguyen et al., who reported decreases on FB<sub>1</sub> and FB<sub>2</sub> of 88% and 98%, respectively (Nguyen et al. 2018). Strub et al. identified a down-regulation of fumonisin genes pathway on *F. verticillioides* in the presence of *Streptomyces* isolates (Strub et al. 2019). This type of mechanism could explain the decrease on fumonisin amount observed during our study. The observed FB<sub>1</sub> and FB<sub>2</sub> reductions during dual cultures could be caused by an inhibition of the mycotoxin biosynthetic pathway, or by a degradation of the toxic molecule once it has been produced by the fungi. However, the fact that bacterial metabolites alone (CFEs) strongly enhanced fumonisins production gives a clue that a potential degradation could occur in the dual culture assay, leading to an apparent reduction of fumonisin accumulation. Furthermore, the negative correlations observed between the impact of bacterial cells and CFEs on fumonisin accumulation, suggest a stimulating effect of *Streptomyces* metabolites that could be masked during dual cultures. Thus, the study of fumonisins degradation by the *Streptomyces* isolates seems necessary to validate this hypothesis. For instance, fumonisin degradation by the action of carboxylesterases and aminotransferases has been already documented for *Sphingomonas* sp. and *Exophiala* sp. (Ji et al. 2016; McCormick 2013; Vanhoutte et al. 2016).

Our results show that CFEs were able to strongly decrease DON and 15-ADON amount without causing any overproduction. The thermal treatment did not eliminate this ability,

which suggests the presence of heat-stable compounds (Boukaew and Prasertsan 2014). A hypothesis for the lack of ZEN observed in the CFEs assay, compared to the huge overaccumulation in dual culture, is that this mycotoxin may be produced by *F. graminearum* as a defense mechanism against the bacteria in dual culture (Chen et al. 2019), while the metabolites present in the CFEs do not trigger this response. The decrease on DON provoked by the bacterial CFEs could be due to the presence of mycotoxin production inhibitors or to a degradation by extracellular compounds. However, the great number of studies reporting microbial degradation of trichothecenes suggests that this explanation is highly probable, especially since trichothecenes can be detoxified by several mechanisms such as de-epoxidation, oxidation or isomerization (Vanhoutte et al. 2016). Indeed, DON degradation was documented for a series of *Nocardioides* strains. Additionally, degradation of DON in wheat and barley grain was assessed for the actinobacteria strain *Marmoricola* sp. Moreover, *Eubacterium* (Biomin® BBSH 797), has been developed into a commercial product to detoxify animal feed from trichothecenes (Ito et al. 2012).

Regarding fumonisins, as mentioned before, a massive increase was caused by bacterial CFEs as compared to the control, probably as a fungal reaction to the stress caused by the *Streptomyces* metabolites (Picot et al. 2010). Similar fumonisin increases were documented during the application of sublethal doses of fungicides to *F. proliferatum* (Cendoya et al. 2020). It is therefore conceivable that the low concentration of bacterial CFEs used in this study (10% v/v) might have stimulated the production of fumonisins in a similar way. Further studies with different concentrations of bacterial CFEs will allow this hypothesis to be explored. Contrasting results were obtained by Dalié et al. when studying the antagonistic capacity of the lactic acid bacteria *Pediococcus pentosaceus* against *F. verticillioides*. They determined that co-inoculation with bacterial cells inhibited fungal growth and in parallel caused an increase on fumonisin production. Inversely, when they used bacterial CFEs, fumonisins biosynthesis was significantly decreased (Dalié et al. 2012). Furthermore, a study performed by Danial et al. depicted an important decrease on fumonisins when *F. verticillioides* was cultured with *Streptomyces*' CFEs (Danial et al. 2020). The previous results and ours, evidence the interest of assessing the impact of BCAs on mycotoxin amount, since a reduction on disease severity is not always positively correlated to a reduction of mycotoxins production (Colombo et al. 2020).



The obtained correlations seem to indicate that, for each pathogen, the accumulation of the two mycotoxins was reduced simultaneously, which suggests that no reconversion or stimulation from one to the other occurred.

Globally, results indicate that several *Streptomyces* isolates in the collection have strong antagonistic properties against *F. graminearum* and *F. verticillioides*, such as IX11 and IX23, which not only inhibited both pathogenic fungi growth but also decreased their mycotoxin accumulation. However, additional research should be conducted to understand the triggering effect on fumonisin production caused by bacterial CFEs, which could lead to the identification of fumonisin biosynthesis regulators.

#### 4.6.5 Conclusion and perspectives

Our work allowed to identify several *Streptomyces* isolates with *in vitro* antifungal properties against *F. graminearum* and *F. verticillioides*. Furthermore, several isolates were able to decrease mycotoxin amount, independently of fungal growth inhibition. This *in vitro* screening represents a first attempt on the identification of potential biocontrol agents. Furthermore, in order to elucidate the antagonistic mechanisms applied by *Streptomyces* during antagonistic assays, a selection of their CFEs was also evaluated. A concerning result obtained during these experiments was the massive increase on fumonisins production. Which raises an alert on the importance of always assessing the impact that potential BCAs have on mycotoxin amount. Complementary studies on the bacterial strains' ability to degrade the studied mycotoxins, especially fumonisins, could help elucidate the mechanism behind mycotoxin decrease. Furthermore, a more detailed analysis of the impact of CFEs on mycotoxin specific production could lead to the identification of metabolites capable of regulating mycotoxin biosynthetic pathway.

*In planta* assays should be implemented in order to validate the observed antagonistic effects. An intermediate experiment performed on detached leaves or spikelets could represent a relatively fast and easy way to approach more natural conditions. Studies on the efficacy of *Streptomyces* as biological control agents will help improve the guidelines for the selection of potential antifungal microorganisms, by using integrated *in vitro* and *in planta* procedures. Furthermore, the comprehension of their antagonistic mechanisms and the optimization of their modes of application will increase their efficacy and guarantee their safe application, so they ultimately constitute a viable alternative to chemical fungicides.

#### 4.7 Synthesis of the effect of *Streptomyces* isolates on all pathogenic models

The heatmap in Figure 56 summarizes the effect of all the *Streptomyces* isolates of the QualiSud collection (plus Mycostop®'s strain) on the growth of the seven fungal pathogens evaluated in this study. Two main Clusters are identified, I and II. The isolates with the strongest antifungal activities are assembled on Cluster II (e.g. IX23, IX49, IX50 and IX51). Red symbols indicate those isolates that were unable to degrade the chitin during our preliminary assays. As we can observe, they are mainly grouped on Cluster I, which exhibits inferior fungal inhibition properties. However, the chitinase activity is not directly correlated with an inhibition of the growth of the various fungi, as several isolates in this cluster have limited antifungal effect, even if their enzymatic activity was detected. As our procedure was only qualitative for chitinase activity, it is still possible that large variations in terms of production level occurred between the isolates. Although, this result does suggest that the degradation of the chitin on the fungal cell walls may be one of the mechanisms behind the antifungal activities observed, but not exclusively in the case of strong inhibitor isolates. In fact, the implementation of several mechanisms is well documented among *Streptomyces* strains (Newitt et al. 2019). For the isolates without chitinolytic activity that still were capable of decreasing fungal growth (IX05 and IX31), two hypotheses can be drawn: the use of an alternative antifungal mechanism, such as antibiosis, or a stimulation of chitin production induced by the presence of chitin in the fungal walls (Nazari et al. 2011).

Pearson correlation coefficients of the impact of *Streptomyces* isolates on fungal growth are presented in Figure 57 (top). Strong correlations were revealed between the effect of *Streptomyces* isolates on the growth of the four mycotoxinogenic fungi and *M. oryzae*, with coefficients ranging from 0.81 to 0.97 ( $p < .001$ ), while *P. nodorum* and *Z. tritici* were slightly less correlated, as their coefficients ranked from 0.33 to 0.63 ( $p < .01$  and  $p < .001$ ). These results suggest that there may be a common mechanism behind the inhibition of non-necrotrophic fungi, and that the interaction of *Streptomyces* isolates with the two necrotrophic fungal strains might be due to a different mode of action. Indeed, singular behaviors were recorded regarding the inhibition of this pathogens. Isolates from Cluster I, mostly inefficient on all other pathogens, were highly active against *P. nodorum* (i.e. IX39, IX42, IX44, IX41 and IX47) and *Z. tritici* (e.g. IX01, IX38, IX57, MYC). Necrotrophic fungi are able to resist mycotoxins, lytic enzymes, and reactive oxygen species, as a mechanism to counterattack host defenses

(Karlsson et al. 2017). These resistances may have modified the profile of bacterial isolates being able to prevent their growth.

