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Unveiling the Link: Exploring the Relationship between Volatile Organic Compounds Emission and Mycotoxin Production in *Aspergillus flavus* and *Fusarium verticillioides*.

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Unveiling the Link: Exploring the Relationship between Volatile Organic Compounds Emission and Mycotoxin Production (Aflatoxins and Fumonisins) in *Aspergillus flavus* and *Fusarium verticillioides*.

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Abstract

Molds are an integral part of our daily lives, and their presence is not always benign. While some molds offer benefits, others produce harmful secondary metabolites called mycotoxins. Approximately 25% of global food supplies are contaminated with these substances, posing a significant threat to human health and contributing to a substantial number of deaths (direct or indirect). The danger lies not only in their high toxicity, even at low concentrations, but also in their remarkable ability to withstand heat during conventional food processing methods. Therefore, they are subject to strict governmental monitoring, control, and regulation.

In addition to mycotoxins, molds produce volatile organic compounds (VOCs), which are essential in intra- and inter-species communication. These VOCs can also serve as biomarkers for identifying the genus, species, and growth stage of the mold. The inherent link between VOCs and fungal metabolism supports the use of these VOCs as a tool for detecting molds and mycotoxin production. Additionally, the study of VOCs helps to identify specific VOCs that have potential as biocontrol molecules.

This thesis has made a significant contribution to the understanding of VOC's profiles emitted by the two harmful fungal pathogen species *Aspergillus flavus* and *Fusarium verticillioides*, known for their respective production of aflatoxins and fumonisins. In addition to an in-depth study of VOCs and mycotoxins during individual inoculation of these fungi, their co-inoculation under different interaction conditions (contact and non-contact) was investigated. Thus, epizonaren and 4-epi-α-arocadiene were associated with contamination by *A. flavus* and *F. verticillioides,* respectively. Additionally, the emission of germacrene D during aflatoxin production and α-cedrene during fumonisin production was reported. On the other hand, ethyl 3-methylbutanaote has been recognized for its antifumonisin property. Its mode of action was studied at the level of gene expression following two applications of this ester in the fungal environment. Two opposite reactions were observed depending on the application, while its efficacy as an antifumonisin compound was confirmed.

Mots-clés: Composés organiques volatils (COVs), Volatolome, Volatilome, Mycotoxines, Aflatoxines, Fumonisines, *Fusarium verticillioides*, *Aspergillus flavus*, expression génique, co-inoculation, contact, non contact, 3-methylbutanoate d'éthyle

Résumé

Les moisissures font partie intégrante de notre vie quotidienne, mais leur présence n'est pas toujours inoffensive. Alors que certaines moisissures offrent des avantages, d'autres produisent des métabolites secondaires nocifs appelés mycotoxines. Environ 25% des denrées alimentaires mondiales sont contaminées par ces substances, ce qui représente une menace significative pour la santé humaine et contribue à un nombre important de décès (directs ou indirects). Le danger réside non seulement dans leur grande toxicité, même à faibles concentrations, mais également dans leur capacité remarquable à résister à la chaleur lors des méthodes conventionnelles de transformation alimentaire. Par conséquent, elles font l'objet d'une surveillance, d'un contrôle et d'une réglementation gouvernementales stricts.

En plus des mycotoxines, les moisissures produisent des composés organiques volatils (COVs), indispensables dans la communication intra- et inter-espèces. Ceux-ci peuvent également servir de biomarqueurs pour identifier notamment le genre, l'espèce et le stade de croissance. Le lien inhérent entre les COVs et le métabolisme fongique, soutient l'utilisation de ces COVs en tant qu'outil de détection des moisissures et de production de mycotoxines. En parallèle, l'étude des COVs contribue aussi à mettre en évidence des COV spécifiques ayant un potentiel en tant que molécules de biocontrôle.

Cette thèse a apporté une contribution significative à la compréhension des profils de COV émis par les deux espèces pathogènes fongiques nuisibles, *Aspergillus flavus* et *Fusarium verticillioides*, connues pour leur production respective d'aflatoxines et de fumonisines. En plus d'une étude approfondie lors de l'inoculation individuelle de ces champignons, leur co-inoculation dans différentes conditions d'interaction (contact et non-contact) a été investigué. Ainsi l'epizonarene et le 4-epi-α-arocadiene ont été respectivement associés à la contamination par *A. flavus* et *F. verticillioides*. De plus, l'émission du germacrene D lors de la production d'aflatoxines et du α-cedrene lors de la production de fumonisines a été reporté. D'autre part, le 3-methylbutanaote d'éthyle a été reconnu pour sa propriété anti-fumonisine. Son mode d'action a été étudié au niveau de l'expression génique à la suite de deux type applications de cet ester dans l'environnement fongique Deux réactions opposées ont alors été observée en fonction de son application alors que de son efficacité comme composé anti-fumonisine a été confirmé.

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List of acronyms and abbreviations

A. flavus	*Aspergillus flavus*
AFB_1	Aflatoxin B1
AFG_1	Aflatoxin G1
F. verticillioides	*Fusarium verticillioides*
FB_1	Fumonisin B1
FB_2	Fumonisin B2
FB_3	Fumonisin B3
FBs	Fumonisins
GC-MS	Gas chromatography coupled at mass spectrometry
HPLC	High performance liquid chromatography
NTS	Non-toxigenic strain
RI	Retention index
SPME	Solid Phase Mobile Extraction
TS	Toxigenic strain
VOCs	Volatile organic compounds

Chapter 1

The Main Protagonists, The Objectives and The Journey

1. Stealthy Menace: The Mycotoxins

1.1. Fungi: Source of secondary metabolites

Fungi produce a wide variety of primary and secondary metabolites. Primary metabolites are essential compounds produced according to the normal metabolic process involved in basic functions and needed for growth, development and reproduction. Secondary metabolites are not directly involved in the core metabolic process and they have specific ecological functions role such as defense, attraction, or competition against other species. In addition, a relation between the secondary metabolites and fungal development has been proved [1].

The secondary metabolites can be gathered in six categories:

- the antibiotics which are compounds that inhibit the growth of bacteria or other microorganisms, such as penicillin from *Penicillium* genus;
- the pigments, like many *Fusarium* species that produce bright red pigments named anthraquinones;
- the antifungal compounds, to limit proliferation of other fungi and/or compete with them;
- the bioactive compounds, often used in industrial and medical applications;
- the signaling molecules such as volatile organic compounds (VOCs), playing a role in fungal inter and intra-species communication;
- the mycotoxins, which are toxic compounds produced by several fungi.

1.2. Maize and Mycotoxins: a worldwide challenge

Maize, a globally cultivated and traded cereal, is the second most widely grown crop and a highly consumed staple, with Europe alone consuming approximately 102 million tons annually [2]. As a major economic and food commodity worldwide, it is crucial to comprehensively understand and optimize all aspects of maize production [3].

Indeed, maize production is subject to numerous variables. The maize cultivation ecosystem encompasses a wide variety of bacteria, viruses, insects, fungi, animals and other plants. Among these, fungi are among the most problematic issue since many of them produce mycotoxins, which are toxic compounds produced through specific secondary metabolism pathways. Additionally, it is not uncommon to find multiple mycotoxin families in maize, each of them associated with specific fungal species. This issue has been known for many years, leading to the development of a wide range of control and detection methods [4], as well as strict legislation to address it [5].

Food and Agriculture Organization (FAO) have estimated the food crop contamination at level of 25% [6] and a study conducted in 2020 confirmed these levels of contamination [7]. In addition, the European Food Safety Authority (EFSA) ranged the mycotoxins as a major concern in the importation of food in EU.

Mycotoxin contamination can occur at various stages along the chain from cultivation to consumption [8].

Actually, around 400 mycotoxins are reported as a significant threat and some of them regulated by the legislation [9]. However, new emergences mycotoxins are described currently and the knowledge of their effects is still investigated and not the subject of a legislation yet [10]. Until now, mycotoxins have been classified into several families primarily based on their chemical structures. Major issues, associated with mycotoxin contamination of agricultural products in Europe, include contamination by *Fusarium* and *Aspergillus* species [11], mainly leading to respectively aflatoxins and fumonisins contamination. Furthermore, the increasing global temperatures inevitably lead to the wider colonization of these species, resulting in a growing contamination of their mycotoxins, worldwide [12,13]. Therefore, the focus of this study is directed towards these specific mycotoxin families.

1.3. The toxicity of aflatoxins and fumonisins is a significant concern.

One of the main concerns in food safety is linked to the consumption of food or feed contaminated by mycotoxins that involve high health risks to humans and animals. While chronic exposition to mycotoxins can lead to chronic diseases such as immune system suppression, organ damages, cancer, the exposure to high contamination lead to acute effects and sometimes to death.

The mode of action of aflatoxins and fumonisins are different. The aflatoxins can exert their toxic effects in the human body through multiple mechanisms. One of the primary mode of action is their ability to establish covalent bonds with DNA, leading to the formation adducts and resulting in DNA damage and mutations, which can disrupt normal cellular processes and increase the risk of developing hepatocellular carcinoma (liver cancer). Aflatoxins are considered to be potent carcinogens. In addition to their genotoxic effects, aflatoxins can also impair liver function. They are primarily metabolized by the liver, and their toxic metabolites can cause liver cell damage, inflammation, and oxidative stress [14,15]. Prolonged exposure to high levels of aflatoxins can lead to liver diseases, including cirrhosis and liver failure. In addition, aflatoxins have immunosuppressive properties, which can weaken the immune system's ability to fight infections and diseases, making individuals more susceptible to various infectious diseases.

Regarding fumonisins, their key mode of action is the ability to inhibit ceramide synthase, an enzyme involved in sphingolipid metabolism [16]. The fumonisins interfere with ceramide synthesis and disrupt strictly cellular processes, including cell membrane integrity, signaling pathways, and lipid metabolism.

Each of these mycotoxins families have a predominant mycotoxin classified by the International Agency for Research on Cancer (IARC). Indeed, fumonisin B_1 is classified as a Group 2B agent, as possibly carcinogenic to humans, but aflatoxin B_1 is classified as a Group 1 carcinogen which means that aflatoxin B_1 is evaluated as a cause of liver cancer (hepatocellular carcinoma), according with the results obtained in numerous studies [17].

1.4. The strength of mycotoxins

The knowledge of chemical and biological aspects of aflatoxins and fumonisins has provided the base for generating an enormous amount of studies leading to achieve valuable insights into the genetic regulation, biosynthesis pathway, the factors influencing their production [18–21] and also their effects on human and animal health [22,23]. On the other hands, several studies were carried out with the aim to know the parameters affecting aflatoxins and fumonisins production and therefore generate tools for their control [24].

The notable threat of aflatoxins and fumonisins stems also from their remarkable thermostability. They are chemically stable and resistant to temperature variations, making them difficult to eliminate. The melting points of fumonisins and aflatoxins vary depending on the specific type, but they generally fall within a range that allows them to withstand conventional food processing methods (aflatoxin B_1: 268-269°C, aflatoxin B2: 286-289°C, fumonisins B_1: 230°C, fumonisin B_2: 230°C) [25]. However, they can be sensitive to factors such as UV light in the presence of oxygen, extreme pH levels (either very acidic or very alkaline), and oxidizing agents. These conditions can lead to degradation or alteration of these mycotoxins [26,27].

1.5. Why do fungi produce mycotoxins?

The role of mycotoxins is an ongoing area of research and remains somewhat of a mystery, although hypotheses have emerged. These toxic compounds are produced as secondary metabolites, potentially serving as a defense mechanism for fungi against competing organisms by inhibiting the growth of other fungi or bacteria. This inhibition could provide a competitive advantage to the toxin-producing fungi, allowing them to outcompete other microorganisms. In addition, mycotoxins may contribute to the ecological adaptation of fungi, potentially protecting them from predation and deterring herbivores and other organisms that could consume them. Furthermore, mycotoxins could be involved in nutrient acquisition, potentially assisting fungi in colonizing nutrient-rich substrates by inhibiting the growth of competing microorganisms. It is also speculated that mycotoxins may play a role in the stress response of fungi, helping them cope with various stressors such as drought, nutrient limitation, or high temperature. Lastly, mycotoxins might have interactions with host plants, potentially affecting the plant's defense responses or facilitating the establishment and spread of fungal infections. Indeed, the recent discovery of glucosylated mycotoxins opens up new perspectives, highlighting the potential harm of these molecules to other living organisms and the need to reduce their toxicity

through biotransformation [28,29]. These hypotheses warrant further investigation to confirm their validity [30].

2. The Targeted Fungi: *Aspergillus flavus* and *Fusarium verticillioides*

This part focused on the main producers of aflatoxins and fumonisins, which are: *Aspergillus flavus*, for aflatoxins [31] and *Fusarium verticillioides,* for fumonisins [32,33].

2.1. Brief Introduction to A. flavus and F. verticillioides

Aspergillus flavus and *F. verticillioides* are filamentous fungi that belong to the phylum *Ascomycota*. Both are plant pathogenic fungi. They are eukaryote heterotrophs and absorbotrophic, that means the fungi absorb the nutriments and carbon through the apex of their hyphens thanks to the enzymatic secretion. They share characteristic as the vegetal cell due to their cell membrane and their turgescence vacuoles in the cytoplasm but also animal cell characteristic for their lack of chloroplasts, chlorophyll, and starch.

Both fungi are mainly pathogens of maize where they cause ear rot and can contaminate deeply the kernels with their respective mycotoxins. In addition, *A. flavus* is widespread and can be found in various habitats, including soil, decaying vegetation, and other crops such as cotton, peanut and pistachio, while *F. verticillioides* can also contaminate a wide number of crops of agro-food interest such as other cereals, sugar cane and banana.

Both thrive in warm and humid conditions and are prevalent in regions where maize is grown. Even if their respective optimum of growth and mycotoxin production are different [34,35], these fungi are often co-occurrent species in maize [36].

F. verticillioides can colonize maize residues, that are a reservoir for inoculum of following year. Therefore, three ways of infection can occur. Seed germinating in *F. verticillioides*-infested soils may acquire an asymptomatic, endophytic infection. At the silking stage, the fungus colonizes developing kernels via a narrow opening called the stylar canal. Insects play a significant role in the disease cycle. The European corn borer is known to produce infection courts for *Fusarium spp*. through feeding on ears. The larvae of the insect feed on stalks, ears, and collar tissues, providing ample infection courts for development of *F. verticillioides* ear rots. Finally, environmental conditions can influence development of Fusarium ear rot, by wet post silking conditions, and drought stress. In particular, warm, dry conditions during grain filling are linked to disease severity and fumonisin contamination [37].

Although *A. flavus* is thought as a storage pathogen, its contamination of maize starts in the field. The fungus survives as conidia and sclerotia in soil and crop debris. Therefore, wind and insects can spread the fungal spores, at certain conditions of temperature and humidity, allowing the fungus to enter in the plant through the silks. Here, germination and colonization of the fungus can cause yellow/brown discoloration, and pollination can be affected because of changes in physiology and structure of silks. At the same time, *A. flavus* can continue to grow as a saprophyte inside the plant, without cause symptoms. A further colonization of the plant occurs through the damages caused on the ears by birds and insects. Broken pericarps allow invasion, and when moisture content drops rapidly <35% of kernels, the *A. flavus* takes advantages and competes successfully with other fungal pathogens such as the *Fusarium spp*, growing at its best with a 17-20% grain moisture. Finally, a further colonization of the maize plants and the aflatoxin production are favored by high temperatures combined with very low rainfall and hydric stressed plants, that show altered nutritional status of developing kernels [38].

2.2. The Uniqueness of A. flavus

A. flavus species can be divided in two categories based, on morphological characteristics of the sclerotia. The sclerotium is a compact structure of fungal mycelium containing food reserves produced when environmental conditions are unfavorable. A production of smaller than 400 µm sclerotia belongs to the category of type S (short) where the strains are toxigenic, that means they produce mycotoxins in large quantities. On the other hand a production of sclerotia superior to 400 µm belongs to the category of type L (large) which contains more variability and gathers toxigenic strains whose mycotoxin concentrations are more variable and non-toxigenic strains which do not produce mycotoxins [39].

Although toxigenic and non-toxigenic strains live in the same environment, they are genetically different. The toxigenic strains possess the entire gene cluster involved in aflatoxin biosynthesis that gives to the fungi the ability to produce aflatoxins, while non-toxigenic strains lack part of the genes [40]. *A. flavus* toxigenic strains and non-toxigenic strains exhibit genetic barriers preventing the transfer of genetic material between them, thereby making it not possible the exchange of mycotoxin-producing capabilities [41].

3. Informative Agents and Active Participants: The Realm of Volatile Organic Compounds

3.1. Concise introduction of the VOCs

Volatile organic compounds (VOCs) are organic chemicals that have a high vapor pressure at room temperature, meaning they easily evaporate into the air as gases. VOCs are composed of carbon-based molecules and can originate from both natural

and human-made sources. VOCs play important roles in the environment and can have both beneficial and detrimental effects.

In the context of fungi, VOCs are produced as secondary metabolites and can serve multiple functions, including communication between organisms, defense against competitors or predators, attraction or repulsion of other organisms, and regulation of fungal growth and development [42]. The specific VOCs produced by fungi can vary depending on the species, environmental conditions, and interactions with other organisms [43].

They are sometimes also referenced using their origin. Therefore, the term "microbial VOCs" is commonly used to refer to VOCs produced by various microorganisms (bacteria, yeast, fungi) [44], while "fungal VOCs" specifically relates to VOCs produced by fungi [45].

VOCs can exhibit a wide range of chemical structures. Each chemical class may have predominant roles associated with it, although many roles are still not well understood or known [46]. Among these classes, terpenes are the most abundant and diverse group of VOCs produced by fungi. They are derived from isoprene units and can be further classified into subgroups such as monoterpenes, sesquiterpenes, diterpenes, and triterpenes. Terpenes have been associated with various biological activities, including antifungal and antimicrobial properties [47,48]. Alcohols play a major role in fungal metabolism and signaling. Aldehydes and ketones have diverse functions, including antimicrobial properties. Esters, formed through the reaction between alcohols and organic acids, contribute to the characteristic aroma and flavor profiles of many fungal species. Alkanes and alkenes are often associated with signaling or protective functions in fungi. Lastly, sulfur compounds contribute to the characteristic odor associated with certain fungal species.

By categorizing these VOCs based on their chemical structure, we can gain insights into their potential functions and roles within the fungal kingdom even if not specific mode of action is associated yet.

3.2. Parameters involved into VOCs profile modifications

The emission of VOCs by fungi presents a specific composition and abundance but can vary depending on factors related to both biotic and abiotic influences.

Inter-species variability linked to genetic variations within fungal populations as well as different growth stages such as germination hyphal growth and sporulation, may produce distinct VOC profiles. The VOC emissions have exhibited notable changes between the early stages of fungal growth and more advanced stages, indicating significant fluctuations (see **Chapter 2**). Furthermore, nutrient availability plays a role and can affect the type and quantity of VOCs produced.

Environmental and cultural conditions, including temperature, humidity, light exposure, atmospheric composition, growth medium composition, pH, aeration, and

incubation conditions, can also impact VOC emissions. Interactions with other organisms, such as bacteria, plants, or other fungi, can induce chemical signaling or competition, further influencing VOC profiles [49]. These factors, including epigenetic influences, can modulate the expression of genes involved in VOC biosynthesis.

Stress and defense responses, such as nutrient limitation, physical damage, or exposure to toxins, can also trigger alterations in VOC emissions [50,51]. These VOCs may serve as signaling molecules or possess antimicrobial properties, aiding the fungus in adapting to its environment [52].

It is important to recognize that although certain VOCs may be commonly emitted, the quantity and composition of VOCs produced by fungi can be influenced by various parameters and factors. Thus, VOCs emitted daily can contribute to species identification, while those emitted in response to external stimuli provide insights into active biosynthetic pathways in the fungus.

The VOC detection methods play a crucial role in the detection of these compounds. Indeed, in addition to the variations associated with the biological model studied, each step from sampling (extraction tool, extraction time, etc.) to detection (flame ionization detector, mass spectrum, etc.) and the various parameters required for separation (column, 1- or 2-dimensional analysis, oven program, etc.) involves factors that affect the chemical families detected, as well as their abundance [53–55].

4. Exploring VOCs in Mycotoxin Contamination: Literature Background, Applications, and Thesis Questions.

4.1. The latest research on A. flavus, F. verticillioides and their VOC emissions

The identification and characterization of VOCs in fungi is an active area of research, increasing these last years (**Figure 1-1**). New VOCs continue to be discovered, enriching the knowledge to improve the identification of fungi via these compounds, but also enabling the study of mycotoxigenic fungi, thereby refining the relationship with mycotoxin contamination.

On the other hand, researches on fungi are still predominantly carried out by considering a single species model, while the studies of species community could better figure out the field conditions (**Figure 1-2**).

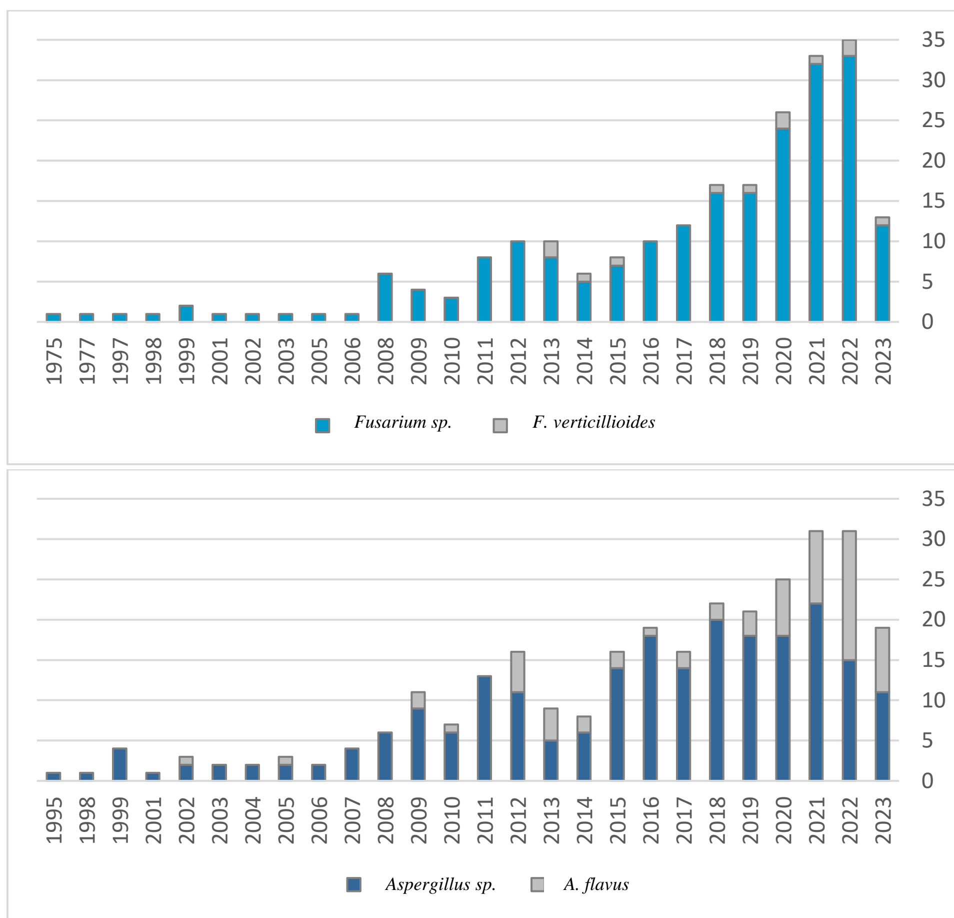


Figure 1-1. Number of articles published since 1995 reported in Medline (PubMed) using the keywords: Volatile organic compounds, *Fusarium, F. verticillioides,* and *Aspergillus*, *A. flavus* using the Boolean operator "and" & "or".

Considering the number of publications reported in **Figures 1-1** and **1-2**, the field remains incomplete, and further studies are needed to refine species identification and monitor the production of specific secondary metabolites.

As the VOCs and mycotoxins cannot be directly related in terms of chemical nature and biosynthesis, a potential relationship between these two kinds of secondary metabolites at metabolic level, also due to some similar function that they share, can be supposed. Indeed, both are produced during a fungal stress response, and can be activated by a common signaling pathways and regulatory system. Both act on other species (insects, bacteria, fungi etc.) to maintain the survival of the fungus. In addition, VOCs impact the mycotoxins production to induce or inhibit them, changing the dynamic among the species in a same ecological niche [56].

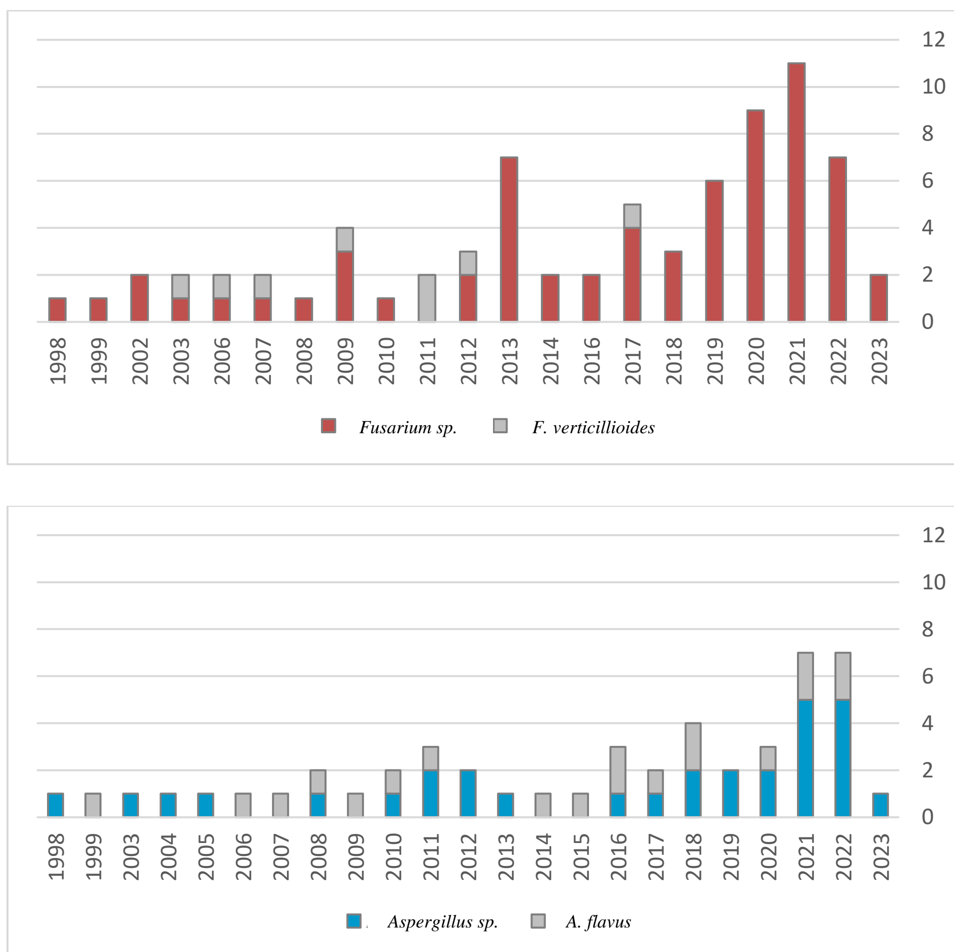


Figure 1-2. Number of articles published since 1995 reported in Medline (PubMed) using the keywords: co-inoculation/ed, *Fusarium*, *F. verticillioides* and *Aspergillus, A. flavus* using the Boolean operator "and" & "or

4.2. Harnessing the power of VOCs: Biomarkers and biocontrol agents

VOCs can be used for two main objectives. Firstly, VOCs can be used as biomarkers with several applications. Firstly, VOCs can be used as biomarkers in several ways: they can be added to and complete the characterization of a fungal species following the identification of VOCs on a particular medium, but they can also become a powerful tool for monitoring different levels of microbial, fungal and mycotoxin contamination, by associating a particular pool of VOCs for each of these levels of contamination (see Chapters 2 and 3). This type of approach could also help to more

rapidly identify the fungal species or genus present, in order to guide subsequent analyses.

On the other hand, VOCs can be useful biocontrol agents as antifungal and/or antimycotoxin agents at different stages of the food process, from the field to storage to the consumer, with the use of these bioactive VOCs in silos or in smart packaging replacing preservatives (see **Chapter 3**).

4.3. The relevant questions to unravel the mystery between VOCs and mycotoxins

In order to highlight the relationship between mycotoxins and VOCs, the species *A. flavus* emerged as the best candidate to be tested, since it is the main aflatoxin B_1 producing species, the most harmful mycotoxin. In addition, its unique characteristic of including both aflatoxin-producing strains and non-aflatoxin-producing strains, which lack the necessary genetic cluster for aflatoxin production, makes it a very suitable candidate for studies on the VOC profiles. It was then evident to include a co-occurring species on maize, such as *F. verticillioides*, also a species producing a harmful mycotoxin, FB_1, to further complicate the biologic model and observe the modifications introduced by this new species in *A. flavus* environment. Considering this, the thesis reached several goals that can be exposed here as questions:

- Do VOCs could be potential biomarkers of these fungi?
- Do VOCs could be potential biomarkers for aflatoxin and fumonisin contamination?
- How can the co-occurrence of fungal species impact the VOCs and mycotoxin production?
- Does the mode of interaction among the species involve different reactions?
- Can single VOC be used as potential antifungal or antimycotoxin agent?

5. The investigations accomplished

This thesis consists of six chapters, in addition to the introduction, aimed at addressing the questions raised in the precedent section. After a short introduction, a comprehensive review of the VOCs emitted by *A. flavus*, including an examination of the factors influencing their production, as well as the VOCs emitted by various sources that impact fungal growth and the production of aflatoxin B_1 was conducted. The subsequent step focuses on monitoring individually the VOCs emitted by *A. flavus* and *F. verticillioides,* along with their respective mycotoxins (aflatoxins and fumonisins), over several days to characterize their temporal dynamics. Following that, co-inoculation experiments of these co-occurrent fungi in maize were conducted to assess the potential synergy and the correlation between mycotoxin production and VOC emissions. Then, the thesis investigates the modifications in gene expression associated with the application of ethyl 3-methylbutanoate using different modes of

application as a potential biocontrol strategy to reduce fumonisin production under *F. verticillioides* contamination. Lastly the mechanism of ethyl 3-methylbutanoate was explored through the monitoring of the gene expression of the fumonisins pathway. The **Figure 1-3** summarizes the practical questions posed throughout the thesis.

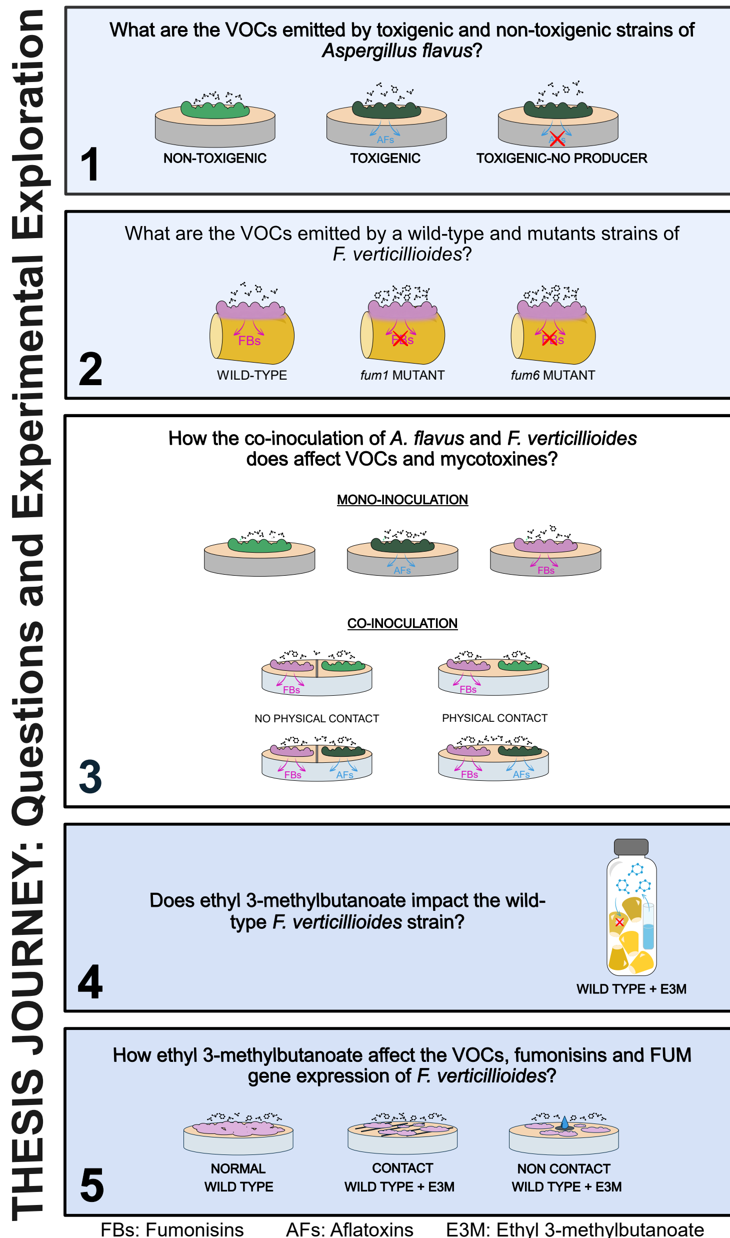


Figure 1-3. Thesis journey

Chapter 2

Impact of Volatile Organic Compounds on the Growth of *Aspergillus flavus* and Related Aflatoxin B_1 Production: A Review

The second chapter of the thesis presents a bibliographic review published in 2022 in *International Journal of Molecular Sciences*:

Josselin, L., De Clerck, C., De Boevre, M., Moretti, A., Fauconnier, M.- L. (2022) **"Impact of Volatile Organic Compounds on the Growth of *Aspergillus flavus* and Related Aflatoxin B_1 Production: A Review."** International Journal of Molecular Sciences, 23(24), pp. 1–24. doi: 10.3390/ijms232415557.

This review presents a report on all the volatile organic compounds (VOCs) emitted by the *Aspergillus flavus* species as well as those emitted by other sources that affect the growth and/or AFB_1 production of *A. flavus*.

In recent years, VOCs have been recognized as an important part of inter- and intra-species communication. It is interesting to carefully assess the VOCs emitted by *A. flavus* and then to study the VOCs affecting it for two specific parameters, which are mycelial growth and AFB_1 production.

In that respect, the first part of the review is dedicated to the VOCs emitted by *A. flavus*. This species can be genetically split into two groups: the toxigenic strains that have the ability to produce aflatoxins due to the presence of the whole *afl* gene cluster, and the non-toxigenic strains that lack part of the gene cluster and cannot produce aflatoxins. The investigation on the differential VOCs emitted by these two groups was performed to use these secondary metabolites as reliable tools in taxonomy but also to link the toxigenic ability of the strains with specific VOC. Such knowledge could improve the risk assessment related to *A. flavus* occurrence in food commodities.

The second part of the review reports the VOCs affecting the growth and/or the AFB_1 production of *A. flavus*. Indeed, several microorganisms, including fungi, bacteria, and yeasts, have been found to affect these two parameters through their emission of VOCs as well as the essential oils, which have recently emerged as a potential alternative in the control of fungi, because of their noteworthy anti-fungal properties. Single VOCs that have been identified among the VOCs emitted by microorganisms or found in the composition of essential oils, have undergone extensive testing against *A. flavus*. Beyond their fungistatic and fungicidal effects, some of these VOCs have demonstrated the remarkable ability to reduce or even halt aflatoxin production. As such, they show significant promise as biocontrol method for use in the field and during crop storage.

However, their mode of action is not yet fully understood, although recent studies have explored their impact in the membrane integrity, the gene expression of the *afl* gene cluster, and few other parameters. All these latter aspects are reported in the third part of the review.

By highlighting all these information, a more comprehensive understanding of how to utilize these VOCs as potent tools in maize cultivation and storage can be achieved. The final part of this review delves into several of the innovative applications that have been developed in recent years.

Abstract

Volatile organic compounds (VOCs) are secondary metabolites of varied chemical nature that are emitted by living beings and participate in their interactions. In addition, some VOCs called bioactive VOCs cause changes in the metabolism of other living species that share the same environment. In recent years, knowledge on VOCs emitted by *Aspergillus flavus*, the main species producing aflatoxin B_1 (AFB_1), a highly harmful mycotoxin, has increased. This review presents an overview of all VOCs identified as a result of *A. flavus* toxigenic (AFB_1-producing) and non-toxigenic (non AFB_1-producing) strains growth on different substrates, and the factors influencing their emissions. We also included all bioactive VOCs, mixes of VOCs or volatolomes of microbial species that impact *A. flavus* growth and/or related AFB_1 production. The modes of action of VOCs impacting the fungus development are presented. Finally, the potential applications of VOCs as biocontrol agents in the context of mycotoxin control are discussed.

Keywords

Volatolome, fungal growth, Aflatoxin B_1 control, bioactive volatile organic compounds

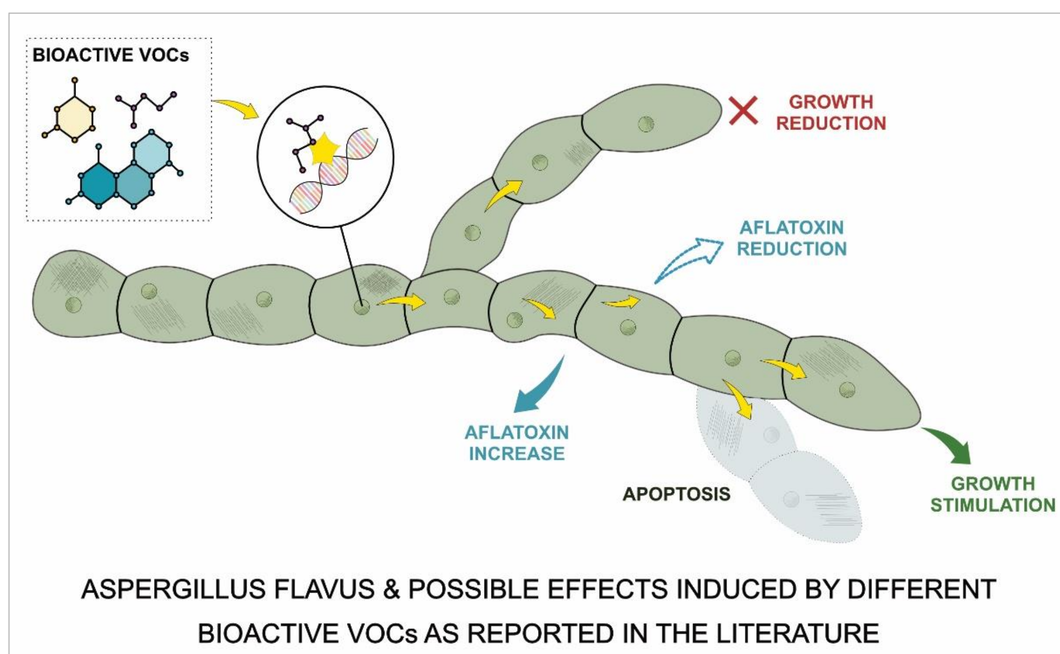


Figure 2-1. Graphical abstract of the Chapter 2

1. Introduction

Recently, volatile organic compounds (VOCs), which are categorized as secondary metabolites, have risen to general attention and been widely studied. VOCs are known to actively participate in inter- and intra- living species communication [57–59]. In particular, VOCs are becoming the new frontier in the metabolomics field. With the development of new technologies, the fields of application of VOCs, such as the biomedical field, have grown over the last few years. In addition, VOCs have been investigated in-depth for the roles they also play in soil, in influencing atmospheric chemistry, and in microbe–microbe, plant–microbe, and plant–plant interactions [60].

Much research has also addressed the influence of certain VOCs or volatolomes (the set of VOCs emitted by a given species) on fungi by observing their antifungal, antibiotic, antimicrobial properties and others [49].

Interestingly, these secondary metabolites oftentimes share the same biosynthetic pathway as some mycotoxins [61]. Thus, much work has been also devoted to analyzing the impact of the VOCs on inhibiting the ability of some mycotoxigenic fungi to produce mycotoxins. In particular, VOCs from several sources have been shown to inhibit the production of Ochratoxin A by *Aspergillus carbonarious* [60] and aflatoxins by *Aspergillus flavus* [62].

Aspergillus flavus is a fungal species that causes serious damage to crops in the field or during storage [63]. Beyond its pathogenic effects on several crops, this fungus is also the main producer of the mycotoxin aflatoxin B_1 (AFB_1) [31]. This mycotoxin contributes to major health problems worldwide as well at sanitary and economic levels [64,65]. AFB_1 remains active even after heat treatments used in conventional food manufactures since it is a thermostable compound [66]. Chronic exposure towards AFB_1 leads to multiple diseases such as hepatocellular cancer, and acute consumption beyond the maximum permissible limits can lead to the death of the individual [9,15,67]. This makes AFB_1, to date, a serious threat to humans, but also the most controlled mycotoxin via European and worldwide legislation [68]. In recent years, the methods of detection have become increasingly powerful and sensitive, and accurate techniques promoting an easier detection have been developed [69,70]. At the same time, the concern and the need to control the contamination and the production of AFB_1 in order to mitigate its occurrence in crops have emerged [71].

In particular, some VOCs identified here as bioactive VOCs have promising ability to affect both *A. flavus* growth and AFB_1 production. Among the works compiled, we can distinguish among those focused on the evaluation of mycelial growth, other works investigating the effects on AFB_1 production, and studies aiming to elucidate the impact of VOCs on the gene expression of the aflatoxin biosynthetic pathway gene cluster.

Among the tools to reduce the impact of mycotoxins on crops, the early detection of both fungi and mycotoxins is a key approach. Such detection can be achieved by a range of different markers, such as DNA-based markers for the fungi, or rapid kits for easy and fast

chemical analyses of mycotoxins [72,73]. A recent trend aims to develop species-specific markers based on specific VOC profiles emitted by fungi. The monitoring of the emission of VOCs over several days of growth of *A. flavus* has revealed many VOCs, some of which are commonly emitted also by other fungal or other microbial species, whereas others are considered specific to this species.

A. flavus includes two kinds of strains based on their ability to produce AFB_1. The biosynthesis of AFB_1 is linked to the presence of a cluster consisting of 30 genes (*afl*) on chromosome 3 [74]. The toxigenic strains (TS) possess the entire gene cluster involved in aflatoxin biosynthesis that gives to strains the ability to produce AFB_1, whereas non-toxigenic strains (NTS) lack some of these genes [40]. In addition, NTS are not genetically identical since they can lack different number and kind of *afl* genes [75]. Finally, although TS and NTS share the same environment, they are genetically incompatible and there are no examples of hybridization between them [76].

NTS and TS of *A. flavus* share the same environment and can be both frequently isolated from same parts of plants or soils. Therefore, it is important to correctly identify TS and NTS to accurately evaluate the risk related to *A. flavus* occurrence. Molecular markers are not available for *A. flavus* since, as mentioned above, several genetic patterns of NTS can occur. On the other hand, chemical analyses, even using rapid kits, can require much time and laboratory resources. Therefore, the identification of specific VOCs for NTS and/or TS strains of *A. flavus* could provide further markers for an early and reliable assessment of strain toxigenicity.

This review will address four main questions related to *A. flavus*, VOCs and AFB_1:

- Which VOCs are emitted by *A. flavus* and are specific to TS or NTS?
- Which bioactive VOCs or volatolomes of various origins affect the growth of *A. flavus* and/or its production of AFB_1?
- What are the modes of action of these bioactive VOCs?
- How can we exploit these VOCs to our advantage to control the growth of *A. flavus* and its AFB_1 production?

2. Which VOCs are produced by *A. flavus* and are specific to TS or NTS?

2.1. The diversity of VOCs emitted by A. flavus

VOCs include a wide range of molecules (alcohols, esters, furans, ketones, aldehydes, terpenes, hydrocarbons, i.a.) with low molecular weight and high vapor pressure. These VOCs are emitted by many natural and anthropogenic sources. Concerning natural VOCs, different terminologies are used depending on their origin. Biogenic volatile organic compounds (bVOCs) are defined as the volatile compounds that are emitted by living

beings [77,78]; the VOCs emitted by microorganisms (including fungi and bacteria) can be referred to as microbial volatile organic compounds (mVOCs) [44]. Finally, in a more specific way, the VOCs produced by fungi are defined as fungal volatile organic compounds (fVOCs) [45].

VOCs are emitted by fungi in order to fulfill different internal or external functions for the fungus [59]. The emission of some VOCs can inhibit certain functions of the fungus or fungal structures [79]. Thus, germination, mycelium growth, and sporulation can be regulated by the emission of VOCs. Other VOCs are involved in interactions with other kinds of living organisms. Some VOCs attract insects to maximize fungal dissemination [57], some interact with the host plants to weaken their defenses [80], and other VOCs have antimicrobial activity and thus limit the colonization of other fungal or microbial species that may compete for the substrate, or even control the population of the microorganism that produces them, a phenomenon called quorum sensing [81,82].

Almost 400 VOCs emitted by *A. flavus* have been reported in the literature so far, as identified from the volatolomes emitted by the various strains analyzed. A synthesis of these VOCs (listed as a whole in the **Table 2-6** (in the supplementary material) (https: //www.mdpi.com/article/10.3390/ijms232415557/s1) is presented in **Table 2-1**. This table illustrates their great diversity from a chemical class standpoint. **Table 2-1** also presents the total number of VOCs emitted for each chemical family and whether these VOCs are emitted more specifically by TS or NTS of *A. flavus*. The strains for which we lack the information on their toxigenicity are reported in the **Table 2-1** as unknown (US). Many studies examined both a TS and a NTS and thus compared their volatolomes.

More than 50 different compounds belonging to four chemical families (alcohol, alkane, alkene and terpene) have been reported. The alcohol class includes the highest number of identified VOCs (3-methylbutan-1-ol, ethanol), as well as those associated with the typical odor of the fungi (oct-1-en-3-ol, octan-3-ol) [54,83]. In the alkane class, there is a predominance of compounds ranging from 5 to 19 carbons, while only three compounds containing more than 30 carbons and 16 cyclic structures have been listed. Within the family of alkenes, aromatic and cyclic compounds such as derivatives of xylene or styrene were often found (up to 40% of the total). The terpene group is composed of monoterpenes and sesquiterpenes, with a great predominance of the latter.

Some recurrent VOCs are always detected as emitted by *A. flavus* strains, such as 3-methylbutan-1-ol, 2-methylpropan-1-ol, ethyl acetate and 2-methylfuran, making them potential markers of *A. flavus* occurrence.

In **Table 2-6** some trends associated with the toxigenicity of *A. flavus* strains are highlighted. From a general point of view, TS emit a greater diversity of chemical families than NTS. Indeed, all chemical families are emitted and are widely represented, especially terpenes with more than 40 specific VOCs, followed by ketones and hydrocarbons (alkane and alkene). Only a single monoterpene emitted exclusively by NTS has been identified: p-mentha-1,3,8-triene. To our knowledge and to date, some VOCs are assimilated as specific to a category of *A. flavus*, as it is the case of pent-2-yn-1-ol for TS [84]. Other VOCs, such as epi-bicyclosequiphellandrene, 2-phenoxyethanol or γ-gurjunene, are

supposed to be specific to TS but due to the lack of information about the studied strains, their exclusivity to this category cannot be fully confirmed.
It is necessary to underline that the specificity of some VOCs for NTS vs TS and vice-versa does not exclude the possibility that some of them are produced by other fungal or microbial species.

2.1. VOCs emission of A. flavus influenced by biotic and abiotic factors

A significant variability in the number and amounts of VOCs emitted by *A. flavus* and in its growth kinetics has been reported. Sun et al. (2014) showed that the VOCs emitted by a NTS were more abundant than those emitted by a TS [54]. Josselin et al. (2021) have observed the opposite trend that TS can emit larger amounts of VOCs compared with a NTS, with the majority of these VOCs belonging to the terpene family. This latter study also highlighted a change in the volatolome of a natural mutant unable to produce AFB_1, obtained from a TS. For this mutant strain, in addition to its loss of AFB_1 production, a concurrent difference in the emission of certain terpenes was observed [85]. In conclusion, the nature of the strain itself brings variability to the volatolome released by *A. flavus*.

The effects of an increase in temperature on VOC emissions in TS of *A. flavus* was also studied by Sun et al. (2014) and showed fluctuations in terpene and alcohol contents (ethanol, butan-1-ol, 3-methylbutan-1-ol and 2-methylbutan-1-ol). For example, a temperature higher than 37°C seems to inhibit the production of terpenes, although they were abundant during the analyses carried out at 30°C and 15°C [86]. Growth temperature is thus an important parameter when considering VOC emission by *A. flavus*. On the other hand, water activity and pH are also frequently mentioned as parameters that influence fungal growth and AFB_1 production [87–89]. However, data that relate these parameters and studies on VOCs are lacking. The growth media also influences VOC emission, as reported by De Lucca et al. [83,84], who pointed out that maize media resulted in a greater number of VOCs compared with PDA medium. In addition, Sun et al. (2016) showed that the number of terpenes emitted increased if the carbon source was more accessible [86].

The method of VOC sampling can also influence the VOCs detected. Among the methods, the most common static method used is the SPME, which leads to adsorbing a large range of chemical families, while the dynamic head space method used is most often performed with a TENAX tube for the same reason. The importance of the SPME parameters was highlighted by Sun et al. (2016) by comparing the number, the amount and the chemical families sampled [86].

Table 2-1. Overview of literature references concerning the Volatile Organic Compounds (VOCs) emitted by *A. flavus* strains according to the chemical family of these VOCs and the ability of the *A. flavus* strains to produce AFB_1 or not.