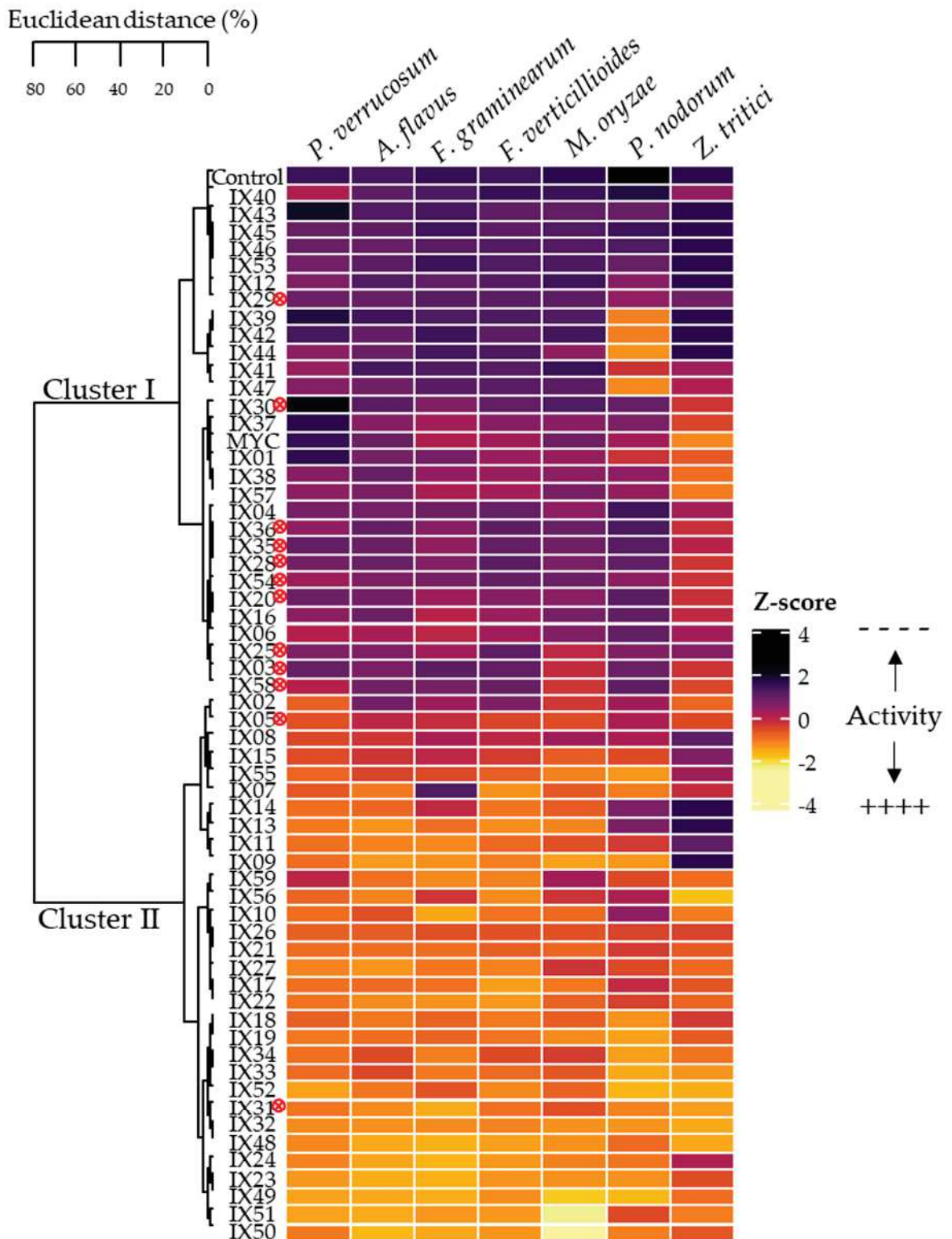


Figure 56. Summary heatmap of the effect of *Streptomyces* isolates on fungal growth of 7 pathogenic fungi. The results are given in a range of colors according to their Z-score where purple to black indicated a weak activity or an increase compared to the control and yellow indicated the strongest activity. Red symbols indicate the isolates without chitinolytic activity.

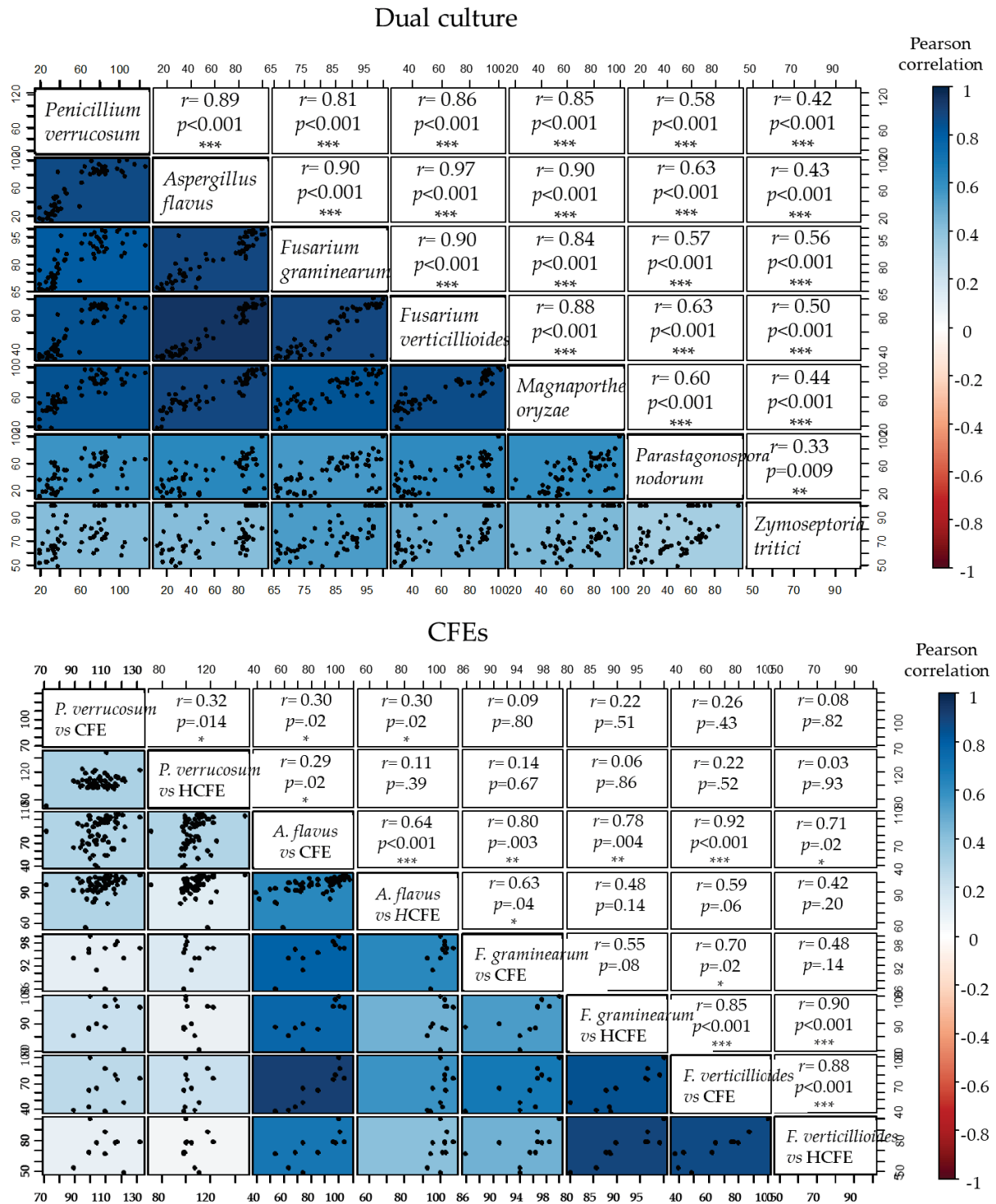


Figure 57. Pearson’s correlation of the effects of *Streptomyces* isolates on fungal growth in dual culture (top) or by their CFEs (bottom). Dual cultures were conducted against 7 fungi while CFEs were evaluated on 4. In the correlation scale, the 1 in blue indicates a strong positive correlation and the -1 in dark red is a strong negative correlation. CFE: Unheated cell free extracts. HCFE: Heated cell free extracts.

The correlations of the effect of bacterial CFEs on the four mycotoxinogenic fungi growth were also calculated (Figure 57, bottom). Results indicate that the effects on *A. flavus*, *F. graminearum* and *F. verticillioides* were generally highly correlated, while those on *P. verrucosum* were not. The difference between the impact of CFEs and dual cultures on *P. verrucosum* growth suggests that its inhibition requires a potential induction of *Streptomyces* isolates by the fungi. Indeed, our complementary results confirmed this assumption, as bacterial CFEs produced by isolates stimulated by fungal metabolites exhibited strong antagonistic properties, while non-stimulated CFEs did not (see 4.2.2 Complementary results).

The correlations of the effects of *Streptomyces* isolates and their CFEs on the accumulation of the four mycotoxins targeted in this study were also calculated (Fig. 57). Results revealed only one significant correlation in dual culture, between specific production of AFB<sub>1</sub> and DON ( $r=0.34$ ,  $p<.05$ ). Regarding CFEs, significant correlations were found almost exclusively between the effect of both CFE treatments (unheated vs heated) on the specific production of the same mycotoxin:  $r=0.83$  for OTA ( $p<0.001$ ),  $r=0.54$  for AFB<sub>1</sub> ( $p<0.001$ ),  $r=0.84$  for DON ( $p=0.001$ ) and  $r=0.67$  for FB<sub>1</sub> ( $p=.02$ ).

Seemingly, the effects of *Streptomyces* isolates on mycotoxin accumulation during the growth of the various fungal pathogens are not conducted by a common mechanism. This could be expected, as the studied mycotoxins possess different structures and biosynthetic pathways (Reverberi et al. 2010; Vanhoutte et al. 2016).

In conclusion, the capacity of *Streptomyces* isolates to decrease fungal growth seems to be linked to the production of chitinases, but not exclusively, as many isolates with chitinolytic activity were ineffective at decreasing fungal growth. This suggests that stronger inhibitors, such as isolates IX23, IX49, IX50 and IX51, might involve other antagonistic mechanisms, which makes them promising candidates for further research on antifungal properties. Moreover, correlations indicate the possible existence of two different mechanisms of interaction between *Streptomyces* and saprophytic/hemibiotrophic fungi and necrotrophic fungi. This is underlined by the singular interactions observed with isolates mostly inactive from Cluster I. Furthermore, the mechanisms behind mycotoxin decrease seem to be different for each mycotoxin.

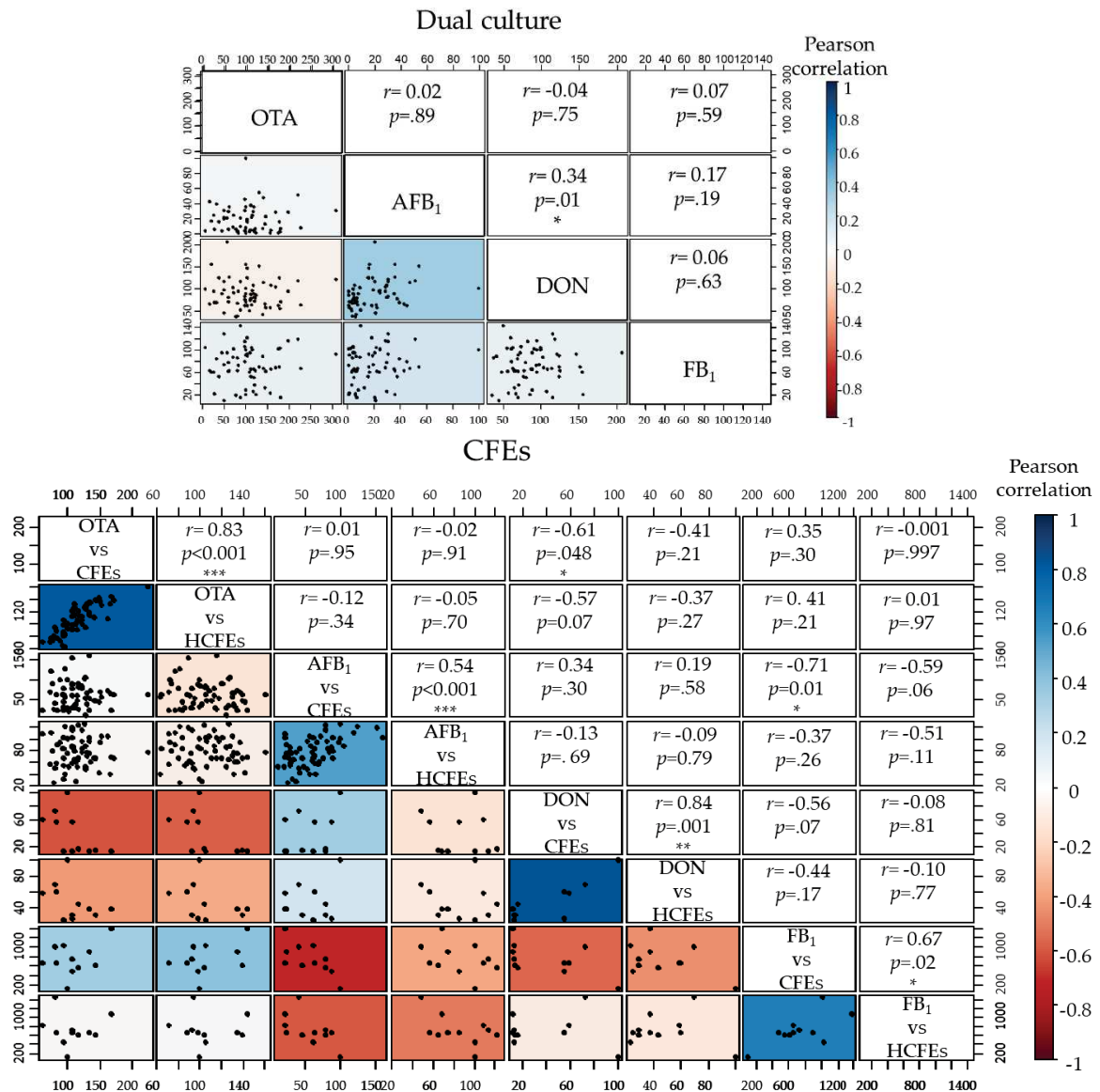


Figure 58. Pearson’s correlation of the effects of *Streptomyces* isolates the accumulation of four mycotoxins (OTA, AFB<sub>1</sub>, DON, FB<sub>1</sub>) in dual culture (top) or by their CFEs (middle). In the correlation scale, the 1 in blue indicates a strong positive correlation and the -1 in dark red is a strong negative correlation.



## 5. General conclusion and perspectives

The objective of our study was to evaluate a collection of *Streptomyces* isolates for their potential as antagonistic agents against various aspects of the control of fungal contamination from the field to the final products.

Initial studies were focused on the interactions of bacterial isolates with four mycotoxinogenic fungi, producers of four of the most prevalent mycotoxin groups: *Fusarium graminearum* and *Fusarium verticillioides*, infecting wheat on the field and producing deoxynivalenol and fumonisins, respectively, and *Aspergillus flavus* and *Penicillium verrucosum*, developing mostly during the storage of grains and producers of aflatoxins and ochratoxin A, respectively. The impact of *Streptomyces* isolates on fungal growth and mycotoxin accumulation, as well as their ability to degrade mycotoxins revealed interesting mechanisms of decontamination, but it also evidenced some singular cases where mycotoxin specific production was enhanced. Afterwards, we focused on foliar phytopathogens of wheat, exhibiting different pathogenic mechanisms: *Parastagonospora nodorum* and *Zymoseptoria tritici*, which are necrotrophic, *Magnaporthe oryzae*, an hemibiotroph, and *Puccinia triticina* an obliged biotroph. A first assessment of the antifungal activity of the *Streptomyces* isolates against these phytopathogens was conducted (except for *P. triticina*), followed by *in planta* assays. The results revealed the lack of correlation between the antifungal capacities measured *in vitro* and the protective capacity once applied to the plant, despite the apparent leaf colonization by *Streptomyces* isolates. Overall, the designed experimental steps allowed us to answer to a series of research questions with gradual increasing complexity, and to identify new research perspectives. Clustering and Pearson correlation analyses allowed us to have a global view of the results and to compare the effect of all the isolates on each parameter measured. These statistical approaches facilitated the comprehension of the antagonistic mechanisms implemented by the *Streptomyces* isolates evaluated, and the identification of the most promising candidates for further research. The most relevant findings will be detailed in this conclusion, as well as some improvement points that will allow a deeper understanding of the interactions between *Streptomyces* and pathogenic fungi.