(a)	Total Number of VOCs	Chemical Family of VOCs Reported in Literature							
		(b) TS VOCs		(c) NTS VOCs		(d) VOCs Shared by TS and NTS		(e) US VOCs	
Alcohol	51	De Lucca et al., 2010 De Lucca et al., 2012 Josselin et al., 2021 Müller et al., 2013 Polizzi et al., 2012 Sun et al., 2014 Sun et al., 2016	[83] [84] [85] [90] [91] [54] [86]	De Lucca et al., 2010 Josselin et al., 2021 Jeleń and Wąsowicz, 1998	[83] [85] [92]	De Lucca et al., 2010 Gao et al., 2002 Josselin et al., 2021 Kamiński et al., 1972 Polizzi et al., 2012 Spraker et al., 2014 Sun et al., 2014	[83] [93] [85] [94] [91] [95] [54]	Jeleń and Wąsowicz, 1998 Kamiński et al., 1972	[92] [94]
Aldehyde	23	De Lucca et al., 2010 De Lucca et al., 2012 Josselin et al., 2021 Müller et al., 2013 Sun et al., 2014 Sun et al., 2016	[83] [84] [85] [90] [54] [86]	De Lucca et al., 2010 Sun et al., 2014	[83] [54]	De Lucca et al., 2010 Josselin et al., 2021 Sun et al., 2014	[83] [85] [54]		
Alkane	96	De Lucca et al., 2010 De Lucca et al., 2012 Josselin et al., 2021 Müller et al., 2013 Spraker et al., 2014	[83] [84] [85] [90] [95]	De Lucca et al., 2010 De Lucca et al., 2012 Josselin et al., 2021 Spraker et al., 2014 Sun et al., 2014	[83] [84] [85] [95] [54]	De Lucca et al., 2010 De Lucca et al., 2012 Farh and Jeon, 2020 Josselin et al., 2021 Sun et al., 2014	[83] [84] [79] [85] [54]	De Lucca et al., 2012	[84]
Alkene	65	De Lucca et al., 2010 De Lucca et al., 2012 Jeleń and Wąsowicz, 1998 Josselin et al., 2021 Polizzi et al., 2012 Sun et al., 2016	[83] [84] [92] [85] [91] [86]	De Lucca et al., 2010 De Lucca et al., 2012 Sun et al., 2014	[83] [84] [54]	De Lucca et al., 2012 Josselin et al., 2021	[84] [85]	Jeleń and Wąsowicz, 1998	[92]

Table 2-1 (continued)

(a)	Total Number of VOCs	Chemical Family of VOCs Reported in Literature			
		(b) TS VOCs	**(c) NTS VOCs**	**(d) VOCs Shared by TS and NTS**	**(e) US VOCs**
Alkyne	9	De Lucca et al., 2012 [84]	De Lucca et al., 2012 [84]		
Amine	8	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Spraker et al., 2014 [95]			
Amide	3	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84]			
Acid	13	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Josselin et al., 2021 [85]	De Lucca et al., 2010 [83] Sun et al., 2014 [54]	Sun et al., 2014 [54]	
Ester	20	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Josselin et al., 2021 [85]	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Sun et al., 2014 [54]	De Lucca et al., 2010 [83]	
Ether	1	De Lucca et al., 2012 [84]			
Furan	10	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Jeleń and Wąsowicz, 1998 [92] Josselin et al., 2021 [85] Sun et al., 2014 [54] Sun et al., 2016 [86]	De Lucca et al., 2012 [84] Sun et al., 2014 [54]	De Lucca et al., 2010 [83] Josselin et al., 2021 [85] Sun et al., 2014 [54]	Jeleń and Wąsowicz, 1998 [92]

Table 2-1 (continued)

(a)	Total Number of VOCs	Chemical Family of VOCs Reported in Literature			
		(b) TS VOCs	(c) NTS VOCs	(d) VOCs Shared by TS and NTS	(e) US VOCs
Furan	10	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Jeleń and Wąsowicz, 1998 [92] Josselin et al., 2021 [85] Sun et al., 2014 [54] Sun et al., 2016 [86]	De Lucca et al., 2012 [84] Sun et al., 2014 [54]	De Lucca et al., 2010 [83] Josselin et al., 2021 [85] Sun et al., 2014 [54]	Jeleń and Wąsowicz, 1998 [92]
Ketone	29	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Josselin et al., 2021 [85] Spraker et al., 2014 [95] Sun et al., 2016 [86]	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Sun et al., 2014 [54]	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Gao et al., 2002 [93] Josselin et al., 2021 [85] Kamiński et al., 1972 [94] Sun et al., 2014 [54]	Polizzi et al., 2012 [91]
Halogen	4	De Lucca et al., 2010 [83]			Jeleń and Wąsowicz, 1998 [92]
Terpene	69	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Josselin et al., 2021 [85] Polizzi et al., 2012 [91] Sun et al., 2016 [86] Zeringue et al., 1993 [96]	De Lucca et al., 2012 [84] Sun et al., 2014 [54]	De Lucca et al., 2010 [83] Josselin et al., 2021 [85]	Gao et al., 2002 [93] Pennerman et al., 2016 [97] Polizzi et al., 2012 [91]
Others	9	De Lucca et al., 2010 [83] De Lucca et al., 2012 [84] Spraker et al., 2014 [95] Sun et al., 2014 [54]		De Lucca et al., 2010 [83] Josselin et al., 2021 [85]	Gao et al., 2002 [93]

If the chemical family (a) of VOC is specifically emitted by a toxigenic strain (TS) in the article, then the reference will be listed in column (b), if it is specifically emitted by the non-toxigenic strain (NTS), then the reference is listed in column (c), if the VOC is non-specific (NS) to one of the categories, then the reference is listed in column (d), and if the toxigenicity of the strain is unknown (US), then the reference is listed in column (e).

3. Which bioactive VOCs or volatolome of various origins affect the growth of *A. flavus* and/or its production of AFB_1?

In order to examine all the bioactive VOCs leading to a modification of the growth of *A. flavus* and/or its production of AFB_1, the VOCs were grouped according to their origin of emission. Thus, the volatolomes of microorganisms such as fungi, bacteria or yeasts, the VOCs from plant extracts such as essential oils and, finally, the individual and pure VOCs are detailed in the three sections below.

Figure 2-2 shows all the studied volatolomes or bioactive VOCs in eight categories, listing the changes observed in the two targeted parameters (growth and AFB_1 production). The majority of the compounds are active mainly on fungal growth, and some also act on AFB1 production. In contrast, the above mention parameters can be stimulated by some given bioactive VOCs or volatolomes, as reported by Cleveland et al. (2009) and Zeringue et al. (1990) [98,99].

Table 2-2. Summary of the major effects on *A. flavus* growth and AFB_1 production of volatolome or bioactive VOCs emitted by microorganisms, essential oils and individual VOC classed by chemical families.

Application Mode		**Contact**		**No Contact**	
Source of Bioactive VOCs		**Growth of *A. flavus***	**AFB_1 Production**	**Growth of *A. flavus***	**AFB_1 Production**
Microorganisms	Bacteria	NA	NA	↓	↓/↑
	Yeast	NA	NA	↓	↓
	Fungi	NA	NA	↓	NA/↓
Essential oil		↓	↓	↓	NA/↓
Individual VOC	Acid	↓	↓	↓	↓
	Alcohol	↓/↑	↓	↓	↓/↑
	Aldehyde	↓	↓	↓	↓
	Alkane	NA	NA	NA	↓
	Ester	↓	↓	↓	↓
	Furan	NA	↓/↑	NA	↓/↑
	Ketone	NA	NA	↓/↑	↓/↑
	Terpene	↓	↓	↓/↑	↓/↑
	Other	NA	NA	NA	↓

(↓) Inhibition; (↑) Augmentation; (NA) no data available.

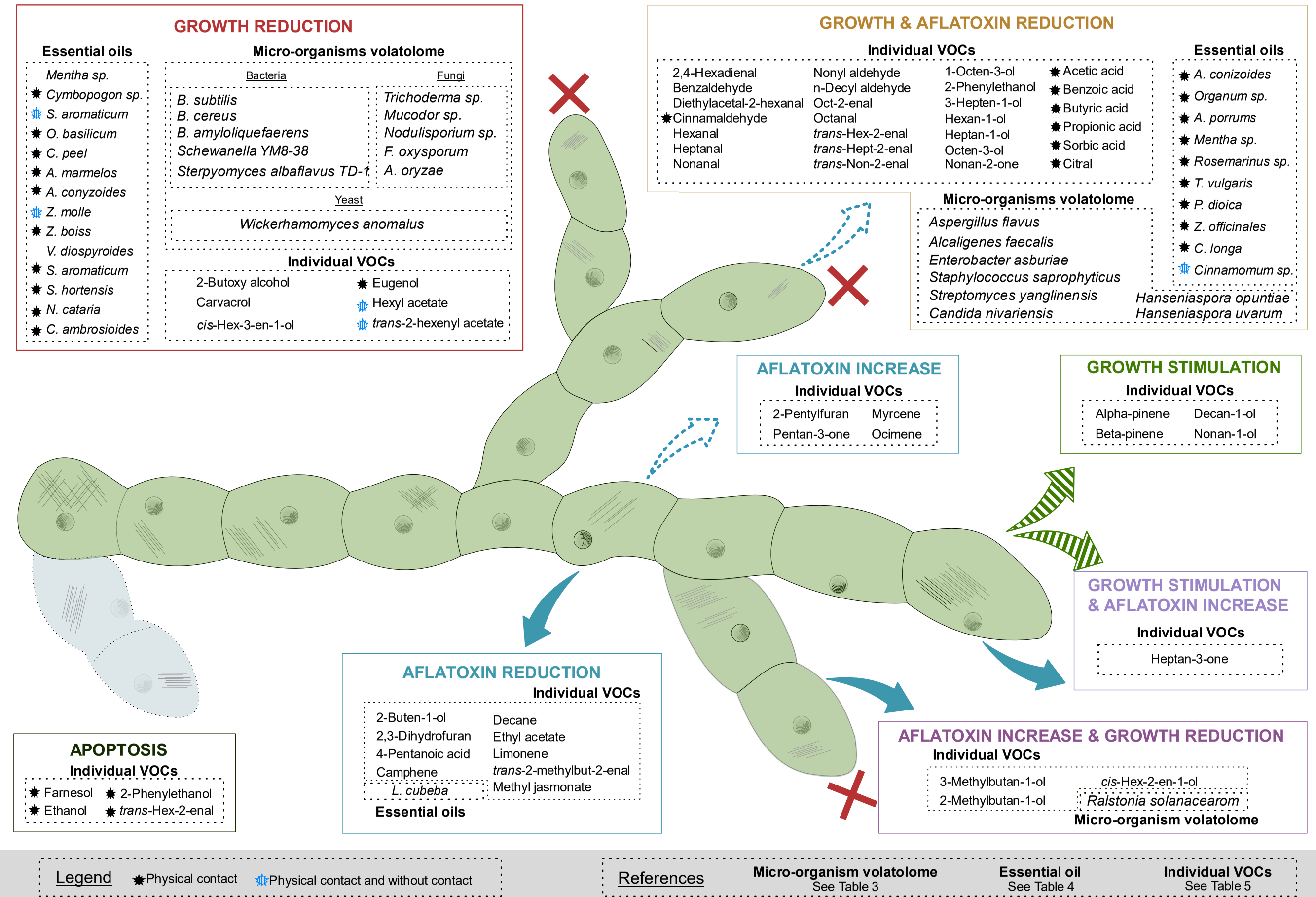


Figure 2-2. Summary of bioactive VOCs and species volatolomes influencing growth and AFB_1 production parameters of *A. flavus*.

The main patterns in the effects of volatolomes, essential oils and individual VOCs on *A. flavus* growth and related AFB_1 production are summarized in **Table 2-2**. According to the information known today, it seems that some families of compounds such as alcohols or terpenes can cause either inhibitory effects on the growth and the production of AFB_1 or stimulate them, although the prevailing tendency of the studied bioactive VOCs or volatolomes is a reduction in the above-mentioned parameters. However, the trends observed for *A. flavus* are not always identical to those found when a wider species or genus of fungi are considered, demonstrating that each species reacts differently to the same bioactive VOCs. This is notably the case for aldehydes that are extremely efficient at reducing *A. flavus* growth and turn out to have an effect of increasing fungal growth of other species such as *F. oxysporum, Colletotrichium fragarie* or *Botrytis cinerea* [100]. **Table 2-2** also shows that the mode of application of the individual VOCs also influences the effects on the two parameters, which will be discussed in the section devoted to them.

3.1. The volatolomes from bacteria, yeast and fungi reduce the growth of A. flavus

The effects on growth and AFB_1 production caused by panels of volatolomes released by microorganisms (bacteria, yeast and fungi) on *A. flavus*, without physical contact between *A. flavus* colonies and the emitting species, are reported in **Table 2-3**. In general, the fungistatic effect is often the primary parameter to be studied; therefore, data regarding the effect on AFB1 production are sometimes not available. In the case of fungi, bioactive VOCs can impact a wide range of parameters, including sporulation, conidia germination and different morphological modifications of their living structures (e.g., hyphae) [101,102].

The VOC-producing species most frequently investigated for their effects against *A. flavus* belong to the *Muscodor* and *Trichoderma* fungal genera, and *Streptomyces* and *Bacillus* bacterial genera [103,104]. Following a screening of 75 *Bacillus* strains, significant reductions in the growth of *A. flavus* in the presence of *Bacillus subtilis, B. cereus* and *B. amyloliquefaciens* volatolomes were noted [105]. In addition, the volatolomes of the above-mentioned *Bacillus* species had a significant impact also on other toxigenic fungi, such as *Aspergillus niger*, *Fusarium graminearum, F. oxysporum* and *F. verticillioides*, on other fungal pathogens, and even on other organisms such as nematodes.

The fungal species reported in **Table 2-3** are all endophytic fungi [106]. Concerning endophytic fungi, the experiments were carried out by physically separating the strains in order to evaluate only the action of VOCs, which is indeed a little different from the conditions found in the plant where non-volatile compounds could also play a role. The most abundant VOC produced by *A. oryzae*, 1-octen-3-ol, was found to increase AFB_1 production with a dose-dependent effect. Moreover, *A. faecalis* [107], *E. asburiae* [108], *Staphylococcus saprophyticus* [109] and *A. flavus* itself [110] produced VOCs that induced a reduction in AFB_1 production, whereas VOCs of *Ralstonia solanacearum* stimulated its production.

Bacterial and fungal volatolomes can also affect other developmental parameters of *A. flavus*. Several effects were reported by Gong et al. (2020) and Braun et al. (2012) including inhibition of pectin methylesterase, cellulase and polyphenol oxidase secretion, conidial germination, sexual development and cell damage [59,62]. Interestingly, the effects are reciprocal, as was the case with *Ralstonia solanacearum* where a reduction in the growth of the bacterium and its melanin production was observed, probably induced by an increase in AFB_1 production by *A. flavus* [110]. A characterization of the volatolomes of some species has been performed, making it possible to relate the effects observed on *A. flavus* and the VOCs with bioactive potential [46,103,107,108,111–113].

Table 2-3. Volatolomes and their major compounds when identified from bacteria, yeast and fungi impacting *A. flavus* growth and/or its AFB_1 production without physical contact.

(+) Increase, (-) Reduction, (NA) data not available

	Species and Main VOCs	Impact		References	
		Growth	Aflatoxin		
Bacteria	***Alcaligenes faecalis*** Dimethyl disulfide Methyl 3-methylbutanoate	-	-	Gong et al., 2019	[107]
	Bacillus subtilis	-	NA	Chaves-López et al., 2015	[105]
	Bacillus cereus	-	NA	Chaves-López et al., 2015	[105]
	Bacillus amyloliquefaciens	-	NA	Chaves-López et al., 2015	[105]
	Enterobacter asburiae 1-Methoxy-3-methylbutane Pentan-1-ol 2-Phenylethanol	-	-	Gong et al., 2019	[108]
	Ralstonia Solanacearum	-	+	Spraker et al., 2014 Singh et al., 2020 Suwannarach et al., 2013	[95] [103] [111]
	***Schewanella* algae** Dodecan-2-ol 2,4-bis(1,1-Dimethylethyl)-phenol 2,2-Dimethyl-oxazole Butylated hydoxytoluene Nonane Dimethyl trisulfide	-		Gong et al., 2015	[113]
	Staphylococcus saprophyticus 3,3-dimethyl-1,2-epoxybutane	-	-	Gong et al., 2020	[109]
	Streptomyces philanthi	-		Boukaew and Prasertsan, 2020	[104]
	Streptomyces yanglinensis	-	-	Lyu et al., 2020	[114]

Table 2-3 (continued)

	Species and Main VOCs	Impact: Growth	Impact: Aflatoxin	References	
Yeast	***Candida nivariensis*** 2-Methylpropan-1-ol 3-Methylbutan-1-ol Pentan-1-ol	-	-	Jaibangyang et al., 2020	[115]
Yeast	***Hanseniaspora opuntiae*** Acetic acid 2-Methylbutanoic acid 2-Phenylethyl acetate	-	-	Tejero et al., 2021	[116]
Yeast	***Hanseniaspora uvarum*** Ethyl acetate 3-Methylbutan-1-ol 2-Methylbutan-1-ol 2-Phenylethyl acetate	-	-	Tejero et al., 2021	[116]
Yeast	***Wickerhamomyces anomalus*** 2-Phenylethanol	-	NA	Tilocca et al., 2020	[46]
Fungi	***Streptomyces alboflavus***	-	NA	Yang et al., 2019	[117]
Fungi	***Fusarium oxysporum*** Limonene	-	NA	Suwannarach et al., 2013	[111]
Fungi	***Muscodor genus*** 2-Methylpropanoic acid 2- Methylbutan-1-ol 3-Methylbutan-1-ol	-	NA	Braun et al., 2012 Singh et al., 2020 Suwannarach et al., 2013	[112] [103] [111]
Fungi	***Nodulisporium sp.*** 1,8 Cineole Terpinen-4-ol	-	NA	Suwannarach et al., 2013	[111]
Fungi	***Trichomderma genus***	-	NA	Singh et al., 2020	[103]
Fungi	***Aspergillus flavus***	-	-	Sweany and Damann, 2020	[110]
Fungi	***Aspergillus oryzae*** Octa-1,3-diene Octa-1,5-diene-3-ol 1-Octene-3-ol Octan-3-one Octanal Oct-2-enal 1-Octene-1-ol Octa-2,4-dieneal	-	NA	Singh et al., 2020	[103]

3.2. Blends of VOCs from essential oils show antifungal properties and regulation effects on AFB_1 production in A. flavus

For many years, essential oils have been the subject of numerous studies on their properties, including their efficiency as antifungals. With regard to *A. flavus*, the efficiency of this property has been by using two modalities: (i) during a contact between *A. flavus* and the essential oil (by using discs or by introducing it directly into the culture medium),

or (ii) without direct physical contact between the fungus and the essential oil (by fumigation or by introducing a volume of essential oil in a closed space containing the colony of *A. flavus*) (**Table 2-4**). An essential oil is a mixture of VOCs, often consisting of mono- and sesquiterpene, benzoids and other classes of molecules, resulting from the natural extraction from a plant. Many terpenes discovered in recent decades that are components of essential oils, have various associated activities such as anti-phytopathogenic, immunosuppressive, anti-inflammatory, anti-bacterial, cytotoxic, antifungal, anti-viral activities as well as enzyme inhibition, among others [118].

All the tested essential oils produced a fungistatic effect regardless the mode of application (contact or not) with the fungus, with the exception of *Litsea cubeba*, although this essential oil produced an inhibition of AFB_1 production. The essential oils in the Table 4 are non-specific to *A. flavus* and also affect other fungal species, including those belonging to the *Aspergillus* genus.

Two opposing approaches have been tested. On the one hand, the complexification of the mixtures to improve the synergy of the constituent molecules of the essential oils has been investigated. *Cinnamomum, Origanum* and *Thymus*, taken individually, have been shown to have a significant impact. However, it is the combination of the three that induced a much more effective synergy, causing a down-regulation of aflatoxin biosynthesis genes (70% inhibition of aflatoxins) and an associated decrease in the total growth of *A. flavus* colonies [119–121]. On the other hand, the simplification of mixtures by determining the VOCs associated with antifungal and anti-aflatoxigenic effects, starting with their major compounds, has been studied. In this case, the antifungal effect of the essential oil of *Cinnamomum cassia* was compared with its main compound, cinnamaldehyde. Both showed inhibition of the development of *A. flavus* and *A. oryzae*, but according to the data presented, the single molecule was more effective than the whole essential oil [120].

Some of the *A. flavus* antagonistic molecules emitted by the microorganisms listed in **Table 2-3** are also present in the essential oils listed in **Table 2-4**. This is the case for 1,8-cineole and limonene, the latter of which appears as a constituent of six of the essential oils observed here.

Table 2-4. Essential oils and their major VOCs impacting the growth of *A. flavus* and/or its production of AFB_1.

(a) Latin Name and Major VOCs	(b) Impact		(c) Application Mode	References	
	Growth	Aflatoxin			
Aegle marmelos D and L-Limonene *	-	NA	Contact	Adorjan and Buchbauer, 2010	[122]
Ageratum conyzoides Precocene I and II Dimetoxy ageratocromene Ageratocromene	-	-	Contact	Adorjan and Buchbauer, 2010 Esper et al., 2014	[122] [119]

Table 2-4 (continued)

(a) Latin Name and Major VOCs	(b) Impact		(c) Application Mode	References	
	Growth	Aflatoxin			
Allium porrums Diallyl trisulfide Diallyl disulfide Methyl allyl trisulfide 5-Ethylthiazole	-	-	Contact	Kocevski et al., 2013 Abd El-Aziz et al., 2015	[120] [123]
Capsicum Not available	-	NA	No contact	Boukaew et al., 2017	[124]
Chenopodium ambrosioides (Z)-Ascaridole	-	NA	Contact	Adorjan and Buchbauer, 2010	[122]
Cinnamomum Cinnamaldehyde (E)-2-methoxycinnamaldehyde Carveol α-Cadinol	-	-	Contact No contact	Abd El-Aziz et al., 2015 Boukaew et al., 2017 Manso et al., 2013 Kocevski et al., 2013 Xiang et al., 2020	[123] [124] [125] [120] [121]
Citrus peel Limonene* Linalool Citral	-	NA	Contact	Taguchi et al., 2015	[126]
Curcuma longa L Ar-Tumerone α –Tumerone β-Tumerone Ar-Curcumene β -Sesquiphellandrene	-	-	Contact	Ferreira et al., 2013 Hu et al., 2017	[127] [128]
Cymbopogon (Z)-Citral (E)-Citral Limonene*	-	NA	Contact	Xiang et al., 2020	[121]
Litsea cubeba essential (Z) and (E)-Limonene oxide D-Limonene*	NA	-	Contact No contact	Li et al., 2016	[129]
Mentha Menthol Menthone Menthyl acetate Menthofurane	-	-	Contact	Abd El-Aziz et al., 2015 Beyki et al., 2014 Taguchi et al., 2015	[123] [130] [126]
Nepeta cataria 4aa,7a,7ab-Nepetalactone	-	NA	Contact	Adorjan and Buchbauer, 2010	[122]
Ocimum basilicum Linalool Methylchalvicol Eugenol Methyl eugenol Methyl cinnamate 1,8- Cineole Caryophyllene *	-	NA	Contact	Taguchi et al., 2015 Xiang et al., 2020	[126] [121]

Table 2-4 (continued)

(a) Latin Name and Major VOCs	(b) Impact		(c) Application Mode	References	
	Growth	Aflatoxin			
Origanum Carvacrol Thymol 4-Terpineol Linalool γ-Terpinene α-Terpineol	-	-	Contact	Esper et al., 2014 Xiang et al., 2020	[119] [121]
Pimenta dioica α-Terpinoel β-Linalool γ-Terpinene Eucalyptol	-	-	Contact	Kumar Chaudhari et al., 2022	[131]
Pogostemon cablin Patchouli alcohol 4-Oxo-14-norvitrane δ-Guaiene	-	NA	Contact	Kocevski et al., 2013	[120]
Rosemary Camphor 1,8-Cineole α-Pinene* Verbenone Camphene Limonene * Bornyl acetate α-Terpineol β-Pinene	-	-	Contact	Abd El-Aziz et al., 2015 Taguchi et al., 2015	[123] [126]
Satureja hortensis Thymol Carvacrol	-	NA	Contact	Adorjan and Buchbauer, 2010	[122]
Syzygium aromaticum Eugenol Eugenyl acetate Caryophyllene Benzenemethanol	-	NA	Contact No contact	Adorjan and Buchbauer, 2010 Boukaew et al., 2017 Taguchi et al., 2015 Xiang et al., 2020	[122] [124] [126] [121]
Thymus vulgaris p-Cymene γ-Terpinene Thymol	-	-	Contact	Abd El-Aziz et al., 2015 Khalili et al., 2015	[123] [132]
Vatica diospyroides Symington Benzyl acetate Benzyl benzoate Isoeugenol α-Terpineol	-	NA	No contact	Boukaew et al., 2017	[124]

Table 2-4 (continued)

(a) Latin Name and Major VOCs	(b) Impact		(c) Application Mode	References	
	Growth	Aflatoxin			
Zanthoxylum molle Undecan-2-one Limonene * Terpinen-4-ol	-	NA	Contact No contact	Tian et al., 2014	[133]
Zataria multiflora Boiss Carvacrol	-	NA	Contact	Adorjan and Buchbauer, 2010	[122]
Zingiber officinale β-Phellandrene Zingiberene Geranial Neral	-	-	Contact	Adorjan and Buchbauer, 2010 Nerilo et al., 2016 Taguchi et al., 2015	[122] [134] [126]

(a) Latin name of the plant and majority VOCs identified in essential oils. When a VOC constituting an essential oil is known to be emitted by *A. flavus* species (in accordance with the Table 2-7) it is indicated by an asterisk * in column (a). (b) Compilation of (-) inhibitory or (NA) unavailable data for growth and production of AFB_1. (c) Contact type (contact/non-contact).

3.3. Single bioactive VOCs affecting the growth and/or the AFB_1 production of A. flavus

The individual bioactive VOCs are produced by fungal species, microorganisms and plants, but to our knowledge, no study on the influence of the complete plant volatolome on *A. flavus* or mycotoxin production has been undertaken. Among the 64 individual bioactive VOCs affecting the growth of *A. flavus* and/or its production of AFB_1, there are 27 VOCs known to be emitted by the species *A. flavus* itself (**Table 2-5**). Within these bioactive VOCs, we find nonan-2-one and octan-3-one [98] or trans-2-methylbut-2-enal and 2,3-dihydrofuran [62] specifically emitted by NTS, or decan-1-ol and limonene [98] emitted by TS.

Four molecules with fungicidal action resulting in cell death have been reported. All of them were studied following physical contact with colonies of *A. flavus*. These studies showed that hexanal (0.4 µL/mL) [135], 2-phenylethanol (lethal at 0.3–0.5%) [136], farnesol (400 µM) [137] and nonan-1-ol (20 µL/mL) [102] lead to fungal death due to the loss of its membrane integrity.

All other individual bioactive VOCs have fungistatic effects toward *A. flavus* associated with variable AFB1 production responses. A reduction in the mycelial structure does not necessarily extend to the other fungal structures, as is the case for trans-hex-2-enal (diluted in ethanol) which causes lethality to the mycelia (95% at 20 µM) but does not affect conidia viability [138].

Total inhibition of AFB_1 and fungal growth was observed with benzaldehyde, hexanal, nonyl aldehyde, trans-non-2-enal, heptanal and octanal by using different concentrations in

a contactless approach [86,90,92]. In particular, Cleveland et al. (2009) showed a significant influence on the AFB_1 production of the VOCs concentration used, highlighting that the mechanisms leading to AFB_1 production are more sensitive than those involved in growth reduction [99]. Additionally, the inhibition of spore germination with trans-hex-2-enal, hexanal, trans-non-2-enal and 2-methylpropionic acid was observed and further damage by their hydroperoxide metabolites via lysis of hydroperoxides was also noted [112,139]. A positive correlation was established between AFB_1 and the amount of 1-octen-3-ol, although this compound induced a reduction in *A. flavus* growth, sclerotia and conidia density [103]. In addition, an increase in alpha-amylase production by *A. flavus* was also observed as a consequence of 1-octen-3-ol presence [103].

Furthermore, it has been proved that each molecule has its own minimum concentration that affects the growth of *A. flavus* colonies and/or AFB_1 production, and this concentration can be highly variable [99,140]. In addition, for each molecule, the frequency of exposure (punctual or cyclic) is also important [138,139].

Even if no changes are observed in the growth of the mycelium of *A. flavus*, other effects may be observed in the colonies, such as suppression of spore germination, changes in mycelial pigments (notably, observed for methyl jasmonate) [139], and reduction of AFB_1 [98]. Modifications due to the substrates on which *A. flavus* was grown were also observed. The growth inhibition when *A. flavus* was grown on maize seeds or PDA medium are similar, but differences concerning AFB_1 production were observed [119,123].

Some VOCs can exacerbate the production of AFB_1. 3-Methylbutan-1-ol, 2-methylbutan-1-ol, cis-hex-2-en-1-ol, myrcene, ocimene, 2-pentylfuran and hexan-3-one did not affect *A. flavus* growth, but increased AFB_1 production up to 50%, with a higher trend for the first two mentioned alcohols [98,99]. In particular, 3-methylbutan-1-ol and 2-methylbutan-1-ol are mainly emitted by fungal species that are competitors of *A. flavus*. Thus, one hypothesis is that their presence could stimulate the “defense system” of *A. flavus*, leading to the synthesis of AFB_1.

Only a fungistatic effect for *A. flavus* has been reported on vaporization of decan-1-ol, alpha and beta-pinene [98] or with 2-butoxy alcohol [98] and furfural [141]. However, it is interesting to note that, three of these compounds are naturally emitted by the same *A. flavus*, specifically, decan-1-ol, furfural and alpha-pinene.

The expression of divergent effects triggered by the same VOC has also been underlined by Zhang et al. (2021). They found that the growth of *A. flavus* showed a negative correlation with an increasing concentration of sprayed nonan-1-ol [102]; however, the opposite trend was detected by Zeringue et al. (1990), who showed that vaporization increased the mycelium growth, in addition to creating oxidative stress in the mycelium [98].

A comparison between fumigation and contact mode reveals that experiments carried out using fumigation required lower concentrations than those performed using contact, with respect to mycelium inhibition. Ma et al. (2017) determined that a fumigation with a 50-fold lower concentration of trans-hex-2-enal than the concentration used by physical

contact was required for growth inhibition of *A. flavus* [142], and the same trend was noted with the essential oils [124,129,133].

Table 2-5. Individual VOCs impacting the growth of *A. flavus* and/or its production of AFB_1.

(a)	(b) Name	(c) Source	(d) Impact		(e) Application Mode	References	
			Growth	Aflatoxin			
Alcohol	1-Octen-3-ol	○ ●	-	-	No contact	Singh et al., 2020	[103]
	2-Buten-1-ol	○	NA	-	No contact	Zeringue and McCormick, 1990	[98]
	2-Butoxy alcohol	○	-	NA	No contact	Zeringue and McCormick, 1990	[98]
	2-Methylbutan-1-ol	○ ●	-	+	No contact	Braun et al., 2012 Zeringue and McCormick, 1990	[112] [98]
	2-Phenylethanol	○	-	-	No contact Contact	Chang et al., 2015 [1] Gong et al., 2019 Hua et al., 2014	[143] [108] [136]
	3-Hepten-1-ol	○	-	-	No contact	Zeringue and McCormick, 1990	[98]
Alcohol	Cis-hex-3-en-1-ol	○	-	NA	No contact Contact	Ma et al., 2017	[142]
	3-Methylbutan-1-ol	○ ●	-	+	No contact	Braun et al., 2012 Zeringue and McCormick, 1990	[112] [98]
	Cis-hex-2-en-1-ol	○	-	+	No contact Contact	Ma et al., 2017 Zeringue and McCormick, 1990	[142] [98]
	Decan-1-ol	○ ●	+	NA	No contact	Zeringue and McCormick, 1990	[98]
	Ethanol	○ ●	-	-	Contact	Ren et al., 2020	[101]
	Heptan-1-ol	○	-	-	No contact	Zeringue and McCormick, 1990	[98]
	Hexan-1-ol	○ ●	-	-	No contact Contact	Cleveland et al., 2009 Ma et al., 2017	[99] [142]
	Nonan-1-ol	○	+	NA	No contact Contact	Zeringue and McCormick, 1990 Zhang et al., 2021	[98] [102]
	Octan-3-ol	○ ●	-	-	No contact	Cleveland et al., 2009	[99]
	Pentan-1-ol	○	+/-	+/-	No contact	Cleveland et al., 2009 Zeringue and McCormick, 1990	[99] [98]
Acid	2-Methylpropanoic acid	○ ●	-	NA	No contact	Braun et al., 2012	[112]
	4-Pentanoic acid	○	NA	-	No contact	Zeringue and McCormick, 1990	[98]
	Benzoic acid	○ ●	-	-	Contact	Moon et al., 2018	[140]
	Sorbic acid	○	-	-	Contact	Moon et al., 2018	[140]
	Acetic acid	○ ●	-	-	Contact	Moon et al., 2018	[140]
	Propionic acid	○	-	-	Contact	Moon et al., 2018	[140]
	Butyric acid	○	-	-	Contact	Moon et al., 2018	[140]

Table 2-5 (continued)

(a)	(b) Name	(c) Source		(d) Impact		(e) Application Mode	References	
				Growth	Aflatoxin			
Aldehyde	**Trans-hept-2-enal**	○		-	-	No contact	Cleveland et al., 2009 Zeringue and McCormick, 1990 Zeringue et al., 1996	[99] [98] [144]
	2,4-Hexadienal	○		-	-	No contact	Cleveland et al., 2009 Zeringue and McCormick, 1990	[99] [98]
	(E,E)-2,4-Heptadienal	○		-	NA	No contact	Ma and Johnson, 2021	[145]
	Oct-2-enal	○		-	-	No contact	Cleveland et al., 2009 Zeringue and McCormick, 1990 Zeringue et al., 1996	[99] [98] [144]
	Benzaldehyde	○	●	-	-	No contact	Cleveland et al., 2009	[99]
	Cinnamaldehyde	○		-	-	Contact	Liang et al., 2015 Yin et al., 2015 Wang et al., 2019	[146] [147] [148]
	Citral	○		-	-	Contact	Liang et al., 2015	[146]
	Diethylacetal 2-hexenal	○		-	-	No contact	Zeringue and McCormick, 1990	[98]
	n-Decyl aldehyde	○		-	-	No contact	Wright et al., 2000	[149]
	Furfural	○	●	-	NA	No contact	Zeringue, 2000	[141]
	Heptanal	○	●	-	-	No contact	Zeringue and McCormick, 1990 Zeringue et al., 1996	[98] [144]
	Hexanal	○	●	-	-	No contact Contact	Cleveland et al., 2009 Li et al., 2021 Ma et al., 2017 Wright et al., 2000 Zeringue and McCormick, 1990 Zeringue et al., 1996	[99] [135] [142] [149] [98] [144]
	Nonanal	○	●	-	-	No contact	Cleveland et al., 2009	[99]
	Nonyl aldehyde	○		-	-	No contact	Zeringue and McCormick, 1990 Zeringue et al., 1996	[98] [144]
	Octanal	○	●	-	+/-	No contact	Cleveland et al., 2009 Wright et al., 2000 Zeringue and McCormick, 1990 Zeringue et al., 1996	[99] [149] [98] [144]
	Sorbaldehyde	○		-	NA	No contact	Ma and Johnson, 2021	[145]
	Trans-hex-2-enal	○	●	-	-	No contact	Cleveland et al., 2009 De Lucca et al., 2011 Ma et al., 2017 Zeringue and McCormick, 1990 Zeringue et al., 1996	[99] [138] [142] [98] [144]
	Trans-2-methylbut-2-enal	○	●	NA	-	No contact	Moore et al., 2021	[62]
	Trans-non-2-enal	○		-	-	No contact	Zeringue and McCormick, 1990 Zeringue et al., 1996	[98] [144]

Table 2-5 (continued)

(a)	(b) Name	(c) Source		(d) Impact		(e) Application Mode	References	
				Growth	Aflatoxin			
Alkane	**Decane**	○	●	NA	-	No contact	Moore et al., 2021	[62]
Ester	**Ethyl acetate**	○	●	NA	-	No contact	Zeringue and McCormick, 1990	[98]
	Hexyl acetate	○		-	NA	No contact Contact	Ma et al., 2017	[142]
	Trans-2-hexenyl acetate	○		-	NA	No contact Contact	Ma et al., 2017	[142]
Furan	**2,3-Dihydrofuran**		●	NA	-	No contact	Moore et al., 2021 Moore et al., 2022	[62] [56]
	2-Pentylfuran	○	●	=	+	No contact	Cleveland et al., 2009	[99]
Ketone	**Heptan-3-one**	○		+	+	No contact	Cleveland et al., 2009	[99]
	Hexan-3-one	○		=	-	No contact	Cleveland et al., 2009	[99]
	Nonan-2-one	○	●	-	-	No contact	Zeringue and McCormick, 1990	[98]
	Octan-3-one	○	●	-	-	No contact	Moore et al., 2021 Moore et al., 2022 Zeringue and McCormick, 1990	[62] [56] [98]
	Pentan-3-one	○		NA	+	No contact	Zeringue and McCormick, 1990	[98]
	3-Octen-2-one	○	●	-	-	No contact	Cleveland et al., 2009	[99]
Terpene	**α-pinene**	○	●	+	(-)	No contact	Zeringue and McCormick, 1990	[98]
	β-pinene	○		+	(-)	No contact	Zeringue and McCormick, 1990	[98]
	Camphene	○		NA	-	No contact	Zeringue and McCormick, 1990	[98]
	Carvacrol	○		-	NA	Contact	Yin et al., 2015	[147]
	Eugenol	○		-	NA	Contact	Liang et al., 2015	[146]
	Farnesol	○		-	-	Contact	Wang et al., 2014	[137]
	Limonene	○	●	NA	-	No contact	Zeringue and McCormick, 1990	[98]
	Myrcene	○		NA	+	No contact	Zeringue and McCormick, 1990	[98]
	Ocimene	○		NA	+	No contact	Zeringue and McCormick, 1990	[98]
Other	**Methyl jasmonate**	○		NA	-	No contact	Goodrich-Tanrikulu et al., 1995	[139]

(a) Chemical family. (b) IUPAC name. (c) When a VOC is known to be emitted by *A. flavus* species (in accordance with the Table 2-7) it is indicated by ● symbol. The ○ symbol is present when the standard VOC was used in the study. (d) Compilation of (-) inhibitory, (+) stimulating, (=) no significant variation or (NA) unavailable data for growth and production of AFB_1. (e) Contact type (contact/non-contact).

4. What are the modes of action of these bioactive VOCs?

Although fungicidal, fungistatic, or AFB_1-reducing effects induced by several bioactive VOCs or volatolomes have been proved, few of these have been further investigated for the mechanisms that are involved in such activities. Regarding the AFB_1 production, some studies have focused on the gene expression of some selected *afl* genes. In addition, the impact of VOCs on mycelial growth, sporulation and the germination of conidia or on physiological functions and genetic mechanisms have been rarely studied. To date, investigations have focused on mechanisms such as the loss of fungal membrane integrity and the regulation of the AFB_1 biosynthetic gene cluster (**Figure 2-3**).

4.1. Loss of membrane integrity of A. flavus

The loss of membrane integrity is the result of several forms of deregulation of the physiological functions of *A. flavus*. A systematic observation of the endomembrane system, mainly of the plasma membrane and mitochondria, of *A. flavus* cells rapidly detected the induction of structural changes after exposure to some VOCs. 2-Phenylethanol, farnesol, hexanal, nonan-1-ol and *Ageratum conyzoides* essential oil caused shrinkage and detachment of the cell wall in the cytoplasm. An alteration of the mitochondria membrane, which became less defined and discontinuous or absent, was observed due to changes in their lipid and fatty acid composition, in addition to the down regulation of the mitochondrial dehydrogenases [102,135–137,143,150]. On the other hand, essential oils (*Zanthoxylum molle, Ageratum conyzoides*) that are mixtures of several compounds could also disrupt all membranes by crossing the layers of polysaccharides, fatty acids and phospholipids, changing the pH, and dramatically modifying the physiological functions of the cell [122,133]. According to Basak et al. (2018), the main mode of action of essential oils was related to the permeability of organelles [151]. A further impact of *Mentha cardiaca* essential oil on *A. flavus* was the leaking of Ca^{2+}, K^+ and Mg^{2+} ions from cell membranes, as indicated by measurements of the electrical conductivity [102,135,137,152]. This caused accumulation of ROS (reactive oxygen species), disruption of the Krebs cycle (or TCA) and reduction of ATPase [102,137]. Considered together, the effects of essential oils show an enormous capability to alter several cellular functions in *A. flavus* and thereby affect its fitness and survival possibilities.

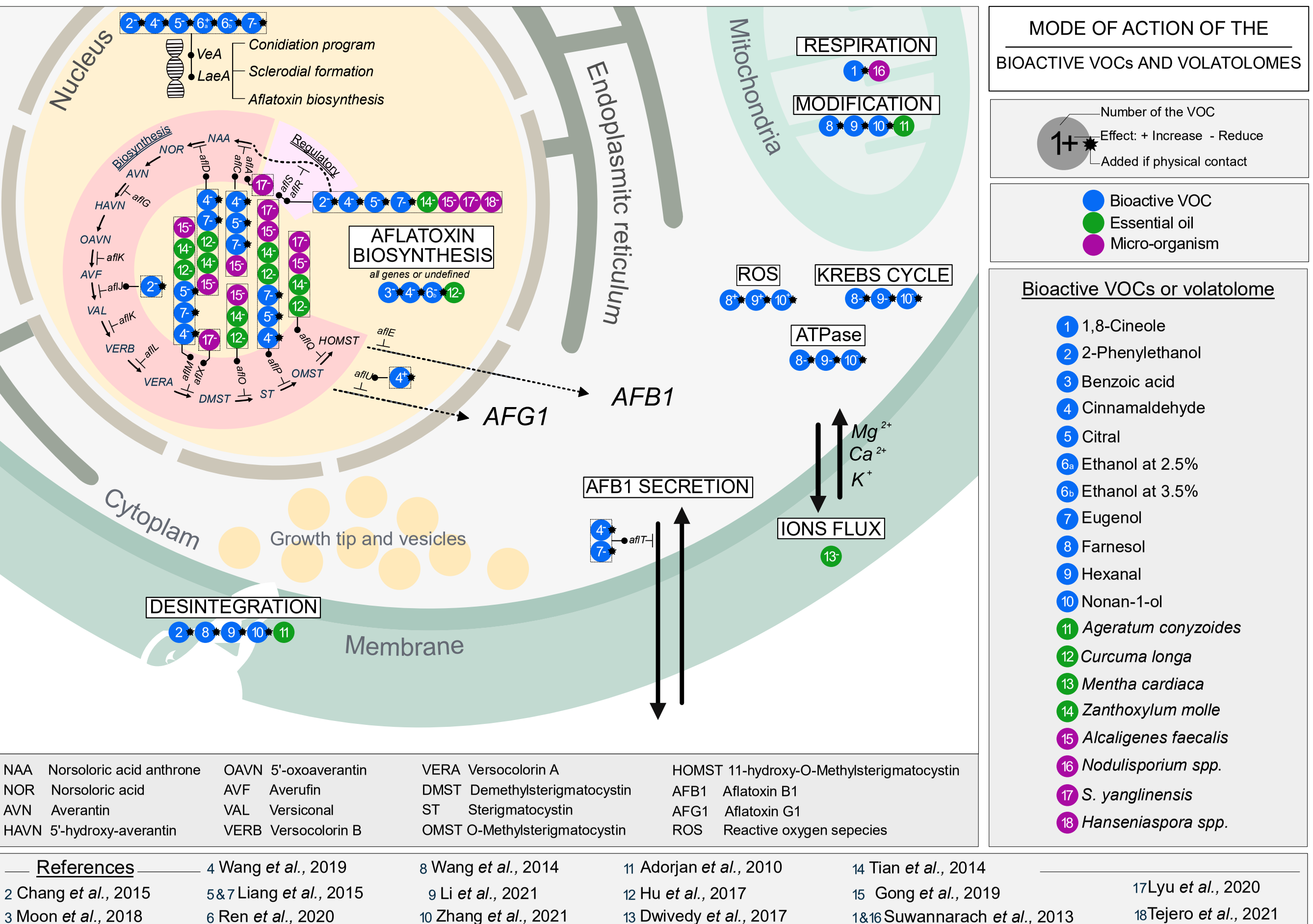


Figure 2-3. Mode of action of the bioactive VOCs and volatolomes on *A. flavus.*

4.2. Modification of afl gene expression

The studies on AFB_1 biosynthesis gene expression are not all focused on the same genes and are not exhaustive.

Some studies proved that 2-phenylethanol, cinnamaldehyde, citral, eugenol and ethanol act on global regulatory genes such as the velvet complex (*VeA*) or the *LaeA* gene in *A. flavus* [99,146,148]. In addition to the aflatoxin biosynthetic pathway, these regulatory genes are also involved in the regulation of sexual development, sclerotia formation, and conidia programming [74].

Interestingly, the concentration of a single applied molecule can differentially affect gene expression. While 2.5% ethanol increased the regulation of the velvet complex, a concentration of 3.5% of the same compound induced its down-regulation [101].

Two regulatory genes of aflatoxin biosynthesis, *aflR* and *aflS*, which are positive regulator of the whole gene cluster as main activator and enhancer in the regulatory biosynthesis process, respectively, were also inhibited by the exposure to 2-phenylethanol, cinnamaldehyde, citral, eugenol, benzoic acid, and ethanol; they were also inhibited by the essential oils of *Zanthoxylum molle* and *Curcuma longa* and the volatolome of the fungal *Nodulisporium spp*. [111,128,133,136,143,146–148]. The volatolome of the bacteria *A. faecalis* and *S. yanglenensis*, as well as the two yeasts *Hanseniaspora sp.*, inhibited the regulation activity of *aflS* and *aflR* [46,105,114].

Some compounds such as benzoic acid or ethanol also showed a reduction in the expression of all the genes involved in the biosynthesis pathway, but this was not explicitly stated [140].

Complete inhibition of aflatoxin production required only 3-4% ethanol. Ethanol at 3.5% and the *A. faecalis* volatolome resulted in down-regulation of all aflatoxin group genes except *aflC* (which controls polyketide synthase) [74,101,107].

As with ethanol, the concentration of cinnamaldehyde always led to a reduction in AFB_1 production, but the genes affected were variable. A constant inhibition of *aflT*, which regulates AFB_1 secretion, was observed. A specificity was noted at 0.60 mM because the *aflU* was upregulated. In general, with 0.8 mM cinnamaldehyde, 25 of the 30 genes in the aflatoxin group were down-regulated [148]. At a concentration of 0.60 mM, the *aflF* and *aflU* genes were more expressed everywhere, except *aflT, aflS* and *aflR,* compared with the control [148]. Finally, five genes (*aflP, aflC, aflM, aflD, aflT*) were down-regulated by cinnamaldehyde at 0.40 mM [146]. These last five genes were also down-regulated by eugenol at 0.80 mM, whereas only the first three genes were affected by citral 0.56 mM [146,147].

When focusing on the modes of action, no successions of mechanisms seem to be attributed to a particular chemical family of VOCs. This could be attributable to the lack of information gathered in this field. However, in general, we can conclude that many VOCs produced by both microorganisms and plants can down-regulate several biosynthetic *afl* genes with different targets and intensity. Therefore, we need more studies to obtain more in-depth knowledge on the links between specific VOCs and specific genes affected.

4.3. Impact on the fungal growth and ergosterol production

The growth of *A. flavus* was proved to be affected by cinnamaldehyde and 2-phenylethanol which completely inhibited the fungal growth [143,148]. Since a related effect on *A. flavus* caused by cinnamaldehyde was the lack of AFB_1 production, the fungal physiology and metabolism, particularly the metabolism of certain amino acids required at the hypha apex for fungal growth, were altered [143].

In addition, the volatolomes of species belonging to the *Nodulisporium* genus were shown to interfere with *A. flavus* physiology. In particular, 1,8-cineole inhibited the mitochondrial respiration as well as different stages of mitosis. This last molecule was shown to penetrate through the cell membrane and cause oxidative damage to cell organelles [111]. Finally, among other effects, the essential oil of *Curcuma longa* also induced a considerable reduction in the amount of ergosterol [128].

In summary, although strong effects on the growth of *A. flavus* and its ergosterol production have been shown in some experiments, few studies are available on the functions and mechanisms of VOCs that enable these effects. Therefore, more in-depth investigations are needed to provide the knowledge for possible practical applications of VOCs in the biological control of *A. flavus*.

5. How can we exploit these VOCs to our advantage to control the growth of *A. flavus* and its AFB_1 production?

In the previous paragraphs and in **Tables 2-3** to **2-5**, we have outlined the effects of bioactive VOCs on the growth of *A. flavus* and on the production of AFB_1. In order to limit the fungal contamination and AFB_1 production, both the early harvesting of maize and quick and controlled storage are recommended [8,153]. However, a further tool that potentially can be integrated in the fight against mycotoxin production at the harvesting phase is the use of bioactive VOCs. Therefore, the selection of bioactive VOCs according to the time of their application in the food chain is also critical to ensuring their antifungal (inhibition of the growth of *A. flavus*) and anti-aflatoxigenic (inhibition of AFB_1 production) properties.

Fumigation or pulverization, using bioactive antifungal VOCs could be also considered to dramatically reduce the presence of unfavorable microorganisms on the surface of the grains during harvest and before storage. This approach, which was applied by Sharon et al. (2009) and Hamann et al. (2008), also causes damage to and destruction of the survival structures of the fungus, eventually initiating apoptotic-like cell death [154,155]. However, for a higher efficacy, higher concentrations of VOCs were used by Li et al. (2016) and Tian et al. (2014) [129,133].

In addition, in order to inhibit *A. flavus* growth during storage, using an antifungal compound combined with a selected anti-aflatoxigenic bioactive VOC applied by diffusion

could be of interest. In general, fumigation requires a lower concentration than contact, although some exceptions do exist [127,131,136]. Currently, the majority of the bioactive VOCs identified have been shown to have a punctual action due to their fungistatic effect. This means that as soon as the *A. flavus* is no longer subjected to their effects, it regains its virulence and all its faculties to grow and produce AFB_1 [109,117]. Therefore, to improve the impact of VOCs on *A. flavus*, setting up a slow diffusion system capable of diffusing the bioactive VOCs over a long period of time would be extremely useful. This objective could be achieved by [131] using new methods of diffusion such as capsules that by a slow release of VOCs in the environment after their dispersion allow a longer temporal dispersion, as proved by Maes et al. (2019) [156]. On the other hand, to apply a bioactive VOC whose effect is permanent would be a reliable alternative. However, it is essential that such a permanent fungicidal effect is effective against all structures of the fungus to avoid any subsequent fungal development after the VOC application.

A further key issue is to optimize the concentration of each VOC since the antifungal efficacy among the bioactive VOCs is highly variable, as shown in vivo experiments over different periods of time on several kinds of food by several authors [122,131,136,138,145]. In addition, such variability has also been confirmed for the VOCs' anti-aflatoxin activities [126,127,131]. From all these studies, it is evident that, in general, in the vivo experiments a higher concentration was required than in in vitro experiments to completely inhibit *A. flavus* growth and AFB_1 production.

Microbial diversity can also be used to inhibit both *A. flavus* growth and AFB_1 production, integrating the beneficial action of selected microorganisms that, for example, share the atmosphere of stored grains. All the microorganisms listed in **Table 2-3** have shown a fungistatic effect against *A. flavus*, but only three of them were also investigated for their ability to control and inhibit AFB_1 production. The whole volatolomes of *S. saprophyticus* and *A. faecalis* have been tested against other fungal pathogens successfully [107,109]. Dimethyl disulfide, which is one of the major VOCs emitted by these bacteria, is also an effective control, while also promoting plant growth [105,120].

The possible contributions of bioactive VOCs emitted by biological material, such as some crop varieties adapted to local conditions and/or particularly resistant to fungi, have also been shown. Zeringue et al. studied the VOCs emitted by resistant hybrids in order to isolate their specific VOCs and identified mainly aldehydes [144]. Since some maize varieties are less attractive for insects that often are the main vectors of fungal contaminations, the combined use of insect repellent molecules and antifungal complementary bioactive VOCs could be an interesting approach to pursue in future [122].

In addition, since some VOCs have been used as antimicrobial agents in food packaging materials such as polyethylene terephthalate films containing essential oils [125], an extended application of these compounds as new preservation methods could be a further tool to control fungal contamination and mycotoxin production in food packaging.