Fifty-nine actinobacteria isolates were purified from culture plates, issued from preliminary studies of microbial isolation from crop soil and organic amendments. A strain of *Streptomyces griseoviridis* retrieved from the commercial product Mycostop® was added as a



reference to constitute the collection of 60 actinobacteria evaluated during this work. A preliminary 16S identification allowed to determine that the isolates in the collection belonged all to the *Streptomyces* genus. A complete genome sequencing is currently been performed for most active isolates, in order to provide a precise identification for further research. Moreover, the obtention of the complete sequences will allow the application of genomic tools to compare the bacterial isolates exhibiting similar and contrasting features, in order to identify the genes implicated in each specific activity and elucidate the mechanisms of interaction.

The first approach to evaluate fungi-bacteria interactions was performed by *in vitro* dual culture assays of the sixty actinobacteria (including Mycostop®) against all fungal pathogens (except for *Puccinia triticina*, an obliged biotroph). This general assay allowed at first to identify their **fungal growth inhibition capacities**. Results indicated that fungal development inhibitions varied between each bacterial isolate and depended on the pathogen considered. However, growth inhibition of most pathogens remained highly correlated, suggesting that at least one common mechanism could be at play. The chitinolytic capacities exhibited by the bacterial isolates could be implied in such observation. That being said, one could note that the ability to degrade chitin was not always linked to a strong antifungal capacity. Thus, additional mechanisms are probably carried out by the stronger fungal growth inhibitors. An interesting result was the broad antifungal spectrum of several isolates, such as IX23, IX49, IX50 and IX51, which were able to strongly inhibit the growth of all the studied fungi. Interestingly, the *in vitro* growth inhibition of *P. nodorum* and *Z. tritici* was less correlated to the inhibition of the other fungi. This could be explained by the fact that some *Streptomyces* isolates exhibited significant antifungal capacities only against one of these two pathogens and no other fungi. For instance, IX39, IX42, IX44, IX41 and IX47 inhibited only *P. nodorum*, while IX30, IX37, IX38, IX57, IX01 and MYC inhibited exclusively *Z. tritici*. This suggests the implication of particular mechanisms of interaction for these isolates, and these mechanisms seem to be different for each necrotrophic fungus. After this initial antifungal assessment, fungal pathogens were divided into storage pathogens and field pathogens to optimize the subsequent experimental approaches.

The evaluation of ***Streptomyces'* ability to detoxify mycotoxins** was conducted on the AFB<sub>1</sub> producer *A. flavus*, and on the OTA producer *P. verrucosum*. *In vitro* assays were pursued

by evaluating the **impact of bacterial CFEs on fungal development and mycotoxin production**, and through the assessment of their **degradation abilities under various conditions**. As actinobacteria cannot be applied directly into food matrices, the main purpose is to identify bacterial candidates for potential **sources of detoxifying enzymes and metabolites**. However, the use of CFEs was not as efficient, especially with *P. verrucosum*, which may indicate that both fungi react differently to the bacterial constitutive metabolites. Interestingly, the antifungal effect of CFEs produced in the presence of *P. verrucosum* metabolites was drastically increased for some isolates (e.g. IX32, IX56), while no activity was detected without activation. This finding opens new perspectives for the study of the stimulation of *Streptomyces*' metabolism and the production of active CFEs. Further studies on the impact of different concentrations of CFEs on fungal growth, together with the extraction of active compounds are strongly recommended.

The effects of *Streptomyces* isolates on OTA accumulation during dual cultures were diverse, with some isolates decreasing it while others enhanced it. By contrast, AFB<sub>1</sub> accumulation was decreased by all bacterial isolates during dual cultures. Another important consideration is the observed increase on mycotoxin accumulation caused by certain bacterial CFEs, particularly for OTA, and mostly when the fungal growth was inhibited. The overproduction of OTA could be the result of a fungal defense mechanism that was revealed only occasionally and with few CFEs for *A. flavus*. However, a potential AFB<sub>1</sub> overproduction might be hidden by the strong AFB<sub>1</sub> degrading capacities of *Streptomyces* isolates on solid medium but also in their CFEs, while OTA degradation on solid medium and in CFEs was scarce. Thus, even when the reduction of pathogenic development is desired, the **impact on mycotoxin accumulation** caused by the *Streptomyces* isolates must be considered.

Mycotoxin degrading assays revealed that OTA could be degraded by the bacterial isolates almost exclusively in liquid medium, while the culture conditions had few impacts on the isolates AFB<sub>1</sub> degrading capacities. Besides, bacterial CFEs were unable to degrade OTA, but they effectively degraded AFB<sub>1</sub>. Several hypotheses could have explained the previous result, such as an internal metabolization of OTA by the bacterial cells, or the stimulation of degradation mechanisms in the presence of fungal metabolites or even the influence of the pH. In our complementary study, the stimulation of bacterial CFEs by the addition of OTA or fungal metabolites during the growth of *Streptomyces* isolates did not confirm the theory of

induced extracellular degrading enzymes. Indeed, despite the observed modification of bacterial metabolism (i.e. production of pigments), the resulting CFEs remained unable to degrade the mycotoxin. Nevertheless, bacterial cells' degradation capacities were stimulated by the fungal metabolites, which reinforces the hypothesis of an intracellular degradation of OTA. The role of the pH remains not fully elucidated but seemed correlated to a degradation of the toxins only when the pH remained around 7, perhaps linked to a slower growth rate of actinobacteria. Further studies implementing pH variations could help to get insight into OTA degradation mechanisms and to improve the degradation efficiency.

For AFB<sub>1</sub> a subsequent verification of the residual toxicity for the strongest degrading bacteria in liquid medium (IX20, IX45 and MYC) allowed to determine that these ***Streptomyces* isolates effectively reduced the genotoxicity of AFB<sub>1</sub>**. *Streptomyces* isolates that displayed no degradation capacities but decreased mycotoxin accumulation in dual cultures and by their CFEs, might produce **active compounds able to inhibit mycotoxin biosynthetic pathway**. This is the case for IX57, IX47, IX53, IX37, IX38 and IX16 for AFB<sub>1</sub> and IX37, IX01, IX12, IX43 and IX09 for OTA. Further research could target the identification of active fractions and the isolation of enzymes and compounds capable of decreasing aflatoxin accumulation.

Antagonistic assays against *Fusarium graminearum* and *Fusarium verticillioides* showed that *F. graminearum* growth was hardly inhibited, while *F. verticillioides* inhibition was achieved by near half of the *Streptomyces* isolates. Furthermore, mycotoxin accumulation was hardly decreased by most isolates during dual cultures for both pathogens. A restricted number of isolates had strong inhibition properties but some of them also caused an important overaccumulation of the mycotoxins. In the case of ZEN, all bacterial isolates led to a huge overproduction by the fungi. The effect of bacterial CFEs on the growth of both fungi was similar to that of the bacterial cells, and thermal treatment decreased their efficiency for *F. graminearum* but not for *F. verticillioides*. This suggests that antifungal compounds are present in the CFEs and that some of them might be heat-stable. Interestingly, bacterial CFEs caused an important decrease on DON and 15-ADON as compared to the dual culture assay, without any mycotoxin stimulation. This was singular, as for *P. verrucosum* and *A. flavus*, the opposite was generally observed, with a higher decrease of mycotoxin accumulation in dual culture. Whether, the decrease of DON and 15-ADON is due to metabolites inhibiting

mycotoxins biosynthesis, or to a degradation by *Streptomyces* enzymes remains to be elucidated.