Finally, it is important to consider that some of the bioactive VOCs discussed here could have negative effects such as possible cytotoxicity for humans and reductions in seed germination, and therefore, these aspects must be well studied before proposing any VOCs

use. On the other hand, their volatility leads to an absence of residue on the foodstuff, facilitating its transformation in the food chain since no washing would be required. Thus, unpleasant smells for consumers would be limited, which is an important organoleptic parameter.

In conclusion, the main advantages of using VOCs as bio-control agents are as follows. Firstly, they have a wider and easier diffusion mechanism without requiring physical contact to affect the fungus and there is an absence of residues on the crop. Secondly, an application of bioactive VOCs at key points of the food chain could be an efficient solution to control the fungal growth and, therefore, the production of AFB_1 and reduce the use of preservatives that can add unpleasant odors to food. On the other hand, it is necessary to take into account that there is a balance between the fauna and the flora of a given environment and that the eradication of a species such as *A. flavus* can induce a recrudescence of its competitors or other microorganisms. Therefore, the control of the population of *A. flavus*, although worthwhile, should avoid a dramatic increase of other species producing other mycotoxins or causing other diseases in plants.

6. Conclusions

VOCs constitute an elementary chain in inter- and intra-species interactions. The great diversity of VOCs emitted by *A. flavus* strains reported in the literature demonstrates that abiotic factors have a great influence on strain VOC profiles. Interesting VOCs have been isolated and identified as bioactive compounds against the growth of *A. flavus* and/or its production of AFB_1. However, the mechanisms involved are poorly studied. Nevertheless, some researchers have oriented their investigations towards the aflatoxin gene cluster. In addition, it is evident that a standardization of the environmental parameters that influence the VOCs production is necessary. This would generate a robust knowledge base for our proposed use of VOCs as a reliable biocontrol tool.

7. Perspectives

Studies on bioactive VOCs need to consider some issues including the imprecision of certain parameters, such as the application mode, which are often missed in many research studies and have different consequences for the metabolism of *A. flavus*.

A further issue is the accurate evaluation of the effectiveness of bioactive VOCs on the growth or production of AFB_1. The control of abiotic parameters, the type and time of exposure, type of contact and strain of *A. flavus* (TS or NTS) targeted are all key aspects to be assessed. Finally, the possibility that specific VOCs could be identified for TS or NTS of *A. flavus* opens significant opportunities for developing reliable markers that can be used

for an early identification of strain toxigenicity, which is difficult to achieve using molecular markers due to the variability of NTS in their *afl* gene profiles.

8. Supplementary materials

The following supporting information can be downloaded at https: //www.mdpi.com/article/10.3390/ijms232415557/s

Table 2-6. Detailed compilation of VOCs known in the literature to be emitted by *A. flavus* sorted by CAS (Chemical Abstracts Service) number.

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
2,4-dimethylpenta-1,3-diene	1000-87-9	Alkene	T	[84]	Non-sterile cracked maize
octa-1,3-diene	1002-33-1	Alkene	T T U	[83] [92] [91]	Cracked maize Wheat and oats Wallpaper
ethylbenzene	100-41-4	Alkene	U	[92]	/
styrene	100-42-5	Alkene	T + NT/T T NT/T U NT T	[83] [84] [85] [92] [54] [86]	PDA + sterile cracked maize Cracked maize PDA / Maize media CSA, CDL, MEA, CMA
phenylmethanol	100-51-6	Alcohol	T	[83] [84]	Non-sterile cracked maize Cracked maize
benzaldehyde	100-52-7	Aldehyde	T	[83]	Cracked maize
ethyl phenylethanoate	101-97-3	Ester	T T*	[83] [85]	Cracked maize PDA
dodecan-2-ol	10203-28-8	Alcohol	T	[84]	Non-sterile cracked maize
eremophilene	10219-75-7	Terpene	U T	[92] [96]	/ Adye and Mateles liquid medium
2-ethylcyclobutan-1-one	10374-14-8	Ketone	T	[84]	Non-sterile cracked maize
benzeacetic acid	103-82-2	Acid	T	[83]	Cracked maize
4a,8-dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	103827-22-1	Terpene	T*	[85]	PDA
3-phenylpropen-2-al	104-55-2	Aldehyde	NT	[83]	PDA
2-ethylhexan-1-ol	104-76-7	Alcohol	NT/T	[83]	Cracked maize
ethyl propionate	105-37-3	Ester	T NT/T NT	[83] [85] [54]	Cracked maize PDA Maize media
ethyl butyrate	105-54-4	Ester	T NT/T	[85] [54]	PDA Maize media
1-(5-methyl-2-furanyl)propan-1-one	10599-69-6	Ketone	T	[84]	Non-sterile cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
ethyl heptanoate	106-30-9	Ester	T	[83]	Cracked maize
ethyl octanoate	106-32-1	Ester	NT+ T	[83]	PDA + Cracked maize
1,4-dimethylbenzene	106-42-3	Alkene	NT	[54]	Maize media
octan-3-one	106-68-3	Ketone	NT NT T NT/T	[83] [84] [94] [54]	PDA Non-sterile cracked maize Wheat meal sterilized Maize media
2-methylpentane	107-83-5	Alkane	T T	[84] [54]	Non-sterile cracked maize Maize media
pentan-2-one	107-87-9	Ketone	NT/T	[54]	Maize media
1,3-dimethylbenzene	108-38-3	Alkene	T	[84]	Non-sterile cracked maize
ethyl 3-methylbutyrate	108-64-5	Ester	NT/T	[54]	Maize media
1,3,5-trimethylbenzene	108-67-8	Alkene	T	[84]	Non-sterile cracked maize
methylcyclohexane	108-87-2	Alkane	T	[84]	Sterile cracked maize
toluene	108-88-3	Alkene	T T NT T	[83] [85] [54] [86]	Craked maize PDA Maize media CDA, CSA, CDL, MEA,
aromadrendrene	109119-91-7	Terpene	U T	[92] [85]	/ PDA
pentane	109-66-0	Alkane	NT/T	[54]	Maize media
tetrahydrofuran	109-99-9	Furan	T T	[83] [84]	Cracked maize Non-sterile cracked maize
furan	110-00-9	Furan	NT/T	[54]	Maize media
propan-2-yl tetradecanoate	110-27-0	Ester	T	[83]	Cracked maize
alpha-cedrene	11028-42-5	Terpene	U	[91]	Wallpaper
heptan-2-one	110-43-0	Ketone	U U NT/T	[93] [91] [54]	Gypsum board Malt extract agar Maize media
hexane	110-54-3	Alkane	T T NT/T	[83] [84] [85]	PDA Non-sterile cracked maize PDA
pentanal	110-62-3	Aldedyde	NT	[54]	Maize media
octan-2-one	111-13-7	Ketone	NT/T	[54]	Maize media
hexan-1-ol	111-27-3	Alcohol	NT/T T	[95] [84]	Maize media Non-sterile cracked maize
butoxyethene	111-34-2	Ether	T	[84]	Non-sterile cracked maize
2-methylbut-2-enal	1115-11-3	Aldehyde	NT NT NT/T	[83] [84] [85]	PDA Non-sterile cracked maize PDA
octane	111-65-9	Alkane	NT/T T NT/T	[83] [85] [54]	Cracked maize PDA Maize media
oct-1-ene	111-66-0	Alkene	T T	[83] [84]	Cracked maize Non-sterile cracked maize
oct-2-ene	111-67-1	Alkene	T	[84]	Non-sterile cracked maize
2-butoxyethanol	111-76-2	Alcohol	T	[83]	Cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
nonane	111-84-2	Alkane	NT/T	[83]	Cracked maize
octan-1-ol	111-87-5	Alcohol	U	[92]	/
2-(2-ethoxyethoxy)-ethanol	111-90-0	Alcohol	T	[83]	Cracked maize
dodec-1-ene	1120-36-1	Alkene	T*	[85]	PDA
nonanoic acid	112-05-0	Acid	T	[83]	Cracked maize
heptyl acetate	112-06-1	Ester	T	[84]	Non-sterile cracked maize
2-methylcyclopent-2-en-1-one	1120-73-6	Ketone	T	[84]	Sterile cracked maize
2-methylcyclohex-2-en-1-one	1121-18-2	Ketone	T	[84]	Non-sterile cracked maize
decan-1-ol	112-30-1	Alcohol	T	[85]	PDA
decanal	112-31-2	Aldehyde	T T	[83] [86]	Cracked maize CDA, CSA, CDL, MEA, CMA
dodecane	112-40-3	Alkane	NT/T T	[83] [86]	Cracked maize CDA, CSA, CDL, MEA, CMA
13-docosenamide	112-84-5	Amide	T	[83]	PDA
eicosane	112-95-8	Alkane	NT/T	[83]	Cracked maize
propene	115-07-1	Alkene	T	[84]	Sterile cracked maize
beta-humulene	116-04-1	Terpene	T	[86]	CSA, CDL, MEA
2-methylbutanoic acid	116-53-0	Acid	NT	[54]	Maize media
(Z)-1,2-dimethylcyclopentane	1192-18-3	Alkane	T	[84]	Sterile cracked maize
benzeneacetaldehyde	122-78-1	Aldehyde	T	[83]	Cracked maize
2-phenoxyethanol	122-99-6	Alcohol	T U	[83] [91]	Cracked maize Wallpaper
4-ethylphenol	123-07-9	Alcohol	T	[84]	Non-sterile cracked maize
3-methylbutan-1-ol	123-51-3	Alcohol	NT/T U U NT/T NT/T T	[83] [93] [92] [85] [54] [96]	PDA + cracked maize Gypsum board / Maize media PDA MEA + Gypsum board
ethyl hexanoate	123-66-0	Ester	T	[83]	Cracked maize
non-1-ene	124-11-8	Alkene	T T	[83] [84]	Cracked maize Non-sterile cracked maize
octanal	124-13-0	Aldehyde	T T	[83] [84]	Cracked maize Non-sterile cracked maize
decane	124-18-5	Alkane	NT T NT	[83] [84] [54]	Cracked maize Non-sterile cracked maize Maize media
nonanal	124-19-6	Aldehyde	NT/T	[83]	Cracked maize
isobornyl acetate	125-12-2	Ester	T	[83]	Cracked maize
2,6-dimethyldecane	13150-81-7	Alkane	T	[84]	Sterile cracked maize
4-methyldec-1-ene	13151-29-6	Alkene	T	[84]	Non-sterile cracked maize
3a,4,7,7,7a-Tetrahydro-4,7-methano-1H-indene	13257-74-4	Terpene	T	[83]	Cracked maize
6-methyltridecane	13287-21-3	Alkane	T	[84]	Non-sterile cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
pentadec-1-ene	13360-61-7	Alkene	T	[84]	Non-sterile cracked maize
(E)-alpha-bergamotene	13474-59-4	Terpene	T	[86]	CDA, CSA, CDL, MEA, CMA
9-octyleicosane	13475-77-9	Alkane	NT	[84]	Non-sterile cracked maize
2,2,4,6,6-pentamethylheptane	13475-82-6	Alkane	NT/T	[85]	PDA
2,5-dimethylcyclohexa-2,5-diene-1,4-dione	137-18-8	Ketone	T	[84]	Sterile cracked maize
2-methylbutan-1-ol	137-32-6	Alcohol	NT/T U NT/T U	[83] [93] [85] [95]	Cracked maize MEA PDA Maize media
beta-cubebene	13744-15-5	Terpene	U T T	[92] [86] [96]	/ CDA, CSA, CDL, CMA Adye and Mateles liquid medium
limonene	138-86-3	Terpene	T T U T	[83] [84] [92] [86]	Cracked maize Sterile cracked maize / CSA, CDL, MEA, CMA
ethyl acetate	141-78-6	Ester	NT+T NT NT/T NT/T	[83] [84] [54] [85]	PDA + cracked maize Sterile cracked maize Maize media PDA
1,3-diethylbenzene	141-93-5	Alkene	T	[84]	Non-sterile cracked maize
ethyl acetoacetate	141-97-9	Ester	T	[84]	Sterile cracked maize
heptane	142-82-5	Alkane	NT/T NT/T NT/T	[83] [85] [54]	Cracked maize PDA Maize media
(2E,4E)-hexa-2,4-dienal	142-83-6	Aldehyde	T T	[83] [84]	Non-sterile cracked maize Sterile cracked maize
(Z)-9-octadecen-1-ol	143-28-2 cis	Alcohol	T	[83]	Cracked maize
2,3,3-trimethylcyclobutanone	1449-49-6	Ketone	T	[84]	Non-sterile cracked maize
cadina-1(10),6,8-triene	1460-96-4	Terpene	T	[86]	CDA, CSA, CDL, MEA
beta-himachalene	1461-03-6	Terpene	NT/T U	[85] [91]	PDA Wallpaper
2,6,10-trimethyltetradecane	14905-56-7	Alkane	T	[83]	Cracked maize
ylangene	14912-44-8	Terpene	T	[86]	CDA, CSA, CDL, MEA
methylcyclooctane	1502-38-1	Alkane	T T*	[84] [85]	Non-sterile cracked maize PDA
bicyclo[4.4.0]dec-1-en,2-isopropyl-5-methyl-9-methylene	150320-52-8	Terpene	T T	[86] [96]	CDA, CDL, MEA, CMA Adye and Mateles liquid medium
5,5-dimethylhexa-1,3-diene	1515-79-3	Alkene	NT	[83]	PDA
2-methyloctadecane	1560-88-9	Alkane	T	[84]	Non-sterile cracked maize
2-methylheptadecane	1560-89-0	Alkane	T	[84]	Non-sterile cracked maize
(Z)-muurola-3,5-diene	157374-44-2	Terpene	T	[85]	PDA

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
4-ethyloctane	15869-86-0	Alkane	T	[84]	Non-sterile cracked maize
2,5-dimethyloctane	15869-89-3	Alkane	T	[84]	Non-sterile cracked maize
docos-1-ene	1599-67-3	Alkene	T	[84]	Non-sterile cracked maize
2,3-dimethylpenta-1,3-diene	1625-49-6	Alkene	NT	[84]	Non-sterile cracked maize
1,1-dimethylcyclopentane	1630-94-0	Alkane	T	[84]	Non-sterile cracked maize
5-methylundecane	1632-70-8	Alkane	T	[84]	Non-sterile cracked maize
4-ethyldecane	1636-44-8	Alkane	T	[84]	Non-sterile cracked maize
undecan-2-ol*	1653-30-1	Alcohol	T	[84]	Non-sterile cracked maize
1-methyl-3-prop-1-en-2-ylcyclohexene	16580-24-8	Alkene	T	[84]	Non-sterile cracked maize
ethylcyclohexane	1678-91-7	Alkane	T	[84]	Non-sterile cracked maize
heptadecan-2-ol	16813-18-6	Alcohol	T	[84]	Non-sterile cracked maize
alpha-cuparene	16982-00-6	Terpene	U	[91]	Wallpaper
beta-selinene	17066-67-0	Terpene	T	[85]	PDA
2,6-dimethylundecane	17301-23-4	Alkane	T	[84]	Non-sterile cracked maize
4,5-dimethylnonane	17302-23-7	Alkane	T T	[83] [84]	Cracked maize Non-sterile cracked maize
2,5-dimethylnonane	17302-27-1	Alkane	T	[83]	Cracked maize
2,6-dimethylnonane	17302-28-2	Alkane	T	[84]	Non-sterile cracked maize
2,5-dimethyldecane	17312-50-4	Alkane	T	[83]	Cracked maize
3,7-dimethyldecane	17312-54-8	Alkane	T	[84]	Non-sterile cracked maize
alpha.-cubebene	17699-14-8	Terpene	T T T	[84] [85] [86]	Non-sterile cracked maize PDA CDA, CSA, CDL, MEA, CMA
p-mentha-1,3,8-triene	18368-95-1	Terpene	NT	[84]	Non-sterile cracked maize
beta-chamigrene	18431-82-8	Terpene	T*	[85]	PDA
5-methylhexanal	1860-39-5	Aldehyde	T	[84]	Non-sterile cracked maize
hept-2-enal	18829-55-5	Aldehyde	T NT	[84] [54]	Non-sterile cracked maize Maize media
hexacos-1-ene	18835-33-1	Alkene	T	[84]	Non-sterile cracked maize
dec-3-ene	19398-37-9	Alkene	T	[84]	Non-sterile cracked maize
3-dodec-2-enyloxolane-2,5-dione	19780-11-1	Ketone	T	[83]	Cracked maize
tau-muurolol	19912-62-0	Terpene	T*	[85]	PDA
epi-cubeno-1-ol	19912-67-5	Terpene	T	[85]	PDA
alpha-chamigrene	19912-83-5	Terpene	U	[91]	Wallpaper
alpha-corocalene	20129-39-9	Terpene	T*	[85]	PDA
1-2-(2-methyloxy-1-methylethoxy)-1-methylethoxy]-propan-2-ol	20324-33-8	Alcohol	T	[83]	Cracked maize
1-iododecane	2050-77-3	Halogen	T	[83]	Cracked maize
2-ethylbut-2-enal	20521-42-0	Aldehyde	T	[84]	Non-sterile cracked maize
2,2-dimethylheptane-3,5-dione	20734-29-6	Ketone	T	[86]	CSA, CDL, MEA, CMA
4-methylpent-2-yne	21020-27-9	Alkyne	T	[84]	Non-sterile cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
2-octadecoxyethanol	2136-72-3	Alcohol	T	[84]	Non-sterile cracked maize
alpha-calacorene	21391-99-1	Terpene	T	[85]	PDA
4-methyloctane	2216-34-4	Alkane	T T	[83] [84]	Cracked maize Non-sterile cracked maize
gamma-gurjunene	22567-17-5	Terpene	T U U	[85] [92] [96]	PDA / Adye and Mateles liquid medium
1-hepten-3-yne	2384-73-8	Alkyne	NT	[84]	Non-sterile cracked maize
germacrene-d	23986-74-5	Terpene	T T	[85] [86]	PDA CDA, CSA, CDL, MEa
n-methyloctan-1-amine	2439-54-5	Amine	T	[84]	Non-sterile cracked maize
alpha-cadinene	24406-05-1	Terpene	T	[85]	PDA
1,2-dimethylcyclopene	2452-99-5	Alkene	T	[84]	Non-sterile cracked maize
(3E)-3-methylhexa-1,3,5-triene	24587-26-6	Alkene	T	[84]	Non-sterile cracked maize
2-methylhexadecan-1-ol	2490-48-4	Alcohol	T	[84]	Non-sterile cracked maize
5-methyltridecane	25117-31-1	Alkane	T	[84]	Non-sterile cracked maize
(Z)-1,3-dimethylcyclopentane	2532-58-3	Alkane	T	[84]	Sterile cracked maize
(E)-2-octanal	2548-87-0	Aldehyde	T	[90]	Maize silk
hept-3-yne	2586-89-2	Alkyne	T	[84]	Sterile cracked maize
(Z)-oct-2-en-1-ol	26001-58-1	Alcohol	T U	[83] [92]	Cracked maize /
tetrahydro-6,6-dimethyl-2h-pyran-2-one	2610-95-9	Ketone	T	[84]	Non-sterile cracked maize
4-methylhept-6-en-3-one	26118-97-8	Ketone	T	[95]	Glucose minimal medium (GMM)
4-methylheptadecane	26429-11-8	Alkane	T	[84]	Non-sterile cracked maize
7-methyltridecane	26730-14-3	Alkane	T	[84]	Non-sterile cracked maize
(Z)-hexadec-2-ene	26741-29-7	Alkene	T	[86]	CDA, CSA, MEA, CMA
4,4-dimethyl-1,2-pentadiene	26981-77-1	Alkene	T	[84]	Non-sterile cracked maize
1,2-benzisothiazole	272-16-2	Other	T	[83]	Cracked maize
cyclopropene	2781-85-3	Alkene	T T	[84] [83]	Sterile cracked maize Non-sterile cracked maize
1-methoxybut-1-en-3-yne	2798-73-4	Alkyne	T	[84]	Non-sterile cracked maize
hexa-2,4-diyne	2809-69-0	Alkyne	T	[84]	Non-sterile cracked maize
4-methyldecane	2847-72-5	Alkane	NT/T	[83]	Cracked maize
beta-acoradiene	28477-64-7	Terpene	U	[91]	Wallpaper
gamma-selinene	28624-23-9	Terpene	U T	[92] [96]	/ Adye and Mateles liquid medium
1,3-dimethyl-2-ethylbenzene	2870-04-4	Alkene	U	[92]	/
3-methylhexa-2,4-diene	28823-42-9	Alkene	T	[84]	Non-sterile cracked maize
gamma-curcumene	28976-68-3	Terpene	U	[91]	Wallpaper
cyclobutanol	2919-23-5	Alcohol	T	[84]	Sterile cracked maize
cyclodecane	293-96-9	Alkane	T	[83]	Cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
cyclododecane	294-62-2	Alkane	T	[83]	Cracked maize
4-methylundecane	2980-69-0	Alkane	T	[83]	Cracked maize
o-decylhydroxylamine	29812-79-1	Amine	T	[84]	Non-sterile cracked maize
gamma-muurolene	30021-74-0	Terpene	U T	[92] [85]	/ PDA
(Z)-9-octadecenamide	301-02-0	Amide	T	[83]	PDA
2,6,11-trimethyldodecane	31295-56-4	Alkane	T T	[83] [84]	Cracked maize Non-sterile cracked maize
alpha-muurolene	31983-22-9	Terpene	T T	[96] [85]	Adye and Mateles liquid medium PDA
2-ethylfuran	3208-16-0	Furan	T T	[84] [54]	Non-sterile cracked maize Maize media
2,4,5-trimethyl-1,3-dioxolane	3299-32-9	Other	NT/T	[85]	PDA
decanoic acid	334-48-5	Acid	T	[83]	Cracked maize
1-octen-3-ol	3391-86-4	Alcohol	T U NT/T	[94] [91]* [54]	/ Malt extract agar Maize media
(Z)-hexadec-3-ene	34303-81-6	Alkene	T	[86]	CDA, CSA, CDL, MEA, CMA
thiochroman-4-one	3528-17-4	Ketone	T*	[85]	PDA
(E)-hexadec-7-ene	35507-09-6	Alkene	T	[86]	CSA, CDL, MEA, CMA
2-methoxyethylbenzene	3558-60-9	Alkene	T	[84]	Non-sterile cracked maize
alpha-guaiene	3691-12-1	Terpene	T	[86]	CDA, CSA, CDL, CMA
2,4-dimethylfuran	3710-43-8	Furan	T	[54]	Maize media
2-nonenoic acid*	3760-11-0	Acid	T	[84]	Non-sterile cracked maize
2-pentylfuran	3777-69-3	Furan	T NT/T	[83] [54]	Cracked maize Maize media
5,6,7-trimethoxy-2,3-dihydroinden-1-one	38472-90-1	Ketone	T	[83]	Cracked maize
alpha-copaene	3856-25-5	Terpene	U T T	[92] [85] [96]	/ PDA Adye and Mateles liquid medium
gamma-cadinene	39029-41-9	Terpene	T T T	[85] [91]* [96]	PDA Adye and Mateles liquid medium, Malt extract agar
2-butyloctan-1-ol	3913-02-8	Alcohol	T	[85]	PDA
(2E,5E)-hepta-2,5-diene	39619-60-8	Alkene	T	[84]	Non-sterile cracked maize
1,3-cycloheptadiene	4054-38-0	Alkene	T	[84]	Non-sterile cracked maize
4-methoxy-2,5-dimethylfuran-3-one	4077-47-8	Ketone	T	[83]	Cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
epizonaren	41702-63-0	Terpene	NT U T T	[84] [92] [85] [96]	Non-sterile cracked maize / PDA Adye and Mateles liquid medium
but-2-enal	4170-30-3	Aldehyde	T	[84]	Sterile cracked maize
eicos-9-ene	42448-90-8	Alkene	T	[84]	Non-sterile cracked maize
1,1,2-trimethylcyclopentane	4259-00-1	Alkane	T	[84]	Non-sterile cracked maize
butan-2,3-dione	431-03-8	Ketone	NT/T	[54]	Maize media
2-heptyl-1,3-dioxolane	4359-57-3	Other	T	[95]	Glucose minimal medium (GMM)
mono(2-ethylhexyl) phthalate	4376-20-9	Acid	T	[83]	Cracked maize
2-butylfuran	4466-24-4	Furan	T	[84]	Sterile cracked maize
valencene	4630-07-3	Terpene	T T* T T	[92] [85] [86] [96]	/ PDA CDA, CSA, CDL, MEA, CMA Adye and Mateles liquid medium
3,3-dimethylbutane-2-ol	464-07-3	Alcohol	T	[84]	Non-sterile cracked maize
cedrene	469-61-4	Terpene	T	[84]	Non-sterile cracked maize
thujopsene	470-40-6 (cis)	Terpene	U	[92]	/
1-methyl-4-propan-2-yl-7-oxabicyclo2.2.1]heptane (1,4-Cineole)	470-67-7	Alkane	T	[83]	Cracked maize
eucalyptol	470-82-6	Terpene	T	[83]	Cracked maize
alpha-selinene	473-13-2	Terpene	T T	[85] [86]	PDA CDA, CSA, CDL, MEA
alpha-cadinol	481-34-5	Terpene	T T	[85] [86]	PDA CDA, CSA, CDL, MEA
delta-cadinene	483-76-1	Terpene	U NT/T U T T	[92] [85] [91] [86] [96]	/ PDA MEA CSA, CDL, MEA, CMA Adye and Mateles liquid medium
1,6-dimethyl-4-(1-methylethyl)naphthalene	483-78-3	Alkene	T	[86]	CSA, CDL, CMA
1-cyclohexene-1-methanol	4845-04-9	Alkene	T	[84]	Non-sterile cracked maize
tetramethylbenzene	488-23-3	Alkene	T T	[92] [84]	Agar Non-sterile cracked maize
6,10,11,11-tetramethyltricyclo6.3.0.1e2, 3]undec-1(7)ene	489-39-4	Terpene	T	[96]	Adye and Mateles liquid medium

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
alpha-gurjunene	489-40-7	Terpene	U T T T	[92] [85] [86] [96]	/ PDA CDA, CSA, CDL, MEA Adye and Mateles liquid medium
alpha-farnesene	502-61-4	Terpene	T	[86]	CDA, CSA, CDL, CMA
3,7-dimethylocta-1,3,7-triene	502-99-8	Alkene	T	[86]	CDA, CDL, MEA
3-methylbutanoic acid	503-74-2	Acid	T	[83]	Cracked maize
hex-2-enal	505-57-7	Aldehyde	T	[84]	Non-sterile cracked maize
(E)-9-octadecen-1-ol	506-42-3	Alcohol	T	[84]	Non-sterile cracked maize
2-methylbut-2-ene	513-35-9	Alkene	T	[84]	Non-sterile cracked maize
butan-2,3-diol	513-85-9	Alcohol	NT/T NT/T	[83] [85]	Cracked maize PDA
3-hydroxybutan-2-one	513-86-0	Ketone	NT/T NT	[85] [54]	PDA Maize media
beta-elemene	515-13-9	Terpene	T T	[85] [86]	PDA CDA, CSA, CDL, MEA, CMA
beta-cadinene	523-47-7	Terpene	T T	[85] [86]	PDA CDA, CSA, CDL, MEA
cadinene	523-47-7	Terpene	U T	[92] [96]	/ Adye and Mateles liquid medium
3,4,5-trimethylphenol	527-54-8	Alcohol	T	[84]	Non-sterile cracked maize
9-butyldocosane	5282-14-9	Alkane	T	[83]	Cracked maize
2-methylfuran	534-22-5	Furan	NT/T T U T T T	[83] [84] [92] [85] [54] [86]	Cracked maize Sterile cracked maize / PDA Maize media CDA, CSA, CDL, MEA, CMA
2-methylnon-3-ene	53966-53-3	Alkene	T	[84]	Non-sterile cracked maize
2, 2, 4-trimethylpentane	540-84-1	Alkane	T	[95]	Glucose minimal medium (GMM)
2,6-dimethylheptadecane	54105-67-8	Alkane	T	[84]	Non-sterile cracked maize
3-methylbutanamide	541-46-8	Amide	T	[84]	Non-sterile cracked maize
epi-bicyclosequiphellandrene	54274-73-5	Terpene	T U T T	[84] [92] [85] [96]	Non-sterile cracked maize / PDA Adye and Mateles liquid medium
heptan-2-ol	543-49-7	Alcohol	T	[54]	Maize media
1-chloropentane	543-59-9	Halogen	T	[83]	Cracked maize
hexadecane	544-76-3	Alkane	NT+NT/T T	[83] [84]	PDA + Cracked maize Non-sterile cracked maize
dotriacotane	544-85-4	Alkane	T	[83]	Cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
decyl formate	5451-52-5	Ester	T	[84]	Non-sterile cracked maize
beta-cedrene	546-28-1	Terpene	U	[91]	Wallpaper
10-methyleicosane	54833-23-7	Alkane	T	[83]	Cracked maize
6-propyltridecane	55045-10-8	Alkane	T	[83]	Cracked maize
viridiflorol	552-02-3	Terpene	T	[85]	PDA
5,14-dibutyloctadecane	55282-13-8	Alkane	T	[83]	Cracked maize
3-ethyltetracosane	55282-17-2	Alkane	T	[83]	Cracked maize
11-decylheneicosane	55320-06-4	Alkane	T	[84]	Non-sterile cracked maize
3,7,7-trimethylbicyclo4.1.0]hept-2-ene	554-61-0	Terpene	T T	[83] [84]	Cracked maize Non-sterile cracked maize
methyl 3-methylbutyrate	556-24-1	Ester	NT/T	[54]	Maize media
2,3,3-trimethylpentane	560-21-4	Alkane	NT/T	[54]	Maize media
o-(2-methylpropyl)hydroxylamine	5618-62-2	Amine	T	[95]	Glucose minimal medium (GMM)
3-methylbut-1-ene	563-45-1	Alkene	T	[84]	Sterile cracked maize
2-methylbut-1-ene	563-46-2	Alkene	T	[84]	Non-sterile cracked maize
3-methylpentan-2-one	565-61-7	Ketone	T	[84]	Non-sterile cracked maize
beta-panasinsene	56684-97-0	Terpene	T	[86]	CDA, CSA, CDL, MEA
2,3-dimethylhexane	584-94-1	Alkane	NT/T	[54]	Maize media
2,4-dimethylhexane	589-43-5	Alkane	NT	[54]	Maize media
octan-3-ol	589-98-0	Alcohol	U	[92]	/
3-methylbutanal	590-86-3	Aldehyde	NT/T NT/T	[85] [54]	PDA Maize media
hexan-2-one	591-78-6	Ketone	NT/T	[84]	Non-sterile cracked maize
penta-1,4-diene	591-93-5	Alkene	T T	[84] [86]	Sterile cracked maize CSA, CDL, MEA, CMA
penta-1,2-diene	591-95-7	Alkene	T	[84]	Sterile cracked maize
2,5-dimethylhexane	592-13-2	Alkane	NT	[54]	Maize media
hept-1-ene*	592-76-7	Alkene	T	[84]	Non-sterile cracked maize
heptacosane	593-49-7	Alkane	T	[86]	CDA, CDL, MEA, CMA
2,2,3,3-tetramethylbutane	594-82-1	Alkane	NT/T	[54]	Maize media
palustrol	5986-49-2	Terpene	T	[85]	PDA
3-methylbutan-2-amine	598-74-3	Amine	NT	[83]	PDA
phenylethyl alcohol	60-12-8	Alcohol	T	[83]	Cracked maize
2,2’-dimethylbiphenyl	605-39-0	Alkene	T	[83]	Cracked maize
(Z)-don-2-enal	60784-31-8	Aldehyde	T	[84]	Non-sterile cracked maize
4,6-dimethyldodecane	61141-72-8	Alkane	T	[85]	PDA
4-methyldodecane	6117-97-1	Alkane	T	[84]	Non-sterile cracked maize
4-methylheptan-3-one	6137-11-7	Ketone	T	[84]	Non-sterile cracked maize
4-methyl-2-propyl furan	6148-37-4	Furan	T	[84]	Non-sterile cracked maize
1-penten-3-ol	616-25-1	Alcohol	U	[92]	/
3-methylthiophene	616-44-4	Other	T	[83]	Cracked maize
7-methylpentadecane	6165-40-8	Alkane	T	[84]	Non-sterile cracked maize
3-phenoxypropan-1-ol	6180-61-6	Alcohol	T	[83]	PDA + Cracked maize
2,3,6-trimethyloctane	62016-33-5	Alkane	T	[83]	Cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
2,4,6-trimethyldecane	62108-27-4	Alkane	T T	[83] [84]	Cracked maize Non-sterile cracked maize
1-ethyl-4-methylbenzene	622-96-8	Alkene	NT	[84]	Non-sterile cracked maize
benzenamine	62-53-3	Amine	T	[83]	Cracked maize
2,5-dimethylfuran	625-86-5	Furan	T NT/T	[83] [85]	Cracked maize PDA
pent-2-yn-1-ol	6261-22-9	Alcohol	T	[84]	Non-sterile cracked maize
1-methoxy-3-methylbutane	626-91-5	Alkane	T	[84]	Non-sterile cracked maize
hexan-2-ol	626-93-7	Alcohol	T	[84]	Sterile cracked maize
pent-1-yne	627-19-0	Alkyne	T	[84]	Non-sterile cracked maize
1,4-cyclohexadiene	628-41-1	Alkene	T	[84]	Non-sterile cracked maize
nonan-2-ol	628-99-9	Alcohol	T	[84]	Non-sterile cracked maize
tridecane	629-50-5	Alkane	NT/T T	[83] [84]	Cracked maize Non-sterile cracked maize
tetradecane	629-59-4	Alkane	T	[83]	Cracked maize
pentadecane	629-62-9	Alkane	NT/T T	[83] [84]	Cracked maize Sterile cracked maize
heptadecane	629-78-7	Alkane	T T T	[83] [84] [85]	PDA Non-sterile cracked maize PDA
nonadecane	629-92-5	Alkane	T T/NT	[83] [84]	Cracked maize Non-sterile cracked maize
heneicosane	629-94-7	Alkane	NT/T	[83]	Cracked maize
eicosan-1-ol	629-96-9	Alcohol	T	[83]	Cracked maize
docosane	629-97-0	Alkane	NT	[83]	Cracked maize
octacosane	630-02-4	Alkane	T T	[83] [84]	Cracked maize, Sterile Non sterile cracked maize
nonacosane	630-03-5	Alkane	T T	[83] [84]	Cracked maize Sterile cracked maize
isopropyl butanoate	638-11-9	Ester	T	[83]	Cracked maize
2,6,10,14-tetramethylhexadecane	638-36-8	Alkane	T	[84]	Non-sterile cracked maize
ethanol	64-17-5	Alcohol	T T NT/T T	[83] [84] [85] [86]	Cracked maize Sterile cracked maize PDA CDA, CSA, CDL, MEA, CMA
3-methylhexadecane	6418-43-5	Alkane	T T	[83] [84]	Cracked maize Non-sterile cracked maize
3-methyleicosane	6418-46-8	Alkane	T	[84]	Sterile cracked maize
acetic acid	64-19-7	Acid	NT/T NT/T T*	[83] [85] [54]	Cracked maize PDA Maize media
(Z)-5-octen-1-ol	64275-73-6	Alcohol	T	[84]	Non-sterile cracked maize
2-methylbiphenyl	643-58-3	Alkene	T	[83]	Cracked maize
3-methyl-1,1’’-biphenyl	643-93-6	Alkane	NT/T	[83]	Cracked maize
alpha-curcumene	644-30-4	Terpene	U	[91]	Wallpaper

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
(E)-pent-2-ene	646-04-8	Alkene	T NT/T	[83] [84]	Non-sterile cracked maize Sterile cracked maize
Tetracosane	646-31-1	Alkane	T T	[83] [84]	Cracked maize Sterile cracked maize
alpha-isocomene	65372-78-3	Terpene	T	[85]	PDA
benzoic acid	65-85-0	Acid	NT T	[83] [84]	PDA Non-sterile cracked maize
hexanal	66-25-1	Aldehyde	NT T	[54] [83]	Maize media Cracked maize
(4E,6E)-2,6-dimethylocta-2,4,6-triene	673-84-7	Alkene	T	[84]	Non-sterile cracked maize
2,6-dimethylocta-5,7-dien-4-one	6752-80-3	Ketone	NT	[83]	PDA
propan-2-ol	67-63-0	Alcohol	NT T	[85] [86]	PDA CDA, CSA, MEA, CMA
acetone	67-64-1	Ketone	NT/T T	[54] [86]	Maize media CSA, CDL, MEA, CMA
bicyclogermacrene	67650-90-2	Terpene	T	[84]	Non-sterile cracked maize
trichloromethane	67-66-3	Halogen	NT/T T	[85] [95]	PDA Glucose minimal medium (GMM)
1,1,2,3-tetramethylcyclohexane	6783-92-2	Alkane	T	[84]	Non-sterile cracked maize
aristolene	6831-16-9	Terpene	U T T	[92] [86] [96]	/ CDA, CSA, CDL, MEA, CMA Adye and Mateles liquid medium
dipropan-2-yl hexanedioate	6938-94-9	Acid	T	[83]	Cracked maize
bicyclo[4.2.0]octa-1,3,5-triene	694-87-1	Alkene	T NT	[83] [84]	Cracked maize Sterile cracked maize
pentatriacont-17-ene	6971-40-0	Alkene	T	[83]	Cracked maize
propan-1-ol	71-23-8	Alcohol	NT/T NT/T	[95] [85]	Maize media PDA
butan-1-ol	71-36-3	Alcohol	T T*	[95] [85]	Glucose minimal medium (GMM) PDA
pentan-1-ol	71-41-0	Alcohol	NT/T	[95]	Maize media
benzene	71-43-2	Alkene	T	[84]	Non-sterile cracked maize
2,3-dimethyloctane	7146-60-3	Alkane	T	[84]	Non-sterile cracked maize
di-epi-1,10-cubenol	73365-77-2	Terpene	T*	[85]	PDA
ethyl 2-methylbutyrate	7452-79-1	Ester	NT NT/T* NT/T	[83] [85] [54]	PDA PDA Maize media
nonyl-cyclopropane	74663-85-7	Alkane	T	[85]	PDA
1-ethyl-2-heptylcyclopropane	74663-86-8	Alkane	T	[84]	Non-sterile cracked maize
acetaldehyde	75-07-0	Aldehyde	T	[85]	PDA
2,6-dimethyloctadecane	75163-97-2	Alkane	T	[84]	Non-sterile cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
dimethyl sulfide	75-18-3	Other	T U T	[83] [93] [54]	Cracked maize Gypsum board Maize media
cyclopropane	75-19-4	Alkane	U	[84]	Non-sterile cracked maize
Nitromethane	75-52-5	Halogen	U	[92]	/
3-methylbut-3-en-1-ol	763-32-6	Alcohol	T	[84]	Non-sterile cracked maize
2,2-dimethylbutane-1,3-diol	76-35-7	Alcohol	T	[84]	Non-sterile cracked maize
5-methylhexa-1,4-diene	763-88-2	Alkene	T	[84]	Non-sterile cracked maize
bicyclo2.2.0]hexa-2,5-diene	7641-77-2	Alkene	T*	[85]	PDA
hex-2-yne	764-35-2	Alkyne	T	[84]	Non-sterile cracked maize
1-phenoxypropan-2-ol	770-35-4	Alcohol	NT	[92]	PDA
4-ethenyl-2-methoxyphenol	7786-61-0	Alcohol	T	[83]	Cracked maize
alpha dehydro-ar-himachalene	78204-62-3	Terpene	T	[85]	PDA
2-methylpropan-1-ol	78-83-1	Alcohol	NT/T T U U T NT/T	[83] [93] [92] [85] [95] [96]*	Maize media PDA + Cracked maize Gypsum board + MEA / PDA /
2-methylpropanal	78-84-2	Aldehyde	NT	[54]	Maize media
2-methylpropenal	78-85-3	Aldehyde	T	[84]	Sterile + Non-sterile cracked maize
butan-2-one	78-93-3	Ketone	T*	[85]	PDA
2,3-dimethylbutane	79-29-8	Alkane	T	[83]	Cracked maize
2-methylpropanoic acid	79-31-2	Acid	T T* NT/T	[83] [85] [54]	Cracked maize PDA Maize media
terpineol	8006-39-1	Terpene	U	[93]	Gypsum board
alpha-pinene	80-56-8	Terpene	T NT	[84] [54]	Non-sterile cracked maize Maize media
1,5-hexadien-3-yne	821-08-9	Alkyne	NT	[84]	Non-sterile cracked maize
nonan-2-one	821-55-6	Ketone	NT/T	[54]	Maize media
(E)-2-octenoic acid	871-67-6	Acid	T	[84]	Non-sterile cracked maize
(E)-caryophyllene	87-44-5	Terpene	T T T	[92] [85] [96]	Adye and Mateles liquid medium PDA /
isobazzanene	88661-59-0	Terpene	U	[91]	Wallpaper
2,3,4-trimethylhexane	921-47-1	Alkane	NT/T	[54]	Maize media
biphenyl	92-52-4	Alkane	NT	[83]	PDA
hex-3-yne	928-49-4	Alkyne	T	[84]	Non-sterile cracked maize
6-methylheptan-2-one	928-68-7	Ketone	U	[91]*	Malt extract agar
1,3,6-octatriene	929-20-4	Alkene	T	[84]	Non-sterile cracked maize
1-methoxyoctane	929-56-6	Alkane	T	[84]	Non-sterile cracked maize
1-ethenoxyoctadecane	930-02-9	Alkane	T	[84]	Non-sterile cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
3-methylfuran	930-27-8	Furan	U NT	[92] [84]	/ Non-sterile cracked maize
butylcyclopropane	930-57-4	Alkane	T	[84]	Non-sterile cracked maize
4-ethyl-1,2-dimethylbenzene	934-80-5	Alkene	T	[84]	Non-sterile cracked maize
dimethylbenzene	95-47-6	Alkene	U	[92]	/
1,2-dimethylbenzene	95-47-6	Alkene	T	[84]	Non-sterile cracked maize
3,4-dimethylphenol	95-65-8	Alcohol	T	[84]	Non-sterile cracked maize
isoledene	95910-36-4	Terpene	T	[86]	CSA, CDL, MEA, CMA
3-methylpentane	96-14-0	Alkane	T	[84]	Non-sterile cracked maize
2-methylbutanal	96-17-3	Aldehyde	NT/T NT/T	[54] [85]	Maize media PDA
butyrolactone	96-48-0	Other	T	[83]	Cracked maize
ethyl 2-methylpropionate	97-62-1	Ester	T NT/T NT/T	[84] [54] [85]	Non-sterile cracked maize Maize media PDA
furfural	98-01-1	Aldehyde	T T	[83] [84]	Crackedv+Sterilecracked maize Non-sterile cracked maize
acetophenone	98-86-2	Ketone	NT/T	[83]	Cracked maize
(Z)-(1s,3s,6r)-4-carene	NA	Alkene	T	[84]	Non-sterile cracked maize
1-(3-propoxyphenyl)propan-2-amine	NA	Amine	T	[84]	Sterile cracked maize
1-(4-aminophenyl-3-phenyl-2-propen-1-one	NA	Ketone	NT	[83]	PDA
1-butylhexene	NA	Alkene	T	[84]	Non-sterile cracked maize
1-ethyl-methylbenzene	NA	Alkene	NT	[84]	Non-sterile cracked maize
1-iodo-2-methylnonane	NA	Halogen	T	[83]	Cracked maize
1-methoxypentadecane	NA	Alkane	T	[84]	Sterile cracked maize
2-butanoic acid	NA	Acid	T	[83]	Cracked maize
3-fluoro-a, 5-dihydroxy-N-methyl-benzeneethanamine	NA	Amine	T	[95]	Glucose minimal medium (GMM)
3-methyl-1-butyl acetate	NA	Ester	T	[83]	Cracked maize
4-(1-methylethyl)phenol	NA	Alcohol	T	[84]	Non-sterile cracked maize
alpha-chamipinene	NA	Terpene	T	[91]	Wallpaper
benzaldehyde, 4-(methyoxyethyl) acetate	NA	Ester	T	[84]	Non-sterile cracked maize
beta-germacrene	NA	Terpene	T	[86]	CDA, CSA, CDL, MEA
esorubin hydrochloride*	NA	Other	T	[84]	Non-sterile cracked maize
hexaosan-1-ol	NA	Alcohol	T	[84]	Non-sterile cracked maize
methoxyphenyl oxime	NA	Other	NT/T	[83]	Cracked maize
n-(4-phenylazo)phenyl-2-phenylcyclopropionamide	NA	Ester	T	[83]	Cracked maize
4-methyl-1-91-methylethyl)bicycle3.1.0]hexan-3-ol	NA	Alcohol	T	[83]	Cracked maize
tetracyclo[3.3.1.1(1,8).0(2,4)]decane	NA	Alkane	T	[84]	Non-sterile cracked maize

(a) Name	(b) Cas number	(c) Chemistry family	(d) *Strains*	(e) Ref	(f) Substrate
6-isopropenyl-1,2,3,4-tetramethyl-1,4-cyclohexadiene	NA	Alkene	NT	[84]	Non-sterile cracked maize
(E,Z)-1,2-diethylidenecyclopentane	NA	Alkane	NT	[85]	PDA
2-(3,3-diphenyl-propylamino)ethanol	NA	Alcohol	T	[84]	Sterile cracked maize
1,4-methanonaphtalene	NA	Alkene	T	[83]	Cracked maize
1-(2,5-dimethoxy-4-methylsulfonylphenyl)propan-2-amine	NA	Amine	T	[95]	Glucose minimal medium (GMM)
(7a-isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol	NA	Terpene	T*	[85]	PDA
pyrrolidine,1-8-(3-octyloxiranyl)-1-oxooctyl]-	NA	Other	T	[84]	Non-sterile cracked maize

(a) IUPAC or common name of the VOC (b) VOC case number (c) Chemical family of the VOC (d) Toxigenicity of the *A. flavus* strain: (T) toxigenic, (NT) non-toxigenic, (T*) natural mutant and (U) data unknown (e) The literature reference in relation to (f) the substrate type [No data available (/); Potato dextrose agar (PDA); Malt extract agar (MEA), Czapek solution agar (CSA) and corn meal agar (CMA) chemical defined agar (CDA) chemical defined liquid (CDL)]

Chapter 3

Volatile Organic Compounds Emitted by *Aspergillus flavus* Strains Producing or not Aflatoxin B_1

The detection of fungal contamination and mycotoxins has always been at the center of food monitoring. These come mainly from filamentous fungi of the genera *Aspergillus*, *Fusarium* and *Penicillium* during the growth in the field or during the storage of foodstuffs, especially cereals.

Among the many mycotoxins listed to date, AFB_1 is the most abundant and the most harmful inducing, depending on the frequency and concentration of exposure, the development of cancers but also a mortality of the individual. Its main producers are *Aspergillus parasiticus* and *Aspergillus flavus.*

The species of *A. flavus* can be subdivided in two groups, according to its ability to produce aflatoxins which is conferred by the presence of a cluster of genes on chromosome 3 coding for aflatoxins. The presence of this cluster dissociates the strains referred to as aflatoxigenic (or toxigenic) from the strains named non-aflatoxigenic (or non-toxigenic) resulting from a total or partial absence of this cluster in some strains.

The genetic difference within the *A. flavus* species makes it an ideal candidate for the study of VOCs from aflatoxigenic and non-aflatoxigenic strains directly highlighting biomarker VOCs to identify the presence of *A. flavus* and to differentiate the aflatoxin producing population.

On the basis of the VOCs emitted by the fungi, would it be possible to detect, identify them and determine the presence of mycotoxins?

The third chapter of the thesis presents a research article published in 2021 in the special issue *New Insight into Fusarium Toxins and Aflatoxins* in *Toxins*:

Josselin, L., De Clerck, C., De Boevre, M., Moretti, A., Jijakli, M-H., Soyeurt, H. and Fauconnier, M-L. (2021) **“Volatile Organic Compounds Emitted by *Aspergillus Flavus* Strains Producing or Not Aflatoxin B_1.”** *Toxins* 13 (10). https://doi.org/10.3390/toxins13100705.

Abstract

Aspergillus flavus is a phytopathogenic fungus able to produce aflatoxin B_1 (AFB_1), a carcinogenic mycotoxin that can contaminate several crops and food commodities. In *A. flavus*, two different kinds of strains can co-exist: toxigenic and non-toxigenic strains. Microbial-derived volatile organic compounds (mVOCs) emitted by toxigenic and non-toxigenic strains of *A. flavus* were analyzed by solid phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC-MS) in a time-lapse experiment after inoculation. Among the 88 mVOCs emitted, 44 were previously listed in the scientific literature as specific to *A. flavus*, namely alcohols (2-methylbutan-1-ol, 3-methylbutan-1-ol, 2-methylpropan-1-ol), aldehydes (2-methylbutanal, 3-methylbutanal), hydrocarbons (toluene, styrene), furans (2,5-dimethylfuran), esters (ethyl 2-methylpropanoate, ethyl 2-methylbutyrate), and terpenes (epizonaren, trans-caryophyllene, valencene, α-copaene, β-himachalene, γ-cadinene, γ-muurolene, δ-cadinene). For the first time, other identified volatile compounds such as α-cadinol, cis-muurola-3,5-diene, α-isocomene, and β-selinene were identified as new mVOCs specific to the toxigenic *A. flavus* strain. Partial Least Square Analysis (PLSDA) showed a distinct pattern between mVOCs emitted by toxigenic and non-toxigenic *A. flavus* strains, mostly linked to the diversity of terpenes emitted by the toxigenic strains. In addition, the comparison between mVOCs of the toxigenic strain and its non-AFB_1-producing mutant, coupled with a semi-quantification of the mVOCs, revealed a relationship between emitted terpenes (β-chamigrene, α-corocalene) and AFB_1 production. This study provides evidence for the first time of mVOCs being linked to the toxigenic character of *A. flavus* strains, as well as terpenes being able to be correlated to the production of AFB_1 due to the study of the mutant. This study could lead to the development of new techniques for the early detection and identification of toxigenic fungi.

Keywords

Aflatoxin B_1 (AFB_1); *Aspergillus flavus*, microbial volatile organic compounds (mVOCs), solid phase microextraction (SPME), toxigenic, terpenes, mycotoxins, semi-quantification

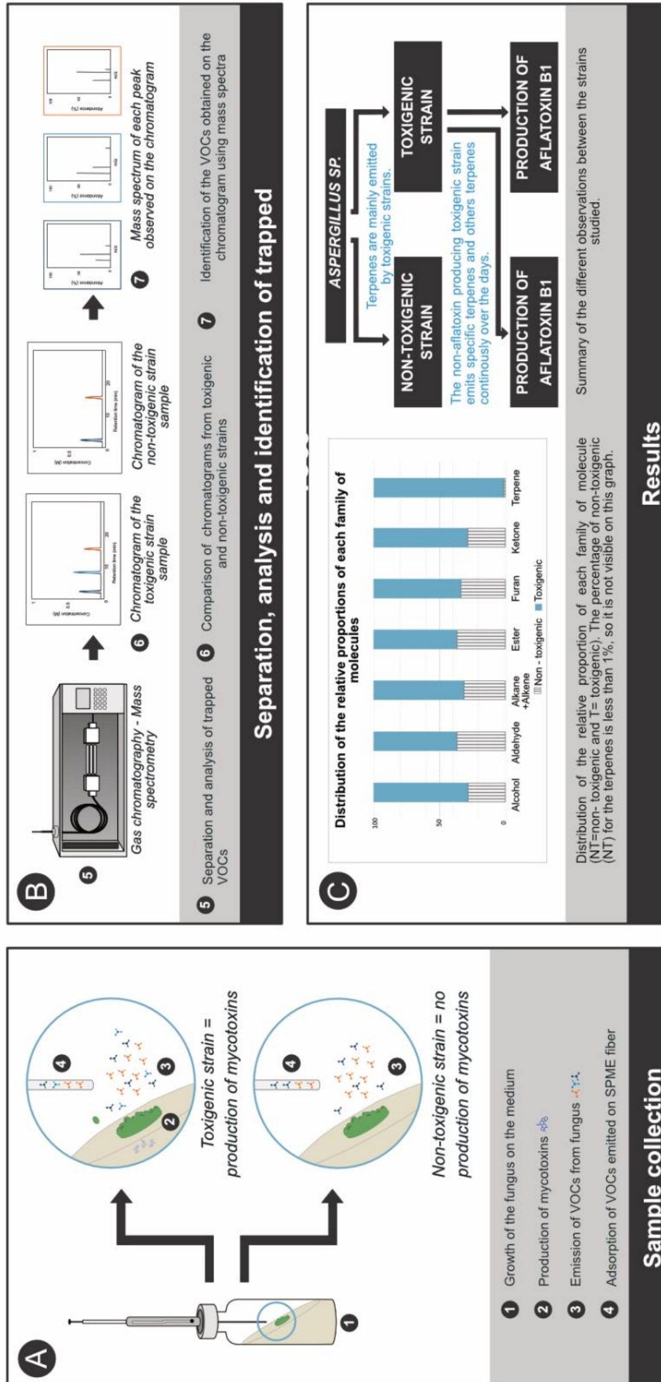


Figure 3-1. Graphical abstract of the Chapter 3

1. Introduction

Contamination by filamentous fungal species such as *Aspergillus*, *Fusarium*, and *Penicillium* in different types of agricultural commodities such as grains is a common phenomenon [67,157]. Many species of these genera have the ability to produce small, non-volatile, secondary metabolites, namely mycotoxins, which are (possibly) harmful for humans and more generally to all vertebrates, even at low concentrations. These fungi have the ability to produce mycotoxins during pre- or post-harvest conditions, and could possibly exert adverse health effects upon dietary consumption by both animal and humans. Besides the latter, the damage of fungi on the agricultural crop leads to residual crop and subsequent trade loss for the agricultural entrepreneurs [64,158,159]. In particular, many species of *Aspergillus* can colonize cereals [160], including *Aspergillus flavus*, which occurs frequently on maize at different stages of both pre-harvest and post-harvest conditions [161,162]. The *A. flavus* species includes toxigenic strains producing mycotoxins and non-toxigenic strains not producing mycotoxins. Their difference is linked to the presence of a gene in the toxigenic strains that gives them the ability to produce aflatoxins [74,163]. The main mycotoxin produced by *A. flavus* is aflatoxin B1 (AFB_1) [31]. Studies have shown that human chronic exposure to AFB_1 may lead to hepatocellular cancer, and that a single acute exposure could result in the death of the consumer [23,164].

The determination of the aflatoxins content in cereals is commonly performed using liquid chromatography coupled to mass spectrometry (LC-MS/MS), however, un-der certain conditions, such as in the field, rapid immuno-chromatographic competitive assay strips are used to enable a fast decisive result [165,166]. When applying quantitative LC-MS/MS procedures, an extensive sample clean-up is required, and the analysis itself is expensive, complex, and requires trained staff. These tests are therefore not suitable to analyze large numbers of samples in, for example, a remote setting where fast results are required [167–170].

Several scientific reports have already shown that there is a correlation between the occurrence of certain volatile organic compounds (VOCs) and the presence of fungi in foodstuffs [45,171], as well as in indoor buildings [93,172]. These VOCs have been referred to as microbial VOCs (mVOCs) [173]. Even if mycotoxins are not volatile contaminants, we hypothesize that their production is possibly linked to the emission of mVOCs, produced through common parts of biosynthetic pathways linked to the mycotoxin production [74]. Citron et al. highlighted the correlation between the secondary metabolism of Actinomycetes that are rich in terpenoids and their genome [61]. Keller et al. studied the synthesis of molecules from the secondary metabolism of fungi and the production of toxins [24].

In *A. flavus*, not all strains have the same toxigenic potential, it is common to isolate and identify strains that produce AFB_1 and strains that do not. These latter strains are called non-toxigenic strains and, since they lack different genes of the AFB_1 biosynthetic gene cluster, are used as biological control agents on several crops to reduce the incidence of AFB_1-producing strains [174].

The objective of this work is to determine if specific mVOCs are emitted when mycotoxins are produced in the setting of *A. flavus* strains, with the final aim of developing rapid online detection systems. These specific mVOCs could allow a faster and indirect detection of AFB_1 produced by *A. flavus*. In this study, we have analyzed the mVOCs emitted by non-toxigenic and toxigenic strains of *A. flavus*. In addition, we have compared the emission of a toxigenic strain producing AFB_1 (ITEM 8111) with its non-aflatoxigenic mutant (ITEM 8111*).

2. Results

The results are derived from the detection of mVOCs emitted at different days of fungal growth of the three strains studied, as well as their AFB_1 concentration. The method and experimentation are summarized in **Figure 3-2**.

2.1. Aflatoxin Concentrations

AFB_1 concentrations in the toxigenic AFB_1-producing strain (ITEM 8111) were: 70.30 $\mu g.kg^{-1}$ on day 3; 82.20 $\mu g.kg^{-1}$ on day 5; 2321.60 $\mu g.kg^{-1}$ on day 7; and 149.20 $\mu g.kg^{-1}$ on day 9 after inoculation. AFB_1 concentrations in the toxigenic non-aflatoxin producing strain (ITEM 8111*) were below the limit of quantification ($<3 \mu g.kg^{-1}$).
As expected, no aflatoxins were detected in the non-toxigenic samples (ITEM 8088).

2.2. mVOCs Screening

The 88 compounds identified to be emitted by the three *A. flavus* strains (35 terpenes, 3 ketones, 2 furans, 4 alkenes, 9 alkanes, 4 aldehydes, 11 alcohols, 6 esters, 2 acids, 2 others, and 9 non-identified) are listed in **Table 3-1**.

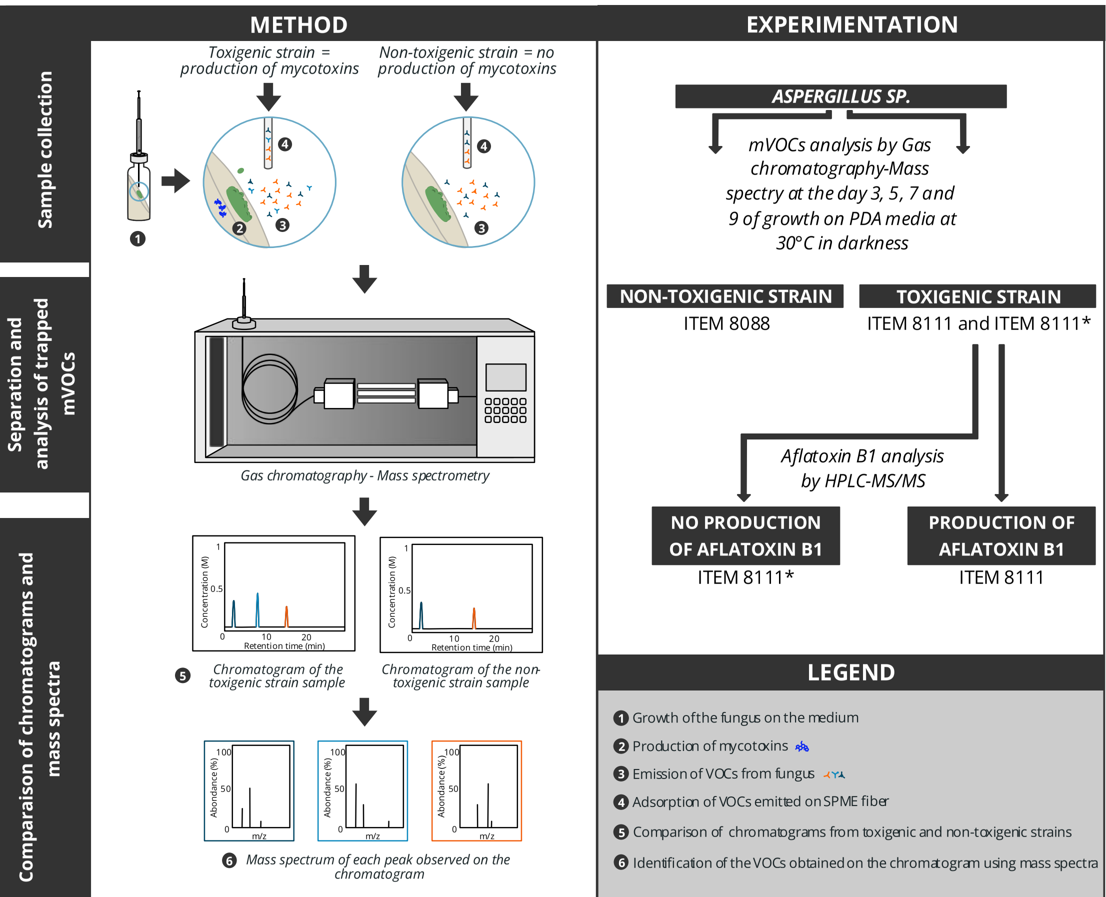


Figure 3-2. General outline of the experiment and the methods used.

Table 3-1. List of mVOCs present at least in two replicates. The values are the percentage of the total area. Identification by comparison with the NIST17 and Wiley298 mass spectra libraries, as well as by comparison between the literature retention index (RI (HP-5ms)), according to the method of Van Den Dool and Kratz on a non-polar HP-5ms column, and the calculated retention index (RI) for each mVOC. The retention index is established using a mixture of n-alkanes under the same chromatographic conditions.

						ITEM 8088				**ITEM 8111**				**ITEM 8111* (mutant)**			
Name			**# cas**	**RI (HP-5ms)**	**RI**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 3**	**Day 5**	**Day 7**	**Day 9**
Acetic acid	ac	**Acid**	64-19-7	-	625	-	-	-	-	-	-	-	-	-	-	17.79	-
2-Methylpropanoic acid		**Acid**	79-31-2	785	767	-	-	-	-	-	-	-	-	-	-	-	0.51
2-Butyloctan-1-ol*		**Alcohol**	3913-02-8	-	1286	-	-	-	-	0.65	-	-	-	0.43	-	-	-
2-Methylbutan-1-ol	ac	**Alcohol**	137-32-6	736	720	13.2	7.84	9.32	17.8	16.25	8.87	10.5	10.5	32.21	16.84	11.34	11.35
2-Methylpropan-1-ol	bc	**Alcohol**	78-83-1	622	624	11.39	27.10	37.01	40.07	26.67	32.76	50.92	29.47	27.10	25.08	36.50	35.68
3-Methylbutan-1-ol	ac	**Alcohol**	123-51-3	734	724	13.3	11.6	11.9	14.1	13.09	13.04	8.48	16.6	21.18	11.54	8.57	9.11
Butan-1-ol	ab	**Alcohol**	71-36-3	668	648	-	-	-	-	-	-	-	-	-	8.57	9.45	9.30
Butan-2,3-diol	abc	**Alcohol**	513-85-9	804	809	-	0.37	0.43	-	-	-	1.04	-	-	-	-	-
Butan-2,3-diol (enantiomer)	abc	**Alcohol**	24347-58-8	-	816	-	-	-	-	-	-	0.41	-	-	-	-	-
Decan-1-ol	b	**Alcohol**	112-30-1	1272	1272	-	-	-	-	0.53	-	-	-	0.36	-	-	-
Ethanol	abc	**Alcohol**	64-17-5	-	575	94.95	100	100	90.40	100	100	80.97	97.52	62.03	100	100	100
Propan-1-ol	abc	**Alcohol**	71-23-8	-	595	-	26.2	31.4	-	36.42	39.75	13.4	33.04	51.76	14.77	37.05	34.78
Propan-2-ol	a	**Alcohol**	67-63-0	-	584	-	-	-	72.3	-	-	-	-	-	-	-	-
2-Methylbut-2-enal		**Aldehyde**	497-03-0	737*	723	4.22	2.19	3.39	-	4.30	3.14	5.84	6.70	3.62	-	-	-
2-Methylbutanal	abc	**Aldehyde**	96-14-0	660	649	12.54	12.64	15.16	-	10.26	10.99	10.03	17.09	9.77	-	-	-
3-Methylbutanal	abc	**Aldehyde**	590-86-3	649	643	5.21	5.76	-	-	-	10.01	7.32	11.77	-	-	-	-
Acetaldehyde	ab	**Aldehyde**	75-07-0	-	566	-	-	-	-	-	-	6.82	-	-	-	-	-

Table 3-1 (continued)

Name			# cas	RI (HP-5ms)	RI	ITEM 8088				ITEM 8111				ITEM 8111* (mutant)			
						Day 3	Day 5	Day 7	Day 9	Day 3	Day 5	Day 7	Day 9	Day 3	Day 5	Day 7	Day 9
(E,Z)-1,2-diethylidenecyclopentane*		Alkane	Not available	-	975	-	-	0.36	-	-	-	-	-	-	-	-	-
2,2,4,6,6-pentamethylheptane	b	Alkane	13475-82-6	997	984	1.22	-	-	-	0.52	0.63	-	-	-	-	-	-
4,6-Dimethyldodecane*		Alkane	61141-72-8	-	1277	-	-	-	-	0.57	-	-	-	-	-	-	-
Heptadecane	bc	Alkane	629-78-7	1700	1696	-	-	-	-	1.39	-	-	-	1.61	0.80	0.47	0.54
Heptane	ac	Alkane	142-82-5	700	677	1.57	0.26	0.10	-	-	-	-	0.48	-	-	-	-
Hexane	c	Alkane	110-54-3	600	612	4.68	17.18	-	8.62	-	17.97	-	13.93	-	-	-	-
Methyl-cyclooctane*	c	Alkane	1502-38-1	-	1386	-	-	-	-	-	-	-	-	-	-	-	0.33
Nonyl-cyclopropane*		Alkane	74663-85-7	-	1273	-	-	-	-	0.49	-	-	-	-	-	-	-
Octane	c	Alkane	111-65-9	800	788	-	-	-	-	-	-	-	1.76	-	-	-	-
Bicyclo[2.2.0]hexa-2,5-diene*		Alkene	7641-77-2	-	1380	-	-	-	-	-	-	-	-	-	-	-	0.33
Dodec-1-ene		Alkene	1120-36-1	1187	1188	-	-	-	-	-	-	-	-	0.50	-	-	0.27
Styrene	abc	Alkene	100-42-5	898	882	8.31	-	-	6.10	3.73	-	-	-	63.64	42.46	32.57	29.06
Toluene	abc	Alkene	108-88-3	762	745	-	-	-	-	-	-	-	2.37	-	-	-	-
Ethyl 2-methylbutyrate	bc	Ester	7452-79-1	-	840	2.28	-	0.60	2.84	-	-	-	-	-	-	0.31	0.35
Ethyl acetate	bc	Ester	141-78-6	612	618	5.87	12.9	18.7	17.1	3.62	14.5	-	12.7	11.93	9.00	13.08	11.67
Ethyl butyrate	c	Ester	105-54-4	802	795	-	-	-	-	-	-	-	0.36	-	-	-	-
Ethyl isobutyrate	c	Ester	97-62-1	-	740	1.01	0.42	1.20	2.09	0.41	0.31	-	1.28	1.51	0.62	0.25	0.43
Ethyl phenylethanoate	c	Ester	101-97-3	1248	1242	-	-	-	-	-	-	-	-	-	-	-	0.39
Ethyl propanoate	c	Ester	105-37-3	714	695	1.66	-	-	0.32	0.57	-	-	0.30	-	0.64	0.54	0.42

Table 3-1 (continued)

						ITEM 8088				ITEM 8111				ITEM 8111* (mutant)			
Name			**# cas**	**RI (HP-5ms)**	**RI**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 3**	**Day 5**	**Day 7**	**Day 9**
2,5-Dimethylfuran	c	**Furan**	625-86-5	-	689	-	-	0.14	0.21	-	-	-	0.15	-	-	0.25	0.27
2-Methylfuran	abc	**Furan**	534-22-5	603	615	-	-	-	-	-	-	10.71	-	-	-	-	-
3-Hydroxybutan-2-one	c	**Ketone**	513-86-0	-	695	-	0.37	0.40	-	-	2.03	1.26	-	-	-	-	-
Butan-2-one	a	**Ketone**	78-93-3	605	609	-	-	-	-	-	-	-	-	23.00	12.42	-	19.29
Thiochroman-4-one*		**Ketone**	3528-17-4	-	1124	-	-	-	-	-	-	-	-	0.56	-	-	-
NI 640		**Other**	-	-	640	-	-	12.7	-	-	-	-	-	-	-	-	-
NI 649		**Other**	-	-	649	-	-	-	22.1	-	-	-	-	-	-	-	-
NI 689		**Other**	-	-	689	0.75	-	-	-	-	-	-	-	-	-	-	-
NI 729		**Other**	-	-	729	-	-	1.74	-	-	-	-	-	-	-	-	-
NI 756		**Other**	-	-	756	-	-	0.11	0.47	-	-	-	-	-	-	-	-
NI 1271		**Other**	-	-	1271	-	-	-	-	-	-	-	-	0.41	-	-	-
NI 1323		**Other**	-	-	1323	-	-	-	-	0.46	-	-	-	-	-	-	-
NI 1386		**Other**	-	-	1386	-	-	-	-	-	-	-	-	1.49	-	-	-
NI 1476		**Other**	-	-	1476	-	-	-	-	-	-	-	-	-	0.46	0.68	-
NI 1501		**Other**	-	-	1501	-	-	-	-	-	-	-	-	0.45	-	-	-
2,4,5-Trimethyl-1,3-dioxolane*		**Other**	3299-32-9	752*	708	-	-	0.32	-	-	-	0.31	-	-	-	-	-
Trichloromethane	c	**Other**	67-66-3	-	623	3.76	-	-	-	8.20	-	-	-	-	-	-	-

-

Table 3-1 (continued)

Name			# cas	RI (HP-5ms)	RI	ITEM 8088 Day 3	ITEM 8088 Day 5	ITEM 8088 Day 7	ITEM 8088 Day 9	ITEM 8111 Day 3	ITEM 8111 Day 5	ITEM 8111 Day 7	ITEM 8111 Day 9	ITEM 8111* (mutant) Day 3	ITEM 8111* (mutant) Day 5	ITEM 8111* (mutant) Day 7	ITEM 8111* (mutant) Day 9
4a,8-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene*			103827-22-1	-	1476	-	-	-	-	-	-	-	-	-	-	-	0.57
(7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol*			Not available	-	1738	-	-	-	-	-	-	-	-	0.54	-	-	-
Di-epi-1,10-cubenol			73365-77-2	1623	1611	-	-	-	-	-	-	-	-	-	-	-	0.16
Aromadendrene	c		109119-91-7	1444	1443	-	-	-	-	0.86	-	-	-	0.70	-	-	-
cis-Muurola-3,5-diene*	b		157374-44-2	1447*	1448	-	-	-	-	2.43	-	-	-	1.97	-	0.34	0.26
Epi-bicyclosesquiphellandrene	abc	Terpene	54274-73-5	1478	1463	-	-	-	-	6.27	-	-	-	4.04	1.25	0.85	0.68
Epi-cubeno-1-ol*			19912-67-5	1619*	1611	-	-	-	-	0.94	-	-	-	0.79	-	-	-
Epizonaren	abc		41702-63-0	1497	1494	-	-	-	-	23.22	6.38	4.22	1.27	17.54	7.17	5.15	4.50
Germacrene-D	ab		23986-74-5	1480	1480	-	-	-	-	4.24	0.63	-	-	3.03	0.92	0.59	0.46
Palustrol			5986-49-2	1569	1565	-	-	-	-	0.34	-	-	-	0.45	-	-	-
trans-Caryophyllene	abc		87-44-5	1418	1414	-	-	-	-	1.63	-	-	-	1.12	-	-	-
Valencene	abc		997297	1490	1491	-	-	-	-	-	-	-	-	-	-	-	0.69
Viridiflorol			552-02-3	1589	1589	-	-	-	-	0.51	-	-	-	0.74	-	-	-
α.-Dehydro-ar-himachalene			78204-62-3	1522	1537	-	-	-	-	-	-	-	-	0.36	-	-	-
α-Cadinene	b		24406-05-1	1538	1534	-	-	-	-	1.59	-	-	-	0.78	0.24	-	0.09
α-Cadinol			481-34-5	1656	1654	-	-	-	-	-	-	-	-	0.53	-	-	-

Table 3-1 (continued)

						ITEM 8088				ITEM 8111				ITEM 8111* (mutant)			
Name			**# cas**	**RI (HP-5ms)**	**RI**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 3**	**Day 5**	**Day 7**	**Day 9**
α-Calacorene	b		21391-99-1	1540	1540	-	-	-	-	0.55	-	-	-	0.56	-	-	-
α-Copaene	ac		3856-25-5	1372	1365	-	-	-	-	1.16	-	-	-	1.20	-	-	-
α-Corocalene	bc		20129-39-9	1629	1620	-	-	-	-	-	-	-	-	0.32	-	-	-
α-Cubebene	a		17699-14-8	1348	1342	-	-	-	-	0.69	-	-	-	0.79	-	-	-
α-Gurjunene	ac		489-40-7	1408	1401	-	-	-	-	2.02	-	-	-	1.45	-	0.26	0.22
α-Isocomene			65372-78-3	1392	1380	-	-	-	-	3.51	0.47	-	-	2.21	0.65	0.40	-
α-Muurolene	bc		31983-22-9	1472	1471	-	-	-	-	0.88	-	-	-	0.64	-	-	-
α-Selinene	ab		473-13-2	1494	1491	-	-	-	-	6.73	-	-	-	4.73	1.42	0.88	-
β-Cadinene	c		523-47-7	-	1489	-	-	-	-	1.11	-	-	-	0.72	-	-	-
β-Chamigrene	a	**Terpene**	18431-82-8	1472	1476	-	-	-	-	-	-	-	-	1.05	-	-	-
β-Elemene (E)	abc		515-13-9	1382	1376	-	-	-	-	1.85	-	-	-	0.93	-	3.05	1.83
β-Elemene (Z)	abc		515-13-9	1382	1384	-	-	-	-	35.71	4.15	-	-	17.10	5.13	-	-
β-Himachalene	abc		1461-03-6	1498	1497	0.54	-	-	-	2.53	1.47	-	-	6.41	5.24	4.46	5.61
β-Selinene	a		17066-67-0	1479	1483	-	-	-	-	3.30	-	-	-	2.35	0.65	0.42	0.28
γ-Cadinene	abc		39029-41-9	1513	1508	-	-	-	-	18.85	1.93	-	-	9.95	3.30	2.09	1.57
γ-Gurjunene	ac		22567-17-5	1476	1472	-	-	-	-	10.06	-	-	-	6.37	1.66	1.17	0.95
γ-Muurolene	abc		30021-74-0	1477	1477	-	-	-	-	2.80	-	-	-	0.99	-	-	-
δ-Cadinene	abc		483-76-1	1524	1520	-	-	-	-	26.06	5.91	2.92	0.92	18.11	6.58	4.43	3.68
τ-Muurolol			19912-62-0	1641	1644	-	-	-	-	-	-	-	-	0.38	-	-	-

NI: not identified, *: potentially identified based on the mass spectra libraries or retention index only, a: compound listed as being of filamentous fungal origin, b: compound listed as being of the genus Aspergillus, c: compound listed as being of the species *Aspergillus flavus* in accordance with the literature [54,74,83,84,86,91,92,96,97,172,175–180]

We observed that the non-toxigenic strain emits a smaller number of mVOCs than the toxigenic strains. A total of 22 mVOCs common to toxigenic and non-toxigenic strains were released (**Figure 3-3**).

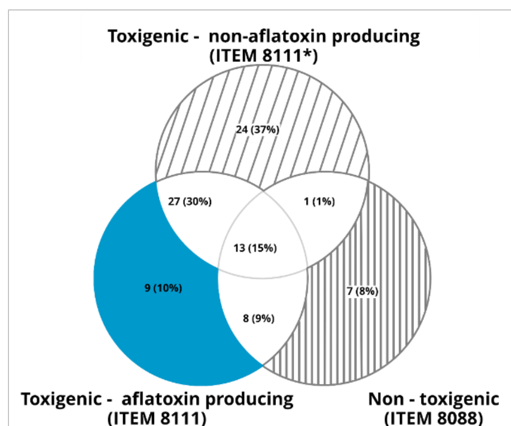


Figure 3-3. Number of compounds (and their percentage) emitted only by one of the three strains and compounds common to two or three strains.

Ethanol, 2-methylpropan-1-ol, 2-methylbutan-1-ol, 3-methylbutan-1-ol, and propan-1-ol were predominant for each day studied with similar values for each strain. Some hydrocarbons (2,2,4,6,6-pentamethylheptane, hexane, heptane, styrene), aldehydes (2-methyl-2-butenal, 2-methylbutanal, 3-methylbutanal), and esters (ethyl isobutyrate, ethyl propanoate, ethyl acetate, ethyl 2-methylbutyrate) were also detected in common, as well as 2,5-dimethylfuran, 3-hydroxybutan-2-one, 2,4,5-trimethyl-1,3-dioxolane, trichloromethane, and a single terpene (β-himachalene) (**Table 3-1**).

Some compounds were specifically and punctually emitted by the non-toxigenic strain (ITEM 8088):

- on day 3: NI 689;
- on day 7: (E,Z)-1,2-diethylidenecyclopentane, NI 640, NI 729;
- on day 9: propan-2-ol with a large relative area of 72.3%;
- no specific compound emission is recorded on day 5.

Interestingly, with the exception of β-himachalene emitted punctually on day 3, no terpene emission was detected during the 9 days of analysis for the non-toxigenic strain.

The main difference that characterizes the toxigenicity of the strains is the abundance of terpenes emitted by the toxigenic strains (**Figure 3-4**).

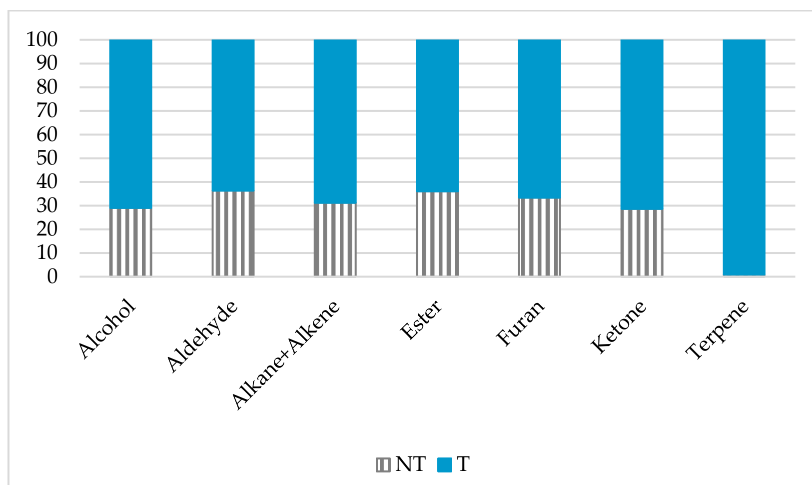


Figure 3-4. Distribution of the relative proportion of each family of molecule (NT: non-toxigenic, T: toxigenic). The percentage of non-toxigenic (NT) for the terpenes is less than 1%, so it is not visible on this graph.

In the case of the toxigenic strains (ITEM 8111 and ITEM 8111*), 60 different mVOCs have been identified, among which 27 as β-cadinene or viridiflorol were emitted in common (**Figure 3-2**).

For both strains, we observed a similar punctual emission on day 3 of decan-1-ol, 2-butyloctan-1-ol, aromadendrene, epi-cubeno-1-ol, palustrol, trans-caryophyllene, viridiflorol, α-calacorene, α-copaene, α-cubebene, and β-cadinene.

Unlike in the non-toxigenic strain, a constant emission of epizonaren and δ-cadinene was recorded.

Like in ITEM 8088, styrene was detected on day 3, but unlike in the non-toxigenic strain, where emissions were punctual, styrene emissions in the strain ITEM 8111 producing AFB_1 persisted during the 9 day period considered.

In the AFB_1- producing strain (ITEM 8111), 2 -methylbutanal and 2-methylbut-2-enal were continuously emitted, while they were emitted only on day 3 by the non-AFB_1-producing strain (ITEM 8111*). Cis-muurola-3,5-diene, germacrene-D, α-cadinene, α-gurjunene, α-isocomene, β-elemene, and γ-cadinene were emitted more or less regularly by the two toxigenic strains.

Interesting differences were spotted between the two toxigenic strains. Several molecules (heptadecane, γ-gurjunene, epi-bicyclosesquiphellandrene, and α-selinene) were punctually emitted (usually on day 3) for the AFB_1-producing strain (ITEM 8111), while the emissions persist in time for the non-AFB_1-producing strain (ITEM 8111*).

Butan-2,3-diol, nonyl-cyclopropane, 4,6-dimethyldodecane, octane, toluene, ethyl butyrate, NI 1323, and 2-methylfuran were only detected for the AFB_1-producing strain (ITEM 8111).

Hydrocarbons (methyl-cyclooctane, dodec-1-ene, bicyclo[2.2.0]hexa-2,5-diene), one ester (ethyl benzeneacetate), one ketone (butan-2-one), one alcohol (butan-1-ol), terpenes (4a,8-dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene, (7a-Isopropenyl-4, 5-dimethyloctahydroinden-4-yl)methanol, di-epi-1,10-cubenol, valencene, α-corocalene, β-chamigrene, τ-muurolol), and other (including unidentified) compounds (thiochroman-4-one, NI 1271, NI 1386, NI 1476, NI 1501) were only detected for the non-AFB_1-producing mutant of strain ITEM 8111.

In comparison with the other strains, AFB_1-producing strain (ITEM 8111) has the lowest terpene diversity. These terpenes emissions decreased over time until their total absence at day 9. In addition, the total number of terpenes emitted by the non-AFB_1-producing mutant of strain ITEM 8111 was higher than for the AFB_1-producing strain (ITEM 8111), on all days considered (**Figure 3-5**).

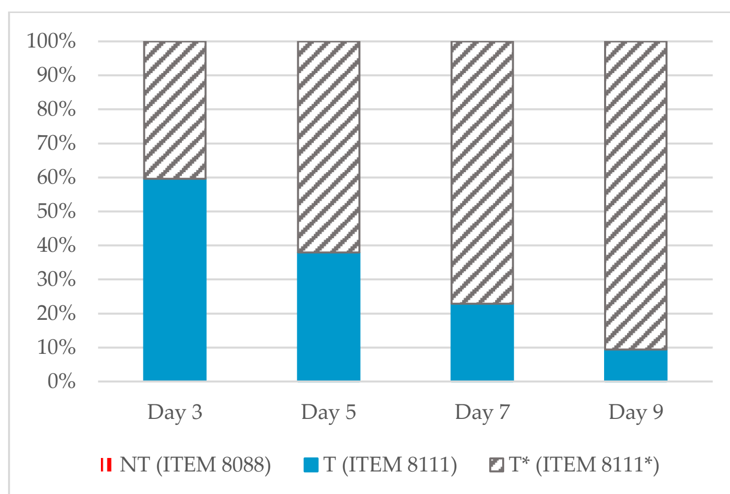


Figure 3-5. Terpenes distribution between the strains (NT: non-toxigenic strain, T: toxigenic aflatoxin-producing strain, T*: toxigenic non-aflatoxin-producing strain) emitted of each time point (day 3, 5, 7, 9). The percentage of non-toxigenic (NT) is only present.

The majority of terpenes' highest emissions were detected at the 3rd day. Among the 32 terpenes emitted, 26 were in common and were emitted in similar proportions in both toxigenic strains. However, 6 compounds were specific to the non-AFB_1-producing mutant of strain ITEM 8111 (**Figure 3-6**).

2.3. mVOCS Related to Toxigenic Characteristic

Partial Least Square Analysis (PLSDA) shows the presence of a split according to the toxigenicity of the strain (**Figure 3-7a**). This division is mainly related to the terpenes emitted by the toxigenic strains. The indicator molecules that can be used for toxigenicity are epizonaren, δ-cadinene, germacrene-D, β-himachalene, γ-cadinene,

β-selinene, γ-gurjunene, α-isocomene, and α -cadinene. Ethyl 2-methylbutyrate and heptane can be linked with the non-toxigenic strain.

Notable discrepancies were confirmed in the group of the toxigenic strain and its mutant (**Figure 3-7b**). Indeed, styrene, β-selinene, and γ-gurjunene emissions separated the AFB_1-producing strain (ITEM 8111) and the non-AFB_1-producing strain (ITEM 8111*).

For the most interesting molecules, identified through **Table 3-1** and PLSDA, concentrations were determined on day 3, in order to emphasize qualitative as well as quantitative differences (**Table 3-2**).

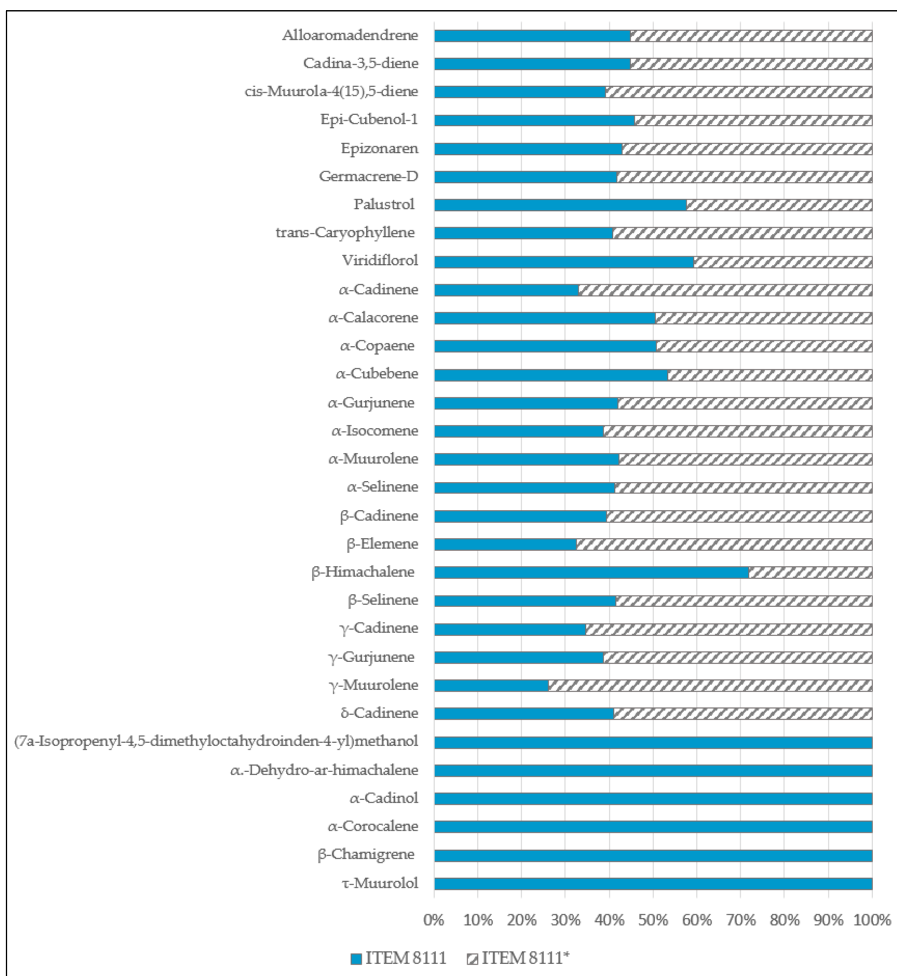


Figure 3-6. Proportion (%) of terpenes released during day 3 by the toxigenic aflatoxin B_1-producing (AFB_1) (ITEM 8111) and the toxigenic non-aflatoxin-producing (ITEM 8111*).

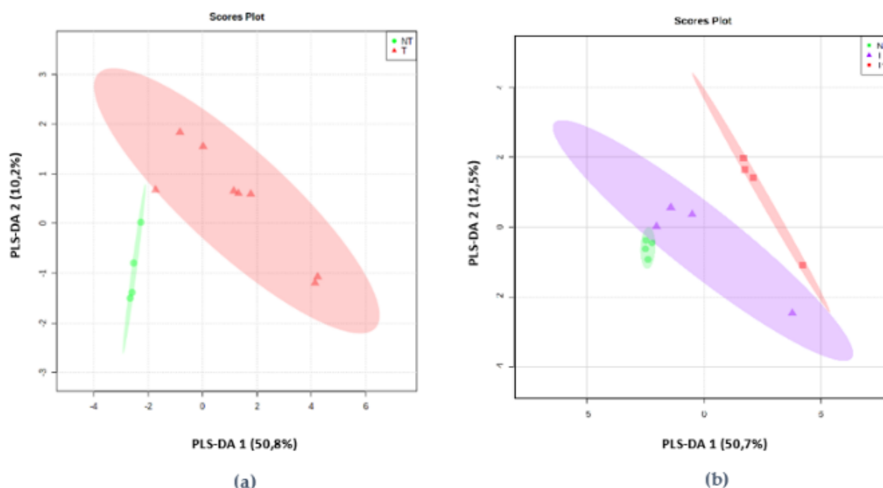


Figure 3-7. PLSDA (Partial Least Square Analysis) applied on the data (a) of the toxigenic (T-Δ) and non-toxigenic (NT-○) strains, (b) of the AFB_1-producing (T-Δ), the non-AFB_1-producing (T*-□), and non-toxigenic (NT-○).

Table 3-2. Compounds quantification (ppb) emitted by the strains on day 3.

Compound	ITEM 8111	ITEM 8111*
α-Cadinene	0.432	0.277
α-Cadinol	-	0.175
α-Isocomene	0.950	0.720
α-Muurolene	0.282	0.209
α-Selinene	1.817	1.565
β-Chamigrene	-	0.370
β-Elemene	8.897	5.181
β-Himachalene	0.737	2.590
δ-Cadinene	6.042	7.874
γ-Gurjunene	2.615	1.895
γ-Muurolene	0.769	0.381
τ-Muurolol	-	0.105
Aromadendrene	0.205	0.255
Epi-cuben-1-ol	0.311	0.360
Epizonaren	7.128	5.948
Germacrene-D	1.132	0.996
Styrene	261.75	$29.8x10^6$
2-Methylbutan-1-ol	2.223	0.888
3-Methylbutan-1-ol	0.934	0.440

3. Discussion

3.1. mVOCs

In our study, we have identified 57 compounds already known to be emitted by fungi (**Table 3-1**). In particular, we have identified 13 compounds known to be associated with the fungal presence (a in **Table 3-1**) and/or the genus *Aspergillus* (b in **Table 3-1**), and more precisely, 44 compounds known in the literature to be involved with the presence of *A. flavus* strains (c in **Table 3-1**) [54,74,83,84,86,91,92,96,97,172,175–181]. In addition, 20 compounds (not counting the 9 unidentified compounds) were identified for the first time to be emitted by *A. flavus.*

Among them: 2-butyloctan-1-ol, α-cadinene, α-calacorene, cis-muurola-3,5-diene, α-cubebene, α-selinene, β-cadinene, epi-cuben-1-ol, palustrol, viridiflorol, and α-isocomene, known to be emitted by fungi, were listed for the first time as specific volatiles of toxigenic strains of *A. flavus* (**Table 3-1**).

In addition, one new compound was systematically detected, in all strains and in significative proportions: 2-methylbut-2-enal, which was known to be emitted only from non-toxigenic strains, as per De Lucca et al. [83].

Like Sun et al. [54], we have observed that, unlike other chemical families, all strains emit the same alcohol proportions, whether toxigenic nor non-toxigenic.

The main difference between the toxigenic and the non-toxigenic strains was the presence/absence of terpenes (**Figure 3-4**). This correlation was already suggested in another study [176]. The terpenes identified are exclusively sesquiterpenes.

Terpenes are known to play several roles in nature. In fungi, they have been found to attract certain worms to defend them (trans-caryophyllene), to repel herbivores (trans-caryophyllene, α-muurolene, γ-muurolene) [91], and to be involved in inter- and intraspecific communication [57,182].

We observed that terpenes were only emitted in the case of the toxigenic strain and its mutant. Interestingly, these emissions tend to be continuous over time in the case of the non-AFB_1-producing strain, while they are punctual (mainly on day 3) in the AFB_1-producing strain.

Several studies have already shown that the toxigenicity of *A. flavus* could be associated with punctual emissions of terpenes, like trans-caryophyllene, α-gurjunene, α-muurolene, and γ-muurolene (that we detected in our study on day 3) [54,91,96,172,176–178], and with constant emissions of epizonaren, γ-cadinene, and γ-gurjunene [92,96,97,175], which we detected during the 9 days of growth. These compounds were not listed in the literature as being emitted by a non-toxigenic *A. flavus* strain. This was not the case with δ-cadinene and valencene, which were detected in our study only in the toxigenic strain and its mutant, although they have been detected in the non-toxigenic strain in other studies [54,176,177].

Interestingly, we detected the presence of β-selinene and α-selinene, which are known to be precursors to the presence of mycotoxins [172]. As with other terpenes, these compounds are only emitted by toxigenic strains. However, we observed

different patterns of emission between the toxigenic strain 8111 and its non-AFB1-producing mutant: punctual emission (at day 3) for the AFB_1-producing strain and continuous emission during the 9 days for the mutant strain (ITEM 8111*). We also observed this emission profile for β-himachalene, γ-cadinene, germacrene-D, α-gurjunene, and epi-bicyclosesquiphellandrene, suggesting that these compounds could, in the same way, be involved in the toxin production.

Terpenes could also act as inhibitors of AFB_1 synthesis, as was shown in Holmes et al. [183]. In our study, six terpenes are specifically emitted by the non-AFB_1-producing mutant strain (ITEM 8111*) and could act as inhibitors. Among them, α.-dehydro-ar-himachalene, τ-muurolol, and α-cadinol, present in some essential oils, have shown antimicrobial and/or fungicidal activities [184]. However, whether such production of terpenes was the cause of the lack of AFB_1 synthesis or was triggered by this loss of mycotoxin production needs to be better evaluated.

In fungi, aflatoxins are supposed to be involved in defense against other external pathogens (bacteria, fungi, etc.) but also host-related defenses. In our non-AFB_1-producing mutant strain (ITEM 8111*), the absence of AFB_1 production could be compensated by an important and continuous emission of terpenes, playing similar roles.

Other interesting compounds were detected. (E,Z)-1,2-diethylidenecyclopentane was only emitted by the non-toxigenic strain and is a known compound of *Laurus nobilis* essential oil, which has shown antifungal activities and caused inhibition of AFB_1 in vitro [184]. The thiochroman-4-one emitted by the non-AFB_1-producing strain was known to be an antifungal agent involved in population regulation [185].

Ethyl 2-methylbutyrate is the only volatile that can be related to the absence of AFB_1 production for both non-toxigenic and toxigenic non-AFB_1-producing strains. It has been identified as specific to the genus *Aspergillus* [184].

3.2. Potential mVOCs Markers

Several studies have already considered the use of mVOCs as potential biomarkers to detect the presence of fungi [186] and even mycotoxin contamination [187].

However, this kind of dispositive for the detection of *A. flavus* is not available yet, to the best of our knowledge.

Our study provides, for the first time, a group of potential marker molecules that could be considered to determine the presence of *A. flavus* and its AFB_1 production.

Based on our results, some volatiles emitted in significant proportions, like 3-methylbutan-1-ol and 2-methylbutanal, could be used to detect the presence of a fungal contamination. Other volatiles like 2-methylbut-2-enal, ethyl isobutyrate, ethyl acetate, and δ-cadinene are specific to *A. flavus* and can be used to detect a specific contamination by this fungus.

More interestingly, some volatile compounds can be used to specifically detect the presence of *A. flavus* toxigenic strains. Among them, epizonaren is a good candidate, as it is emitted in significant proportion (5 ppb) continuously on every day of growth

only by toxigenic strains. In other studies, this compound was already used as a fungal indicator [92] related to *A. flavus* [178] and has been detected for several *A. flavus* toxigenic strains [184].

Heptadecane, 2-methylfuran, and toluene were only detected for the toxigenic strain and could also be used as potential biomarkers. These compounds are already known as common fungal volatiles and used as indicators of fungal growth [54,86].

We did not show any mVOCs related to AFB_1 production but rather to the absence of production in the non-AFB_1-producing strain (ITEM 8111*). To determine the AFB_1 production potential, mVOCs that are specifically emitted by strains not producing toxins will also need to be targeted: ethyl 2-methylbutyrate, α.-dehydro-ar-himachalene, τ-muurolol, dodec-1-ene, 2,4,5-trimethyl-1,3-dioxolane, di-epi-1,10-cubenol, (7a-isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol, α-corocalene, and β-chamigrene. However, as some of these are emitted in low amounts, this will require the development of highly sensitive captors [86]. In this case, it could be interesting to consider the development of an electronic sensor, making the detection of productive strains in silos possible quickly and without any sample preparation [74,173,188]. The essential parameters such as selectivity and sensitivity for their design must also be taken into account [188–190].

Semi-quantification of terpenes showed similar amounts for the toxigenic strain ITEM 8111 and its mutant. Values ranged for day 3 from 0.1 for τ-muurolol to 8.89 ppb for β-elemene (**Table 3-2**).

The β-himachalene was detected in all our tested strains. However, its concentration was significantly higher for the non-AFB1-producing mutant (ITEM 8111*) with a peak of 2.59 ppb (against 0.74 ppb for the AFB1-producing strain) at day 3.

Styrene is common to both toxigenic strains (ITEM 8111 and 8111*) and non-toxigenic ITEM 8088 [178]. However, the amount of styrene released could be a good indicator of the absence of AFB_1 production. Indeed, 29.8×10^6 ppb was released for the non-AFB1-producing mutant (ITEM 8111*), against 261.75 ppb for the AFB_1-producing strain (ITEM 8111). This molecule was already detected for other fungal genera like *Penicillium sp.,* but the detected concentrations were much lower [86,187].

If the developed captors allow temporal and quantitative observations, γ-gurjunene, γ-cadinene, β-elemene, and α-selinene could act as additional indicators, as they are emitted in high proportions on the 3rd day of growth of the AFB_1-producing strains (ITEM 8111).

In order to confirm and refine the relevance of these molecules, further research is in progress on a wider variety of toxigenic and non-toxigenic strains of *A. flavus*. In vivo tests will also be needed to confirm the emission of the volatiles in real agronomical conditions. Several studies have indeed shown that mVOCs emitted by fungi vary with the substrate used [172,176].

To better understand the potential correlation between sesquiterpenes and aflatoxins production, a focus on metabolic pathways is needed. The origin of the terpene biosynthesis pathway is acetyl-CoA, which is then converted to malonyl-CoA by acetyl-CoA carboxylase. On the one hand, the combination of acetate and malonyl-

CoA leads to the formation of hexanoyl units and then to norsolorinic acid, which is the first stable precursor of the aflatoxin biosynthetic pathway [191]. On the other hand, the farnesene backbone, the basis of many fungal sesquiterpenes, is derived from the isoprenoid biosynthetic pathway from the same acetyl-CoA molecule [192].

Recent studies are progressing to detect the genes involved in of each of the sesquiterpenes' production [193], as well as studies on the aflatoxin biosynthetic pathway, which is being analyzed to better understand its functioning and genetic structure [18,194–196].

4. Conclusions

In conclusion, new mVOCS were associated with *A. flavus,* in addition to those already known in the literature to be common to *A. flavus* and other species of the genus *Aspergillus*. Comparison of non-toxigenic and toxigenic strains identified potential biomarkers, mainly terpenes, to differentiate these two categories (**Figure 3-8**). Comparison of the volatiles emitted by the toxigenic AFB_1-producing strain and its non-AFB_1-producing mutant surprisingly allowed the detection of a dramatic variability in terpene production between these two strains related to the lack of AFB_1 production. Studies to identify genomic as well as stability assessment of this mutation that inhibited AFB_1 production in the ITEM 8111* mutant strain will be performed. An approach focused on the metabolic pathways of mVOCs specific to toxigenic strains, and in particular those of certain terpenes emitted by the non-AFB_1-producing toxigenic strain could be proposed in order to clarify their impact on the expression of the AFB_1 biosynthesis genes, and thus determine their influence at a different scale of the fungi [195].

Finally, the semi-quantification of some molecules allowed the definition of detection thresholds for the conception of a future molecular fingerprint sensor.

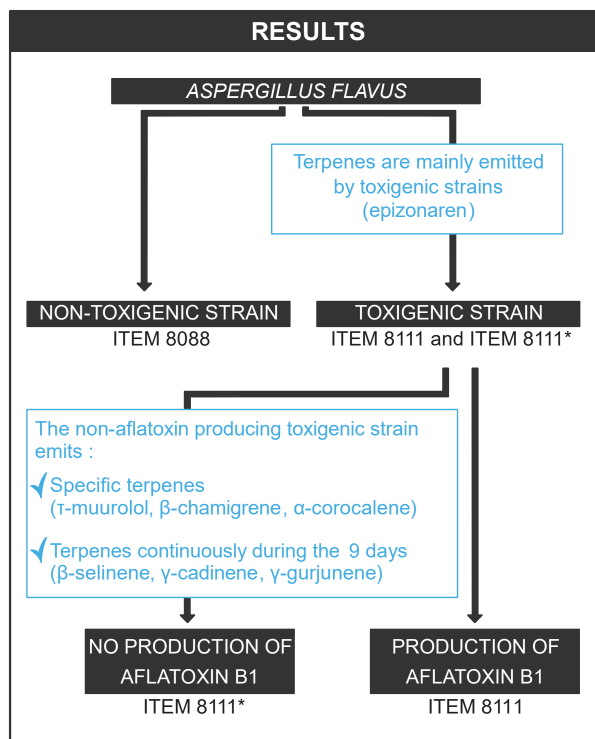


Figure 3-8. Summary of the results.

5. Materials and Methods

5.1. *Fungal Strains*

In order to investigate the above-mentioned hypotheses, fungal strains were provided by CNR-ISPA (Research National Council of Italy—Institute of Sciences of Food Production, Bari, Italy). The strains of *Aspergillus flavus* belong to the official collection of fungi of the Institute of Sciences of Food Production ITEM Collection, where they are available. The ITEM is recognized by the International Organization of European Culture Collections and the World Federation of Culture Collections.

Two categories of *A. flavus* strains were studied: a non-toxigenic strain as negative control for the aflatoxin B_1 (AFB_1) production (designated as ITEM 8088), and a toxigenic strain which produces AFB_1 (designated as ITEM 8111), as well as its mutant (ITEM 8111*), which does not produce AFB_1.