Contrasting results were obtained for FB<sub>1</sub> and FB<sub>2</sub>, for which specific production was stimulated from 4 to 15 times by all the bacterial CFEs, while it was generally decreased during the dual culture assay. The strong stimulation by the CFEs suggests that the reduction of FB<sub>1</sub> and FB<sub>2</sub> in dual culture could be due to a degradation mechanism rather than an inhibition of the fumonisins pathway. The hypothesis of an induced metabolism of the *Streptomyces* by the fungi cannot be ignored either. As already done for *P. verrucosum*, further studies on the induction of *Streptomyces* metabolism by *F. graminearum* and *F. verticillioides* metabolites seems to be an interesting approach for a deeper comprehension of the interactions occurring during bacteria-fungi confrontation. Moreover, it could be a promising strategy to promote the production of potential bioactive compounds.

The occurrence of single toxins in nature is uncommon. They appear mostly as a mix of produced by a consortium of fungi, which often cause additive and synergistic effects. For this reason, isolates capable of decreasing several mycotoxins are of great interest. Further research should include the evaluation of bacterial detoxifying properties of a combination of mycotoxins to get as close as possible to natural conditions. For this, degradation assays of artificially contaminated media with a mix of mycotoxins could be conceived.

*In planta* biocontrol potential was assessed by foliar application of selected *Streptomyces* isolates on wheat plants, which resulted in different levels of protection following the pathogen. SNB disease incidence was significantly decreased by two isolates: IX23 and MYC. STB was decreased by MYC but an undesirable increment on *Z. tritici* pycnidia was also reported. Finally, a significant protective effect against *P. triticina* was only exhibited by MYC. The evaluation of the **influence of *Streptomyces* on the levels of expression of plant defense genes** indicated that their biocontrol properties were not linked to an increase of plant immune response but to a more direct antifungal action. This hypothesis was reinforced by the observation of ***Streptomyces* installation on the surface of wheat leaves**. As the results of the *in vitro* and *in planta* assays on the selected isolates were not correlated, more isolates from the collection should be evaluated *in planta*, as other potentially bioactive isolates could have been neglected. For further research, the study of the **efficiency of various modes of application** (such as soil treatment or the addition of culture filtrates) and the **persistence of**

**the protective effect** represent interesting perspectives. Due to the number of isolates, *in planta* evaluation can be time consuming and demand a lot of effort, so an intermediate *in vitro* assay using detached leaves or spikelets can be conducted, as they have shown to be accurate at predicting *in planta* protection.

Thanks to the identification of active isolates, further studies on the medium-scale culturing of promising candidates could be explored. Such as solid-state fermentation for the production of bacterial spores, and submerged culture for the obtention of secondary metabolites and enzymes. Additionally, the optimal culture conditions should be assessed, as well as the verification of the conservation of the antagonistic/detoxifying activities. These assays could eventually lead to the development of biological products.

In conclusion, this work presents a comprehensive approach for the screening of *Streptomyces* isolates, and suggests optimization steps to achieve an integral evaluation of their antifungal and/or detoxifying properties. The various assays processed simultaneously helped to understand the mechanisms involved in fungal growth inhibition and mycotoxin decrease. We highlighted the relevance of assessing the impact of fungi-bacteria interactions on mycotoxin accumulation, in order to avoid overproduction of toxic metabolites. We also made an emphasis on the lack of correlation between *in vitro* and *in planta* assays and the importance of considering the tripartite interaction BCA-Pathogen-Host by pursuing screenings up to *in planta* validation.

The use of a large set of fungi and bacteria allowed us to analyze a great variety of scenarios and to identify not only *Streptomyces* isolates having a wide spectrum of activity, but also those exhibiting particular behaviors, encouraging additional research. To sum up, we validated the potential of *Streptomyces* isolates in the collection as promising sources of active enzymes and metabolites for the decontamination of mycotoxins in food matrices. Our results contribute to the search for more ecological solutions and to increase the availability of biological alternatives to substitute the chemical management of fungal pathogens and mycotoxins risk.



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## 7. Supplementary material

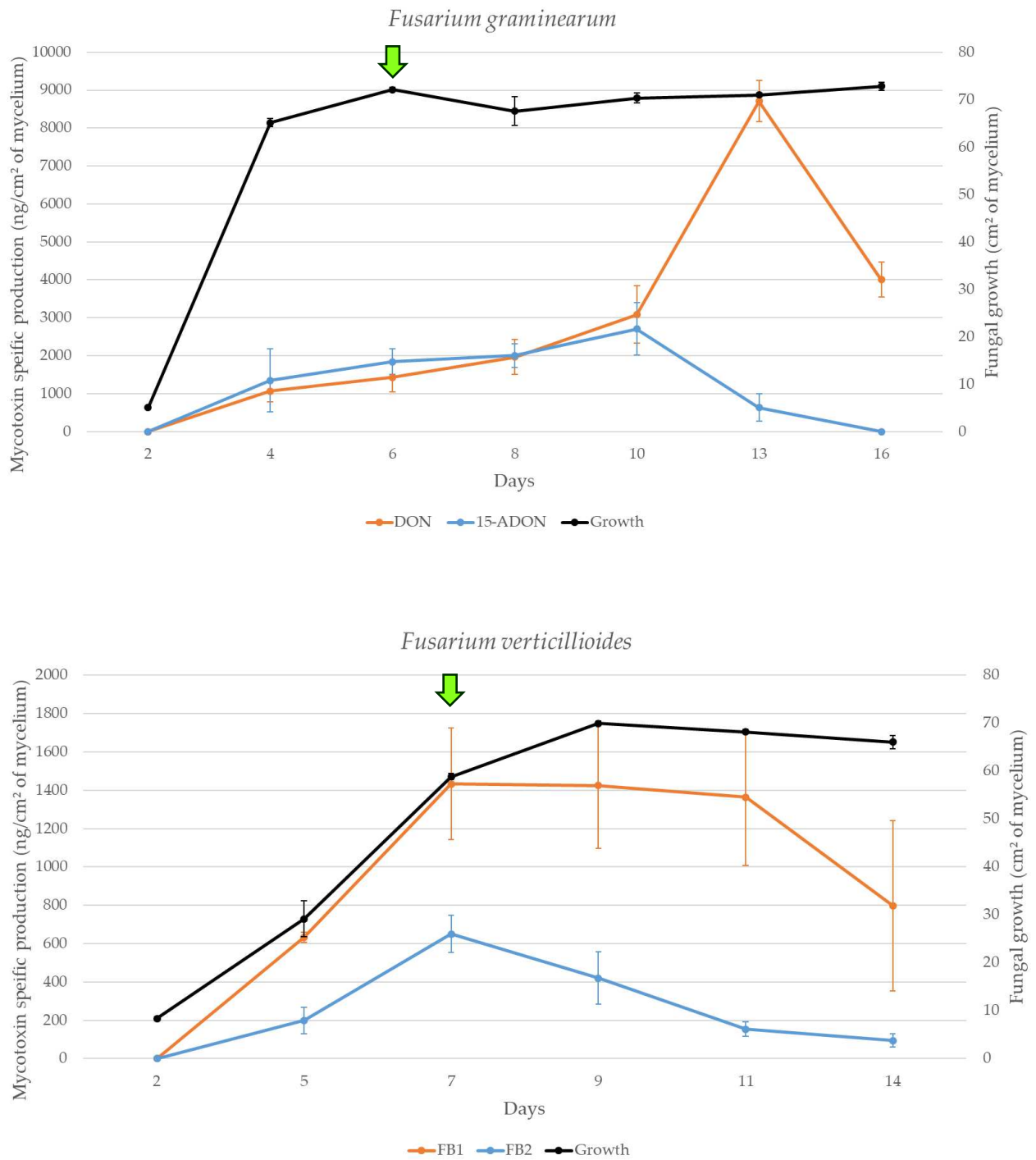


Figure S1. Kinetics of fungal growth and mycotoxin production of *F. graminearum* (up) and *Fusarium verticillioides* (bottom) grown on CYA medium at 25°C. Green arrow indicates the chosen incubation time for the antagonistic assays described in this work



Figure S2. Kinetics of fungal growth and mycotoxin production of *Aspergillus flavus* (up) and *Penicillium verrucosum* (bottom) grown on CYA medium at 25°C. Green arrow indicates the chosen incubation time for the antagonistic assays described in this work.