5.2. *Fungi Inoculation*

Fungi were grown on SNA medium (for 1 L, 1 g KH_2PO_4; 1 g KNO_3; 0.5 g $MgSO_4.7H_2O$; 0.5 g KCl; 0.2 g glucose; 0.2 g sucrose; 20 g agar) and stored at -80 °C in glycerol. They were incubated at 30 °C during 7 days in darkness. The spore suspensions were prepared with Tween 20 and sterile water. The concentrations were determined using a Bürker cell and adjusted to centrally inoculate 1.15×10^3 spores.mL^{-1}. The inoculation was carried out in 20 mL vials containing slanted PDA to provide a larger growth surface for the fungus. The vials inoculated were incubated at 30 °C during 3, 5, 7, and 9 days in darkness before sampling. Three replicates were systematically prepared [96,141,182].

5.3. *Aflatoxin Analysis*

The aflatoxin incidence was determined using liquid chromatography coupled to mass spectrometry (LC-MS/MS) according to an in-house validated protocol. One gram of sample was taken and transferred into an extraction tube. Blanks and unknown samples were spiked with the volume as indicated in the following **Table 3-3**. The samples were left in the dark for approximately 15 minutes for re-equilibration. Five mL of acidified ethyl acetate (ethyl acetate + 1% formic acid, v/v) was added, and vortexed accordingly. The samples were shaken on an overhead shaker for 15 minutes, and centrifuged at 3600 rpm for 15 minutes. The supernatant was transferred onto a filter with a plastic Pasteur pipette, preconditioned with acidified ethyl acetate. Then, 5 mL of dichloromethane was added to all samples. The samples were vortexed, and centrifuged again at 3600 rpm for 15 minutes. Then, the supernatant was transferred onto a filter with a plastic Pasteur pipette, and preconditioned with dichloromethane. The residue was then evaporated completely in a warm water bath at 40 °C. The remaining fraction was dissolved in 200 µL of injection solvent. To fully dissolve the matrix, the dilution was vortexed for 2 minutes. Afterwards, 200 µL of hexane was added, and transferred to a centrifugal filter (0.22 µm). The sample was centrifuged for 10,000 rpm for 5 minutes, and 100 µL of the bottom layer was transferred into an LC-MS/MS vial. The samples were run according to a validated methodology, and the instrumental parameters were as described in Monbaliu et al. [197].

Table 3-3. Treatment of blank and unknown samples for LC-MS/MS analysis.

Samples	Aflatoxin mixture 2.5 ng.µL^{-1}	Zearalenone 10 ng.µL^{-1}	Deepoxydeoxynivalenol 50 ng.µL^{-1}
Blank	-	20	10
Spike 0.5 X	10	20	10
Spike 1 X	20	20	10
Spike 1.5 X	30	20	10
Spike 2 X	40	20	10

5.4. GC-MS Parameters

The volatile organic compounds (VOCs) analyses were performed on an Agilent Technologies GC 7890B fitted with a Gerstel MPS (MultiPurposeSample, (MPS, Gerstel©, Mülheiman der Ruhr, Germany) robotic autosampler with the SPME tool for SPME fibers modules and MSD 5977B (USA). The inoculated vials were incubated at 40 °C for 30 minutes and extracted for one hour at 40 °C with SPME fibers (Supelco, Darmstadt, Germany, DVB/CAR/DDMS, 50/30µm, 24 Ga). The VOCs separation was performed on an HP-5ms column (Agilent Technologies, Santa Clara, CA, USA, 5%-phenylmethylpolysiloxan, non-polar, 30 m × 0.250 mm × 0.25 µm) with a constant helium flow rate of 1.2 mL.min−1. The inlet SPME fibers were desorbed at 250 °C by splitless injection using an SPME inlet coating of 78.5 mm × 6.5 mm × 0.75 mm (Supelco Inc., Bellefonte, PA, USA). The temperature programs were applied as follows: 45 °C for 7 minutes, 5 °C.min−1 up to 70 °C, 70 °C for 3 minutes, 3 °C.min−1 up to 120 °C, 120 °C for 3 minutes, 10 °C.min−1 up to 270 °C, and a final hold at 270 °C for 5 minutes. The mass spectral analysis was performed using the electron ionization (EI) mode at 70 eV and scan mass range from 35 to 350 amu. The ion source and MS source temperatures were 250 ˚C and 280 ˚C, respectively [54,83,86,93].

5.5. Identification of GC-MS Analysis

The identification was made by mass spectra comparison with NIST17 and WILEY298 libraries, and using the retention indices of Kovat (standard solution of saturated n-alkane C6-C30 (1000 mg.mL^{-1} in hexane, Supelco, Belgium)) in order to calculate the retention indices of each molecule, then using the indices associated with the Van den Dool and Kratz method. Some identifications were confirmed by injecting pure analytical standards purchased from Sigma-Aldrich (Overijse, Belgium). Some terpenes, not commercially available were confirmed by injecting in the same chromatographic conditions an essential oil (Pranarôm, Belgium) typically containing this compound as the main compound [91,198]. In this perspective, γ-gurjunene, δ-cadinene, γ-cadinene, and viridiflorol have been identified with the essential oil of *Cistus ladaniferus*; the δ-cadinene, α-selinene, α-copaene, and τ-muurolol with the essential oil of *Cedrelopsis grevei*; and finally, the β-himachalene with the essential oil of *Cedrus deodara*.

5.6. Statistical Model

Statistics were performed using metaboanalyst (http://www.metaboanalyst.ca, 26th march 2021 accessed on 26 march 2021) [199]. Partial Least Square Analysis (PLSDA) models were built using four components (1) to discriminate the toxigenic versus non-toxigenic strains and (2) to discriminate three classes: AFB_1-producing strain, non-AFB_1-producing strain, and non-toxigenic strain. For all models, the features (i.e., GCMS profiles) were log transformed and mean centered. The discrimination was visualized by plotting the first PLSDA components.

5.7. Semi-Quantification

In order to semi-quantify the compounds of a sample, a mixture composed of the molecules of interest as well as the five most abundant molecules present in this sample of fungi was carried out by preserving the relative area proportions between each molecule (stock solution). The standards used were bought from Sigma-Aldrich (Overijse, Belgium) when commercially available as 2-methylbutan-1-ol, 3-methylbutan-1-ol, styrene, valencene (70% purity), and heptadecane. Terpenes not commercially available were semi-quantified using valencene as a reference standard. The construction of the calibration curves was established by successive dilution of the initial mixture in ethanol (D1 = 300µL of the mother solution, D2 = 1/2D1, D3 = 1/2D2, D4 = 150µL of the mother solution, D5 = 1/2D4). After stirring, a volume of 1µL of the diluted solutions was deposited at the bottom of a 20 mL vial and analyzed concomitantly as the samples [200–212].

Chapter 4

Does Alteration of Fumonisin Production in *Fusarium verticillioides* Lead to Volatolome Variation?

The previous chapter showed a difference in VOCs emission depending on the toxigenic character of *A. flavus* strains and their ability to produce AFB_1. Indeed, the major difference in VOCs emitted is associated with terpene diversity, particularly emitted by toxigenic strains of *A. flavus*. The study of the natural mutant strain of the toxigenic *A. flavus* strain then enabled us to observe the VOC profiles when a toxigenic strain no longer produces aflatoxin. In this situation, the chemical family most involved in the variations observed was also linked to terpenes. Indeed, new terpene VOCs were generated by the mutant strain, a variant of the toxigenic strain, in addition to a change in emission temporality. While most of them were emitted exclusively on day 3 in the case of AFB_1 production, they were then detected every day when AFB_1 production of the strain was lost.

Following the work described in the previous chapter, a similar study concerning the VOCs and the fumonisins of *F. verticillioides* was conducted. This time, the link between VOCs and mycotoxins, which was suggested in the previous work with *A. flavus*, was investigated using a wild-type strain *of F. verticillioides* producing fumonisins B_1, B_2, and B_3, as well as two of its mutants. The mutants of *the F. verticillioides* strains were genetically modified in their fumonisin biosynthetic pathway to halt their production. Thus, in addition to the wild-type strain, the *fum1* and *fum6* mutants, in which the *fum* 1 and *fum* 6 genes were respectively deleted, were monitored for 21 days with measurements taken every three days using maize as a substrate.

In this case, the use of maize kernels was more representative of agricultural reality, but its use may involve added variability linked in particular to kernel composition.

The fourth chapter of the thesis presents a research article submitted in 2023 in *Food chemistry*:

Josselin, L., Proctor R.H., Lippolis V., Cervellieri S., Hoylaerts J., De Clerck C., Fauconnier, M-L and Moretti A. (2023) **“Does Alteration of Fumonisin Production in *Fusarium verticillioides* Lead to Volatolome Variation?”** Submitted in *Food Chemistry.*

Abstract

Fusarium verticillioides is a main maize fungal pathogen and produces fumonisins, mycotoxins raising global food safety concern. It is important to mitigate fumonisins occurrence on maize by using innovative control tools. We investigated volatile organic compounds (VOCs) emitted by a fumonisin-producing wild-type strain of *F. verticillioides* and two mutant strains blocked in the fumonisin biosynthetic pathway. VOCs were analyzed by gas chromatography-mass spectrometry from day 3 to day 21 and fumonisins were detected by high-performance liquid chromatography. The mutants emitted specific VOCs, especially ethyl 3-methylbutanoate, that the wild-type strain was not able to produce. The emission of these specific VOCs was significantly related to the absence of fumonisin production in the mutants. Ethyl 3-methylbutanoate reduced both wild-type strain growth and fumonisin production showing potential as a biocontrol agent. These findings offer valuable insights into potential biocontrol strategies and interaction between VOCs and fumonisins in *F. verticillioides* biology.

Keywords

Fusarium verticillioides, Fumonisins, Volatile organic compounds, Ethyl 3-methylbutanoate, Mutant, Bioactive compound

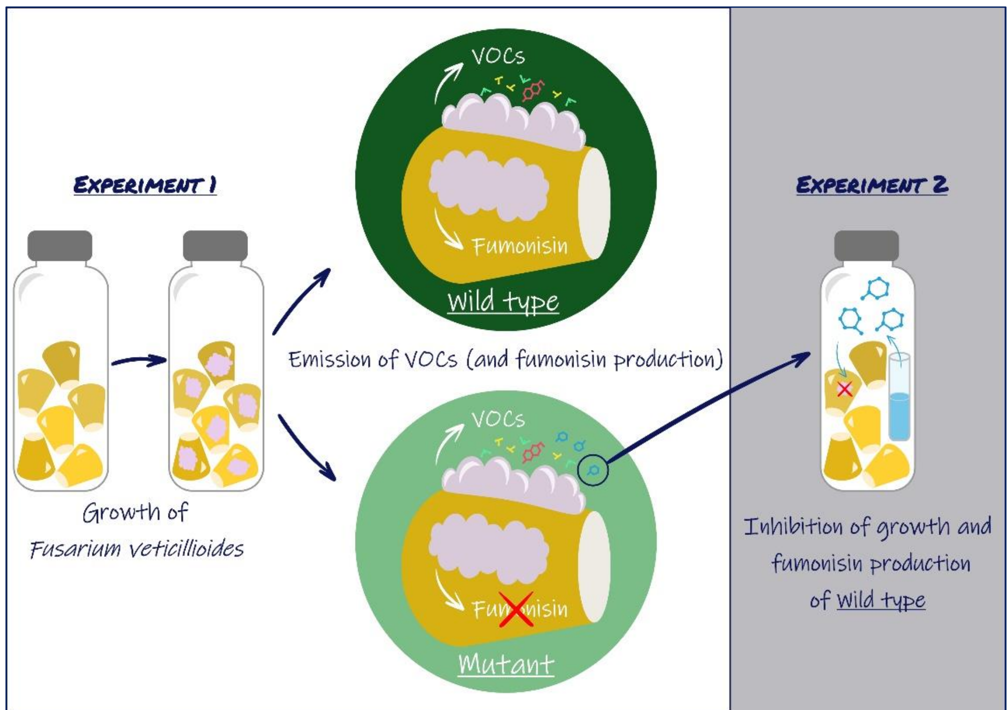


Figure 4-1. Graphical abstract of the Chapter 4

1. Introduction

Fusarium verticillioides (Sacc.) Nirenberg is an agriculturally important fungal pathogen of maize worldwide and is the most significant contributor of contamination of maize with the carcinogenic mycotoxins fumonisins [213]. This species can infect all tissues and developmental stages of maize, causing seed rot, seedling blight, stalk rot, and ear rot, thus being able to contaminate all plant parts with fumonisins [214]. At least 28 fumonisin analogs are known, but the most important are the B series, especially fumonisin B1 (FB_1), B2 (FB_2), and B3 (FB_3) [215]. In particular, the International Agency for Research on Cancer (IARC) classified FB_1 as a possible carcinogen to humans (group 2B) [17] and several studies indicate associations of FB_1 with increased incidence of human esophageal and liver cancers [215]. Due to the impact that fumonisins have on maize crops and the occurrence of *F. verticillioides* as a dominant species in maize-cultivation regions, diagnostic tools are urgently needed for early detection in the field in order to develop robust and reliable management strategies.

Analysis of VOC emissions by microorganisms is of growing interest because of the potential of VOCs as non-destructive diagnostic tools. In addition, studies of chemical structures and biological impacts of VOCs are providing a wide range of information on their biological significance for the organisms that emit them [42]. Some studies indicate that VOCs play a crucial role in interactions of fungi with their environment. In addition, in fungi, some toxins have been linked to emission of volatile organic compounds (VOCs), notably with terpenes [48,61]. Thus, might be possible to use VOCs as biomarkers to detect fungal infection and mycotoxin contamination in crops [216]. However, because VOC production in fungi can be affected by multiple abiotic and biotic factors, including metabolic pathways [217], much work remains to be done to identify VOCs that can serve as reliable biomarkers of fungal infection and mycotoxin contamination. Further, the use of VOCs as markers for fungal species is a subject of multiple investigations [218].

In addition, studies on the role and impact of VOCs on fungal biology have been made for several species and have provided new biocontrol tools to control fungal growth and mycotoxin production [52].

In the current study, we analyzed VOCs emitted by a wild-type strain of *F. verticillioides* and two mutants derived from it that were unable to produce fumonisins due to deletion of one of two fumonisin biosynthetic genes (*FUM1* or *FUM6*). The aims of the study were: a) to compare the volatolomes of the wild type (fumonisin producer) and mutants (fumonisin-nonproducers); b) to identify possible VOCs specific to the fumonisin-producing strain; c) to select a specific VOC to be used as possible biocontrol agent against *F. verticillioides* growth and fumonisin production.

2. Results

2.1. The mycelial development of F. verticillioides overtime

Visual examination of maize kernel cultures revealed that all *F. verticillioides* strains produced abundant mycelium that completely covered the kernels by day 6 of incubation. However, cultures of *fum1* and *fum6* mutants exhibited a purple pigmentation that was not observed in cultures of their wild-type progenitor strain (**Figure 4-7, Supplementary material**). This purple pigmentation was visible by day 6 in cultures of the *fum6* mutant and became darker as the experiment progressed. In comparison, the purple pigmentation of the *fum1* mutant was not observed until day 15 of incubation and never reached the intensity of cultures of the *fum6* mutant.

2.2. The variation of the VOCs emitted by the three F. verticillioides strains

Seventy-five VOCs were detected in GC-MS analysis of the headspace of cultures of the three *F. verticillioides* strains (**Table 4-1**). Of these 75 VOCs, 41 were commonly detected in the headspace of cultures of all three strains; 7 were detected only in the headspace of the wild type; 14 were detected in the headspace of the two mutants but not in that of the wild type; 2 and 9 VOCs were detected in the headspace of only the *fum1* or *fum6* mutant, respectively. Interestingly, 2 VOCs were detected in the headspace of both the wild type and the *fum6* mutant but not the *fum1* mutant. In contrast, no VOCs were detected in headspaces of both the wild type and the *fum1* mutant but not the *fum6* mutant (**Figure 4-2-A**). All VOCs were categorized into 10 chemical families (**Figure 4-2-B**). The three most prevalent of these families, regardless of *F. verticillioides* strain were alcohols, esters, and terpenes. The 7 wild-type-specific VOCs were distributed among all the families except acids, esters, and furans. In general, both mutants emitted more acids, alcohols, and esters than the wild type. The mutant-specific VOCs were 2 acids, 4 alcohols, 1 alkane, 5 esters, 1 ketone and 1 unidentified VOC (**Figure 4-2-B** and **Table 4-1**). The 9 VOCs emitted only by the *fum6* mutant were ethyl heptanoate, heptyl acetate, butane-2,3-diyl diacetate, 2-methoxy-4-vinylphenol, 2,3,5-trimethylfuran, UI 1019 and UI 1034, while the 2 VOCs emitted only by the *fum1* mutant were methyl 2-methylbutanoate and UI 679. Of the 17 VOCs previously reported to be emitted by *F. verticillioides* (Table 4-1), only 12 were detected in the emissions of all the three strains analyzed in the current study, whereas the others 5 VOCs were detected in cultures of only one of the three strains studied. These 12 VOCs consisted of terpenes (D-limonene, β-acorenol and α-cedrene), an ester (ethyl acetate), alkanes (octane and 2,4-dimethylheptane), and alcohols (propan-1-ol, ethanol, 4-ethyl-2-methoxyphenol, 3-methylbutan-1-ol, 2-methylpropan-1-ol and 2-methylbutan-1-ol). Eight VOCs have been previously reported to be produced by *Fusarium* species but not by *F. verticillioides*. These eight metabolites are hexan-1-ol, dodecane, styrene, 2-methylpropyl ethanoate, ethyl 2-

methylbutanoate, ethyl 2-methylpropanoate, ethyl butanoate and α-pinene. As far as we are aware, the current study is the first report of emission of several VOCs by *F. verticillioides*, including β-copaene, butane-2,3-diyl diacetate and 2,4-dimethylhept-1-ene. Interestingly, 2 terpenes ((1R,6R,7R)-2,2,6,8-tetramethyltricyclo[5.3.1.01,6] undec-8-ene and α-pinene) were emitted by the wild type and *fum6* mutant, which both have the first step of the fumonisin pathway intact.

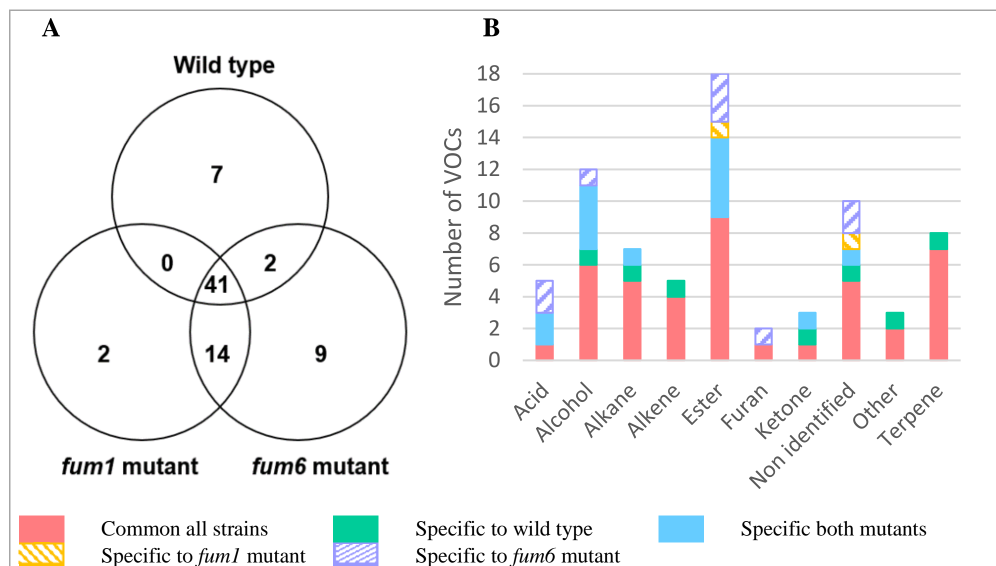


Figure 4-2. (A) Venn diagram showing the number of VOCs detected in the headspace of cultures of the three strains of *F. verticillioides* growing on autoclaved maize kernels. (B) Numbers of VOCs detected categorized by chemical family. Data on all VOCs detected are included (all strains, all timepoints).

Table 4-1. Identification and occurrence of VOCs detected in headspaces of cultures of three *F. verticillioides* strains grown on autoclaved maize kernels.

	Compound Name [a]	Lit [b]	CAS number	RI exp [c]	RI ref [d]	Occurrence of Compounds [e]		
						Wild type	*fum1* mutant	*fum6* mutant
Acid	2-Methylbutanoic acid	3-5	116-53-0	848	846	-	21	18, 21
	2-Methylpropanoic acid		79-31-2	751	NA	-	-	18
	3-Methylbutanoic acid	4-5	503-74-2	841	834	-	9, 12, 18, 21	9, 12, 15, 18
	Acetic acid	3-5	78-93-3	613	602*	3, 6, 12, 18, 21	3	3, 6, 21
	Butanoic acid	4-5	107-92-6	785	790	-	-	21
Alcohol	2-Methoxy-4-vinylphenol		7786-61-0	1318	1318	-	-	3
	2-Methylbutan-1-ol	1-2-3	137-32-6	722	NA	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	2-Methylpropan-1-ol	1-2-3	78-83-1	626	622	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18	3, 6, 9, 12, 15
	3-Ethyl-4-methylpentan-1-ol		38514-13-5	1021	1023	3	-	-
	3-Methylbutan-1-ol	1-2-3	123-51-3	720	734	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	4-Ethyl-2-methoxyphenol	1	2785-89-9	1283	1280	3, 9	3, 6, 9	3, 6, 9, 12
	4-Ethylphenol		123-07-9	1166	1161	-	3, 6	3, 6, 9
	Butan-2,3-diol		513-85-9	782	782	-	21	21
	Butan-2,3-diol E1		24347-58-8	770	782	-	3, 6, 12, 15, 18	3, 6, 9, 15, 18, 21
	Ethanol	1	64-17-5	580	<600	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	Hexan-1-ol	2-3	111-27-3	865	867	-	3	3
	Propan-1-ol	1	71-23-8	599	NA	3	3	3

-

Table 4-1. (continued)						Occurrence of Compounds [e]		
	Compound Name [a]	**Lit [b]**	**CAS number**	**RI exp [c]**	**RI ref [d]**	**Wild type**	***fum1* mutant**	***fum6* mutant**
Alkane	2,2,4,6,6-Pentamethylheptane		13475-82-6	988	995*	3, 6, 9	3	3
	2,3,7-Trimethyloctane		62016-34-6	1007	NA	3	-	-
	2,4-Dimethylheptane	1	2213-23-2	814	NA	3	3	3
	4-Methyloctane		2216-34-4	858	NA	3	3	3
	Dodecane	2	112-40-3	1199	1200	3	3	3
	Octane	1	111-65-9	791	800	3, 6	3, 6	3, 6
	Tridecane	5	629-50-5	1298	1300	-	3	3
Alkene	1,3-bis(1,1-Dimethylethyl)benzene		1014-60-4	1257	NA	3	3	3
	2,4-Dimethylhept-1-ene		19549-87-2	835	NA	3	3	3
	Methylbenzene	1-3	108-88-3	750	NA	3	-	-
	p-Ethylanisole	4	1515-95-3	1115	1104	3	3, 6, 9	3, 6, 9, 12
	Styrene	2-3	100-42-5	887	890	3, 6	3, 6	3, 6, 9
Ester	2-Methylbutyl acetate	3	624-41-9	877	880	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	2-Methylpropyl ethanoate	2-3	110-19-0	762	NA	3, 6, 9, 12	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	3-Methylbutyl acetate	1-3-4-5	123-92-2	874	876	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	3-Methylbutylpropanoate		105-68-0	968	969*	-	3, 6, 9, 12	3, 6, 9, 12, 15, 21
	3-Octyl acetate	3	4864-61-3	1124	1126	3	3	3, 6
	3-Oxobutan-2-yl acetate		4906-24-5	890	NA	-	12	9, 12
	Butane-2,3-diyl diacetate		1114-92-7	1074	1065	-	-	3, 6, 12

Table 4-1. (continued)

	Compound Name [a]	Lit [b]	CAS number	RI exp [c]	RI ref [d]	Occurrence of Compounds [e]		
						Wild type	*fum1* mutant	*fum6* mutant
Ester	Ethyl 2-methylbutanoate	2-3	7452-79-1	847	842	-	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	Ethyl 2-methylpropanoate	2-3	97-62-1	746	755	-	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	Ethyl 3-methylbutanoate	4-5	108-64-5	850	847	-	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	Ethyl acetate	1-2-3	141-78-6	621	611	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	Ethyl butanoate	2-3	105-54-4	796	802	9	6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	Ethyl heptanoate		106-30-9	1097	1095	-	-	12
	Ethyl propanoate	3	105-37-3	694	714	3	3, 6, 9, 12	3, 6, 9, 15, 21
	Heptyl acetate		112-06-1	1112	1110	-	-	6
	Hexyl acetate	5	142-92-7	1013	1011	3, 21	3, 6	3, 6, 12, 15, 21
	Methyl 2-methylbutanoate	4	868-57-5	780	780	-	18	-
	Propyl acetate	3	109-60-4	697	712	3, 6, 9	3, 6, 9, 12	3, 6, 9, 12, 15
Furan	2,3,5-Trimethylfuran	3	10504-04-8	807	NA	-	-	12
	2-Pentylfuran	3	3777-69-3	991	993	3	3, 6	3, 6, 9
Ketone	3-Hydroxybutan-2-one	1-4-5	513-86-0	692	712	-	21	18
	4-Methoxy-2,5-dimethylfuran-3(2H)-one		4077-47-8	1061	1065	3, 18	3-	3, 12
	Hexan-3-one	3	589-38-8	774	NA	9	-	-

Table 4-1. (continued)

	Compound Name [a]	Lit [b]	CAS number	RI exp [c]	RI ref [d]	Occurrence of Compounds [e]		
						Wild type	*fum1* mutant	*fum6* mutant
Terpene	(1R,6R,7R)-2,2,6,8-tetramethyltricyclo[5.3.1.01,6]undec-8-ene		79562-96-2	1409	1405	3	-	3
	3-p-Menthen-7-al		27841-22-1	1204	1197	3	3, 6	3, 6, 9, 12
	4-epi-α-Acoradiene		729602-94-2	1475	1475	3, 6, 9	3, 6, 9	3, 6, 9, 12
	α-Cedrene	1	469-61-4	1425	1416	3, 9	3, 6	3, 6, 9
	UI sesquiterpene (mz=119)		5989-08-2	1485	NA	3	-	-
	α-Pipene	1-2	80-56-8	931	939	3	-	3
	β-Acorenol	1-2	28400-11-5	1645	NA	3	3, 6	3, 6
	β-Cedrene	1-2-3	546-28-1	1434	1413	3	3, 6	3, 6
	β-Copaene		18252-44-3	1442	1436	3	3	3, 6
	D-Limonene	1-3	5989-27-5	1029	1031*	3	3	3
Other	3-Methylbutyl nitrite		110-46-3	836	NA	9	-	-
	Carbon dioxide		124-38-9	<600	NA	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	D-Methyl N-hydroxybenzenecarboximidate		67160-14-9	901	NA	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
Undentified	UI 587			587	-	3	3, 6	3
	UI 679			679	-	-	3	-
	UI 867			867	-	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	UI 928			928	-	-	9, 12	6, 9, 12
	UI 955			955	-	3, 6, 9, 12, 15, 18	3, 6, 9, 12, 15, 18, 21	3, 6, 9, 12, 15, 18, 21
	UI 1019			1019	-	-	-	3

Table 4-1. (continued)

	Compound Name [a]	Lit [b]	CAS number	RI exp [c]	RI ref [d]	Occurrence of Compounds [e]		
						Wild type	***fum1* mutant**	***fum6* mutant**
Undentified	UI 1034			1034	-	-	-	3
	UI 1057			1057	-	3, 12, 15, 18	3	3, 12
	UI 1101			1101	-	3, 12, 18	3	3
	UI 1280			1280	-	6	-	-

[a] The identity of compounds was facilitated by comparisons of mass spectra with the NIST17 and Wiley298 mass spectra libraries and retention times of each compound. The UI designation in the last 10 rows of the table indicates unidentified compound.

[b] Lit = Literature, Numbers indicate whether the compounds have been previously reported in *Fusarium* and or *F. verticillioides*. 1 indicates the compound has been previously reported to be produced by *F. verticillioides*; 2 indicates the compound has been previously reported to be produced by *Fusarium* species but not *F. verticillioides*; 3 indicates that a compound has been reported to be produced by a filamentous fungus but not a *Fusarium* species; 4 indicates that the compound has been reported to be produced by fungus but not a filamentous fungus; and 5 indicates that the compound has been previously reported to be produced by a bacterium but not by a fungus [48,92,217,226,256,282–284].

[c] RI exp = Retention indices determined in the current study.

[d] RI ref = Retention indices reported according to the method of Van Den Dool and Kratz on a non-polar HP-5ms column (* in case of HP-5 column) reported in the NIST web book (https://webbook.nist.gov). When no retention index information is reported in the literature, the mention NA (not applicable) is added.

[e] Detection of compounds in cultures of the wild-type strain, f*um1* mutant or *fum6* mutant of *F. verticillioides*. Numbers in individual rows indicate the timepoints (in days) at which the corresponding compound was detected. The occurrence of a compound was reported only if the compound was detected in at least two replicate cultures of a strain at a given timepoint in at least 2 sets.

For all strains, the highest numbers of VOCs were detected on day 3. Thereafter, the numbers tended to decrease over time such that by day 21 the number of VOCs detected was approximately half the number detected on day 3 (**Figure 4-3**). On day 12 and thereafter, fewer VOCs tended to be detected in cultures of the wild type than in cultures of the mutants. In general, more VOCs were detected in cultures of the *fum6* mutant than cultures of the other two strains.

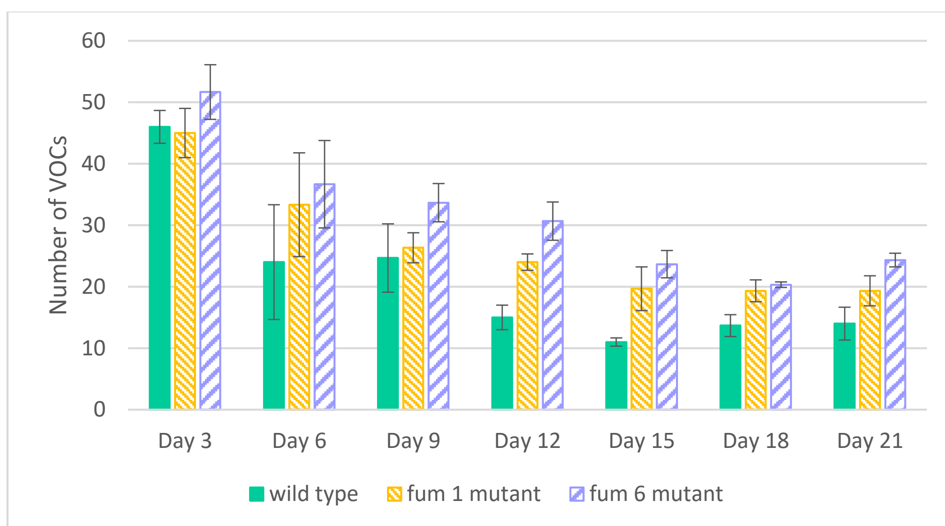


Figure 4-3. Number of VOCs detected in the headspace of three *F. verticillioides* strains over time. For each strain and timepoint, values are mean and standard error based on three replicate cultures from three experiments (n=9).

Figures 4-4-A and **4-4-B** show the results of Partial Least Square Analysis (PLS-DA) applied to VOC data from the three *F. verticillioides* strains at all time points examined. PLS-DA revealed that VOC emission on day 3 was distinct from other days, mostly due to the greater number of VOCs emitted (**Figure 4-4-A**). PLS-DA of the data generated by the three strains (**Figure 4-4-B**) revealed that the wild-type pattern of VOC emission differed from patterns of the two mutants. Moreover, this difference was apparent at all timepoints. The difference suggests that the ability to produce fumonisins can affect VOC emission. Confirmed by the ANOVA results (**Figure 4-4-C**), which indicate the emission of ethyl butanoate, ethyl 3-methylbutanoate, ethyl 2-methylpropanoate and ethyl 2-methylbutanoate by the mutants in contrast to the wild-type. Thus, these 4 esters constitute a significant difference in VOC emissions that distinguishes the fumonisin-producing wild type from the two fumonisin-nonproducing mutants. Moreover, the analysis of emission over the length of the experiment revealed a tendency of increased emission of the 4 esters over time. The results indicate that the mutants emitted the four esters at each timepoint regardless of the gene that was deleted.

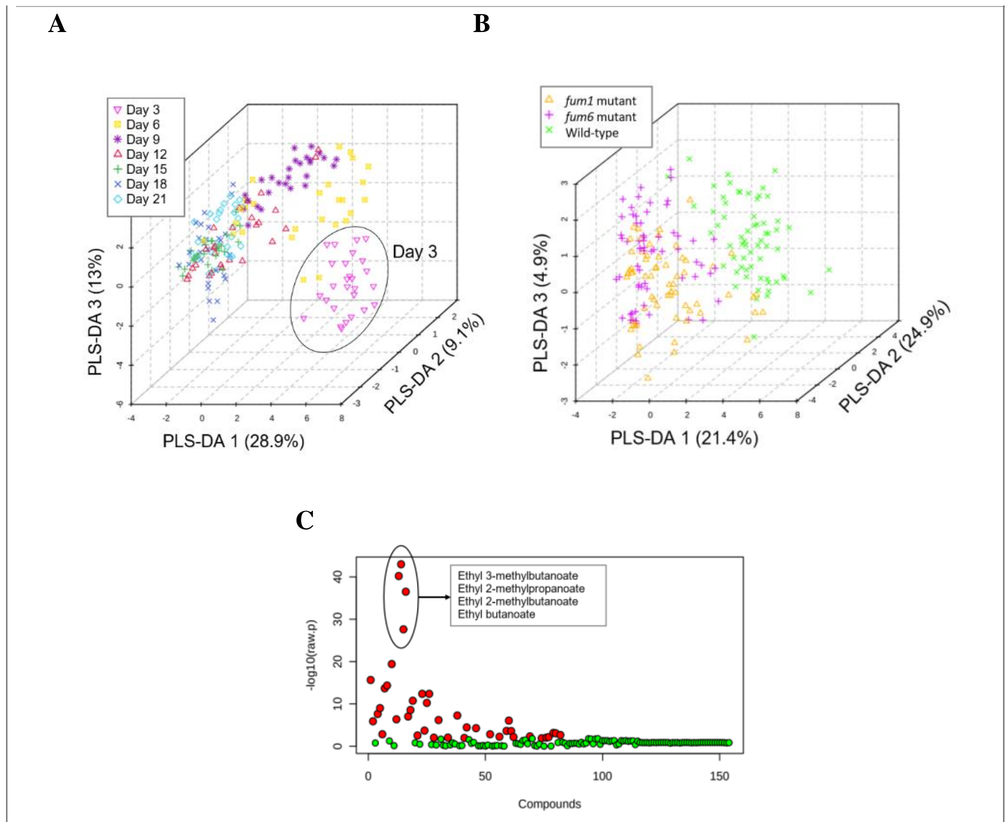


Figure 4-4. (A) Partial Least Square Analysis (PLS-DA) applied to the VOC data by day, with data for the three *F. verticillioides* strains combined; (B) PLS-DA applied on the VOC data by strain, with data from different days combined. (C) ANOVA of VOC data related to the *fum1* and *fum6* mutants and wild-type strain of *F. verticillioides*. Red circles indicate statistically significant differences (p-value =0.05)

2.3. The fumonisins production of *F. verticillioides* wild type strain

Fumonisin production by the wild-type strain was analyzed in three replicates cultures at each time point in three experiments. The analysis revealed that FB_1, FB_2 and FB_3 were present at low levels at 3 – 9 days and began increasing at 12 days to levels as high as 27.7, 6.0 and 5.7 $mg.kg^{-1}$, respectively, at 21 days. Analysis of cultures of the *fum1* and *fum6* mutants confirmed that both mutants do not produce FB_1, FB_2 or FB_3. The levels of FB_1, FB_2 and FB_3 produced by the wild-type strain tended to increase over time in all three replicate experiments, but the levels of FB_2 and FB_3 were lower than those of FB_1 (**Figure 4-5**). After day 12, the levels of fumonisins increased to much higher levels in Experiment 2 (orange curve) than in Experiments 1 and 3.

In Experiment 2, where the overall fumonisin levels were higher, the ratio of FB_1 to FB_2 or FB_3 was 5:1, whereas in Experiments 1 and 3, where the overall fumonisin levels were lower, the ratio of FB_1 to FB_2 or FB_3 was 2.5:1. The higher levels of fumonisins in Experiment 2 were not associated to the presence or absence to a specific VOC. The volatolomes detected in the 3 experiments were similar.

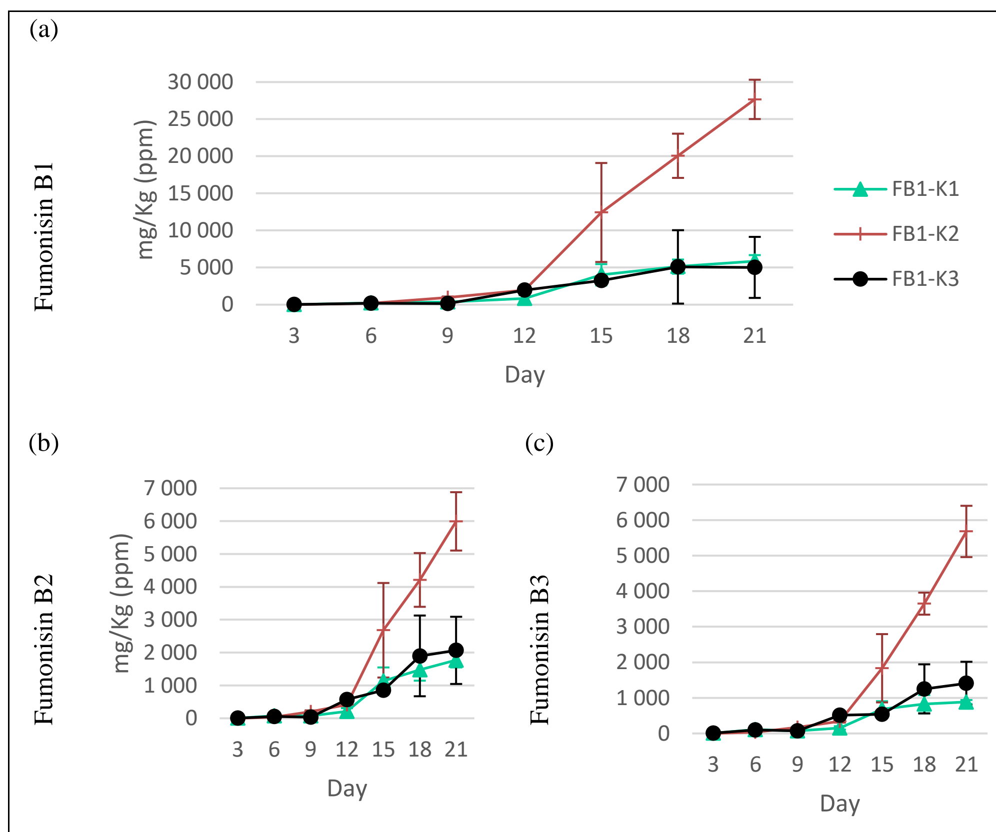


Figure 4-5. Production of fumonisin B1 (FB_1), B2 (FB_2) and B3 (FB_3) on the three experiments (K1 = Experiment 1; K2 = Experiment 2; K3= Experiment 3) of wild-type *F. verticillioides* over a 21-day incubation period.

2.4. The ethyl 3-methylbutanoate reduces growth and fumonisin production

According to the ANOVA and PLS-DA analyses, the four VOCs that exhibited the greatest differences in emission by wild-type versus mutant cultures were esters of butanoate or propanoate (**Figure 4-4-C**). We examined the effects of one of these VOCs, ethyl 3-methylbutanoate (ethyl isovalerate), on growth and fumonisin production in wild-type *F. verticillioides*. After a 6-day incubation period, maize

kernel cultures of the wild type exposed to 10 and 100 µL of ethyl 3-methylbutanoate had less visible mycelial growth and less fumonisins than control cultures that lacked ethyl 3-methylbutanoate (**Figure 4-6**). In contrast, cultures exposed to 0.1 and 1 µL ethyl 3-methylbutanoate were not visibly different and had similar levels of fumonisins as control cultures.

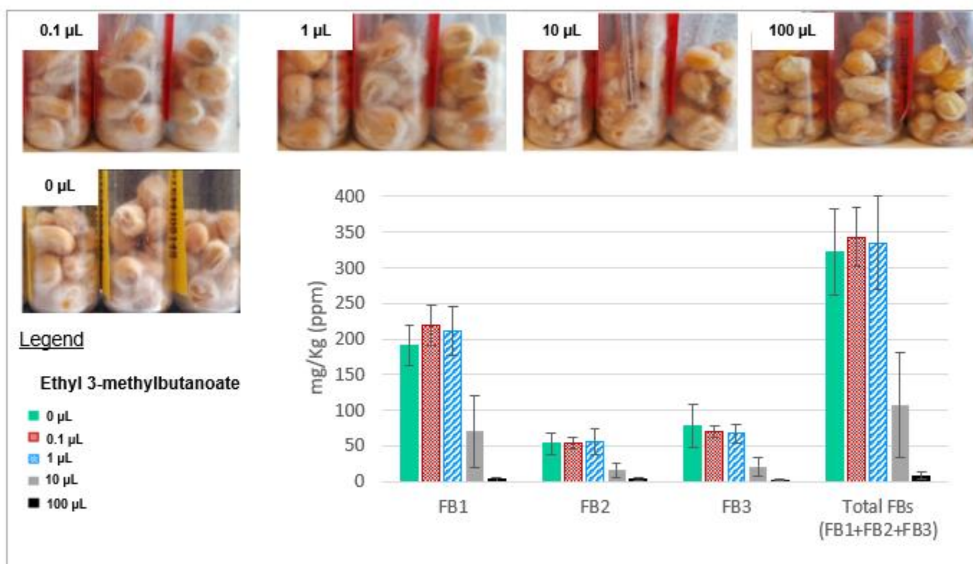


Figure 4-6. Effect of ethyl 3-methylbutanoate exposure on wild-type *F. verticillioides* strain ITEM 10514. Upper panel – physical appearance of maize cultures after a 6-day incubation period. Lower panel – fumonisin production in the same maize cultures and incubation period. Exposure was facilitated by inserting a test tube containing 0 (Control), 0.1, 1, 10, or 100 µL ethyl 3-methylbutanoate into each culture at the beginning of the incubation period (n=3).

3. Discussion

VOCs have been largely examined to characterize fungi with the perspective of developing diagnostic tools for their early identification. However, limited investigations have been devoted to the volatolome profiles of toxigenic fungi. The current study is the first to examine how inactivation of mycotoxin biosynthetic genes can impact on VOC emissions.

3.1. VOCs, biomarkers for maize contaminated by F. verticillioides

Analysis of volatolomes in autoclaved maize cultures inoculated with the three *F. verticillioides* strains revealed potential VOC biomarkers for early detection of *F.*

verticillioides. Among the VOCs commonly reported for the strains we tested, several ones were already known to be emitted by *F. verticillioides*, by other species of *Fusarium*, and even by other microorganisms. On the other hand, we identified some new VOCs for the first time, such as the 4-epi-α-acoradiene sesquiterpene emitted by the wild-type and the mutant strains of *F. verticillioides*. Esters, alcohols and terpenes were the prominent chemical families of VOCs emitted by the three *F. verticillioides* strains. Among the terpenes emitted, we identified the α-cedrene sesquiterpene, previously reported by Dickschat et al. (2011), as component of *F. verticillioides* volatolome. Compounds of terpene family proved to be reliable markers of toxigenic fungi due to their wide diversity [85]. However, because of their planar chemical structure and their common core skeleton, their differentiation can be challenging since certain biosensors can detect only three-dimensional structures. Therefore, would be worthwhile to combine terpenes with other compounds with chirality and greater structural diversity, before using them as biomarkers. Based on the results of the current study, a possible mixture of VOCs to detect *F. verticillioides* could be composed by : the terpenes D-limonene, β-acorenol, α-cedrene, and 4-epi-α-acoradiene, the hydrocarbons p-ethylanisole, octane, 2,4-dimethylheptane, the esters 2-methylbutyl acetate and 3-methylbutyl acetate, and the alcohols 4-ethyl-2-methoxyphenol, 3-methylbutan-1-ol, 2-methylpropan-1-ol, and 4-ethylphenol. Other VOCs which have been emitted only at a specific day, could provide useful information not only on *F. verticillioides* occurrence, but also on its growth stage (**Table 4-1**). However, further investigations need to be carried out using additional strains of *F. verticillioides*, other *Fusarium* species, and other toxigenic fungi to confirm the specificity of this pool of biomarker VOCs toward *F. verticillioides*. Beyond identifying the fungus contamination, pinpointing VOC biomarkers associated with fumonisin production was also our strategic objective in order to improve risk assessment related to *F. verticillioides* occurrence in the field. Falasconi et al. (2005) used an electronic nose successfully, and could distinguish fumonisin-producing and nonproducing strains of *F. verticillioides*, after six days of growth, demonstrating the specificity of different VOC profiles. In our study, we were able to detect differences in VOC emissions by fumonisin-producing and nonproducing strains as early as the third day of inoculation. The distinction between the two types of strains was further facilitated by the accumulation of 4 esters, ethyl 3-methylbutanoate, ethyl 2-methylbutanoate, ethyl 2-methylbutylpropanoate, and ethyl butanoate.

Esters are formed by the condensation of their respective acids with ethanol. Since ethanol is a constant and abundant compound emitted by all three strains, the difference in ester emission can be attributed to differences in the production of the corresponding acids, which were detected exclusively in the mutants (**Table 4-1**). Interestingly, 3-methylbutanoic acid was detected only after day 9 in both mutants, suggesting that during the initial nine days, the acid was rapidly converted to the corresponding ester, detectable from day 3 (**Table 4-1**). Therefore, analysis of production of 3-methylbutanoic acid and 2-methylbutanoic acid, rather than the

corresponding esters themselves, could provide insight into metabolic and VOC differences between the mutants and the wild type strains.

In addition, the volatolomes of the mutant strains consisted of a high number of VOCs (**Figure 4-3**), with higher numbers of esters, alcohols, and acids than wild type (**Figure 4-2**). The fourteen specific VOCs observed in both mutants indicate that the deletion of genes required for fumonisin biosynthesis can impact on the VOC emissions and trigger the same fungal reaction in both mutants (**Figure 4-2**). Certain VOCs, like 3-methylbutanoic acid and butane-2,3-diol, exclusively detected in the mutant emissions, were also reported to be emitted by certain bacterial species belonging to genus *Enterococcus*. These two compounds have been tested for their potential antifungal activity and for their ability to reduce fumonisin production in *F. verticillioides*. Butane-2,3-diol exhibited greater antifungal activity than 3-methylbutanoic acid and caused a considerable reduction in FB_1 levels when applied at a high concentration [219]. In addition, some VOCs were specific for each mutant, suggesting that the suppression of fumonisin production at different points of the pathway and the accumulation of certain intermediates can differentially impact VOC emission.

3.2. Ethyl 3-methylbutanoate, a fungistatic and antifumonisin agent

Recent studies have explored the VOCs emitted by bacteria, yeasts, other fungi and plants, including their derivatives such as essential oils, in order to select reliable biocontrol agents with antifungal and antimycotoxin effects [214]. Our study showed that the ethyl 3-methylbutanaote could be a potential biocontrol agent, since the exposure by fumigation of the *F. verticillioides* wild-type to it, led to a significant reduction in fungal growth and fumonisin production on maize kernels since an introduction of 10 µL (for informational purposes 3.3 mM) of ethyl 3-methylbutanoate into the fungal atmosphere. Moreover, the higher the volume introduced, the greater the reduction in these two parameters was noted. A similar observation was reported with different volumes of cinnamon essential oil, and where the most effective volume over the 21 days of the study was 40 µL (for informational purposes 6.2 mM) (i.e. the large volume tested) [220]. In addition, several VOCs were tested towards maize kernels infected with *F. verticillioides*. While the *trans*hex-2-enal demonstrated an antifungal property and a lack of efficiency in fumonisin reduction, the transhex-2-en-1-ol showed both fungal and fumonisin inhibition, demonstrating that each compound has a variable impact on the fungal metabolism of *F. verticillioides* [221,222]. The chemical structure of a compound is a key factor in its efficacy, as it determines its mode of action and thus induces more or less significant effects at different levels [222]. Indeed, although a positive correlation between mycelial growth and fumonisin production has been established [34], certain VOCs can sometimes increase mycotoxin production, even if a fungistatic effect has been observed [223].

The efficiency of the ethyl 3-methylbutanoate could be attributed to various mechanisms. These may include mycelial damage, alterations in physiology and metabolism, or changes in gene expression, including those regulating fumonisin production. Xing et al. reported that the application of essentials oils such as cinnamon one caused inhibitory effects on *F. verticillioides* growth that they related to morphological alterations in cell membranes [220]. On the other hand, Ferrigo et al. studied the reduction of fumonisin by monitoring of *fum* genes. The contact application of carvacrol and ellagic acid downregulated certain genes, such as *fum19*, involved in fumonisin secretion [224]. However, from our study we could not yet asses the mode of action of ethyl 3-methylbutanoate that remains unknown.

Although this, the antifungal activity and the reduction in fumonisin production, induced by the ethyl 3-methylbutanoate, combined with its very low cytotoxicity to humans and animals, make it, a potential candidate for biological control of *F. verticillioides* [225].

3.3. VOC specificity and pigmentation of mutants

A purple pigmentation was constantly associated in all experiments only for the mutant mycelia grown on maize kernels. The purple pigmentation appeared evident starting in different days for the two mutants. On the contrary, the wild-type mycelium appeared always white (supplementary figure). A link between production of monoterpenes and secretion of naphthoquinone pigments (bikaverin and fusarubin) in *F. verticillioides* has been previously reported [226]. However, in our study, the esters were the compounds specifically correlated with the mutants. A link between pigmentation and toxicity has already been proved in *F. verticillioides,* showing that this species can produce a wide range of pigments, involved in important ecological functions due to their antifungal and antibacterial properties [227–230]. Although both biosynthetic pathways of pigments and fumonisins use acetyl-CoA and malonyl-CoA precursors, they follow different biochemical ways. Therefore, the suppression of fumonisin biosynthesis, due to some *fum* gene deletions, could address the use of acetyl-CoA and malonyl-CoA in other metabolic pathways [231]. Deletion of *FUM1* gene blocks fumonisin production at the beginning of the biosynthetic pathway, before the fumonisin polyketide is synthesized [232]. As a result, *FUM1* deletion could result in accumulation of fumonisin precursors such as malonyl-CoA and/or tricarboxylic acid (TCA) precursors of the fumonisin TCA esters [230]. On the other hand, deletion of *FUM6* blocks the pathway after the polyketide has been synthesized and has undergone condensation with alanine, leading to an accumulation of fumonisin intermediates that could be toxic for the fungus [233–236].