## 8. Summary in French



## Des actinobactéries pour le contrôle des champignons phytopathogènes et/ou mycotoxinogènes du blé

### **Contexte et objectifs de l'étude**

Les céréales sont la principale source d'aliment dans le monde grâce à leur apport élevé en glucides, protéines, vitamines et minéraux essentiels et aux zones étendues de culture. L'Organisation des Nations unies pour l'alimentation et l'agriculture (FAO) estime une production globale d'environ 2719 millions de tonnes de céréales en 2019. Presque deux tiers de la production céréalière mondiale sont destinés à l'alimentation animale, un tiers à l'alimentation humaine et environ 3% à la production de biocarburant. Le blé est la deuxième céréale la plus produite et consommée au monde, et la France est le principal pays producteur en Europe, avec approximativement 58 millions de tonnes par an. En vue de l'augmentation de la population, le rendement de la production céréalière doit être renforcée, afin de garantir l'approvisionnement des futures générations. Ceci implique aussi d'assurer la sécurité des graines à chaque étape de leur transformation. En effet, durant leur développement, les cultures de blé sont exposées à des stress biotiques (pathogènes, ravageurs) et abiotiques (sécheresse, aridité du sol, carences...) qui menacent les rendements de production. Une des principales menaces est causée par des champignons phytopathogènes qui provoquent des maladies sévères, entraînant environ 20% des pertes de production chaque année. En outre, certains de ces champignons sont capables de produire des métabolites secondaires toxiques, appelés mycotoxines, qui peuvent apparaître au champ, mais dont la principale source de risque d'accumulation se produit lors des mauvaises conditions de stockage de grains. L'apparition des mycotoxines dans les grains et produits dérivés présente un risque considérable à la santé humaine et animale, dû à leurs effets génotoxiques, hépatotoxiques, immunosuppresseurs, et pour certaines d'entre elles, cancérigènes. Pour cette raison, la Commission Européenne a établi des limites maximales pour chaque mycotoxine dans les différentes denrées alimentaires, notamment les céréales comme le blé, le maïs et l'orge. Par conséquent, le contrôle de maladies fongiques constitue un des principaux enjeux pour l'augmentation de la production de blé tout en maintenant sa qualité sanitaire.

Les principales maladies foliaires du blé en Europe incluent les rouilles, les septorioses, la fusariose et la pyriculariose. Elles sont causées par des champignons tels que *Puccinia triticina*, *Parastagonospora nodorum*, *Zymoseptoria tritici*, *Fusarium graminearum*, *Fusarium verticillioides* et *Magnaporthe oryzae*. *F. verticillioides* et *M. oryzae* sont des pathogènes se manifestant plus récemment dans le blé, leurs cibles étant principalement le maïs et le riz, respectivement. Au niveau des champignons mycotoxinogènes, *F. graminearum* est l'un des principaux producteurs de déoxynivalenol (DON) et *F. verticillioides* est le majeur producteur de fumonisines. D'autres champignons tels qu'*Aspergillus flavus* et *Penicillium verrucosum* infectent le blé au champ mais se développent surtout lors du stockage, où ils provoquent l'accumulation d'aflatoxines et d'ochratoxine A, respectivement. Le réchauffement climatique tend cependant à augmenter leurs activités toxigènes en pré-récolte.

Limiter la contamination par des champignons phytopathogènes est devenu une priorité pour les agriculteurs à cause de leur impact économique et leurs effets nocifs sur la qualité et la sécurité alimentaire. Les bonnes pratiques agricoles comme la rotation de cultures, le labourage, et une irrigation appropriée, contribuent à limiter la prolifération de maladies fongiques. Néanmoins, jusqu'à présent, le principal moyen de lutte reste l'utilisation des fongicides chimiques. Or, ces produits chimiques comportent eux aussi des risques considérables pour la santé et l'environnement. C'est pourquoi des nouvelles alternatives sont actuellement en développement, tels que l'utilisation de variétés de plantes résistantes et l'utilisation de microorganismes bénéfiques, avec des capacités antagonistes vis-à-vis des champignons pathogènes.

En ce qui concerne les mycotoxines, la recherche sur des méthodes de décontamination progresse rapidement. En effet, une diminution considérable de la quantité de mycotoxines a été documentée lors des procédés physiques de séparation, décorticage, broyage, macération et chauffage, ainsi que grâce à des nouvelles technologies telles que la lumière pulsée, l'irradiation et l'utilisation des matrices liantes. Les moyens chimiques de décontamination incluent principalement l'acidification, l'ammoniation, l'ozonation, l'oxydation et la réduction. Cependant, aucun traitement chimique ayant pour seul objet la décontamination des mycotoxines n'est autorisé dans l'Union Européenne. De plus, les méthodes chimiques comme physiques provoquent des modifications de la matrice alimentaire, pouvant entraîner des pertes de qualités sensorielles et nutritionnelles, ainsi que

l'apparition de résidus. Une alternative plus écologique et spécialisée est la biodégradation, qui consiste en l'utilisation des microorganismes et/ou enzymes capables de décontaminer les mycotoxines présentes dans la matrice, soit en les liant à la surface de leur membrane ou en les dégradant en sous-produits généralement moins toxiques. La première méthode n'est pas considérée comme une vraie détoxification, car les molécules toxiques peuvent être libérées lors de la digestion. Néanmoins, une décontamination par cette méthode peut avoir lieu si les cellules microbiennes peuvent être éliminées facilement de la matrice. Pour que la mycotoxine soit effectivement détoxifiée, il est nécessaire que sa structure chimique soit décomposée, usuellement par des enzymes comme des hydrolases, des laccases et des peroxydases. La vérification de la toxicité résiduelle est primordiale afin de garantir l'innocuité des sous-produits de dégradation. Quelques exemples des produits de dégradation incluent le DOM-1 pour la DON, le ZOM-1 pour la zéaralénone, l'HFB<sub>1</sub> pour la fumonisine B<sub>1</sub>, l'OT $\alpha$  pour l'OTA et l'aflatoxicol pour l'aflatoxine B<sub>1</sub>.

Les microorganismes représentent des possibilités d'utilisation variées en tant qu'agents de biocontrôle en pré et post-récolte avec des inhibitions de croissance et toxigenèse mais aussi des capacités de biodégradation de mycotoxines.

Parmi les agents potentiels, les actinobactéries sont des bactéries filamenteuses, gram-positives se trouvant principalement dans des environnements terrestres mais aussi aquatiques. Dans le sol, les actinobactéries se développent au niveau de la rhizosphère des plantes et dans certains cas à l'intérieur de celles-ci. Les actinobactéries se développent par des hyphes formant du mycélium végétatif. Puis, en réponse à la pénurie de nutriments, entre autres signaux, elles peuvent former du mycélium aérien contenant des spores. La différenciation des actinobactéries durant leur croissance submergée ou en surface, ainsi que la variation dans les conditions de culture, entraînent des modifications dans leur métabolisme donnant lieu à la production des divers composés.

Le genre *Streptomyces* est le plus étudié d'entre les actinobactéries dû à sa capacité à produire une grande variété de métabolites secondaires. En effet, environ 60% des composés agricoles microbiens dérivent des *Streptomyces*. L'efficacité antagoniste des *Streptomyces* contre des champignons pathogènes a été largement documentée. Leur mode d'action est basé sur une série de mécanismes plus ou moins élucidés, mis en place lors des confrontations

contre des agents phytopathogènes, tels que la production d'antibiotiques et d'enzymes, la compétition par l'espace et les nutriments, le parasitisme, etc. Additionnellement, certaines souches sont capables de stimuler les défenses immunitaires des plantes et de faciliter leur développement grâce à des processus de fixation d'azote ou de fer, de solubilisation du phosphate, de production des phytohormones, entre autres. La capacité des *Streptomyces* spp. à combattre des champignons mycotoxinogènes a été également étudiée. En effet, ces bactéries sont capables non seulement d'inhiber la croissance fongique, mais aussi de réduire l'apparition de mycotoxines, soit en les dégradant, soit en inhibant leurs voies de biosynthèse.

Par conséquent, les actinobactéries du genre *Streptomyces* ont un grand potentiel comme des agents de biocontrôle. Elles représentent également une source prometteuse de métabolites et enzymes avec des propriétés détoxifiantes. De plus, leur aptitude à coloniser des plantes, ainsi que leur capacité à former des spores, leur donnent des avantages par rapport à d'autres moyens de biocontrôle et facilite leur formulation et application ultérieure.

L'objectif de ces travaux a été d'évaluer la capacité antagoniste d'une collection d'actinobactéries vis-à-vis divers champignons pathogènes du blé. La capacité antifongique des souches bactériennes a été mesurée *in vitro*. Des essais *in planta* ont permis de vérifier l'installation des actinobactéries sur la plante et d'évaluer leur effet protecteur contre certaines maladies du blé. Finalement, pour les champignons mycotoxinogènes, l'impact des actinobactéries sur la production de mycotoxines a été étudié, ainsi que la capacité de celles-ci à dégrader les mycotoxines. Les résultats obtenus ont permis de mettre en évidence la capacité détoxifiante des bactéries étudiées, ainsi que les divers facteurs ayant un impact sur cette aptitude. Finalement, ces travaux ont servi à l'identification de plusieurs perspectives de recherche pour la compréhension des mécanismes impliqués dans la décontamination.

## Résultats expérimentaux

### Conformation et caractérisation de la collection d'actinobactéries.

Cinquante-neuf souches d'actinobactéries ont été isolées à partir d'échantillons de sol de l'Hérault (notamment de cultures céréalières) et d'amendements organiques. Celles-ci, plus une souche de *Streptomyces griseoviridis* isolée du produit commercial Mycostop®, constituent une collection de soixante isolats préservée à l'UMR Qualisud. Une extraction du génome 16S a permis une identification préliminaire indiquant que les isolats de la collection appartiennent au genre *Streptomyces*. L'évaluation *in vitro* de leurs propriétés PGP (« plant growth promotion », en anglais) indique que plusieurs souches de la collection sont capables de solubiliser le phosphate, ainsi que de produire de la chitinase, des sidérophores, de l'ammonium et de l'acide indole-3-acétique (AIA), la plupart des souches ayant deux ou plusieurs de ces capacités.

### Réduction de la contamination par ochratoxine A en utilisant des actinobactéries et leurs molécules actives

Dans cette étude, publiée dans le journal Toxins de MDPI en 2020, la capacité de la collection d'actinobactéries et leurs surnageants à inhiber la croissance de *P. verrucosum in vitro* a été évaluée, ainsi que leur impact sur la production d'ochratoxine A (OTA) et leur capacité à dégrader cette mycotoxine. Lors des confrontations sur milieu CYA, 17 souches ont inhibé fortement la croissance fongique. Néanmoins, cette inhibition était associée à une stimulation dans la production spécifique d'OTA. Dix-sept souches ont cependant diminué la production spécifique d'OTA jusqu'à 4% par rapport au témoin (soit 96% de réduction). Onze surnageants d'actinobactérie ont réduit la production spécifique d'OTA jusqu'à 62% du témoin, alors que la croissance n'a pas été impactée, sauf pour deux souches qui l'ont inhibé à 72% du témoin. Trente-trois souches ont été capables de dégrader l'OTA quasi totalement en milieu CYB liquide, alors que seulement cinq l'ont dégradé en milieu solide, mais pour deux d'entre elles entièrement. Les surnageants de culture ont été incapables de dégrader la mycotoxine, ce qui interroge sur le mécanisme de détoxification. Aucun produit de dégradation connu n'a été identifié par HPLC-MS/MS, ce qui pourrait être expliqué par une dégradation subséquente en produits inconnus. La clusterisation des résultats a permis d'identifier des traits communs entre les isolats bactériens et de les classer en fonction de

leurs effets, ainsi que de faire ressortir les corrélations entre les différents paramètres permettant d'obtenir des indications sur le mode d'action des actinobactéries.

### Recherche complémentaire sur la stimulation des actinobactéries

Comme décrit précédemment, les surnageants des actinobactéries n'ont pas été capables de dégrader l'OTA, alors que les bactéries dégradent la mycotoxine lors de leur croissance, surtout en milieu liquide mais aussi dans certains cas en milieu solide. Une explication pourrait être que les enzymes capables de dégrader l'OTA ne sont pas produites de façon constitutive lors de la croissance des actinobactéries en milieu liquide sans mycotoxine. Et donc, que la présence de la mycotoxine dans le milieu de croissance des bactéries lors des essais de dégradation aurait pu stimuler le métabolisme nécessaire pour la dégrader. Afin de tester cette hypothèse, les surnageants bactériens ont été produits sous plusieurs modalités de « stimulation », soit avec l'OTA, soit avec des métabolites du *P. verrucosum* ou en culture mixte bactérie-champignon. Puis, les surnageants stimulés ont été testés à nouveau pour leur capacité à dégrader la mycotoxine.

Les résultats obtenus indiquent que les bactéries étaient incapables de dégrader l'OTA lors de leur développement en milieu liquide, ce qui diffère des résultats obtenus précédemment, où la majorité des souches pouvaient la dégrader. Cette différence peut être due à l'utilisation d'Erlenmeyers en verre dans le test actuel au lieu des récipients en plastique dans le test précédent, ce qui aurait pu modifier le ratio d'aération. Dans un test additionnel comparant les deux méthodes de culture, on a observé que dans les récipients en plastique les valeurs de pH restaient plutôt neutres et les souches dégradent la mycotoxine, alors que dans les Erlenmeyers le pH était autour de 9 et les souches n'exhibaient pas de capacité à dégrader. Il semblerait donc qu'une croissance sous conditions non optimales et/ou à pH neutre pourrait pousser les bactéries à dégrader l'OTA. Des recherches complémentaires sont nécessaires afin d'expliquer ce phénomène.

Deuxièmement, on a constaté que l'addition de surnageant fongique stimulait fortement la dégradation d'OTA lors de la croissance des actinobactéries en milieu liquide, y compris à pH basique. Finalement, pendant la culture mixte, les actinobactéries se sont

développées plus rapidement que le champignon, dont le mycélium apparaissait rarement lors des observations microscopiques, ce qui pourrait expliquer la faible quantité d'OTA dosée. Les surnageants obtenus après les modalités de stimulation n'ont pas été capables de dégrader l'OTA. Néanmoins, les surnageants de certaines souches bactériennes ont montré des capacités antifongiques accrues lors qu'ils ont été ajoutés au milieu de croissance de *P. verrucosum*, ainsi qu'une diminution de la production spécifique d'OTA pour certains et une augmentation pour d'autres. Ces résultats complémentaires ouvrent des nouvelles perspectives de recherche afin de mieux comprendre les mécanismes impliqués dans la dégradation d'OTA par des actinobactéries, ainsi que le potentiel isolement et identification des métabolites et enzymes d'intérêt à partir de leurs cultures.

#### Inhibition de la croissance d'*Aspergillus flavus* et décontamination de l'aflatoxine B<sub>1</sub> par des *Streptomyces* spp. et leurs métabolites.

Vus des résultats prometteurs obtenus lors des tests précédents. La collection de 60 actinobactéries, dont l'identification préliminaire suggère être des *Streptomyces* spp., a été évaluée à nouveau, cette fois-ci contre *Aspergillus flavus* et sa production d'aflatoxines. Leurs surnageants de culture ont été également testés pour leur effet sur la croissance et la production d'aflatoxines. Un traitement thermique a été appliqué aux surnageants pour déterminer la part d'effet enzymatique dans les résultats observés. Finalement, la capacité à dégrader l'aflatoxine B<sub>1</sub> (AFB<sub>1</sub>) a été évaluée pour les bactéries et leurs surnageants. Une clusterisation a permis d'identifier des isolats d'intérêt pour des postérieures études. L'article issu de ces travaux a été soumis au journal Toxins de MDPI et est en cours d'évaluation.

Les résultats ont montré que 58 isolats ont été capables d'inhiber la croissance de *A. flavus* lors des tests de confrontation, avec une réduction allant jusqu'à 13% du témoin. De plus, la production d'aflatoxines B<sub>1</sub> et B<sub>2</sub> a été diminuée au moins à 54% du témoin par tous les isolats de *Streptomyces*. Les surnageants bactériens ont été moins efficaces pour inhiber la croissance fongique, avec des pourcentages maximaux de 40% du témoin pour les surnageants non chauffés et 55% du témoin pour les surnageants chauffés. La diminution de la production d'aflatoxines causée par les surnageants a été aussi moins importante, et certains surnageants ont provoqué une augmentation de celle-ci. La quasi-totalité des isolats

de *Streptomyces* ont été capables de dégrader l'AFB<sub>1</sub>, à la fois en milieu solide et en milieu liquide. Une dégradation totale a été accomplie par Mycostop® *Streptomyces griseoviridis* en milieu solide, et l'isolat IX20 a dégradé l'AFB<sub>1</sub> jusqu'à 6% du témoin en milieu liquide. La dégradation par les surnageants de la souche IX09 a diminué l'AFB<sub>1</sub> jusqu'à 37% du témoin. La recherche des produits de dégradation a montré la présence de certaines molécules inconnues et l'évaluation de la toxicité résiduelle par un SOS chromotest a indiqué une détoxification d'au moins 68% de la génotoxicité de l'AFB<sub>1</sub>.