As is the case with most secondary metabolite biosynthetic genes in fungi, all fumonisin biosynthetic genes are located adjacent to one another in a gene cluster, to prevent the accumulation of toxic biosynthetic intermediates [237]. Inactivation of *FUM1* and *FUM6* in the two mutants and the resulting accumulation of potentially toxic precursors or intermediates could induce stress in *F. verticillioides*. Therefore, the altered pigmentation could be associated with interruption of the fumonisin

biosynthesis pathway. We hypothesize that such stress caused the emission of a higher number of VOCs and production of a higher number of pigments by the mutants compared to the wild type, as a mechanism by which *F. verticillioides* compensates for the loss of fumonisin production. In order to confirm such hypothesis, further experiments using additional mutants generated independently of those generated in the current study should be carried out. With respect to the *fum1* mutant, other naturally occurring variants of *F. verticillioides* that have mutations that render *FUM1* nonfunctional have been identified [238]. So, future VOC studies could compare the laboratory-generated *fum1* mutant examined in the current study to the naturally occurring *fum1* mutants identified [232].

4. Conclusion

The comparison of volatolomes of the three *F. verticillioides* strains has revealed the potential of VOCs to distinguish fumonisin producers from fumonisin nonproducers, based on emissions of four esters. Furthermore, the deletion of *FUM1* or *FUM6* resulted in the lack of fumonisin production and led to metabolic changes reflected in the specific volatolomes of mutant strains, along with changes in pigmentation. Ethyl 3-methylbutanoate was among the VOCs associated with the lack of fumonisin production. When applied exogenously to cultures of the *F. verticillioides* wild type, this VOC inhibited both mycelial growth and fumonisin production. Additionally, the high levels of pigment produced by the *fum6* mutant could be the consequence of the accumulation of biologically active fumonisin biosynthetic intermediates, which could have induced stress and subsequently increased VOC production compared to the wild type. Further studies are necessary to elucidate the mechanism of action of ethyl 3-methylbutanoate on fungal metabolism and the eventual link between pigments and block in the fumonisin biosynthetic pathway. Moreover, this research has demonstrated the potential of VOCs to be used as a tool to monitor genetic modifications and to control fumonisin contamination, selecting a non-cytotoxic ester.

For the first time, this study used *F. verticillioides* mutants with targeted gene deletions to compare the emission of VOCs between fumonisin-producing and non-fumonisin-producing strains. As far as we are aware, the current study is pioneering in the evaluation of the effects of FUM gene inactivation on *F. verticillioides* metabolism, not only with respect to fumonisin production, but also with respect to its VOC emissions. Finally, we showed potential VOC biomarkers for *F. verticillioides* diagnosis, and the possible use of a new VOC as biocontrol agent against *F. verticillioides*.

5. Supplementary materials

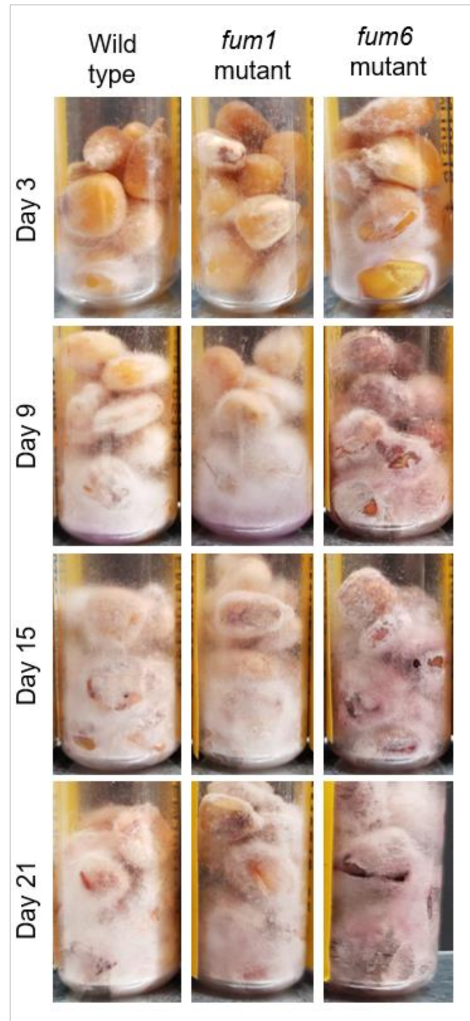


Figure 4-7. Visual observation of the mycelium growth and pigmentation of the three strains of *F. verticillioides* at day 3, 9, 15 and 21

6. Material and method

6.1. The F. verticillioides strains

F. verticillioides strains ITEM 10514, ITEM 10515 and ITEM 10516 were examined in this study and are deposited in the Culture Collection ITEM of the Institute of Sciences of Food Production (ISPA), National Research Council of Italy (CNR), Bari, Italy. ITEM 10514 [NRRL 20956 in the ARS Culture Collection (NRRL), FRC M-3125 for the *Fusarium* Research Center at Pennsylvania State University, and FGSC 7600 in Fungal Genetics Stock Center Culture Collection Kansas State University, Manhattan, KS, USA] is a wild-type strain that produces FB_1, FB_2 and FB_3. Strain ITEM 10515 (GFA2364) was derived from ITEM 10514 by partial deletion of *FUM1*, the gene that encodes the polyketide synthase required for fumonisin production [232]. Mutants resulting from the deletion of all or part of *FUM1* (*fum1* mutants) do not produce fumonisins or fumonisin biosynthetic intermediates [232]. Strain ITEM 10516 (GFA3075) was derived from ITEM 10514 by deletion of the gene that encodes the cytochrome P450 monooxygenase that catalyzes formation of the hydroxyl groups at carbon atoms 14 and 15 of B-series fumonisins [235]. The hydroxyl groups are necessary for formation of the tricarballylic ester groups of fumonisins. Mutants resulting from deletion of *FUM6* (*fum6* mutants) do not produce fumonisins but instead produce some early intermediates in the fumonisin biosynthetic pathway [234]. During this study strains were stored at -80 °C in 25% glycerol. The maize seeds used are native maize varieties from the Wallonie area Namur region, Belgium, and were provided by Walagri.

6.2. The chemical material for the fumonisin analysis

Analytical-grade acetonitrile (ACN), methanol (MeOH), o-phthaldialdehyde (OPA), 2-mercaptoethanol, phosphate buffered saline (PBS) tablet, sodium tetraborate ($Na_2B_4O_7$), glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy) and Sigma (St. Louis, MO, USA). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Standards of FB_1 and FB_2 were obtained from Biopure (Romer Labs Diagnostic GmbH, Tulln, Austria). Glass microfiber filters (Whatman GF/A) and paper filters (Whatman No. 4) were bought from Whatman (Maidstone, UK). FumoniTest^{TM} Wide Bore immunoaffinity columns (IACs) were purchased from Vicam L.P. (Milford, MA, USA). OPA reagent solution was prepared by dissolving 40 mg OPA in 1 mL of MeOH and diluting with 5 mL 0.1 M sodium tetraborate solution. Then, 50 µL 2-mercaptoethanol were added and the solution was mixed for 1 min and stored in the dark up to 1 week at room temperature [239].

6.3. The experiment: fungal inoculation and maize

Conidial suspensions were prepared by growing strains of *F. verticillioides* on SNA medium (for 1L: 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose and 20 g agar) at 23°C in darkness for 7 days. After, conidia were harvested from the resulting cultures using 0.5% Tween 20 in water. Concentrations of conidia were determined using a Bürker cell counter. A total of 1×10^7 conidia of were inoculated in the 20 mL vial containing 4 g of maize kernels that had been previously hydrated to 45% (mass/mass) and autoclaved. The resulting maize kernel cultures were incubated at 23 °C in darkness.

6.4. The VOCs separation and identification of the three strain of F. verticillioides

After 3, 6, 9, 12, 15, 18 and 21 days of incubation, VOCs of inoculated samples were collected with a Solid Phase Micro-Extraction (SPME) divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Darmstadt, Germany, DVB/CAR/DDMS, 50/30µm, 24 Ga) for one hour while the samples were incubated at 25°C in a heating block. After the SPME fiber desorption at 250 °C, injection by splitless using an SPME inlet coating (78.5 mm a 78.5 mm × 6.5 mm × 0.75 mm, Supelco, Bellefonte, PA, USA) was performed. VOCs were separated with a HP-5ms column (30 m × 0.250 mm × 0.25 µm) with a nonpolar 5%-phenylmethylpolysiloxan solid phase (Agilent Technologies, Santa Clara, CA, USA) and helium as a mobile phase with a flow rate of 1 $mL{\cdot}min^{-1}$, using a gas chromatography-mass spectrometry (GC-MS) system, Agilent Technologies GC 7890B (Agilent Technologies, Palo Alto, CA, USA) equipped with a robotic multi-purpose sampler (MPS) Gerstel (Gerstel©, Mülheiman der Ruhr, Germany) that included an SPME tool unit. The GC temperature program was as follows: 40 °C for 5 minutes, 8 $°C{\cdot}min^{-1}$ up to 240 °C, 30 $°C{\cdot}min^{-1}$ up to 290 °C, and a final hold at 290 °C for 5 minutes. VOCs were detected by MS with Agilent MSD 5977B (Agilent Technologies). The MS analysis employed electron ionization (EI) mode at 70 eV over a scan mass range of 35 to 350 amu. The ion source temperature was 250 °C and MS source temperature at 280 ˚C. VOCs were identified based on comparisons with mass spectra of the NIST17 and Wiley298 libraries and retention indices. Retention indices (RI) were calculated using a standard solution of saturated n-alkane C6-C30 (1000 $mg{\cdot}mL^{-1}$ in hexane, Supelco, Overijse, Belgium), analyzed under the same conditions as the samples. Calculated RI were compared with those reported in literature.

Three independent experiments were performed and three replicates for each sample were carried out and analyzed. The VOCs detected in at least 2 experiments by at least two of their respective replicates and after deleting molecules emitted by the blank (i.e. the system containing only the maize kernels not inoculated) were reported **Table 4-1**. However, the figures generated by the statistical analysis included all the raw data.

6.5. The fumonisin analysis

After collection of VOCs, maize kernel cultures were stored at -20 °C until processing for fumonisin analysis. Frozen cultures were dried in an oven at 65°C for 48 h and then powdered. Extraction of fumonisins from maize was carried out according to the AOAC Official method 2001.04 [239] for determination of FB_1 and FB_2 in maize and cornflakes with some modifications. Briefly, aliquots of sample (1 g) were extracted with a mixture (10 mL) of methanol:acetonitrile:water (25:25:50, v/v/v) by shaking for 40 min. The extract was filtered through a filter paper. An aliquot of filtrate (3 mL) was diluted with PBS (12 mL) and filtered through a glass microfiber filter. Then a volume of filtered extract (10 mL) was cleaned up through FumoniTest^{WB} immunoaffinity column (IAC). An appropriate dilution of diluted extract, before loading onto the IAC, was performed with PBS when fumonisin concentrations in the diluted extract were higher than the maximum IAC binding capacity. After elution, the column was washed with 10 mL PBS and then fumonisins were eluted with 2 x 1 mL methanol followed by 2 x 1 mL water. Then the extract was dried under a nitrogen stream at about 50°C and reconstituted with 500 µL of water:acetonitrile (70:30, v/v). Sample extracts were derivatized with OPA reagent and analyzed by High-Performance Liquid Chromatography (HPLC) according to the procedure described by De Girolamo et al. (2011), with some modifications [240]. In particular, the HPLC apparatus was an Agilent 1100 Series chromatographic system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, autosampler, column thermostat set at 30°C and a spectrofluorometric detector (model G7121A, λ_{ex} = 335 nm, λ_{em} = 440 nm). The analytical column was a LUNA C18 (150 mm x 4.6 mm, 5 µm) (Phenomenex, Torrance, CA, USA) with a 3mm i.d., 0.45 µm pore size guard filter (Rheodyne, Cotati, CA, USA). Quantification of fumonisins (FB_1, FB_2) was performed by measuring peak areas at FB_1 and FB_2 retention times and comparing them with the relevant calibration curves. FB_3 was quantified by comparing with the calibration curve of FB_2 as reported by Palacios et al. (2015) [241]. The autosampler was programmed to mix 50 µL of sample extracts or standard with 50 µL of OPA reagent, mix for 50 s, incubate for 2 min and then inject all the derivatized mixture. The mobile phase was a mixture of water:methanol:acetic acid (26.5:72.5:1, v/v/v) eluted at a flow rate of 1 $mL.min^{-1}$. With these conditions, retention times of FB_1, FB_2 and FB_3 were about 8.9, 23.0 and 20.3 min, respectively. The LOD values were 5 $\mu g.kg^{-1}$, while LOQ values were 16 $\mu g.kg^{-1}$.

6.6. The effect of ethyl 3-methylbutanoate on growth and fumonisin production

Maize kernel cultures of the wild-type *F. verticillioides* strain (ITEM 10514) were prepared as described in section 2.3. Immediately after the cultures were started, a 31 x 6 mm (0.1 mL) glass test tube containing 0.1, 1, 10 or 100 µL of ethyl 3-methylbutanoate (Sigma-Aldrich, Schnelldorf, Germany, ≥98%) was placed into each culture. This method of exposure was used to avoid physical contact of liquid ethyl 3-

methylbutanoate and *F. verticillioides*. Each experiment for each volume was carried out in 4 replicates. The cultures were then incubated for 6 days in darkness at 23°C. Fumonisin production was thereafter assessed by the HPLC method described in section 6.5.

6.7. The statistical model

Principal Component Analysis (PCA), Partial Least Squares Analysis (PLS-DA) and ANOVA models were applied using metaboanalyst (http://www.metaboanalyst.ca). PCA and PLS-DA were built using four components to discriminate the three strains of *F. verticillioides*: wild type, *fum1* mutant, and *fum6* mutant. For all models, the features (i.e., GCMS profiles using their percentage of area) were log transformed and mean centered. The discrimination was visualized by plotting the first PLS-DA components (or PCA (data not shown in the results)). The ANOVA was used to compare differences among strains and timepoints (p-values ≤ 0.05 were considered significant).

Chapter 5

Interplay of VOCs and Mycotoxin Production in *Aspergillus flavus* and *Fusarium verticillioides*: Exploring Individual and Co-inoculation Strategies in Contact and Non-contact Conditions

Several observations were highlighted by the previous chapter on the *F. verticillioides* and its *fum* mutants. The use of genetically modified mutants allowed us to compare the volatolomes produced and attribute their differences to these specific genetic modifications, involving the fumonisin biosynthetic pathway, thus clarifying the potential link between these two biological pathways (VOCs and mycotoxins).

The first observation revealed the appearance of a purple pigmentation in the mycelium of the mutants. Furthermore, the comparison of VOCs emitted by these three strains on different days demonstrated that the mutants produced a greater number of VOCs than the wild-type strain, and it was possible to differentiate the fumonisin-producing strain from the other non-producing strains based on four esters specifically emitted by the mutants.

Studying fungal species individually provides insights into their functioning and helps us to understand their basic biological mechanisms. However, the environment consists of a multitude of fungal, bacterial, and other species coexisting in the same ecological niche, and interactions between species are numerous, shaping their behavior, to ensure survival and proliferation. Therefore, after studying fungal species individually by monitoring their respective mycotoxins and VOCs, to introduce them into competition, to examine the fluctuations associated with their co-inoculation, could offer useful tools for achieving insights on the biological basis of their interaction always with the aim of approximating realistic agronomic conditions.

The competition between *A. flavus* and *F. verticillioides* was carried out using two modes of interaction: contact interaction, where the fungi shared their substrate and atmosphere, and non-contact interaction, where the fungi shared only their atmosphere. This allowed us to understand the contribution solely attributed to VOCs without competition for substrate. To reflect the diversity existing within *A. flavus*, both toxigenic (aflatoxin producers) and non-toxigenic (non-aflatoxin producers) strains were used in competition with *F. verticillioides* (fumonisin producer).

The fifth chapter of the thesis presents the work which will be published in a research article in 2023:

Josselin, L., Howa-Lopez F., Proctor R.H., De Clerck C., De Boevre M., Fauconnier, M-L and Moretti A. (2023) **"Interplay of VOCs and Mycotoxin Production in *Aspergillus flavus* and *Fusarium verticillioides*: Exploring Individual and Co-inoculation Strategies in Contact and Non-contact."**

Abstract

Nature is a complex and wise network of interactions among various organisms, and recreating these intricate relationships in vitro conditions is challenging. To gain a deeper understanding of these complex interactions, we looked at two mycotoxigenic fungal pathogens species often co-occurring on maize: *Aspergillus flavus* and *Fusarium verticillioides*. These fungi can produce secondary metabolites (volatile organic compounds (VOCs) and mycotoxins) known to help them to establish dominance in their ecological niche. Hence, an examination of the VOCs, which have a crucial role in inter and intra-species communication, along with mycotoxins (specifically aflatoxins and fumonisins) was conducted. To assess the modifications resulting from the interaction between these species, we initially grew their colonies separately. Then, *F. verticillioides* and *A. flavus* were co-inoculated under two conditions: contact and non-contact. In the contact condition, the two species shared both their substrate and atmosphere, while in the non-contact condition, they only shared their atmosphere, thus avoiding possible interference caused by soluble molecules diffused in the substrate. In addition, the non-contact condition allowed to observe the specific impact of VOCs on the mycotoxin production and volatolomes of the opposite species. For *A. flavus*, two strains were used: a toxigenic strain, capable of producing aflatoxins, and a non-toxigenic strain. The primary objective was to unravel the intricate relationship between VOCs, mycotoxins, and the interplay between two species. A general modification in the emitted VOCs was observed, including the emergence of specific VOCs related to the toxicity of the *A. flavus* strain, such as benzenemethanol and aristolochene, when it is in competition with *F. verticillioides*. Moreover, specific VOC corresponding to the mode of interaction were detected. In the non-contact condition, 4-ethylbenzamide was identified, while in the contact condition, junipene and α-neoclovene were detected. Concomitantly, significant variations in mycotoxin production were recorded. This comprehensive investigation provides insights into the dynamic nature of species interactions and their potential integration in the field management.

Keywords

Interaction, contact, non-contact, *Aspergillus*, *Fusarium*, Fumonisins, Aflatoxins, VOCs, co-occurrence, co-inoculation

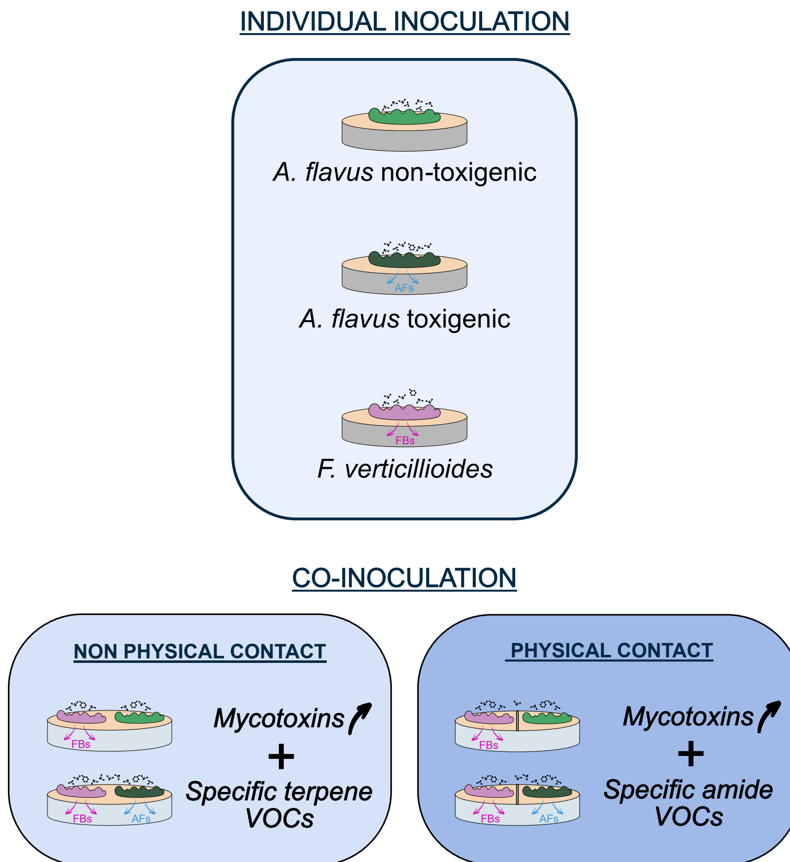


Figure 5-1. Graphical abstract of the Chapter 5

1. Introduction

Co-occurring organisms often exhibit intricate ecological interactions that shape living community dynamics and influence ecosystem functioning [11]. Understanding the nature of these interactions is crucial for untangling the complexities of ecological systems. In this study, we investigate the interaction between two harmful mycotoxigenic fungal pathogens, often co-occurring on maize: *A. flavus* and *F. verticillioides*. These species are known to produce two harmful mycotoxin families both under EU legislation [242,243]: aflatoxins in the case of *A. flavus* and fumonisins for *F. verticillioides* [67]. Maize is an important crop worldwide since it represents a staple food for human and animal. However, maize kernels are often contaminated by

aflatoxins and/or fumonisins [244] making them unavailable as food and feed. Prevention remains the best approach to tackle maize contamination by both species, by using prediction models to be implemented with appropriate methods in the field such as agronomic tools or early detection method for both toxigenic fungi and mycotoxins [245,246]. Several studies have shown that these two species can be detected in the maize plant together at different growth stage [247]. Therefore, it is important to evaluate the reciprocal influence on their biological processes, in order to better define management strategies for reducing their impact on crops.

To gain a deeper understanding of the complex interactions between *A. flavus* and *F. verticillioides*, we focus our investigation on two of their secondary metabolite classes: volatile organic compounds (VOCs) and mycotoxins. VOCs serve multiple functions across organisms, such as communication to attract or repel other organisms to provide their needs [52,57], defense against competitors [49,57], and regulation of their life cycle through auto-induction as stimulants or inhibitors [42,52,58].

Several studies have demonstrated that antagonistic species can emit VOCs that impact both the growth and mycotoxin production of mycotoxigenic fungi [223,246]. These findings have led to the discovery of potential bio-control agents, such as *Trichoderma spp.* or *Bacillus spp.* [105]. In addition, among VOCs, essential oils containing cinnamaldehyde, citral and eugenol have been proved to reduce the growth and mycotoxin production of both *A. flavus* and *F. verticillioides* [248]. As the presence of both species together occurs in the realistic condition scenario it seems promising to investigate the reciprocal antagonist effects that could arise from this co-occurrence.

Additionally, since *A. flavus* occurs in the field as aflatoxigenic and non-aflatoxigenic strains which are genetically divergent and not able to interbreed, it also appears interesting to study the interactions between *F. verticillioides* and non-aflatoxigenic strain of *A. flavus*, as this could provide help in the understanding of the role of aflatoxins in these interactions.

To isolate the effects of VOCs independently from all the molecules involved in the interactions, two experimental conditions were considered. Firstly, the species were grown alone to detect their individual blends of VOCs and respective mycotoxin production (individual inoculation). Then, the VOCs and mycotoxins were monitored when the two species were grown together, sharing the same media and atmosphere (contact condition). Finally, the same parameters were measured when the two species were grown in separate substrates but still shared their atmosphere (non-contact condition). The production of VOCs and mycotoxins by each strain growing alone was analyzed, followed by placing the *F. verticillioides* strain in the presence of either a toxigenic or non-toxigenic *A. flavus* strain. The interaction between the two strains was studied under two conditions: non- contact, where the strains share the atmosphere, but the substrates are separated, and contact condition, where both fungi share both the atmosphere and substrate.

2. Results

2.1. *Visual observation of the strains*

Visual observations revealed a slight reduction in the colony size of toxigenic *A. flavus* when co-cultured with *F. verticillioides* in non-contact condition, compared to its individual growth (**Figure 5-2**). Another noteworthy observation was the traits of the zone between the two species in the contact condition, on day 8. An inhibition zone was observed between toxigenic *A. flavus* strain and *F. verticillioides* strain, with the latter appearing to surround *A. flavus*. Conversely, the opposite trend was observed with the non-toxigenic strain of *A. flavus*, with, the attempt of *A. flavus* non-toxigenic to surround the colony of *F. verticillioides* until coming into contact with it (zoom in **Figure 5-2**).

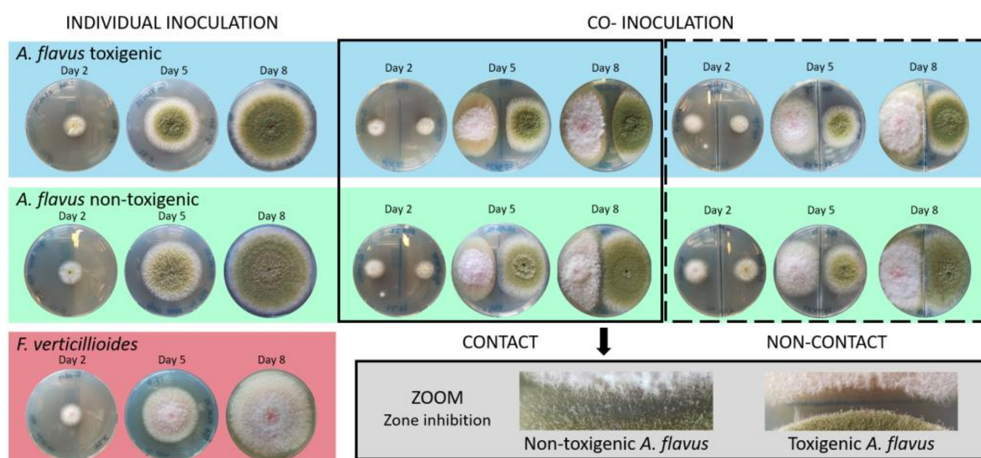


Figure 5-2. Growth evolution of toxigenic *A. flavus*, non-toxigenic *A. flavus* and *F. verticillioides* under the three conditions (individual, non-contact and contact)

2.2. *VOCs emitted by the strains under the different conditions*

Firstly, the observation pertaining the variation in the number of VOCs was noted. **Figure 5-3** illustrates the number of VOCs emitted by each strain over time. When grown alone, the *A. flavus* toxigenic strain exhibited the highest number of emitted VOCs, followed by *F. verticillioides*, and then the *A. flavus* non-toxigenic strain. Both the toxigenic *A. flavus* and *F. verticillioides* strains displayed an increase in the number of VOC emitted until day 5, after which the number of VOCs remained constant. On the other hand, the non-toxigenic *A. flavus* strain recorded a lower number of VOCs on day 5 compared to day 2 and 8, where similar numbers were observed. Interestingly, when *F. verticillioides* was in contact condition with *A.*

flavus, regardless of the strain's toxicity, the contact condition resulted in a higher number of emitted VOCs compared to non-contact condition.

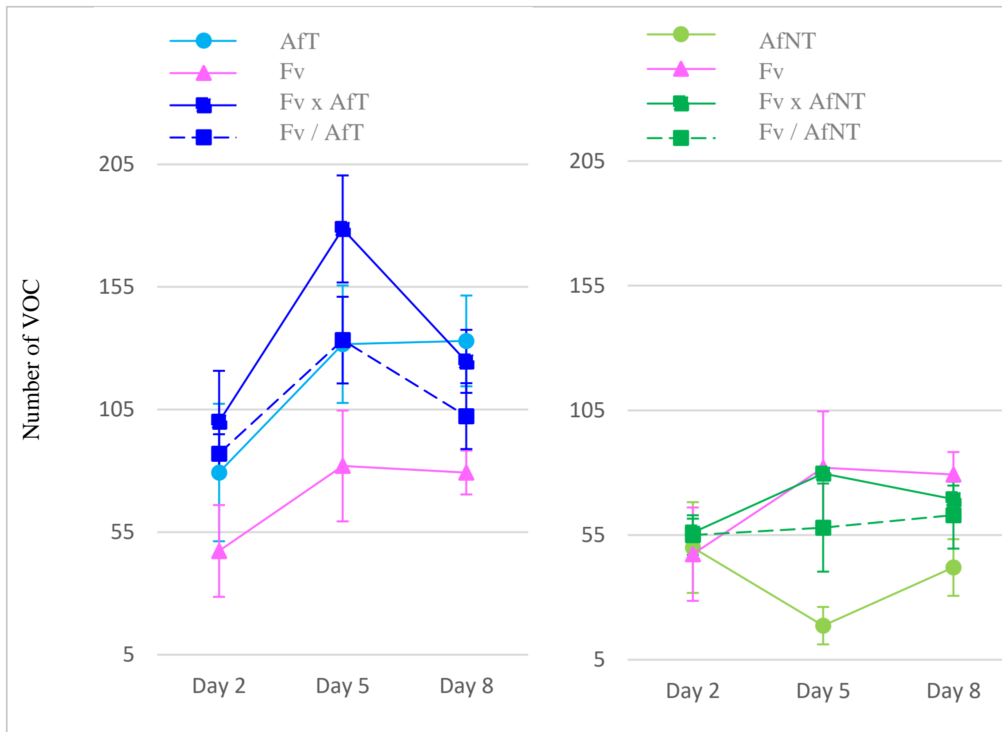


Figure 5-3. Evolution of the number of VOCs emitted by toxigenic *A. flavus* (AfT), non-toxigenic *A. flavus* (AfNT) and *F. verticillioides* (Fv) under the three conditions (individual, non-contact (/) and contact (x)

The distribution of these VOCs in term of chemical classes over the days was compiled in Figure 3. Alcohols and sesquiterpenes were the predominant chemical families emitted by *F. verticillioides* and toxigenic *A. flavus*, whereas alcohols and alkanes were predominant for non-toxigenic *A. flavus* (**Figure 5-4).** A common trend can be observed for sesquiterpenes for both *A. flavus* strains in the co-inoculation condition, in a comparison with the individual inoculation condition (**Table 5-1**). Specifically, their numbers increased in the contact condition, but decrease in the non-contact condition. A peak in sesquiterpene production was also observed on day 5 for *A. flavus* toxigenic in all conditions. The behavior of monoterpenes varied depending on the toxicity of *A. flavus* strains, as monoterpene levels increased with the toxigenic strain but decreased with the non-toxigenic strain (**Table 5-1**).

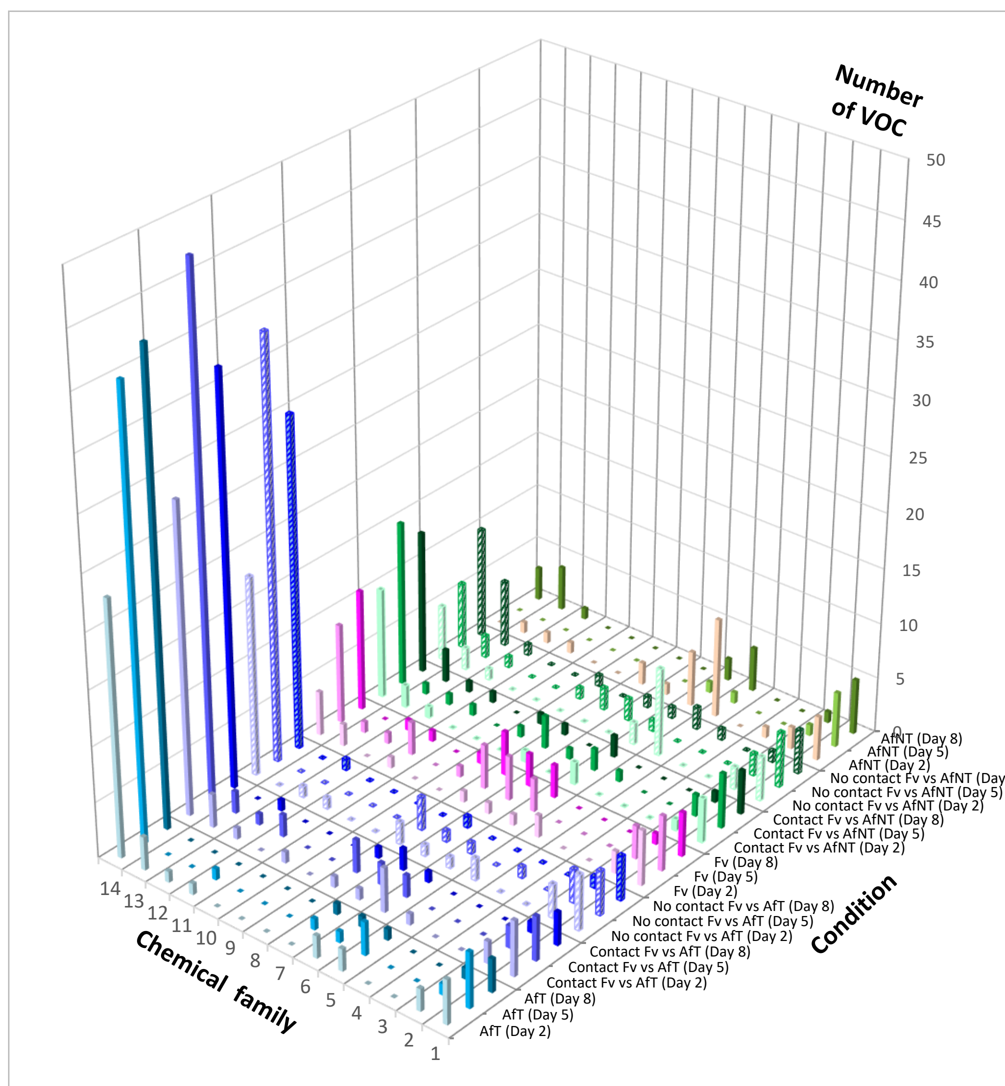


Figure 5-4. Chemical family distribution related to each strain and conditions

Condition: (AfT) toxigenic *A. flavus*, (AfNT) non-toxigenic *A. flavus*, (Fv) *F. verticillioides*, (vs) interaction

Chemical family: (1) Alcohol, (2) Aldehyde, (3) Acid, (4) Amide, (5) Alkane, (6) Alkene, (7) Ketone, (8) Ester, (9) Ether, (10) Amine, (11) Non identified, (12) Other, (13) Monoterpene, (14) Sesquiterpene

Table 5-1. Main observation of the chemical classes depending on the conditions and the nature of interaction

		When F. verticillioides is interacting with	
		A. flavus **toxigenic**	***A. flavus*** **non-toxigenic**
Kind of interaction	**Contact**	Increase in monoterpenes General increase in sesquiterpenes	General increase in sesquiterpenes
	Non-contact	General decrease in sesquiterpenes	Increase in alkanes Increase in monoterpenes since day 5
	Both	All chemical families have the same trend with a peak at day 5	Difference in esters

A clear distinction among the three strains in their individual and co-inoculation conditions can be observed in the PLS-DA analysis of **Figure 5-5-A**. When specifically focused on the data related to the interactions (**Figure 5-5-B**), this analysis confirms their separation, and reveals that, in addition to the distinction based on the type of interaction (contact and non-contact), the toxicity of *A. flavus* strain also induced variations in the volatolome. Therefore, the PLS-DA analysis reinforced the findings presented in **Figure 5-6**, highlighting the same compounds of interest.

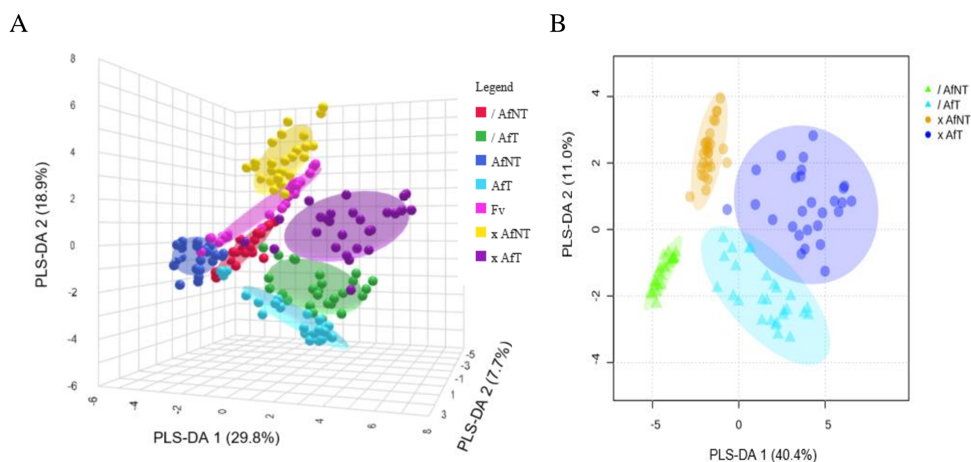


Figure 5-5. PLSDA (Partial Least Square Analysis) applied on the data (A) of the *F. verticillioides* (Fv), *A. flavus* toxigenic (AfT) and non-toxigenic (AfNT) strains single and under the non-contact (/) and contact interaction (x); (B) of the interactions.

When the species were placed in interaction, a modification of the volatolome was noticed, implying a disappearance and an appearance of certain VOCs as we noted

previously. Among the new VOCs emitted, specific VOCs were found to be associated with the type of *A. flavus* strain and/or the type of interaction (**Table 5-1**). Although no VOCs were common to all conditions (i.e. emitted for both interactions and both strains), interesting VOCs were put forward. Indeed, the interaction with a physical contact induced the emission of junipene and α-neoclovene, while non-contact condition resulted in the emission of 4-ethylbenzamide (Class 1 and 2 in **Figure 5-6**) regardless the *A. flavus* strain toxicity. VOCs specifically emitted by a strain were also found, with an emission of benzenemethanol for the *A. flavus* toxigenic strain and the emission of aristolochene for the non-toxigenic strain, independently of the type of interaction (Class A and B in **Figure 5-6**). Interestingly, VOCs in the class 7 and 8 in the **Figure 5-6**were detected in three over the four conditions such as 1,2,3-trimethylbenzene or (-)-isolongifolene for example. Finally, novel compounds exclusive to a unique condition were also detected (Classes 3 to 6 in **Figure 5-6**).

Additionally, we noted the absence (below the detection limit), in the different interaction modalities, of certain individual VOCs. It is the case for example of the (-)-α-panasinsen emitted by the *A. flavus* non-toxigenic strain in its individual inoculation condition or the NI 1183 for *F. verticillioides* (**Table 5-1**, supplementary material).

These specific VOCs highlighted from **Figure 5-6** (Class A, B, 1 and 2), were reported in **Table 5-2** together with their day of emission, in order to evaluate their behavior over time. Several patterns are observed. Indeed, benzenemethanol, the specific VOC linked to the toxigenic *A. flavus* strain was punctually emitted at day 2 in both interactions. Similarly, α-neoclovene and junipene, specific to a contact interaction, were emitted at day 2 and 5 regardless the strains of *A. flavus* involved in the experiment. Aristolochene, specific to the non-toxigenic strain, shows a shift in its occasional emission in case of non-contact condition, and the same observation is noted for 4-ethylbenzamide, specific in case of non-contact condition between strains, which was also punctually emitted earlier in the presence of the toxigenic strain. The presence of toxigenic *A. flavus* generates an alternating constant emission of (-)-isolongifolene, in contact condition with non-toxigenic *A. flavus*, suggesting that toxicity affects its emission. An early emission of 1,2,3-trimethylbenzene, only on day 2, was detected except for the contact and non-toxigenic *A. flavus* condition. Concerning the NI 1397, a constant emission is detected over the days during the contact condition but no in the non-contact condition. Indeed, in the case of the presence of toxigenic *A. flavus* this VOC is detected until day 5 while no detection is recorded with the non-toxigenic *A. flavus*.

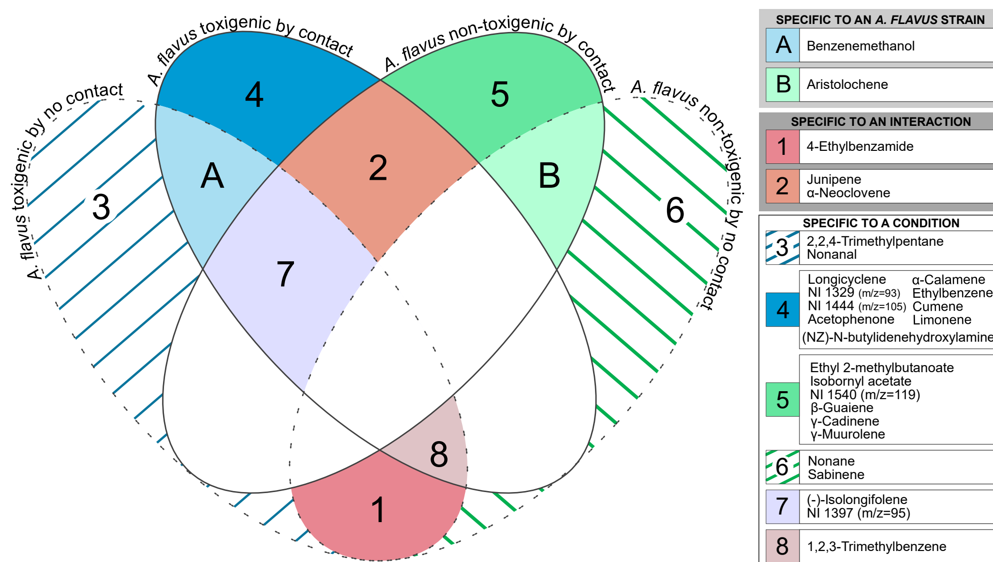


Figure 5-6. Venn of the VOCs that specifically appeared in specific conditions as in function of the nature of interaction (contact and non-contact) and/or the type of *A. flavus* strains (toxigenic or non-toxigenic) in the presence of *F. verticillioides*.

Table 5-2. Days of emission of some specific VOC highlighted by figure 5-6 for the *F. verticillioides* (Fv), toxigenic (AfT) and non-toxigenic (AfNT) *A. flavus* strains under single, non-contact (/) and contact interaction (x).

		Non-contact		Contact	
Name	CAS number	Fv / AfT	Fv / AfNT	Fv x AfT	Fv x AfNT
Specific to an *A. flavus* strain					
Benzenemethanol	100-51-6	2	-	2	-
Aristolochene	26620-71-3	-	8	-	5
Specific to an interaction					
4-Ethylbenzamide	33695-58-8	5	8	-	-
α-Neoclovene	4545-68-0	-	-	2,5	2,5
Junipene	475-20-7	-	-	2,5	2,5
Specific to 3 conditions					
NI 1397 (m/z=95)	-	5,8	-	2,5,8	2,5,8
(-)-Isolongifolene	1135-66-6	2,8	-	2,8	2,5,8
1,2,3-Trimethylbenzene	526-73-8	2	2	2	-

2.3. Mycotoxin production under the different conditions

2.3.1. Evolution of the mycotoxins under the individual condition

The strains of *A. flavus* toxigenic and *F. verticillioides*, which respectively produce aflatoxins (AFB_1 and AFB_2) and cyclopiazonic acid (CPA); and fumonisins (FB_1, FB_2 and FB_3) were monitored over time (**Figure 5-7-A** and **B** – Red curves). The

production of mycotoxins in individual condition was similar in both temporal repetitions. For *A. flavus* toxigenic, AFB_1 was produced to a much greater extent compared to AFB_2 and CPA, which were produced only weakly (Coef < 2.5). A high presence of AFB_1 was detected as early as day 2, indicating the immediate production of the mycotoxin by toxigenic *A. flavus*. This production continued to increase until day 5, reaching its peak, after which, it remained at a steady state. AFB_2 and CPA show the same trend but at much lower levels. No Aflatoxin G is produced by this strain. The production of fumonisins by *F. verticillioides* have evolved continuously and progressively from day 2 to day 8. Fumonisins were produced in decreasing quantities but at the same time, with FB1 being produced predominantly, followed by FB_2 and then FB_3.

2.3.2. Evolution of the *A. flavus* mycotoxins under contact and non-contact conditions

Both temporal repetitions showed the same trend in the evolution of aflatoxin (AFB_1 and AFB_2) production under non-contact conditions (**Figure 5-7-A** - blue light dashed curves). In fact, the same trend as that described for production under individual conditions is observed. Although this production is slightly lower than that under individual conditions, we cannot establish a clear significant difference. We therefore conclude that the non-contact condition did not influence aflatoxins' (AFB_1 and AFB_2) production in our experimental production. However, a significant increase in CPA production seems to be stimulated by the presence of *F. verticillioides*.

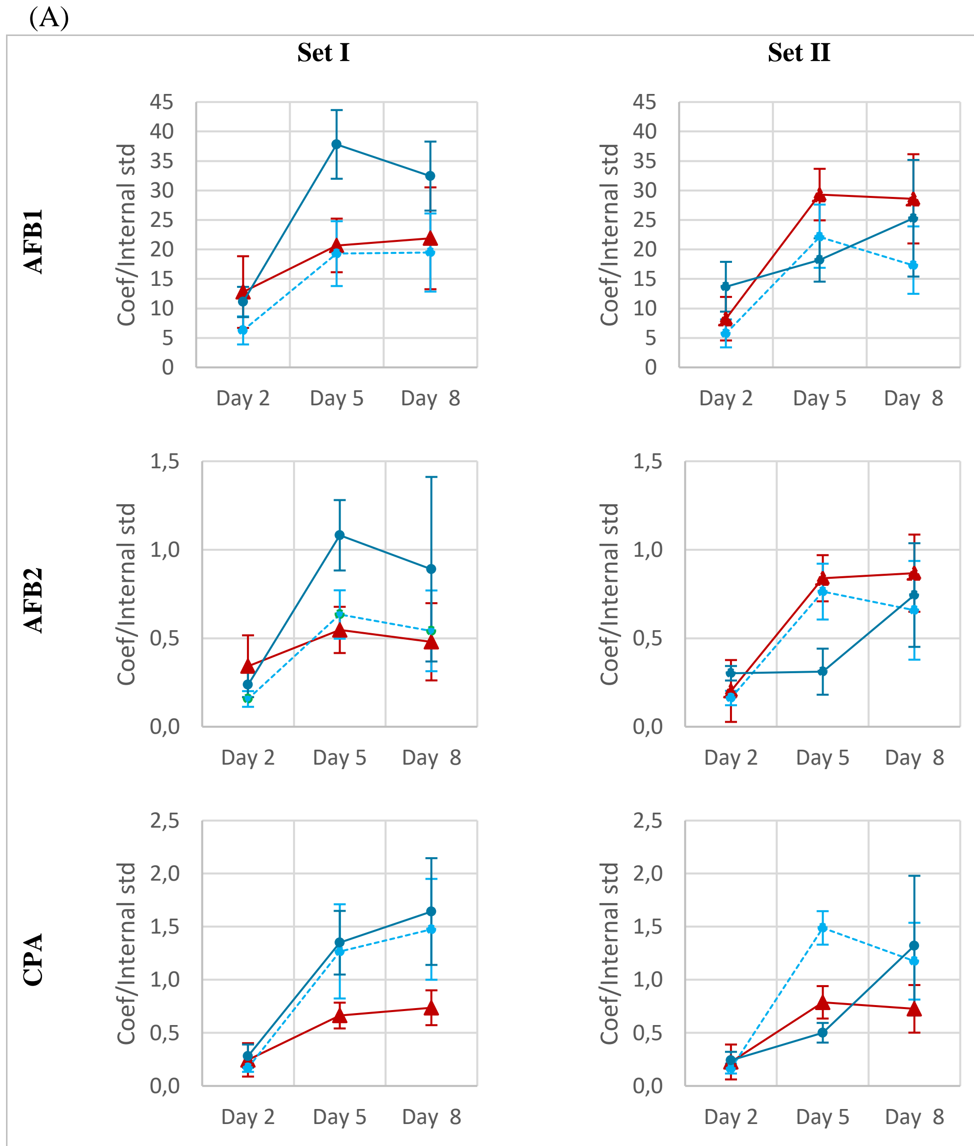
On the other hand, a divergence is observed for the physical contact condition in both sets. While the first temporal repetition (set I) showed a significant increase in the production of all three mycotoxins compared to their individual productions, second temporal repetition (set II) shows a significant decrease on day 5 (**Figure 5-7-A** - blue full curves).

2.3.3. Evolution of the *F. verticillioides* mycotoxins: FB_1, FB_2 and FB_3 under contact and non-contact conditions

A similar increase in fumonisins (FB_1, FB_2 and FB_3) production, in the case of the non-contact condition with the toxigenic *A. flavus* or non-toxigenic *A. flavus* strain, is visible in both sets (**Figure 5-7-B** - Light blue and green dashed curves). However, again in the case of the contact condition, although it leads to an increase in fumonisin production, a divergence in trends between the two sets was noted (**Figure 5-7-B** - Gray and Green full curves). No modification of production is related to the type of *A. flavus* strains present with *F. verticillioides.* Overall, and in both sets, despite a tendency towards an increase, the type of contact and the type of *A. flavus* strains involved in the interaction with *F. verticillioides* do not appear to specifically affect the production of the 3 fumonisins.

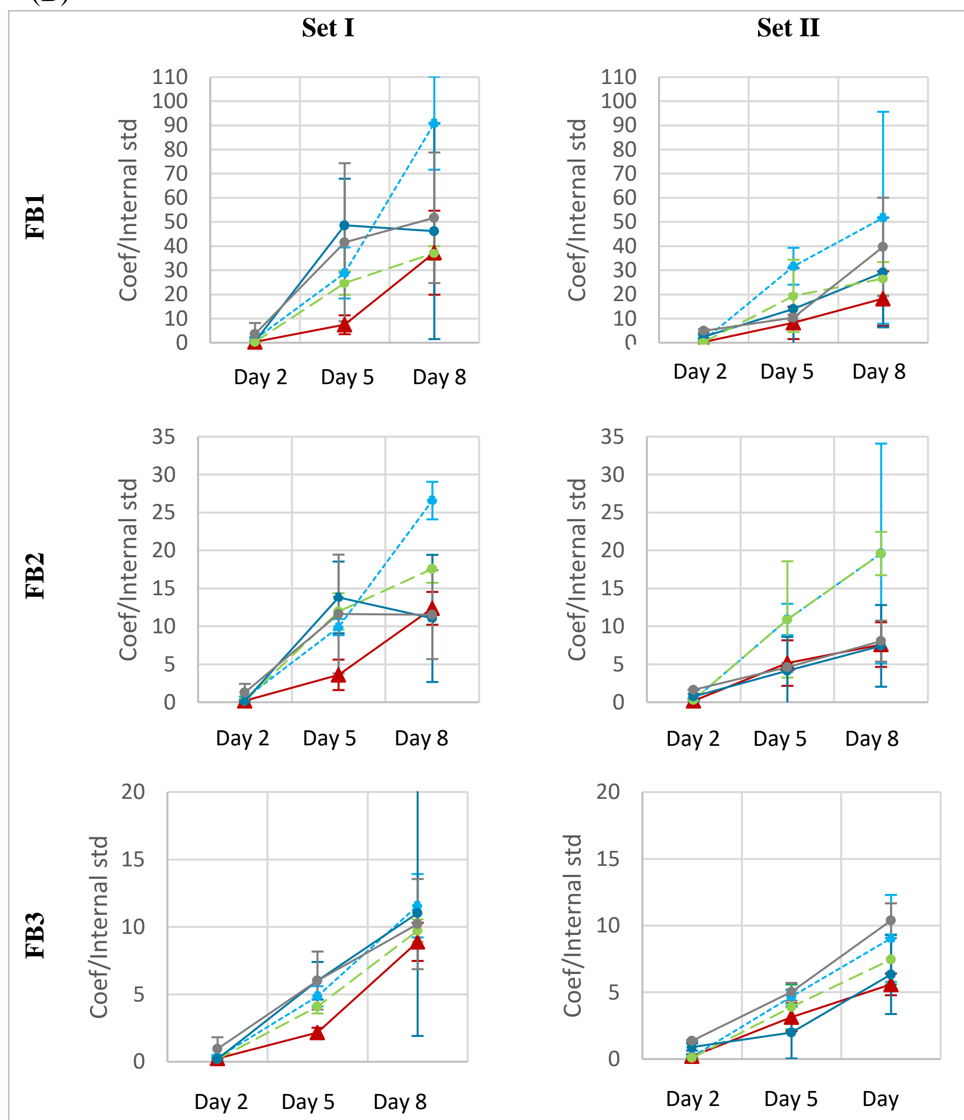
Figure 5-7. Mycotoxin production over time under the different conditions. (A) Production of aflatoxins (AFB_1 and AFB_2) and cyclopiazonic acid (CPA) by toxigenic *A. flavus* (B) Production of fumonisins (FB_1, FB_2 and FB_3) by *F. verticillioides*

(A)



(▲) Mycotoxin production under the individual condition, (---) under non-contact condition, (-) under contact condition, (● and ●) *F. verticillioides* with toxigenic *A. flavus*, (● and ●) *F. verticillioides* with non-toxigenic *A. flavus* using a coefficient calculated with an internal standard.