La comparaison des résultats obtenus contre *A. flavus* et l'AFB<sub>1</sub> et *P. verrucosum* et l'OTA indique l'existence d'isolats dans la collection capables d'inhiber la croissance des deux pathogènes, ainsi que la production spécifique des mycotoxines. Des isolats aptes à dégrader les deux mycotoxines ont été aussi identifiés. En plus de l'identification des isolats avec un large spectre d'action, les différences entre les effets observés en fonction du champignon et des conditions de culture, donnent lieu à de nouvelles perspectives d'étude sur les différents facteurs qui conditionnent le potentiel des *Streptomyces* spp. comme agents de biocontrôle des mycotoxines.

### Contrôle de *Fusarium* et leurs mycotoxines par des *Streptomyces* spp.

Pour finaliser l'étude sur les champignons mycotoxinogènes, la collection de *Streptomyces* a été confrontée à *Fusarium graminearum* et *Fusarium verticillioides*, principaux producteurs de trichothécènes/zéaralénone et fumonisines, respectivement. Les résultats indiquent que *F. verticillioides* a été plus facilement inhibé que *F. graminearum* lors des essais de confrontation (27% et 66% du témoin, respectivement), probablement en raison de la croissance très rapide et invasive de ce dernier. Quant à la production spécifique des mycotoxines, les isolats ont provoqué des effets divers, avec certaines bactéries produisant des diminutions importantes, et d'autres provoquant une augmentation. Notamment pour la zéaralénone (ZEN) produite par *F. graminearum*, pour laquelle les stimulations étaient d'au moins 400%. Les surnageants d'une sélection de dix souches ont été également évalués pour leur effet sur la croissance et la production spécifique des mycotoxines des deux pathogènes. Les résultats montrent une faible inhibition de la croissance fongique pour les deux champignons. Néanmoins, la production de trichothécènes par *F. graminearum* a été



diminuée en moyenne jusqu'à 30% du témoin pour les surnageants non chauffés et 45% du témoin pour les surnageants chauffés. Aucun surnageant n'a provoqué une surproduction de trichothécènes, et la production de ZEN n'a pas été détectée. En revanche, tous les surnageants ont provoqué une importante surproduction de fumonisines (au moins 400%) par *F. verticillioides*. Des tests complémentaires de dégradation des mycotoxines pourront aider à élucider le mécanisme impliqué dans la diminution de mycotoxines observée. Une étude plus approfondie sur les effets de diminution et de surproduction en fonction de la mycotoxine pourrait permettre éventuellement l'identification des composés bioactifs ayant un impact sur la régulation des voies de biosynthèse. Additionnellement, des essais *in planta* pourraient être mis en place.

#### Biocontrôle des phytopathogènes du blé en utilisant *Streptomyces* spp.

Les travaux effectués durant cette thèse s'achèvent par l'étude de la collection des *Streptomyces* spp. contre des champignons phytopathogènes du blé : *Puccinia triticina*, *Parastagonospora nodorum*, *Zymoseptoria tritici* et *Magnaporthe oryzae*. La capacité antifongique des isolats bactériens a été d'abord évaluée *in vitro*, sauf pour *P. triticina*, qui est un biotrophe obligatoire. Ensuite, une sélection d'isolats a été testée *in planta* contre le nécrotrophe *P. nodorum*. Puis, une sélection plus réduite a été évaluée *in planta* contre un autre nécrotrophe, *Z. tritici*, et contre *P. triticina*. Lors des essais *in planta*, l'impact des isolats de *Streptomyces* sur l'expression des gènes de défense de la plante a été mesuré, ainsi que l'installation des bactéries sur les feuilles de blé, à l'aide d'un microscope électronique et par comptage des UFC (unités formant de colonies) sur milieu gélosé.

Les résultats des tests *in vitro* ont montré que *P. nodorum* a été plus facilement inhibé (45% du témoin en moyenne), suivi par *M. oryzae* (67% du témoin en moyenne) et finalement par *Z. tritici* (75% du témoin en moyenne). La clusterisation des résultats a permis d'identifier plusieurs isolats de *Streptomyces* capables d'inhiber la croissance *in vitro* des trois pathogènes. Pour la mise au point des essais *in planta*, une étude comparative a été menée entre l'effet des spores de *Streptomyces* à deux stades de développement (dormant et végétatif) contre *P. nodorum*. Les spores germées, ou en stade végétatif, ont été plus efficaces pour réduire le pourcentage des lésions sur la plante, donc ce mode d'application a été défini

pour la suite des essais. Douze isolats de *Streptomyces* ont été choisis pour leur évaluation *in planta*, en fonction de leur efficacité *in vitro*. Un traitement par des spores germées de *Streptomyces* deux jours avant l'infection par *P. nodorum* a indiqué que seulement 4 isolats donnaient des résultats significativement différents du témoin : IX23 et MYC qui ont diminué le pourcentage des lésions, ainsi que IX19 et IX51 qui ont aggravé les symptômes. Ces 4 isolats ont été testés ensuite contre *P. tritici*, résultant en un effet protecteur contre la maladie par MYC suite au traitement préventif avec des spores germées. Finalement, l'évaluation de ces 4 isolats contre *Z. tritici* a montré encore une fois que seulement MYC était capable de réduire le pourcentage de nécroses. Néanmoins, une augmentation du pourcentage des pycnides (structures reproductives de *Z. tritici*), causée par le même isolat, a été observée en parallèle, ce qui est indésirable car cela augmente le risque de prolifération. L'évaluation de l'impact de l'inoculation par des isolats de *Streptomyces* dans le niveau d'expression des gènes de défense du blé a montré que seulement le gène PAL a été significativement sous-exprimé, pour la plupart des isolats testés. Ce dernier résultat suggère que les effets protecteurs des isolats bactériens ne sont pas provoqués par une augmentation des défenses de la plante, mais plutôt par un mécanisme d'interaction directe entre la bactérie et le champignon, tel que l'antibiose. Cette hypothèse est renforcée par la vérification de l'installation des isolats de *Streptomyces* sur les feuilles de blé, observée par microscopie électronique et validée par le dénombrement des UFC.

## **Discussion et conclusion générales**

Les résultats obtenus tout au long de cette thèse ont permis, d'une part, l'évaluation du potentiel antifongique d'une collection de *Streptomyces* spp. contre des champignons phytopathogènes, allant d'un screening *in vitro* jusqu'à la l'évaluation de leur efficacité *in planta* pour certains isolats. Ces résultats sont en accord avec plusieurs études qui ont mis en évidence le potentiel de certaines espèces du genre *Streptomyces* comme agents de biocontrôle. Cependant, un grand nombre de ces recherches s'arrêtent à l'évaluation *in vitro* ou se concentrent sur un seul pathogène. La diversité de pathogènes cibles utilisés pour évaluer la collection de d'actinobactéries étudiée dans le déroulement de ces travaux de thèse permet une comparaison des différents modes d'action et l'identification d'effets contrastants, qui pourraient aider à mieux élucider les mécanismes antagonistes mis en place par les agents de

biocontrôle. De plus, ces travaux présentent une étude plus approfondie sur l'interaction des isolats de *Streptomyces* avec les principales mycotoxines représentant un risque considérable pour la sécurité sanitaire des aliments à base de céréales. Les résultats obtenus ont montré que, malgré l'impossibilité d'employer directement des *Streptomyces* pour la détoxification des aliments, du fait de leur production de métabolites (antibiotiques, composés organiques volatils), ces bactéries constituent une source potentielle d'enzymes pour la détoxification des mycotoxines et/ou pour l'obtention des métabolites d'intérêt qui pourraient empêcher leur production.

En conclusion, le genre *Streptomyces* représente une alternative prometteuse pour le biocontrôle des maladies du blé, ainsi que pour la prévention de l'accumulation des mycotoxines. Des recherches complémentaires sur leur mode d'action et la durée de leur effet de protection permettront d'optimiser leur application et efficacité, afin de surmonter les limitations qui rendent difficile actuellement le remplacement des fongicides chimiques par ces alternatives biologiques.