(B)



(▲) Mycotoxin production under the individual condition, (---) under non-contact condition, (-) under contact condition, (● and ●) *F. verticillioides* with toxigenic *A. flavus*, (● and ●) *F. verticillioides* with non-toxigenic *A. flavus* using a coefficient calculated with an internal standard.

2.4. Correlation between VOC and mycotoxin

Table 5-3. Table of the correlation (Pearson r) between the mycotoxins' families and the VOCs emitted.

VOCs/Mycotoxins	Aflatoxins	CPA	Fumonisins
Epizonaren	0.98	0.95	
α-Cadinene	0.97	0.94	
trans-Caryophyllene	0.97	0.95	
β-Elemene	0.96	0.92	
β-Selinene	0.95	0.91	
Germacrene D *	0.94	0.91	
α-Gurjunene	0.93	0.90	
α-Copaene	0.93	0.90	
Cadina-3,5-diene	0.93	0.88	
α-Calacorene	0.92	0.90	
δ-Elemene	0.90	0.87	
Cadina-1,4-diene	0.88	0.88	
δ-Cadinene *	0.88	0.86	
Epi-cubenol	0.83	0.83	
NI 1393		0.76	
β-Cadinene		0.75	
α-Corocalene		0.70	
Ylangene		0.69	
(1r,4r,5s)-Alpha-acoradiene			0.89
β-Acorenol			0.75
α-Cedrene			0.71
Styrene			-0.78

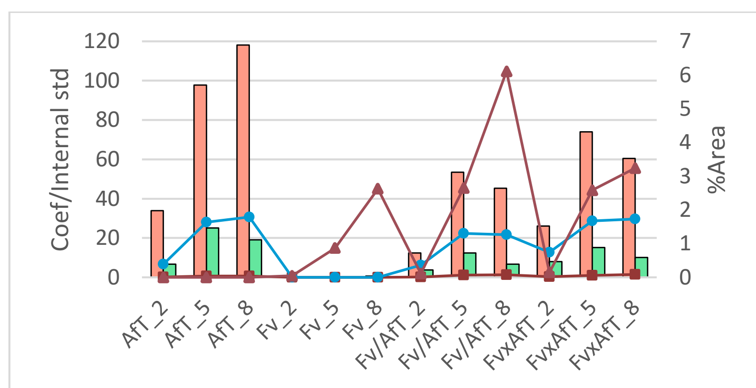
The **Table 5-3** provided insights into the association of 14 terpenes with the presence of aflatoxins and CPA production and4 VOCs exclusively linked to CPA production. On the other hand, 4 VOCs were correlated with fumonisin production, with the styrene negatively correlated. Overall, these VOCs were specifically emitted by their respective producer strains. Except of germacrene D and δ-cadinene, both detected in mycotoxin producer species. Germacrene D was consistently detected in all conditions involving toxigenic *A. flavus*, but also emitted by *F. verticillioides* from day 8 onwards and in its interaction with non-toxigenic *A. flavus*.

Regarding δ-cadinene, predominant in the *A. flavus* toxigenic, its emission was also detected since day 8 for the other strains. **Figure 5-8** demonstrated that these two

VOCs were emitted to a lesser extent by *A. flavus* non-toxigenic and *F. verticillioides*, even in their interaction, while *A. flavus* toxigenic exhibited a significant emission. For *F. verticillioides*, the peak in δ-cadinene coincided with the peak in total fumonisin production at day 8. The production of aflatoxins increased with the amount of these VOCs. In the interaction condition, the production of CPA and aflatoxins followed the evolution of germacrene D. It appears that the decrease in these VOCs was simultaneous with the increase in fumonisins production and a slight reduction in aflatoxin production.

The interaction between *A. flavus* non-toxigenic and *F. verticillioides* resulted in an increase in δ-cadinene production, but only the contact interaction triggered germacrene D production from day 5, whereas it occurred at day 8 for *F. verticillioides* alone.

A



B

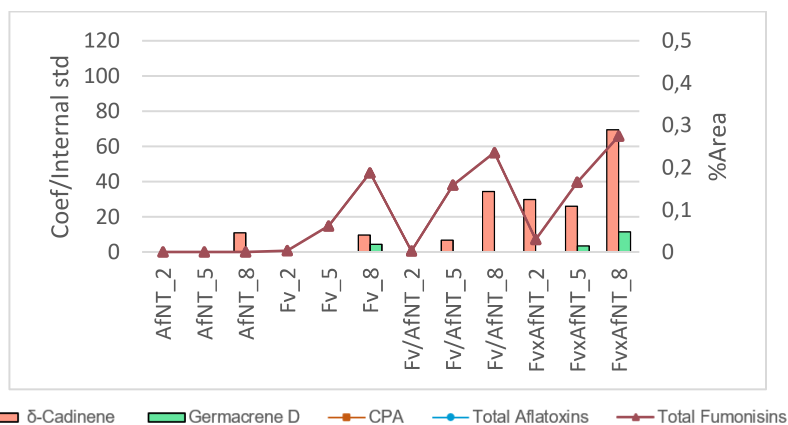


Figure 5-8. Evolution of total aflatoxins and total fumonisins and the amount (% area) of δ-cadinene and germacrene D over the time and in function of the growth conditions (individual, contact (x) and non-contact (/)) (A) for the strains involving the toxigenic *A. flavus* (AfT) and *F. verticillioides* (Fv); (B) for the strains involving non-toxigenic the *A. flavus* (AfNT) and *F. verticillioides* (Fv) at day 2, 5 and 8.

3. Discussion

Studies on fungal-fungal interactions are crucial for understanding ecological dynamic and finding ways to control harmful fungi without altering the existing equilibrium. Several studies have examined the effects of other microorganisms (fugus, yeast, bacteria) on growth and mycotoxin production of toxigenic fungi, however, it is often antagonist species which were investigated. For the first time, the explicit changes in VOC emissions moving from individual to co-inoculation following two modes of interaction (contact and non-contact) have been described in this study using *A. flavus* and *F. verticillioides* species. The duet of VOCs and mycotoxin monitoring was employed to investigate the contact and non-contact interactions between the species and the individual growth of each strain served as a reference for comparison of both parameters. Moreover, the differences between toxigenic and non-toxigenic strains of *A. flavus*, that could be related to the aflatoxin production, were considered, thus providing valuable insights in this study.

The **Figure 5-2** shows the difference in the inhibition zone in the contact condition between the two co-inoculated species, suggesting different mechanism of colonization of the *A. flavus* strains depending on their ability to produce AFB_1. When the toxigenic *A. flavus* strain deliver AFB_1 in the substrate as well as the *F. verticillioides* secrete FB_1, both colonies maintain around 2 mm of distance to one and other [249,250]. On the other hand, the non-toxigenic *A. flavus*, developed to the point of encountering the mycelium of *F. verticillioides* indicating a different strategy to compete. Currently, field management used non-toxigenic *A. flavus* strains to control the toxigenic *A. flavus* strains development and reduce aflatoxins contamination[251,252].

The co-inoculation conditions have shown a clear response in VOC emission to the presence of the other species under two modes of interaction (**Table 5-1**). Principally, contact condition has generated a higher number of VOCs compared to non-contact conditions, which can be explained by the increased production of metabolites due to competition between the two species [249]. Indeed, the diffusion of soluble molecules in the media and the competition for substrate could have stimulated various metabolic pathways, leading to an increase of the diverse metabolite production, although an increase of other secondary metabolites in the substrate cannot be excluded.

In the contact condition, a general increasement of the sesquiterpenes, compounds involved in the defense mechanisms in fungi by acting as antimicrobial and/or antifungal agents [178], was observed. On the contrary, the non-contact condition triggered a drop in sesquiterpene emission in co-inoculation with toxigenic *A. flavus*. These two opposite trends highlighted the differences related to the competition for the substrate as well as the soluble molecules secreted and involved in the fungi reaction to the presence of another species. On the other hand, the co-inoculation with the non-toxigenic *A. flavus* induced a higher production of monoterpenes and alkanes (**Table 5-1**). Interestingly, since the mode of interaction leads to variations in certain chemical families, the toxicity of the *A. flavus* strain involved also variations at the

level of the chemical family elicited. The VOC fluctuations have been already proved for other species co-inoculated [253] as well as the emission of new ones were linked to competition among the fungi [254,255]. Thus, this study reported specific VOCs that can be associated with particular conditions and among the new VOCs detected, mainly belong to the sesquiterpene chemical family (**Figure 5-6**) [47,48].

Indeed, the emission of two sesquiterpenes, junipene and α-neoclovene, were specific to the contact condition. These sesquiterpenes can be found in some essential oils or released by some fungal species [256]. On the other hand, 4-ethylbenzamide was specifically emitted under the non-contact condition. This compound has also been associated, like the sesquiterpenes, to antimicrobial and antifungal activities [257,258].

Additional specific VOCs linked to the *A. flavus* strains were highlighted. Indeed, *A. flavus* toxigenic strain emitted benzenemethanol regardless of the type of interaction [259]. This alcohol is known to induce growth inhibition due to its antifungal properties and acts as a VOC signaling molecule to attract or repel specific fungi or modulate fungal behavior. Its emission has been previously reported to be emitted by toxigenic strains of *A. flavus* during individual inoculation, indicating that other parameters could influence its release [223]. On the other hand, the emission of the aristolochene sesquiterpene was non-toxigenic *A. flavus* strain specific. This volatile bicyclic sesquiterpene is a precursor of aflatoxin. However, since the aflatoxin cluster genes are not present in the *A. flavus* non-toxigenic strain [75], the emission is likely originating from *F. verticillioides*, as sesquiterpenes are commonly produced through the conversion of farnesyl diphosphate (FPP). Although no specific activity to this VOC was clearly found [260]. Although VOCs are characteristic and specific to a particular interaction mode or strain duo, the system for detecting them does not allow them to be attributed to the emitting strain, because the atmosphere studied is the result of the mixture of VOCs emitted by the two interacting strains.

The **Table 5-3** has linked many sesquiterpenes to mycotoxin production, nevertheless, no causality could be proven. Among them, only 3 VOCs were identified for the first time to be emitted by the toxigenic *A. flavus* strain (δ-elemene, cadina-1,4-diene, cadina-3,5-diene) [223]. Remarkably, 4 VOCs were only correlated to CPA production. These distinctive VOCs were always detected from day 5 onwards, coinciding with the peak of CPA production. CPA was identified as being produced when the growth stage decreased to a stop and proved to be another competitive tool for ascendance on other species [261,262].

Finally, the co-detection of germacrene D and δ-cadinene is noteworthy. Both VOCs were previously detected in these same strains in a previous study [85], specifically in relation to the toxigenic strain of *A. flavus*. The detection of δ-cadinene at day 8 in the non-toxigenic *A. flavus* strain, while in Josselin et al. (2021) study, this same strain did not emit this sesquiterpene, show that variations related to the temperature applied during the fungal growth could occur [85,86,172]. Although germacrene D and δ-cadinene are often detected by mycotoxin-producing strains, studies investigating these VOCs are rarely associated with mycotoxin assays, making the idea that a fungi's mycotoxin production can be detected based on the joint presence of these two

VOCs currently impossible to assert. Nevertheless, the combination of germacrene D and δ-cadinene could potentially contribute to early detection of the toxicity and mycotoxins production of the fungal strains (**Figure 5-8**). On the other hand, germacrene D was consistently detected in all conditions, except in the non-contact one between *F. verticillioides* and non-toxigenic *A. flavus*. This suggests that the emission of germacrene D may be supported by other molecules diffused in the substrate, which could be released into the substrate during the colonization and related to the visual fungal growth (**Figure 5-2**) [263,264].

However, it is important not to overlook the VOCs that disappear (below detection limit), as they can serve as good indicators of fungal responses under competitive conditions [265]. It is also important to consider that changes in VOCs can act as both inducers, a molecule capable of activating a biological process, and consequences of stress responses occurring that may include physical alterations in the mycelium.

Regarding the mycotoxins production of the fungi, Leggiero et al. and Giorni et al. reported that when toxigenic *A. flavus* and *F. verticillioides* species grew under contact condition in maize, a reduction in both growth and their respective mycotoxins production, in comparison of individual inoculation was observed [36,244]. However, in our study, the three fumonisins production were increased by the presence of the *A. flavus*, this difference could be associated to the temperature, photoperiod, or substrate parameters applied [19]. Indeed, Chen et al. have been reported different trend *in vitro* and *in planta* experiments with their same *A. flavus* and *F. verticillioides* strains [250]. Also, temperature is an important parameter influencing the VOCs emission, growth and mycotoxins production [86]. The temperature used in this study have certainly favored *F. verticillioides* more than *A. flavus*, which thrives at temperatures above 25°C. In addition, the temperature influences the mycotoxin production through the gene expression. Actually, Lanubile et al. demonstrated that the *FUM1* and *FUM13* genes involved in the fumonisin pathway exhibited an upregulation at 20°C when *A. flavus* and *F. verticillioides* were interacting, whereas a continuous decrease was observed when *F. verticillioides* grew alone. Regarding genes involved in aflatoxin synthesis, the gene expression can be radically influenced not only by the species [266], but also by other parameters such as water activity and carbon source utilization during contact growth conditions. It appeared that *A. flavus* was found to be dominant over *F. verticillioides* only at 30°C and when the water activity was below 0.96, while *F. verticillioides* was dominant under all other conditions tested [267]. The timing of species interactions can impact also on mycotoxin production to varying degrees [36,250,268].

Finally, the plateau observed in AFB_1, AFB_2, and CPA production from day 5 as well as the continuous increase in fumonisins produced by *F. verticillioides* could be due to the ability to utilize carbon sources more effectively under specific conditions trigged by the temperature of the experiment [87].

The analysis of the two temporal repetitions (**Figure 5-7**) revealed a significant variability that warranted closer attention. While the individual and non-contact experiments yielded clear results, the contact conditions raised some questions. In the first temporal repetition (set I), lower levels of mycotoxins were recorded at day 5.

Fumonisins production in co-inoculation was always higher than individual production, confirming the general observation that the presence of *A. flavus* triggers fumonisins production by *F. verticillioides* in both temporal repetitions. On the other hand, a significant decrease was observed at day 5 in the levels of AFB_1, AFB_2 and CPA secreted by *A. flavus* in this scenario. Interestingly, the mycotoxin levels at day 8 were consistently similar in both temporal repetitions, indicating that the toxigenicity of the fungi was not affected, but that a factor inhibited mycotoxin production until day 5 for the second temporal repetition (set II). Even if all precautions have been carefully taken, variations can sometimes be observed between temporal repetitions. Several hypotheses could be proposed, considering that it is well-known that other parameters can influence mycotoxin production. However, the variations in VOCs recorded in these two temporal repetitions do not provide any specific insights or highlights. Fluctuations in the mycotoxin production of the same strains was already observed in **Chapter 4** regarding the fumonisins production of *F. verticillioides* in maize.

4. Conclusion

For the first time, modifications in emitted VOCs were reported for several types of interactions between *F. verticillioides* and *A. flavus* species, using their volatolomes monitored during their individual growth as a reference. Moreover, the toxicity associated to aflatoxin production of *A. flavus* strains co-inoculated with *F. verticillioides* was considered using toxigenic and non-toxigenic strains. Finally, different modes of interactions between these fungal species were compared. This study revealed specific VOCs that reflected the mode of interaction, and the monitored mycotoxins (aflatoxins and fumonisins) showed an increased production in response to the presence of the other species. It appears that *A. flavus* and *F. verticillioides* can trigger their respective mycotoxin production when co-occurring. The parameters used in this study suggest that these species work together to colonize their host. However, it is important to consider that various parameters such as temperature, pH, water activity, and others, play a crucial role in the generation of these VOCs and mycotoxins, which are the results of the activated metabolic pathways within the fungi. Although this study has yielded valuable insights into the co-inoculation dynamics of VOCs and mycotoxins, it is crucial to extend the investigation to real-world scenarios. This entails studying commonly consumed foodstuffs like maize and considering relevant agronomic conditions. By doing so, we can enhance our understanding of the complexity and wise networks that exist in nature.

5. Supplementary material

Table 5-4. Supplementary Table - Day of detection (day 2, 5 and 8) of VOCs by the 3 fungal strains studied: *F. verticillioides* (Fv), *A. flavus* toxigenic (AfT) and *A. flavus* non-toxigenic (AfNT)) under the different conditions (mono or co-inoculation with a physical contact (x) or no physical contact (/)).

					Alone			No contact		Contact	
	Name	CAS number	RI exp	RI ref	Fv	AfT	AfNT	Fv / AfT	Fv / AfNT	Fv x AfT	Fv x AfNT
Acid	2-Methylpropanoic acid	79-31-2	745	-	-	-	2	-	-	-	-
Alcohol	2-Ethylhexan-1-ol	104-76-7	1028	1026	-	2	-	-	-	2	-
Alcohol	2-Methylbutan-1-ol	137-32-6	717	720	2,5,8	5	5,8	2,5,8	2,5,8	2,5,8	2,5,8
Alcohol	2-Methylpropan-1-ol	78-83-1	620	622	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8
Alcohol	3-Methylbutan-1-ol	123-51-3	714	724	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8
Alcohol	Benzenemethanol	100-51-6	1032	1033	-	-	-	2	-	2	-
Alcohol	Dihydromyrcenol	18479-58-8	1071	-	2	-	2	-	-	-	-
Alcohol	Ethanol	64-17-5	< 600	-	2,5,8	2,5	2,5,8	8	2,5,8	-	5
Alcohol	Propan-1-ol	71-23-8	< 600	-	5	5,8	5,8	2,5	5	5	2,5,8
Aldehyde	2-Methylbut-2-enal	1115-11-3	721	-	-	2	-	-	-	-	-
Aldehyde	2-Methylbutanal	96-17-3	< 600	-	5	-	-	-	-	-	-
Aldehyde	2-Methylbutanal	96-17-3	649	-	-	-	-	-	-	5	5
Aldehyde	3-Methylbutanal	590-86-3	639	649	2,5	-	-	2,5	2,5	2	-
Aldehyde	Acetaldehyde	75-07-0	< 600	-	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8	2,5,8
Aldehyde	Benzeneacetaldehyde	122-78-1	1041	1042	-	-	2	-	-	-	-
Aldehyde	Nonanal	124-19-6	1104	1103	-	-	-	2,5	-	-	-
Alkane	1,2-Dipropylcyclopentane	91242-57-8	1059	-	-	-	2	-	-	-	-
Alkane	2,2,3,3-Tetramethylbutane	594-82-1	620	-	-	5	-	-	-	-	-
Alkane	2,2,4,4-Tetramethylpentane	1070-87-7	716	-	-	8	-	-	-	-	-
Alkane	2,2,4-Trimethylpentane	540-84-1	664	-	-	-	5,8	2	2,8	-	5
Alkane	2,4-Dimethylpentane	108-08-7	646	-	-	-	2	-	-	-	-
Alkane	3-Methylhexane	589-34-4	653	-	-	-	2	-	2	-	-
Alkane	3-Methylpentane	96-14-0	646	649	2	-	-	-	-	-	-
Alkane	Cyclohexane	110-82-7	< 600	-	-	5	-	-	-	-	-
Alkane	Cyclohexane	110-82-7	659	-	-	-	8	-	2	-	-
Alkane	Decahydronaphthalene	91-17-8	1048	-	-	-	2	-	-	-	-
Alkane	Decane	124-18-5	998	-	-	-	2	-	2	-	-
Alkane	Dodecane	112-40-3	1199	-	-	-	2	-	2	-	-
Alkane	Heptane	142-82-5	673	677	-	2-	-	-	-	-	-
Alkane	Methylcyclohexane	108-87-2	701	-	2	2	2,8	2	2,8	2	-
Alkane	Nonane	111-84-2	897	-	-	-	-	-	2	-	-
Alkane	Octane	111-65-9	789	788	-	5	8	-	2	-	-
Alkane	Spiro[4.5]decane	176-63-6	1092	-	-	-	2	-	-	-	-
Alkane	Undecane	1120-21-4	1099	1100	-	-	2	-	-	-	-

Table 5-4 (continued)

					Alone			No contact		Contact	
	Name	CAS number	RI exp	RI ref	Fv	AfT	AfNT	Fv / AfT	Fv / AfNT	Fv x AfT	Fv x AfNT
Alkene	1-(3-methyl-2-cyclopenten-1-yl)-Cyclohexene	959061-92-8	1526	-	5,8	-	-	-	5,8	-	8
	1,2,3-Trimethylbenzene	526-73-8	989	-	-	-	-	2	2	2	-
	1-tert-Butyl-4-methoxybenzene	5396-38-3	1240	-	5,8	-	-	5	-	5	2,5
	2-Methyl-4-(2,4,4-trimethylpentan-2-yl)phenol	2219-84-3	1341	-	2,5	-	-	-	2	-	-
	Cumene	98-82-8	920	-	-	-	2	-	-	2	-
	Ethylbenzene	100-41-4	854	-	-	-	2	-	-	2	-
	Hex-2-ene	592-43-8	778	-	8	-	-	-	-	-	-
	ortho/para-Xylene	1330-20-7	862	-	-	-	2	-	-	-	-
	Styrene	100-42-5	884	890	-	2,5,8	2,5,8	-	-	2,5,8	2,5,8
	Toluene	108-88-3	748	745	-	2	2,8	-	-	-	-
Amide	4-Ethylbenzamide	33695-58-8	785	-	-	-	-	5	8	-	-
Amine	(NZ)-N-butylidenehydroxylamine	110-69-0	< 600	-	-	-	-	-	-	2	-
Ester	1-*O*-(2-methylpropyl) 4-*O*-propan-2-yl 2,2-dimethyl-3-propan-2-ylbutanedioate	1000140-77-5	1599	-	2,5	-	2	2	-	-	-
	2-Methylbutyl acetate	624-41-9	874	880	5,8	-	-	5	5	5	5
	2-Methylpropyl acetate	110-19-0	757	-	5,8	-	-	5	5	5,8	5
	3-Methylbutyl acetate	123-92-2	871	876	5,8	-	-	5	-	5	5
	Ethyl 2-hydroxypropanoate	97-64-3	811	-	-	-	2	2	-	2	2
	Ethyl 2-methylbutanoate	7452-79-1	844	842	-	-	-	-	-	-	8
	Ethyl butanoate	105-54-4	794	795	8	-	-	-	-	-	-
Ketone	3-Hydroxybutan-2-one	513-86-0	691	695	5,8	5,8	-	2,5	5	5,8	5
	Acetophenone	98-86-2	1063	1066	-	-	-	-	-	2,5	-
	Hexan-3-one	589-38-8	772	-	5	-	-	-	-	-	-
	Pentan-2-one	107-87-9	627	-	5,8	-	-	-	-	8	-
	Propan-2-one	67-64-1	607	-	2,5,8	-	2	8	5,8	-	-
Monoterpene	Camphor	76-22-2	1141	1147	2	2	8	-	8	-	-
	DL-Menthol	89-78-1	1170	1173	-	2	-	-	-	-	-
	Eucalyptol	470-82-6	1027	1026	5	-	8	-	5,8	5	-
	Isobornyl acetate	125-12-2	1285	1286	-	-	-	-	-	-	8
	Limonene	138-86-3	1025	1025	-	-	2	-	2,8	2	8
	Sabinene	3387-41-5	969	969	-	-	-	-	8	-	-
	Terpinolene	586-62-9	1099	1089	2	-	-	-	-	-	-
	α-Neoclovene	4545-68-0	1453	1451	-	-	-	-	-	2,5	2,5
	α-Pinene	80-56-8	928	931	-	2	8	-	2,5,8	2	2,8
	β-Pinene	127-91-3	971	975	-	-	8	-	8	-	-

Table 5-4 (continued)

	Name	CAS number	RI exp	RI ref	Alone			No contact		Contact	
					Fv	AfT	AfNT	Fv / AfT	Fv / AfNT	Fv x AfT	Fv x AfNT
Non identified	NI 630	-	630	-	2,5	2	-	2	-	-	-
	NI 731	-	732	-	5	-	-	-	-	5	-
	NI 781	-	781	-	-	-	2	-	-	-	-
	NI 1183	-	1183	-	5,8	-	-	-	-	-	-
	NI 1202	-	1202	-	-	-	-	-	-	5	5,8
	NI 1329	-	1329	-	-	-	-	-	-	5	-
	NI 1386	-	1386	-	-	-	-	2	-	2,5,8	-
	NI 1393	-	1393	-	-	2,5,8	-	5,8	-	5,8	-
	NI 1397	-	1397	-	-	-	-	5,8	-	2,5,8	2,5,8
	NI 1414	-	1414	-	5,8	-	-	-	8	5,8	5
	NI 1422a	-	1422	-	5	-	-	5	-	5,8	-
	NI 1422b	-	1422	-	-	5,8	-	-	-	-	-
	NI 1425	-	1425	-	-	-	-	-	-	5	-
	NI 1444	-	1444	-	-	-	-	-	-	5,8	-
	NI 1474	-	1474	-	-	-	-	-	-	5,8	-
	NI 1480	-	1480	-	8	-	-	-	-	-	-
	NI 1488	-	1488	-	-	-	-	-	-	-	2
	NI 1496	-	1496	-	-	-	8	-	8	-	-
	NI 1497	-	1497	-	-	-	-	-	-	-	8
	NI 1499	-	1499	-	-	-	-	-	-	-	2,5,8
	NI 1506	-	1506	-	-	-	-	-	-	5,8	-
	NI 1512	-	1512	-	-	5	-	-	-	-	-
	NI 1540	-	1540	-	-	-	-	-	-	-	5
	NI 1572	-	1572	-	-	5,8	-	-	-	5,8	-
Other	3-Hydroxyphenylurea	701-82-6	1202	-	5,8	-	-	8	5	5,8	5
	Methyl (Z)-N-hydroxybenzenecarboximidate	1000222-86-6	905	-	-	2	2	2	2,8	2	2,8
	Methylene chloride	75-09-2	614	-	-	-	8	-	-	-	-
Sesquiterpene	Italicene ether	104188-25-2	1543	-	5,8	-	-	-	5,8	-	5,8
	(-)-Isolongifolene	1135-66-6	1383	1390	-	-	-	2,8	-	2,8	2,5,8
	(-)-α-Alaskene	28400-12-6	1517	-	2,5,8	-	-	-	2,5,8	-	5
	(-)-α-Panasinsen	56633-28-4	1523	-	-	-	8	-	-	-	-
	(+)-Cyclosativene	22469-52-9	1364	-	-	5,8	-	5	-	5	-
	(+)-β-Funebrene	546-28-1	1419	-	5,8	-	-	-	2,5,8	-	2,5,8
	Cadina-1,4-diene	16728-99-7	1540	-	-	5,8	-	5,8	-	2,5,8	-
	1-Naphthalenol, 1,2,4a,5,6,7,8,8a-octahydro-3,8-dimethyl-5-(1-methylethenyl)-, (1R,4aR,5R,8R,8aS)	1000196-33-2	1425	-	-	8	-	-	-	-	-
	1ξ,7ξ,10β-Cadina-4(14),5-diene	22339-27-1	1466	-	-	8	-	-	-	-	-

Table 5-4 (continued)

	Name	CAS number	RI exp	RI ref	Alone			No contact		Contact	
					Fv	AfT	AfNT	Fv / AfT	Fv / AfNT	Fv x AfT	Fv x AfNT
Sesquiterpene	4-epi-α-Acoradiene	729602-94-2	1469	-	2,5,8	-	-	2,5,8	2,5,8	2,5,8	2,5,8
	Alloaromadendrene	25246-27-9	1443	1453	-	2,5,8	-	5,8	-	-	-
	Aristolochene	26620-71-3	1491	-	-	-	-	-	8	-	5
	Aromadendrene	489-39-4	1440	1440	-	5,8	-	5	-	5	-
	Cadalene	483-78-3	1686	1684	-	5,8	-	-	-	-	-
	Cadina-3,5-diene	267665-20-3	1449	-	-	2,5,8	-	2,5,8	-	2,5,8	-
	cis-Muurola-4(15),5-diene	157477-72-0	1468	1461	-	2,5	-	-	-	-	-
	cis-β-Copaene	18252-44-3	1433	1430	5,8	-	-	5,8	8	5,8	5,8
	Dehydrocyclolongifolene oxide	1000156-11-4	1648	-	-	8	-	-	-	-	-
	Epi-cubenol	19912-67-5	1623	1619	-	5,8	-	5,8	-	5,8	-
	Epizonaren	41702-63-0	1499	1497	-	2,5,8	-	2,5,8	-	2,5,8	-
	Germacrene D	23986-74-5	1488	1490	8	2,5,8	-	2,5,8	-	2,5,8	5,8
	Isocaryophyllene	118-65-0	1412	1408	-	5,8	-	-	-	-	-
	Isoledene	95910-36-4	1455	-	-	8	-	5	-	5	-
	Junipene	475-20-7	1402	1405	-	-	-	-	-	2,5	2,5
	Longicyclene	1137-12-8	1367	1372	-	-	-	-	-	2	-
	Palustrol	5986-49-2	1576	1569	-	2,5,8	-	2,5	-	2,5,8	-
	trans-β-Copaene	20479-06-5	1430	-	-	5,8	-	5,8	-	2,5,8	-
	Viridiflorol	552-02-3	1597	1589	-	5	-	5	-	5	-
	Ylangene	14912-44-8	1369	1368	-	5,8	-	5,8	-	5	-
	α-Cadinene	24406-05-1	1546	1538	-	2,5,8	-	2,5,8	-	2,5,8	-
	α-Cadinol	481-34-5	1666	1656	-	5,8	-	-	-	-	-
	α-Calacorene	21391-99-1	1552	1542	-	2,5,8	-	5,8	-	2,5,8	-
	α-Calamene	1460-96-4	1592	-	-	-	-	-	-	5	-
	α-Cedrene	469-61-4	1410	1410	2,5,8	-	-	2,5	2,5,8	2,5,8	2,5,8
	α-Copaene	3856-25-5	1374	1375	-	2,5,8	-	2,5,8	-	2,5,8	-
	α-Corocalene	20129-39-9	1631	-	-	5,8	-	5	-	5,8	-
	α-Cubebene	17699-14-8	1348	1348	-	2,5,8	-	5,8	-	2,5,8	-
	α-Gurjunene	489-40-7	1408	1409	-	2,5,8	-	5,8	-	2,5,8	-
	α-Isocomene	65372-78-3	1386	1380	-	2,5,8	-	5,8	-	-	-
	α-Muurolene	10208-80-7	1474	1470	-	5,8	-	-	-	-	-
	α-Selinene	473-13-2	1497	1491	-	2,5,8	-	2,5	-	-	-
	β-Acorenol	28400-11-5	1641	-	2,5,8	-	-	2,5,8	2,5,8	2,5,8	2,5,8
	β-Cadinene	523-47-7	1495	-	-	5,8	-	5,8	-	5,8	-
	β-Caryophyllene	87-44-5	1418	1419	-	2,5,8	-	2,5,8	-	2,5,8	-
	β-Elemene	515-13-9	1383	1383	-	2,5,8	-	5	-	5	-
	β-Elemene	515-13-9	1390	1390	-	2,5,8	-	2,5,8	-	2,5,8	-
	β-Guaiene	88-84-6	1497	1491	-	-	-	8	-	2,5,8	-
	β-Himachalene	1461-03-6	1499	1499	5,8	-	-	-	-	-	-

Table 5-4 (continued)

	Name	CAS number	RI exp	RI ref	Alone			No contact		Contact	
					Fv	AfT	AfNT	Fv / AfT	Fv / AfNT	Fv x AfT	Fv x AfNT
Sesquiterpene	β-selinene	17066-67-0	1492	1488	-	2,5,8	-	2,5,8	-	2,5,8	-
	γ-Cadinene	39029-41-9	1518	1513	-	2,5,8	-	2,5,8	-	2,5,8	8
	γ-Elemene	29873-99-2	1435	1435	-	8	-	-	-	5	-
	γ-Gurjunene	22567-17-5	1478	1473	-	2,5,8	-	2,5,8	-	2,5,8	-
	γ-Muurolene	30021-74-0	1483	1484	-	2,5,8	-	5,8	-	2,5,8	8
	δ-Cadinene	483-76-1	1530	1530	8	2,5,8	8	2,5,8	5,8	2,5,8	2,5,8
	δ-Elemene	20307-84-0	1336	1335	-	2,5,8	-	5,8	-	2,5,8	-
	δ-Guaiene	3691-11-0	1506	1505	-	5,8	-	-	-	-	-
	τ-Cadinol	5937-11-1	1654	1653	-	5	-	-	-	-	-

6. Materiel and method

6.1. *Biological material*

Three strains were studied. *Fusarium verticillioides* strain (ITEM 10514), producing fumonisin B_1, B_2 and B_3, and two strains of *Aspergillus flavus* species: a toxigenic strain (ITEM 8111) producing aflatoxin B_1, B_2 and cyclopiazonic acid (CPA) and a non-toxigenic strain (ITEM 8088) which does not produce any of these mycotoxins. Research National Council of Italy—Institute of Sciences of Food Production, Bari, Italy (CNR-ISPA) provided the fungal strains which belong to the CNR-ISPA official microbial ITEM Collection. ITEM is affiliated to by the International Organization of European Culture Collections and the World Federation of Culture Collections. All strains were conserved in a glycerol stock at -80°C.

6.2. *Experimental design and fungal inoculation*

All spore suspensions were prepared in the same way and concentration of spores' suspensions were determined using a Bürker cell. Briefly, 1 mL of Tween 20 (0.5%) and 1 mL of physiologic water were added to a petri dish containing the fungi that had grown on SNA medium (Synthetic Nutriment-poor Agar, for 1L: 1 g KH_2PO_4; 1 g KNO_3; 0.5 g $MgSO_4$; $7H_2O$; 0.5 g KCl; 0.2 g glucose; 0.2 g sucrose; 20 g agar) in darkness at 23°C for 7 days. A volume containing 1 x 10^5 spores, was centrally inoculated on PDA (Potato Dextrose Agar) petri dish.

Three conditions were studied (**Figure 5-9**):

- Individual: only one fugus was used and *F. verticillioides*, or toxigenic *A. flavus* or non-toxigenic *A. flavus* was centrally inoculated on PDA;
- Contact condition: co-inoculation of *F. verticillioides* strain in the presence of either a toxigenic or non-toxigenic *A. flavus* strain at 5 cm to one another on PDA;
- Non-contact condition: co-inoculation of *F. verticillioides* strain in the presence of either a toxigenic or non-toxigenic *A. flavus* strain on PDA in each compartment of a bicompartmental petri dish.

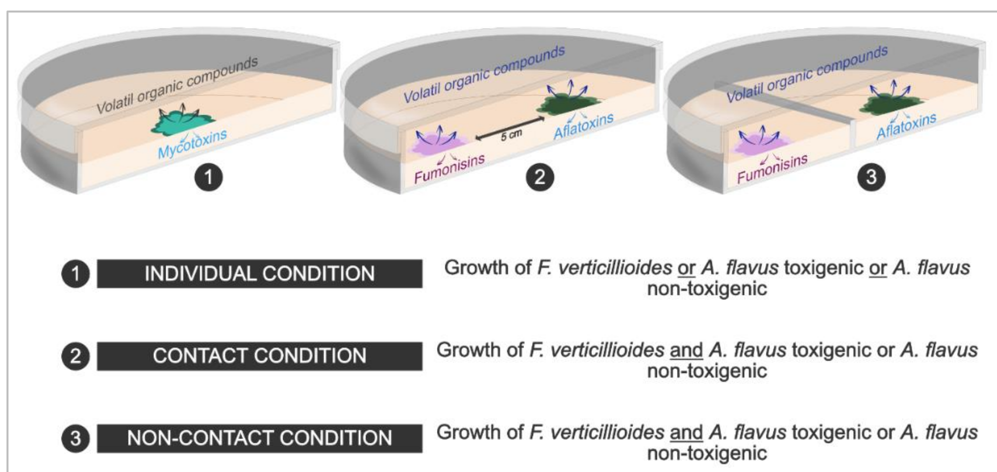


Figure 5-9. Set up of the 3 conditions studied with the three strains (*F. verticillioides, A. flavus* toxigenic and non-toxigenic) growing on PDA during 2, 5 and 8 days in darkness at 23°C.

6.3. VOCs extraction, separation and identification

The petri dishes were opened and placed in VOC extraction system (glass recipient of 1.5L) for 1.5 h to let VOCs disperse and equilibrate through the air volume at 23°C±3°C in darkness. Then, the VOCs were sampled for 1 hour using a SPME fiber (Supelco, Darmstadt, Germany, DVB/CAR/DDMS, 50/30µm, 24 Ga).

On an Agilent Technologies GC 7890B, the VOCs were separated using an HP-5ms column (Agilent Technologies, Santa Clara, CA, USA, 5%-phenylmethylpolysiloxan, non-polar, 30 m × 0.250 mm × 0.25 µm) with a constant helium flow rate of 1 mL·min^{-1}. The desorption at 250 ◦C by splitless injection using an SPME inlet coating of 78.5 mm × 6.5 mm × 0.75 mm (Supelco Inc., Bellefonte, PA, USA) during 5 min was made. The temperature program was applied as follows: 40 °C for 2 minutes, 3 °C·min^{-1} up to 140 °C, 80 °C·min^{-1} up to 300 °C and a final hold at 300 °C for 3 minutes. The mass spectral analysis was performed using the electron ionization (EI)

mode at 70 eV and scan mass range from 35 to 350 amu. The ion source and MS source temperatures were respectively at 250 °C and 280 °C.

The VOCs identification was based on the mass spectra comparison with NIST17 and WILEY298 libraries and the retention indices (RI) according to the method of Van Den Dool and Kratz on a non-polar HP-5ms column. The retention indices were established under the same chromatographic conditions as the samples, using a mixture of n-alkanes (alkane C6-C30 (1000 mg.mL^{-1} in hexane, Supelco, Belgium)). The VOCs detected in at least 2 repetitions by at least two of their respective replicates.

6.4. Mycotoxins analysis

Once the VOCs analysis performed, the petri dishes were stored at -30°C. To extract the FB_1, FB_2 and B_3 produced by the *F. verticillioides* and AFB_1, AFB_2 and CPA produced by the *A. flavus* toxigenic, three plugs of fungus were taken and placed in a tube. The plugs were cut with a scalpel and leaved in darkness for 15 minutes. 5 mL of ethyl acetate/formic acid (99:1) was added before stirred 20 minutes with an overhead shaker. The sample were centrifuged during 15 minutes at 3000g. Then, the supernatant was transferred to a new flask with a plastic pipette. 5 mL of dichloromethane was added again and agitated during 20 minutes with an overhead shaker, the pellet was transferred to the tube using a paper filter to avoid solid agar residues in the extract. The samples extracted were dried at 40°C under a steam of nitrogen.

The mycotoxin was detected by LC-MS/MS. The analysis was performed on a Micromass Quattro Premier XE triple quadruple mass spectrometer (Waters, Zellik, Belgium); Waters Acquity UPLC console (Waters, Zellik, Belgium) equipped with a 5 µm x 2.1 mm x 150 mm Symmetry C-18 column and a 3.5 µm x 2.1 x 10 mm Symmetry C-18 guard column. The samples were run according to a validated methodology and the instrumental parameters were as described in Monbaliu *et al.* [197].

Two temporal repetitions have been shown to demonstrate the variability that can be observed during these analyses.

The mycotoxin production results were represented using a coefficient calculated with the assistance of an internal standard.

6.5. Statistical analysis

For all models, the features (i.e., GCMS profiles) were log transformed and mean centered. The discrimination was visualized by plotting the first PLSDA components and a Pearson r correlation was also applied to find the link between VOCs and specific mycotoxins. All the figures and data were performed using the data reported in the **Table 5-4** (Supplementary material).

Chapter 6

Comparative Analysis of Volatile Organic Compounds, Mycotoxins, and Gene Expression in *Fusarium verticillioides*: Investigating the Role of Ethyl 3-methylbutanoate as a Novel Bioactive VOC under Contact and Non-contact Conditions

As already mentioned in **Chapter 4**, ethyl 3-methylbutanoate is a potential bioactive VOC, given its observed antifungal and antifumonisin effect.

In addition, the **Chapter 5** highlighted the importance of the mode of interaction between fungal species, modulating VOC variations with the emergence of new VOCs depending on the type of interaction (contact or no physical contact) and the strains involved, but also leading to a general increase in mycotoxin production, associated with the triggering of an aggressive response by strains through the release of mycotoxins.

However, several questions arose from the previous work. How does ethyl 3-methylbutanoate contribute to this reduction? Is the reduction of fumonisins a result of growth delay? Is there a direct effect on gene expression, similar to what was observed in the study with bioactive VOCs against aflatoxins produced by *A. flavus*? How can the mode of application influence fumonisin production and in what manner?

This sixth chapter of the thesis investigated the mode of action leading to the reduction of fumonisin produced by *F. verticillioides*, initially studied in the fourth chapter in the presence of ethyl 3-methylbutanoate when applying ethyl 3-methylbutanoate by physical contact with the fungus, introducing the molecule into the growth medium, and by fumigation, introducing it into the growth atmosphere of this fungus.

To explore these questions, a strategy involving **the monitoring of VOCs, fumonisins, and gene expression in the fumonisin biosynthesis pathway** was carried out. *F. verticillioides* was grown in Malt Extract Agar (MEA) media under three conditions:

- Control, where the fungi were grown in MEA without any additional compounds;
- Non-contact application of ethyl 3-methylbutanoate, where the fungi were exposed to an atmosphere containing the compound;
- Contact application of ethyl 3-methylbutanoate, where the compound was directly introduced into the MEA.

Abstract

The use of bioactive VOCs as antifungal and antimycotoxin agents is an expanding field due to the advantages these compounds offer. However, even though fungistatic effects have been observed, as well as reductions in mycotoxin production, their mode of action remains unknown. In an effort to elucidate this understanding, a strain of *F. verticillioides* producing fumonisins and its *fum 6* mutant were studied under three growth conditions: control grown on the medium alone; in the presence of the bioactive VOC ethyl 3-methylbutanoate in its growth atmosphere; in direct contact by introducing the same compound into the medium. During these three applied conditions, the monitoring of emitted VOCs, fumonisin production, and the gene expression of six genes involved in the fumonisin biosynthesis pathway were recorded. Ethyl 3-methylbutanoate induced a reduction of fumonisin production in the wild type, accompanied by the appearance of specific sesquiterpenes under the conditions involving the bioactive VOC. Finally, the analyses of the *fum* genes expression showed that the non-contact condition under-regulated the expression of genes in both strains, while the contact condition registered an over expression of the *fum* genes.

Keywords

Fusarium verticillioides, fumonisins, volatiles organic compounds, gene expression, FUM genes, ethyl 3-methylbutanoate, bioactive compound

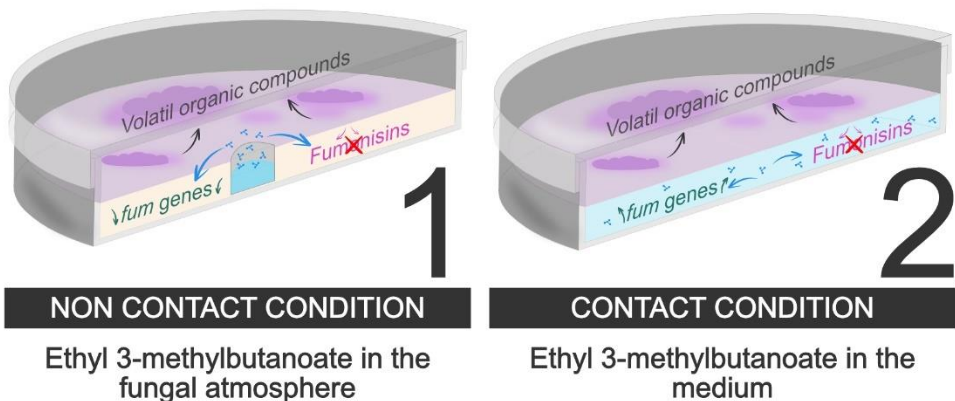


Figure 6-1. Graphical abstract of the Chapter 6

1. Introduction

Bioactive compounds are natural substances produced by living organisms such as plants, and micro-organisms. These compounds have diverse chemical structures and various biological activities that can influence multiple biological processes [269]. Over the years, bioactive compounds have received particular attention due to their potential applications in various fields, notably as an alternative to pesticides [270].

Bioactive compounds include a wide range of soluble compounds (alkaloids, polyphenols, etc.) and volatile organic compounds (VOCs). The latter ones offer several advantages over other bioactive compounds. One of the main advantages is their volatility into the atmosphere, enabling them to function as signaling molecules and travel long distances to ensure communication between organisms. In addition, VOCs often have a low molecular weight, which favors their diffusion and dispersion in the environment [271]. This makes VOCs highly effective in ecological interactions, such as defense against predators or competitors [43,272].

Bioactive VOCs also offer versatile applications for grain protection and storage. As natural fumigants, VOCs provided of insecticidal and fungicidal properties can be applied in closed storage areas to control pathogens, or sprayed directly on cereal crops. VOC-based seed treatments could improve seed germination and protect seedlings against soil-borne pathogens [273]. In addition, VOCs can be mixed with water and applied to the soil, protecting the root zone against pests and pathogens [273], the VOC-based traps and baits can be used to monitor and reduce pest populations in storage facilities (you need citation here), finally, bioactive VOCs can also be incorporated into slow-release devices for continuous protection [274].

Mycotoxin contamination is one of the world's most important and worrying agronomic problems. Indeed, mycotoxin contamination accounts for 25% of the world's foodstuffs [7], particularly cereals. As far as maize is concerned, one of the main contributors to mycotoxin contamination, and more specifically fumonisin contamination, is the pathogenic fungus *Fusarium verticillioides* [275]. This endophytic fungus can colonize any part of the maize plant, causing stalk and ear rot of maize, and accumulate fumonisins in the kernels. Fumonisins are a family of mycotoxins associated with disease in animals, with the development of cancer and disease in humans [233,238]. Using a bioactive VOC to control *F. verticillioides* growth and fumonisin production could be a useful alternative tool to be integrated into field management of the disease. Bioactive compounds affect physiological and biochemical processes of target organisms, and understanding their mode of action is crucial, for optimizing their use, developing effective control strategies against *F. verticillioides* and expand it to other pathogens.

Ethyl 3-methylbutanoate is a naturally occurring VOC, promising candidate as antifungal agent, since recent studies have revealed its bioactivity against certain pests and therefore applied as ecological fumigant.

In this chapter, we explored the efficacy of ethyl 3-methylbutanoate applied as a fumigant and in contact application against *F. verticillioides*, analyzing its specific properties.

In a previous work, we have demonstrated the anti-fungal and anti-fumonisin activity of this ester. However, we could not explain whether the fumonisin reduction was induced by mycelial reduction or by a direct influence on the fumonisin metabolic pathway. Therefore, we carried out a study on the expression of six genes involved in the fumonisin biosynthetic pathway, taking into account the different ways in which ethyl 3-methylbutanoate was applied (fumigation and direct contact). At the same time, the changes caused by the addition of this molecule to the fungal environment were monitored, to record reactions in terms of both VOCs and fumonisin production, and therefore to obtain an overall picture on its impact in the wild type and one mutant of *F. verticillioides*.

Understanding ethyl 3-methylbutanoate mode of action and exploring its potential, could help to the development of effective and sustainable pest management approaches for agricultural and storage systems.

2. Results

2.1. VOCs emission of F. verticillioides under all the conditions

In general, the application of ethyl 3-methylbutanoate caused a reduction in the total number of VOCs emitted on day 4 for both *F. verticillioides* strains studied. An overall decrease in the number of VOCs for chemical family was recorded except for the terpene family showing a different behavior depending on the type of ethyl 3-methylbutanoate application (**Figure 6-2**). While an increase of terpenes was recorded in the case of the wild-type strain and decrease was reported for the *fum 6* mutant. In another hand, the visible increase of esters was linked to the introduction of ethyl 3-methylbutanoate in the fungus environment. Since the purity of the standard of the ester had a value of 98%, we assumed that some of ethyl 3-methylbutanoate derivatives were generated.

Among the common VOCs identified in both strains of *F. verticillioides* a base of 6 VOCs was detected for all of the three conditions, made up of sesquiterpenes (4-epi-α-acoradiene, α-cedrene, β-acorenol), esters (ethyl 3-methylbutanoate, diethyl phthalate) and alcohol (2,6-bis(1,1-dimethylethyl)-4-methylphenol). Specific VOCs such as cembrene were attributed to be always emitted by the wild type regardless the conditions of the fungal growth but none was associated to the *fum 6* mutant.

The **Table 6-1** (supplementary material) reports the VOCs detected on each condition tested for the wild-type and the *fum 6* mutant strains. In general, at day 4, the number of VOCs emitted by the *fum 6* mutant was higher than the number emitted by the wild type, an observation already noted in **Chapter 3**.

Furthermore, a specific mix of VOCs were always identified for each given condition (control, contact and non-contact growth conditions with ethyl 3-

methylbutanoate), showing that even if the strains have different volatolomes, the growth condition could trigger a reaction leading to the emission of same VOCs in both strains. Thus, α-pinacene, β-copaene and δ-elemene were detected by wild-type and mutant *F. verticillioides* strains, during the contact application of the ethyl 3-methylbutanoate, while di-epi-α-cedrene was identified in the case of the presence of the ester only in the fungal growth atmosphere for both strains.

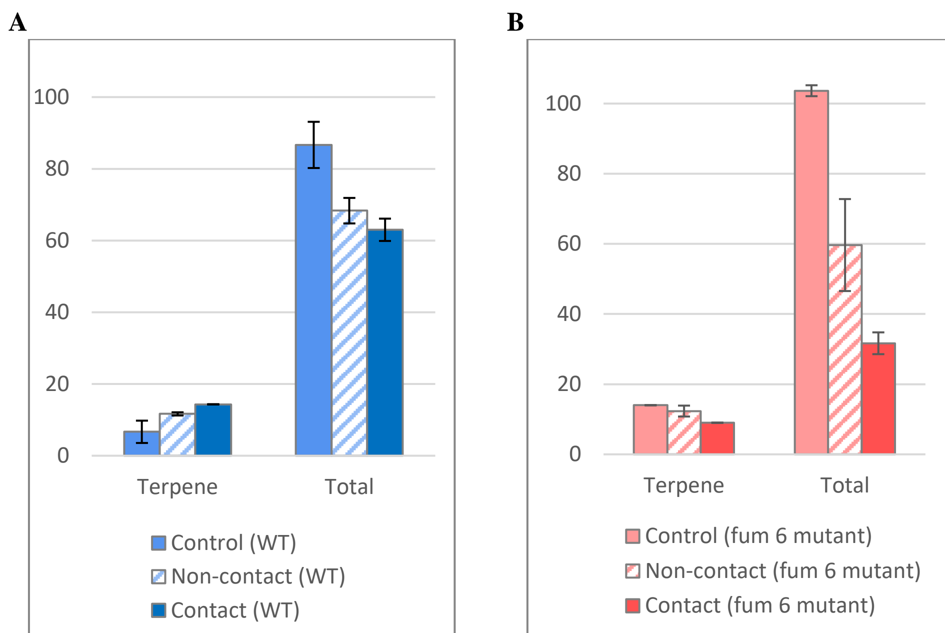


Figure 6-2. Number of VOCs emitted on day 4 under the three growth conditions of (A) the wild-type strain and (B) the *fum6* mutant *F. verticillioides* for the total number of VOC emitted and the terpene chemical family.

2.2. Fumonisins production by F. verticillioides linked to the conditions applied

The production of fumonisins induced during the 3 growth conditions of the fungus is shown in **Figure 6-3**. In the control, the *F. verticillioides* strain produced fumonisins B_1 and B_2 with a predominance of FB_1 up to 10,000 ppb on day 4. In contrast, the two conditions with the application of ethyl 3-methylbutanoate resulted in a significant reduction of both fumonisins. Between the two modes of application, around 600 ppb of FB_1 was observed for the contact application of ethyl 3-methylbutanoate, while a maximum of 20 ppb of FB_1 production at day 7 for the non-contact application. Compared with the fumonisin production of control, the application of the ester

resulted in a 96.4% reduction in production for the direct contact application and 99.9% in the non-contact condition. Concerning the *fum 6* mutants fumonisins production in all the conditions were under the LOQ.

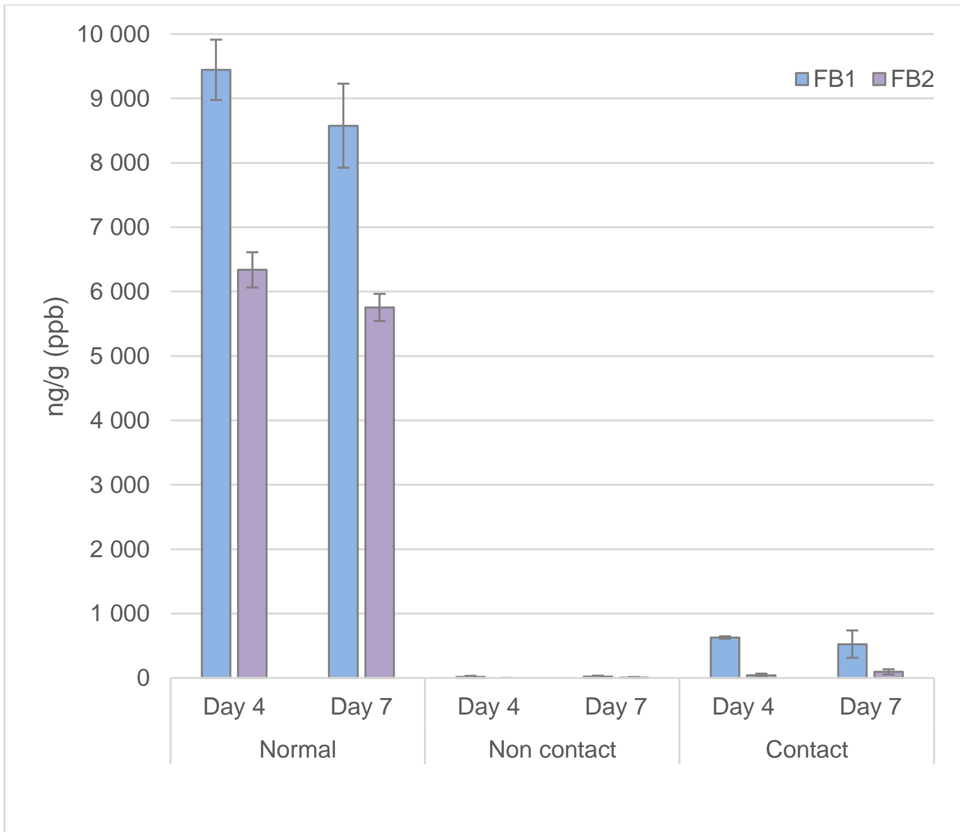


Figure 6-3. Production of fumonisin B_1 and B_2 on days 4 and 7 by *F. verticillioides* strains under different growth conditions. Control (fungal strain grown on MEA), without contact (ethyl 3-methylbutanoate in the atmosphere) and with contact (ethyl 3-methylbutanoate in MEA).

2.3. Expression of the fum genes

The RNA was extracted from *F. verticillioides* mycelium grown under the three different conditions (**Figure 6-4-A**). The mRNAs were then converted into cDNA. The efficiency of this step was verified by comparing the cDNA obtained with the fungal genomic DNA. As shown in **Figure 6-4-B**, the cDNA has a lower number of bp than the fungal genomic DNA, certifying the exclusion of the introns that differentiate these two DNAs.

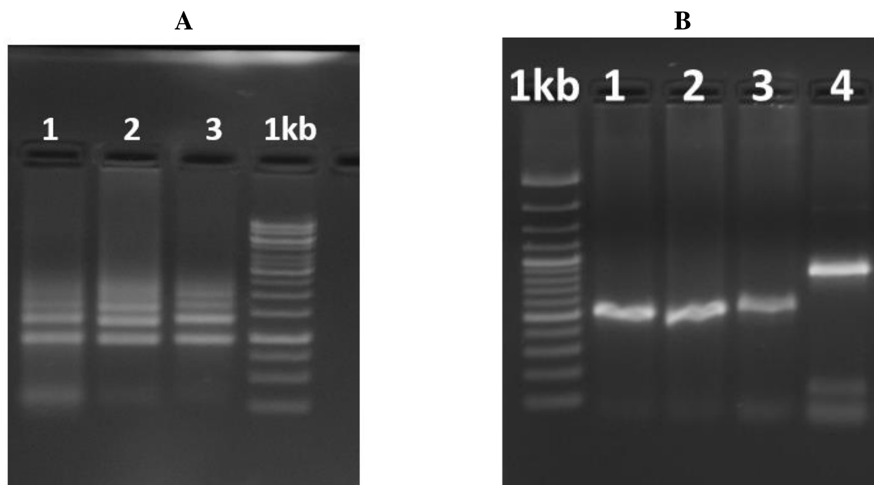


Figure 6-4. (A) RNA extraction using the Rneasy ® plant mini kit (Qiagen). (1) *F. verticillioides* in control condition, (2) grown without contact with ethyl 3-methylbutanoate and (3) grown in contact with ethyl 3-methylbutanoate.

(B) Reverse transcription using the SuperScript™ IV First-Strand Synthesis System (Invitrogen). (1) cDNA of *F. verticillioides* in control condition, (2) cDNA in non-contact condition and (3) cDNA in contact condition, (4) genomic DNA of *F. verticillioides* strain.

Using the level of *fum* gene expression of the control condition as a reference, the modifications due to the two kind of the ethyl 3-methylbutanoate application were determined. For the wild-type strain, a general over expression of the 6 *fum* genes was observed in the contact condition up to 6.4 times higher than in the control condition for *fum* 14. In addition, *fum* 14 has a higher overexpression than *fum* 1, the first gene involved in the biosynthesis of fumonisins (**Figure 6-5-A**). In contrast, the non-contact condition has reported value under 1.0, revealing a down regulation of the 6 *fum* genes implied in the fumonisin pathway (**Figure 6-5-B**).

Concerning the *fum 6* mutant strain, a same over expression of the 6 *fum* genes was noted in the contact condition for all the genes. Indeed, the *fum* 3 is over expressed 18.8 times in comparison to the normal level of expression observed in the control condition (**Figure 6-6-A**). On the other hand, the non-contact condition has revealed also a down regulation but only for *fum* 1, *fum* 3, *fum* 8 and *fum* 14 (**Figure 6-6-B**).

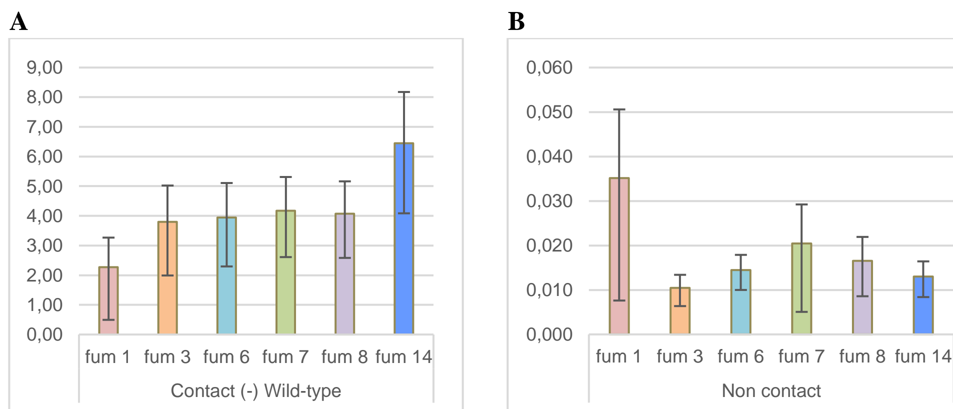


Figure 6-5. (A) Relative quantification (RQ: $2^{-\Delta\Delta Ct}$) vs *fum* gene target for the contact condition (when ethyl 3-methylbutanoate was in the MEA) (B) RQ vs *fum* gene target for the non-contact condition (when ethyl 3-methylbutanoate was present in the fungus atmosphere).

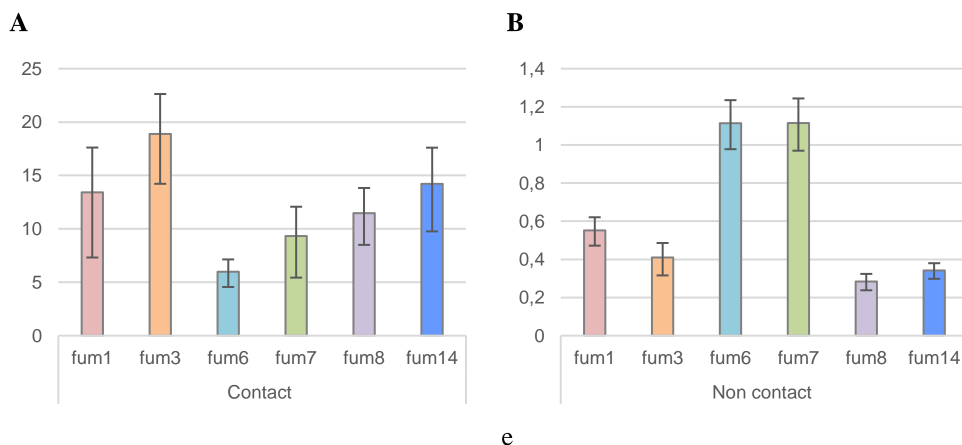


Figure 6-6. (A) Relative quantification (RQ: $2^{-\Delta\Delta Ct}$) vs *fum* gene target for the contact condition (when ethyl 3-methylbutanoate was in the MEA) (B) RQ vs *fum* gene target for the non-contact condition (when ethyl 3-methylbutanoate was present in the fungus atmosphere).

3. Discussion

Several bioactive molecules have been reported to reduce fumonisin production, but their modes of action are still poorly understood (citation here). On the contrary,

previous work on *A. flavus* and its aflatoxin production has highlighted few mechanisms of action on which the activity of some molecules able to control this species is based. These include the loss of membrane integrity, induced by a number of physicochemical modifications essential to the fundamental structure of fungal cells, and variations in the expression of genes involved in the biosynthetic pathway of aflatoxins.

Our work contributes to improve the knowledge on the best possible applications of ethyl 3-methylbutanoate.

This work has therefore been focused on volatolome modifications and *fum* gene expression, two fundamental aspects of biology. The study of gene regulation and RNA transcription provides valuable information on the dynamic nature of living organisms, just as VOCs are now recognized as fundamental vectors in fungi's perception of the environment.

Above all, it is interesting to note that among the VOCs commonly emitted by both *F. verticillioides* wild-type and *fum* 6 mutant strains during their growth under the three conditions, sesquiterpenes such as α-cedrene and β-acorenol were detected as we reported in a previous work, where these sesquiterpenes were correlated with the presence of *F. verticillioides* (see **Chapter 4**). Therefore, these data support the relevance of α-cedrene and β-acorenol as possible VOC biomarkers, as we proposed previously. In our experiment, the wild-type strain of *F. verticillioides* produced ethyl 3-methylbutanoate on MEA, whereas, in a previous work, where *F. verticillioides* was grown on maize, this VOC has been emitted exclusively by the *fum* mutants. These data confirm the versatility of the emission of certain VOCs depending on the substrate [217]. Moreover, the application of ethyl 3-methylbutanoate have led to different modification of terpene emissions in both strains. In this case, a higher number of terpenes was emitted by the wild-type strain in all the kind of application of the ethyl 3-methylbutanoate while we have recorded only this trend in the contact competition experiments between the *F. verticillioides* wild-type and the toxigenic *A. flavus* strains, that are reported in **Chapter 5**.

It is noteworthy that when the wild-type *F. verticillioides* strain grew on MEA, a natural production of a small amount of ethyl 3-methylbutanoate, which was not detected on maize and PDA used in previous chapters, was recorded. This highlights that the concentration level of this ester is important, as demonstrated in the experiments conducted in **Chapter 3**, which evaluated its fungistatic and anti-fumonisin efficiency across varying volumes in the fungal atmosphere.

Thus, the introduction of the ethyl 3-methylbutanoate at a such level into the *F. verticillioides* environment, inevitably induced metabolic changes.

Although there was no effect on mycelial growth (data not reported), the efficacy of ethyl 3-methylbutanoate as anti-fumonisin compound is undeniable. As shown in **Figure 6-3**, as soon as this ester is present in the environment, a drastic reduction of

FB_1 and FB_2 production was recorded. A clear impact of ethyl 3-methylbutanoate on the *fum* gene expression was noted. Indeed, the two opposite scenarios in the *fum* genes expression could be recorded according to the mode of application of the ethyl 3-methylbutanoate.

On one hand, the non-contact *F. verticillioides* growth condition caused a down regulation (**Figure 6-5-B**). Thus, it is likely that the perception of ethyl 3-methybutanoate by the fungal colony induced a reduction of self-defense of the fungus. Indeed, ethyl 3-methylbutanoate is an ester known to be emitted by several microorganisms. Its presence in the atmosphere of *F. verticillioides* reduced its production of fumonisin. The fact that this ester may be used by the antagonistic producing microorganism, to impact the mycotoxin production, making *F. verticillioides* less competitive, is an interesting hypothesis worth to be further investigated. As VOCs are important participants in the inter- and intra-species communication, this hypothesis is relevant. Furthermore, the down-regulation observed in the non-contact condition is consistent with the significant reduction in fumonisin produced (**Figure 6-3**) [276]. Indeed, studies using *Streptomyces* species against *F. verticillioides* have shown down-regulation of *fum* genes associated with fumonisin reduction [277]. The downregulation of *fum* 1 expression has been proposed as a potential efficient indicator for potential biocontrol agents [278]. Additionally, it is interesting to observe that a difference between the two strains was observed regarding the *fum* mainly impacted by the down regulation. Indeed, the *fum* 6 and 7 genes were not affected by the presence of ethyl 3-methylbutanoate in the mutant strain.

On the other hand, when the *F. verticillioides* has grown in contact condition with the ethyl 3-methylbutanoate, the *fum* genes were overexpressed. This overexpression may reflect the need to increase the fungus aggressiveness in response to an external stress, represented by a direct physical contact with the fungal cells. Therefore, the different gene expression behavior confirmed that the contact effect triggers a higher response. On the other hand, despite the overexpression of *fum* genes, fumonisin production was much lower compared the control. Strub et al. (2021) reported that bacterial biocontrol agents had no impact on *fum* gene expression, although a significant reduction of fumonisins under contact conditions was assessed [278]. This suggests that the lower production of fumonisin could be attributed to a post-transcription activity.

In addition, a change in the VOC profile of *F. verticillioides* wild type related to its growth condition was observed, showing the impact of ethyl 3-methylbutanoate on the fungus. In particular, the application of the ester resulted in the emission of specific sesquiterpenes by *F. verticillioides*. Sesquiterpenes are the main chemical family reported in essential oils, which are currently proposed as potential alternative as antifungal to the chemicals [219]. For example, the common and novel emission of trans-α-bergamotene in the presence of ethyl 3-methylbutanoate by the wild-type, a compound found in basil oil, has been reported to reduce both *F. verticillioides* mycelial growth and its fumonisin production on maize grains [279]. In addition, another sesquiterpene, only emitted in the non-contact condition for both strains, 1-

methoxy-4-methylbenzene has been reported to be emitted by *Bacillus* spp. and to act against *Monilinia* ssp. [280]. However, as far as we are aware, the knowledge accumulated does not completely elucidate the function or the role of the sesquiterpenes emitted by the microorganism in the interaction with microflora colonizing the same ecological niche, and, in particular, how compounds such as α-pinacene, highlighted here, are used by the producing microorganisms, remains it an open question.

It is also relevant to note that many of the VOCs emitted under the control conditions were no longer emitted in the presence of the ethyl 3-methylbutanoate, showing that this ester can not only trigger the emission of new VOCs by *F. verticillioides*, but can also suppress the production of selected metabolites (**Figure 6-2**). Finally, it is interesting to note that the fumonisin reduction was related to a number of VOCs diminution.

However, in both conditions, contact and non-contact, the application of ethyl 3-methylbutanoate, even though the gene expression response dramatically differed between the two types of application, inhibited the fumonisin production. This suggests that the mode of action of this ester had an impact on other processes unrelated to gene expression, possibly occurring at a post-transcriptional level. Some studies have reported that the application of cinnamaldehyde [220] or trans-2- hexenal [221] caused morphological alterations in the apical region of the hyphae, as well as in the mitochondria, which affect fumonisin and ergosterol production. If the effect of ethyl 3-methylbutanoate on the fumonisin production act at epigenetic level, is something to be better investigated.

Both application conditions of ethyl 3-methylbutanoate induced a significant reduction of fumonisin production. However, the direct contact condition reduced fumonisin B_1 less than the non-contact condition. Indeed, fumigation into the pathogen's atmosphere with the bioactive compound produced no more than 20 ppb after 7 days of exposure. When the efficacy of the same compound was compared, fumigation always proved to be more effective in reducing mycelia and/or mycotoxin production than contact application [223]. Thus, fumigation of ethyl 3-methylbutanoate seems to be the best option to be used to investigate the control of fumonisin production, in a program of field management of the disease.

4. Conclusion

In conclusion, the modifications induced by the presence of ethyl 3-methylbutanoate in fungal behavior are evident. These are visible through variations in VOC emissions, changes in *fum* gene expression, and reduction in fumonisin production. While a drastic reduction in fumonisin production by *F. verticillioides* following the

application of ethyl 3-methylbutanoate was observed, the expression of the 6 *fum* genes showed an opposite effect, being up-regulated in the case of direct contact and down-regulated in non-contact experiments. The fact that gene over-expression in the case of contact application still results in a reduction of fumonisin production suggests that its intervention impacts regulation at a different level, thereby preventing mycotoxin production. The emergence of new specific VOCs for each type of application raises questions about their contribution to fumonisin reduction. The question remains unresolved, always torn between the possibility that these new VOCs would compensate for the loss of fumonisin production to maintain competitiveness with other species, or the result of stress incurred by the presence of this bioactive ester. New avenues of research are opening up to elucidate the mode of action of this promising bioactive compound.

5. Supplementary material

Table 6-1. VOCs emitted by the wild-type and *fum 6* mutant *F. verticillioides* strains at 23°C on MEA under the three growth conditions without the blank subtraction.

	fum 6 mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Acid						
2,6-Dichlorobenzoic acid			x			x
Acetic acid						x
Hexadecanoic acid					x	x
Tetradecanoic acid		x			x	x
Alcohol						
1-Heptatriacotanol		x			x	
2,3-Dimethylbutan-2-ol	x			x		
2,4-Di-tert-butylphenol		x			x	
2,6,10-Trimethyltetradecane		x				
2,6-Bis(1,1-dimethylethyl)-4-methylphenol	x	x	x	x	x	x
2-Butyloctan-1-ol				x		
2-Methylbutan-1-ol		x				
2-Methylpropan-1-ol		x			x	
3,7-Dimethyloctan-1-ol	x					
3-Methylbutan-1-ol		x				
4,4'-(Hexafluoroisopropylidene)diphenol					x	

Table 6-1 (continued)	*fum 6* mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Alcohol						
4-Ethyl-1-octyn-3-ol			x			x
5-Isopropylidene-4,6-dimethylnona-3,6,8-trien-2-ol	x					
Biphenol A		x			x	x
Tetrahydro-2-furylmethanol				x		
Tetrahydrofurylmethanol			x			
Aldehyde						
3,5-di-tert-Butyl-4-hydroxybenzaldehyde					x	
4-Ethylbenzaldehyde		x				
5,9,13-Trimethyl-4,8,12-tetradecatrienal		x			x	
9,10 Dideutero-octadecanal					x	
Benzeneacetaldehyde		x	x	x	x	x
Cinnamaldehyde		x			x	
Decanal		x	x		x	x
Dodecanal	x	x		x	x	x
(E)-15-Heptadecenal					x	
Hexadecanal					x	
Nonanal		x		x	x	x
Tetradecanal		x	x		x	x
Undecanal		x				x
Alkane						
1,2-15,16-Diepoxyhexadecane			x			
10-Methyleicosane		x				
2,3-Dimethyldodecane		x	x		x	x
2,4-Dimethyldodecane			x			
2,5-Dimethyldodecane			x		x	x
2,5-Dimethyltridecane		x			x	
2,6,10-Trimethyltetradecane		x				
2-Methyltridecane		x			x	
2-Phenyldodecane		x				
3-Methyldodecane		x	x		x	x
3-Methyltridecane		x				

Table 6-1 (continued)

	fum 6 mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Alkane						
4-Methyldodecane		x	x			x
5,8-Diethyldodecane		x			x	
5-Methyltetradecane		x			x	
7-Methylpentadecane		x			x	
Docosane					x	
Dodecane		x	x		x	x
Eicosane	x	x		x	x	
Heneicosane		x		x	x	
Heptacosane					x	
Heptadecane	x	x		x	x	
Heptane		x			x	
Hexacosane					x	
Hexadecane	x			x		
Nonadecane		x			x	
Octacosane		x			x	
Octadecane		x		x	x	
Pentacosane					x	
Tetracosane					x	
Tetradecane		x	x		x	x
trans-1,2-Diphenylcyclobutane			x			
Tricosane				x	x	
Tridecane		x	x	x	x	x
Undecane		x			x	
Alkene						
(1-Butylheptyl)benzene		x				
(1-methylethenyl)-Benzene			x			
1,2,3-Trimethylbenzene		x	x		x	
1,2,4-Trimethylbenzene					x	
1,2-Dimethylbenzene		x			x	
1,4-Dimethyl-2-isopropylbenzene			x			x

Table 6-1 (continued)	*fum 6* mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Alkene						
10,18-Bisnorabieta-8,11,13-triene					x	
1-Ethyl-3-methylbenzene			x			
1-Ethyl-4-methoxybenzene	x			x		
1-Hydroxy-3-methyl-5-ethylbenzene			x			
1-Methoxy-4-methylbenzene			x			x
1-Propenylbenzene			x			
2-Phenyltridecane					x	
Caprolactam	x			x		
Cembrene				x	x	x
Ethylbenzene					x	
Methylbenzene		x		x	x	x
Naphthalene			x		x	
Octahydro-4,7-methano indene		x				
Octahydro-4,7-methano-1H-indene				x		
Styrene		x	x	x	x	x
Amide						
4-Ethylbenzamide		x			x	
Amine						
4-Ethyl-N-[4-(1-methylethyl)phenyl]-4H-3,1-benzoxazin-2-amine		x				
Dioxane						
2,2-Diisopropyl-1,3-dioxolane	x		x	x		x
5-hexadecoxy-2-pentadecyl-1,3-dioxane			x		x	x
Ester						
1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester		x			x	
2,4-Dimethyl-3-pentanol acetate						x
2-Methylbutyl 3-methylbutanoate			x			x
2-Methylpropyl (Z)-tetradec-9-enoate		x				
2-Methylpropyl 3-methylbutanoate	x		x	x		x
3-Methylbutyl 3-methylbutanoate	x		x			
3-Methyltetradecane		x				
Bis(2-hydroxyethyl) benzene-1,4-dicarboxylate		x				

Table 6-1 (continued)	*fum 6* mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Ester						
Di-2-methylpropyl phthalate	x	x		x	x	
Diethyl Phthalate	x	x	x	x	x	x
Dihydro methyl jasmonate		x			x	
Ethyl 2,3-dimethylbutanoate			x			
Ethyl 2-hydroxy-3-methylbutanoate	x		x	x		x
Ethyl 2-methylbutanoate						x
Ethyl 3-hydroxy-3-methylbutanoate	x		x	x		x
Ethyl 3-methylbut-2-enoate	x		x	x		x
Ethyl 3-methylbutanoate	x	x	x	x	x	x
Ethyl Acetate						x
Ethyl butanoate		x				
Ethyl pent-4-enoate			x			x
Furfuryl 3-methylbutanoate			x			
Furfuryl pentanoate	x					
Isobutyl pentanoate	x					
Isopropyl myristate		x			x	x
Isopropyl myristate					x	
Isopropyl palmitate					x	
Methyl 3-methylbut-2-enoate				x		
Methyl 3-methylbutanoate	x		x	x		x
Sorbic acid vinyl ester				x		
Triacetin				x		
Vinyl stearate		x				
Ether						
Decyl ether		x	x			
Octyl ether		x				
Ketone						
1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-1-penten-3-one		x				
3-Octyn-2-one	x					
4-(3-cyclohexen-1-yl)-3-Buten-2-one					x	
4-Hydroxy-4-methylpentan-2-one		x			x	

Table 6-1 (continued)	*fum 6* mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Ketone						
6-Methyl-3,5-heptadiene-2-one			x			
6-Methyl-5-hepten-2-one		x			x	
7-Isopropyl-7-methyl-nona-3,5-diene-2,8-dione				x		
Acetophenone			x			x
Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione					x	
Geranyl acetone		x			x	x
Heptan-4-one					x	
Propan-2-one		x			x	x
Other						
4,4,6b-Trimethyl-2-prop-1-en-2-yl-5,5a,6,6a-tetrahydro-2H-cyclopropa[g][1]benzofuran		x				
Caprolactam				x		
Cyclopenta[g]-2-benzopyran, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-		x			x	
Dichloromethane			x		x	x
Methoxyphenyloxime	x	x	x		x	x
Pyrrole				x		
Terpene						
(1E,3E,7E,11E)-4-Isopropyl-1,7,11-trimethylcyclotetradeca-1,3,7,11-tetraene			x			
(4aE)-2,3-diethyl-6,7,8,9,10,11,12,13,14,14a-decahydrobenzo[12]annulene			x			
18-Norabieta-8,11,13-triene					x	
2,6,10,10-Tetramethyl-1-oxaspiro(4.5)deca-3,6-diene	x			x		
3a,4,5,6,7,7a-Hexahydro-4,7-methanoindene		x		x	x	
4-epi-alpha-Acoradiene	x	x	x	x	x	x
Acora-3(7),14-diene	x			x		
alpha-Alaskene		x	x			x
alpha-Cedrene	x	x	x	x	x	x
alpha-Pinacene	x			x		
alpha-Pinene		x			x	
beta-Acorenol	x	x	x	x	x	x
beta-Cedrene		x	x			x

Table 6-1 (continued)	*fum 6* mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Terpene						
beta-Copaene	x			x		
beta-Elemene				x		
beta-Ionone epoxide					x	x
beta-Sesquiphellandrene		x	x			x
Cadina-3,5-diene		x	x		x	x
cis-Thujopsene				x		
delta-Elemene	x			x		
Di-epi-.alpha.-cedrene			x			x
Geranyl-alpha-terpinene		x	x			
Italicene ether	x	x	x	x		x
Limonene		x		x		
p-Cymene		x				
Phellandranal				x		
Sabinene		x				
trans-alpha-Bergamotene			x	x		x
trans-Calamenene		x	x		x	x
Non identified						
NI 1033		x				
NI 1074					x	
NI 1127					x	
NI 1131	x			x		
NI 1145		x				
NI 1165						x
NI 1166		x				
NI 1170					x	x
NI 1175		x				
NI 1187		x				
NI 1190		x				
NI 1194				x		
NI 1202						x
NI 1207				x		

Table 6-1 (continued)	*fum 6* mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Non identified						
NI 1215			x			x
NI 1233			x			
NI 1244		x				
NI 1262					x	
NI 1265				x		
NI 1266						x
NI 1270				x		
NI 1277						x
NI 1278		x				
NI 1282			x			
NI 1292		x				
NI 1302		x				
NI 1312		x				
NI 1314			x			
NI 1315		x				
NI 1332		x	x			
NI 1339		x				
NI 1352		x				x
NI 1359						x
NI 1368			x			x
NI 1371						x
NI 1372		x				
NI 1380			x			
NI 1405				x		
NI 1413						x
NI 1419		x				
NI 1456					x	
NI 1461		x			x	
NI 1484					x	
NI 1492				x		
NI 1508				x		

Table 6-1 (continued)	*fum 6* mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Non identified						
NI 1553		x			x	
NI 1556					x	
NI 1590					x	
NI 1611				x		
NI 1623		x			x	
NI 1645				x		
NI 1653				x		
NI 1662					x	
NI 1675					x	
NI 1683					x	
NI 1703		x			x	
NI 1705			x			x
NI 1735		x				
NI 1740					x	
NI 1761					x	
NI 1763				x		
NI 1806					x	
NI 1815		x				
NI 1870			x			x
NI 1877		x			x	
NI 1877 bis			x			x
NI 1886				x		
NI 1888		x	x			x
NI 1897						x
NI 1898			x			
NI 1902						x
NI 1947					x	
NI 1950						x
NI 1951		x	x			
NI 1961			x			
NI 1984		x				

Table 6-1 (continued)

	fum 6 mutant			Wild type		
	Contact	Control	Non contact	Contact	Control	Non contact
Non identified						
NI 1990					x	
NI 2001					x	
NI 2056				x		
NI 2092				x		
NI 2112				x		
NI 2120				x		
NI 2138				x		
NI 2154				x		
NI 2162				x		
NI 2174				x		
NI 2237				x		
NI 461					x	
NI 875		x				
NI 978						x
NI 999						x

6. Material and method

6.1. Fungal strain

A strain of *Fusarium verticillioides* (ITEM 10514), producing fumonisin B_1, B_2 and B_3 the *fum 6* mutant (ITEM 10516), no fumonisin producer was used. The CNR-ISPA (Research National Council of Italy—Institute of Sciences of Food Production, Bari, Italy) provided the fungal strain which belong to the official collection of the Institute of Sciences of Food Production ITEM Collection, member of the International Organization of European Culture Collections and the World Federation of Culture Collections.

6.2. Chemical material

Analytical-grade acetonitrile (ACN), methanol (MeOH), o-phthaldialdehyde (OPA), 2-mercaptoethanol, phosphate buffered saline (PBS) tablet, sodium tetraborate ($Na_2B_4O_7$), glacial acetic acid were purchased from Mallinckrodt Baker

(Milan, Italy) and Sigma (St. Louis, MO, USA). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Standards of FB_1 and FB_2 were obtained from Biopure (Romer Labs Diagnostic GmbH, Tulln, Austria). Glass microfiber filters (Whatman GF/A) and paper filters (Whatman No. 4) were bought from Whatman (Maidstone, UK). FumoniTest^{TM} Wide Bore immunoaffinity columns (IACs) were purchased from Vicam L.P. (Milford, MA, USA). OPA reagent solution was prepared by dissolving 40 mg OPA in 1 mL of MeOH and diluting with 5 mL 0.1 M sodium tetraborate solution. Then, 50 µL 2-mercaptoethanol were added and the solution was mixed for 1 min and stored in the dark up to 1 week at room temperature [239]. The ethyl 3-methylbutanoate was purchased by Sigma-Aldrich (purity ≥98%).

6.3. Fungal inoculation

The spore suspension was prepared by adding 1 mL of Tween 20 (0.5%) and 2 mL of distilled water in a petri dish containing the fungi that had grown on SNA medium (Synthetic Nutriment-poor Agar, for 1L: 1 g KH2PO4; 1 g KNO3; 0.5 g MgSO4; 7H2O; 0.5 g KCl; 0.2 g glucose; 0.2 g sucrose; 20 g agar) in darkness at 23°C for 7 days, from the fridge stock. Using a Thoma cell, a volume containing 1 x 105 spores was inoculated and spread on Petri dish containing MEA (Malt Extract Agar).

6.4. Experimental design

The VOCs, genes expression and fumonisins production were analyzed at day 4 and day 7 after the day of the inoculation.

Three conditions were studied: the control, where the fungal strains were grown on MEA, the non-contact application, where a volume of 50 µL of ethyl 3-methylbutanoate was added in an inox caps (15 mm x 15 mm, VWR, Belgium), previously autoclaved, and centrally put in the petri dish containing MEA, the contact application, where 50 µL of ethyl 3-methylbutanoate was added in the MEA before to be pour in the Petri dish. For each sample, three petri dishes were used.

6.5. VOCs analysis

The petri dishes containing the fungal strain at day 4 and day 7 after the inoculation were opened and placed in VOC extraction system (glass recipient of 1.5L) for 1 hour to let VOCs disperse and equilibrate through the air volume at 23°C±3°C in darkness. Then, the VOCs were sampled for 1 hour using a SPME fiber (Supelco, Darmstadt, Germany, DVB/CAR/DDMS, 50/30µm, 24 Ga).

VOCs were separated on an Agilent Technologies GC 8890 System, using an HP-5ms column (Agilent Technologies, Santa Clara, CA, USA, 5%-phenylmethylpolysiloxan, non-polar, 30 m × 0.250 mm × 0.25 µm) with a constant helium flow rate of 1 $mL \cdot min^{-1}$. The desorption at 250 °C by splitless injection using an SPME inlet coating of 78.5 mm × 6.5 mm × 0.75 mm (Supelco Inc., Bellefonte, PA, USA) during 5 min was made. The GC temperature program was as follows: 40

°C for 5 minutes, 8 °C.min^{-1} up to 240 °C, 30 °C.min^{-1} up to 290 °C, and a final hold at 290 °C for 5 minutes. On an Agilent Technologies 5977C GC/MSD, electron ionization (EI) mode at 70 eV and scan mass ranges from 35 to 350 amu was performed for the mass spectral analysis. Ion source and MS source temperatures were respectively at 250 °C and 280 °C.

To identify the VOCs, a mass spectra comparison with NIST17 and WILEY298 libraries and the retention indices (RI) according to the method of Van Den Dool and Kratz on a non-polar HP-5ms column was made. RI were calculated using a mixture of n-alkanes (alkane C5-C29 (1000 mg.mL−1 in hexane, Charleston, SC, USA)). The VOCs detected in at least 2 repetitions were reported in **Table 6-1.**

6.6. Collection of the fungal mycelium for the analyses

The collection of material required for fumonisin, and gene expression analyses was carried out as follows. A quart of the Petri dish was used for the fumonisin quantification and stored at -20°C. The remaining three-quarters were used to collect the mycelium in a 2 mL sterile Eppendorf containing liquid nitrogen and stored at -80°C. A single sample was composed of three petri dishes.

6.7. Fumonisin analysis

5g of MEA was chopped and crushed and then extracted with a mixture (20 mL) of methanol:water (70:30, v/v) by shaking for 1 hour. The extract was filtered through a filter paper. An aliquot of filtrate (10 mL) was diluted with PBS (40 mL) and filtered through a glass microfiber filter. After, a volume of filtered extract (10 mL) was cleaned up through FumoniTestWB immunoaffinity column (IAC). An appropriate dilution of diluted extract, before loading onto the IAC, was performed with PBS when fumonisin concentrations in the diluted extract were higher than the maximum IAC binding capacity. After elution, the column was washed with 10 mL PBS and then FBs were eluted with 2 x 1 mL methanol followed by 2 x 1 mL water. Then the extract was dried under a nitrogen stream at 40°C and reconstituted with 500 µL of water:acetonitrile (70:30, v/v). Sample extracts were derivatized with OPA reagent and analyzed by HPLC according to the procedure described by De Girolamo et al. (2011), with some modifications. The analysis were performed on HPLC apparatus was an Agilent 1100 Series chromatographic system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, autosampler, column thermostat set at 30°C and a spectrofluorometric detector (model G7121A, λex = 335 nm, λem = 440 nm). The analytical column was a LUNA C18 (150 mm x 4.6 mm, 5 µm) (Phenomenex, Torrance, CA, USA) with a 3mm i.d., 0.45 µm pore size guard filter (Rheodyne, Cotati, CA, USA). Quantification of fumonisins (FB1, FB2) was performed by measuring peak areas at FB_1 and FB_2 retention times and comparing them with the relevant calibration curves. FB_3 was quantified by comparing with the calibration curve of FB_2 as reported by Palacios et al. (2015). The autosampler was programmed to mix 50 µL of sample extracts or standard with 50 µL of OPA reagent,

mix for 50 s, incubate for 2 min and then inject all the derivatized mixture. The mobile phase was a mixture of water:methanol:acetic acid (27:73:1, v/v/v) eluted at a flow rate of 1 mL.min-1. With these conditions, retention times of FB_1, FB_2 and FB_3 were about 8.9, 23.0 and 20.3 min, respectively. The LOD values were 5 $\mu g.kg^{-1}$, while LOQ values were 16 $\mu g.kg^{-1}$.

6.8. DNA purification and verification

The DNA purification was carried out from a fresh mycelium incubated at 23°C on PDA, using the Wizard® Magnetic DNA purification system for food (Promega) according to the manufacturer's instructions.

6.9. mRNA isolation and reverse transcription (cDNA)

The RNA was extracted from the *F. verticillioides* mycelium obtained under the three-growth condition studied using the RNeasy® Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The quantity and quality of isolated RNA were assessed using nanodrop measurements and electrophoresis analysis. The nanodrop use the absorbance of light. The 260 nm absorbance (A260) corresponds to the presence of nucleic acids in the sample, while the 280 nm absorbance (A280) indicates the presence of contaminants like proteins. The ratio of A260/A280 is used as a measure of RNA purity. High-quality RNA typically exhibits a ratio close to 2.0, indicating minimal protein contamination. In addition, the 28S and 18S ribosomal RNA bands are commonly used as indicators of RNA integrity. The 28S band should be more intense and larger than the 18S band as it is observed in **Figure 6-4**.

The complementary DNA (cDNA) was synthesized using the SuperScript® IV First-Strand cDNA Synthesis Reaction (Invitrogen) using oligo$(dT)_{20}$ and random hexamers (**Figure 6-5**).

The RNA and cDNA were respectively storage at -80°C and -20°C.

5.10 Real time PCR

A real-time PCR (Polymerase Chain Reaction), a molecular biology technique that allows for the amplification and quantification was performed on the cDNA samples using the Applied biosystems VIIa 7. The primer pairs used for amplification targeted the *fum* 1, *fum* 3, *fum* 6, *fum* 7, *fum* 8, *fum* 14, TUB (β-tubulin) and CALM (calmodulin) and their primers are reported **Table 6-2.** he Platinum SYBR Green qPCR SuperMix-UDG reagent (Invitrogen, Life Technologies) was used as the reaction mixture, according to the manufacturer's instructions. The rt-PCR cycling conditions consisted of an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 1 second, and 60°C for 30 seconds. Fluorescence data were collected during the extension step of each cycle, and melting curve analysis was performed to verify the specificity of the PCR products. All rt-PCR reactions

were run in triplicate, and the mean cycle threshold (Ct) values were used for further analysis.

5.11 Gene expression analysis

A relative quantification (RQ: $2^{-\Delta\Delta Ct}$) based on comparative Ct (ΔΔCt) was applied since the efficiencies of compatibility tests between the average of the reference genes (TUB and CALM). The wild type *F. verticillioides* colony used as control was used as a reference to determine the up or down regulation of the 6 *fum* genes under the application of the ethyl 3-methylbutanoate. The method described in Livak et al. (2001) was applied [281].

Table 6-2. *fum* genes investigated and their function in the fumonisin pathway. (Table adapted form Rocha et al. (2016)

Locus	Primer sequence (5'-3')	Fragment size (bp)	Function in biosynthesis
FUM1F *FUM1R*	GAGCCGAGTCAGCAAGGATT AGGGTTCGTGAGCCAAGGA	90	Synthesis of the polyketide backbone
FUM3F *FUM3R*	CTTGGCGGTGCCCATACTA GGACCAAGAGCGTGGATG	60	C5 hydroxylation
FUM6F *FUM6R*	GATAGACTCGGGGCTGAGA AGCTCGCCGACAGAATC	100	C14 and C15 hydroxylation
FUM7F *FUM7R*	CATCGTATCTACATTGTCGCATC TGTACTCTCCAACAATATGAATGAGTC	100	Reduction of the double bond in TCA side chains
FUM8F *FUM8R*	CAACAGAAATACGCAATGACG TGCTCGACCACTACATCAGG	99	Condensation of alanine and polyketide
FUM14F *FUM14R*	TAGGTCCAGGTCGAGATGCT GGAAGCCAAGAACCCAATCT	99	Esterification of TCAs to C14 and C15 hydroxyls
TUBF *TUBR*	CCGGTATGGGTACTCTGCTC CTCAACGACGGTGTCAGAGA	95	
CALMF *CALMR*	ACGGTTTCATTTCTGCTGCT TCAGCCTCTCGGATCATCTC	97	

Chapter 7

General discussion, conclusions and perspectives

The research conducted and reported in the previous chapters have significantly enriched the understanding of the topic, particularly with regard to VOCs, fungi, and mycotoxin contamination, while also proposing new approaches. Building upon this knowledge, the forthcoming endeavor will involve addressing the five main questions posed in the introduction. Drawing on the insights gleaned from these studies, answers will be provided and proposed, contributing to the field and to our overall understanding.

1. General discussion

This section focuses on the features, interesting VOCs and the results based on the strains and the growth conditions included in this study. Further experiments involving a variety of strains for each species studied grown under different growth conditions could provide additional knowledge on the main parameters that impact the VOCs profile modulation.

1.1. Do VOCs could be potential biomarkers of A. flavus and F. verticillioides?

The best VOC candidates as biomarkers are defined by two parameters: their constant emission over time and their independence from substrates, that make them highly specific intrinsic VOCs.

1.1.1. Carbon Source: Fungal Fuel

As pointed out in the introduction, many factors affect VOC emissions. Considering all the data provided in the previous chapters, we can demonstrate the impact of substrate and temperature on the diversity of chemical families emitted. As the time points of analysis are somewhat staggered in each chapter to match the best experimental design, the information that follows will therefore be overall trends, but conclusive, nonetheless.

The VOC analyses carried out for *F. verticillioides* at 23°C were performed on 2 different media: PDA and maize (**Figure 7-1**). Although the same chemical families were detected, and sesquiterpenes, alcohols and esters were the predominant chemical families in both substrates, their relative abundance differed. While sesquiterpenes were on PDA in the majority, followed by alcohols and esters, on maize, esters and sesquiterpenes are swapped, making esters the majority chemical family detected. The trend in the number of VOCs for each chemical family also diverged. The PDA temporality favors an increase in sesquiterpenes, while on maize, sesquiterpenes recorded a high emission on the first day of analysis, followed by a sharp decrease. Esters, on the other hand, had a similar emission temporality on both substrates.

The use of a synthetic medium has the advantage of rapid preparation and homogeneity of nutrients and their bioavailability, but certain limitations must be

considered, such as the restriction on the number of days during which the experiment can be carried out. In fact, once the fungus has colonized the entire medium, the experiment will be over. This stage of the colony may be associated with the phenomenon called quorum sensing, which represents a new condition of the fungi and induces the emission of specific VOCs. Moreover, with pathogenic fungi, this type of synthetic medium can lead to a loss of toxicity and viability of the fungi, particularly in environments considered as rich. That explain why on PDA analyses were performed only until day 8, while on maize the experiments were carried out until 21 days.

On the other hand, it is conceivable that some VOCs result from the degradation mechanism involved in nutrient uptake by fungi, which involves numerous enzymes produced but commonly found in the fungal kingdom, explaining they emission when the fungus grow under specific carbon source.

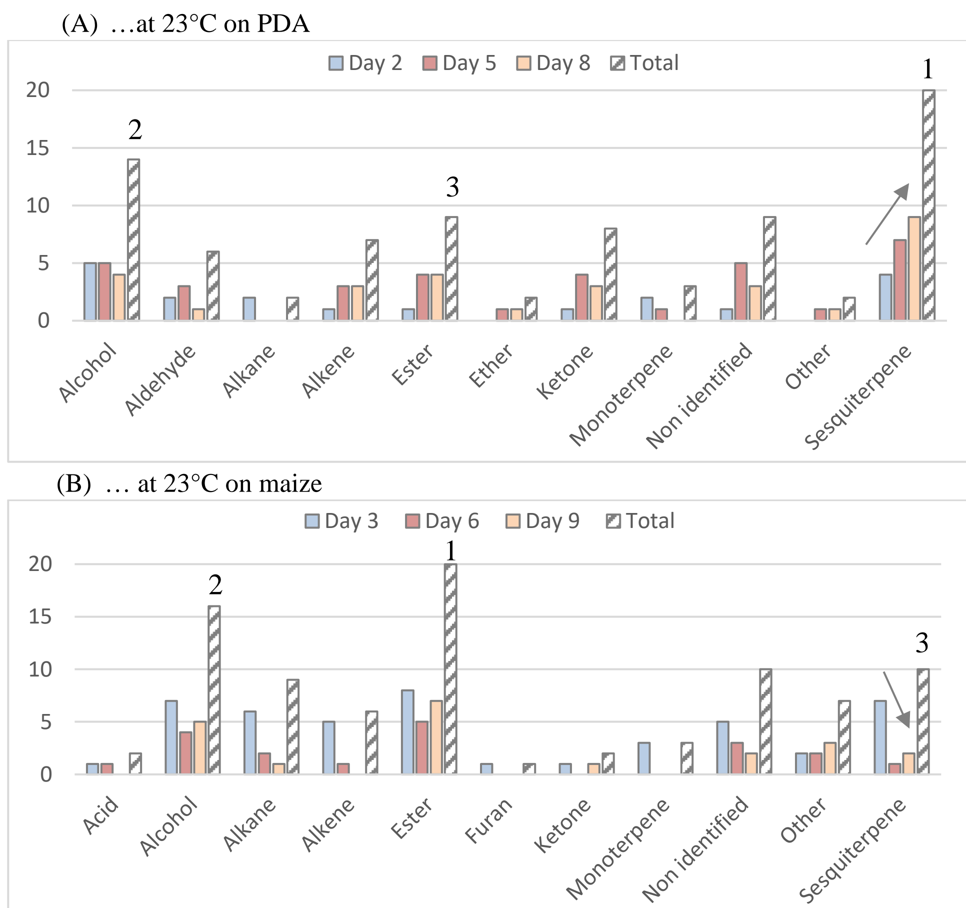


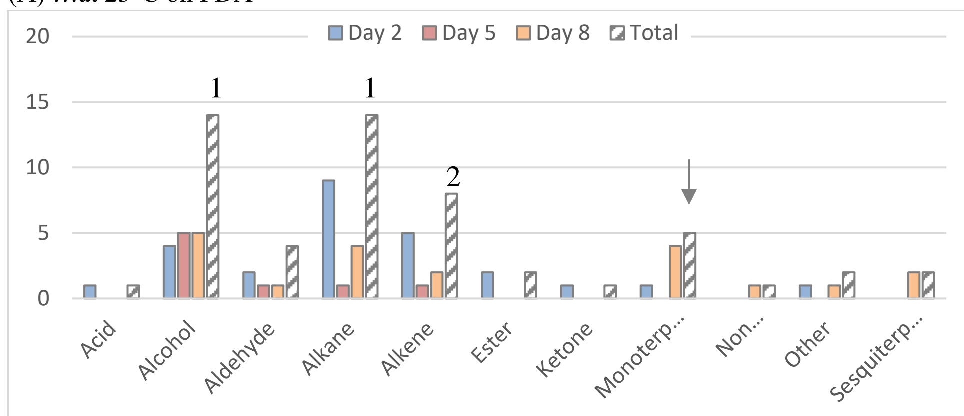
Figure 7-1. Distribution of the chemical classes emitted by *F. verticillioides* at 23°C on PDA (A) and on maize (B) over a ten-day period. (1,2,3: order of predominance and arrow: significant trend)

1.1.2. Temperature: The Orchestra of Fungi

The VOC analyses carried out for toxigenic and non-toxigenic *A. flavus* on PDA were performed at 2 different temperatures: 23°C and 30°C.

At lowest temperature studied, for the non-toxigenic *A. flavus* strain, alcohols and alkanes followed closely by alkenes were the most representative chemical families. But at higher temperature, esters and alcohols are followed by aldehydes and alkenes in equal proportions. The same trend in the number of VOCs for each chemical classes was observed, except for esters. In fact, esters were only emitted on the first day of analysis at 23°C, in contrast to a constant emission at 30°C. In addition, the lower temperature triggered a monoterpene emission in opposition of a sesquiterpene emission at 30°C (**Figure 7-2**).

(A) …at 23°C on PDA



(B) …at 30°C on PDA

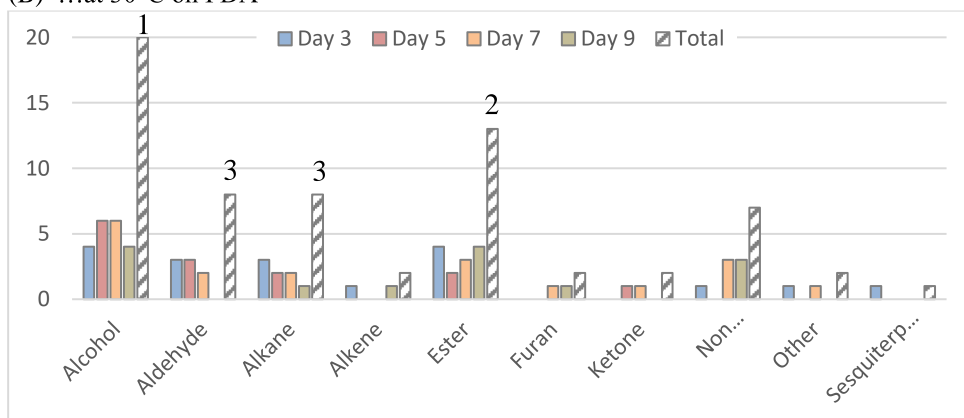
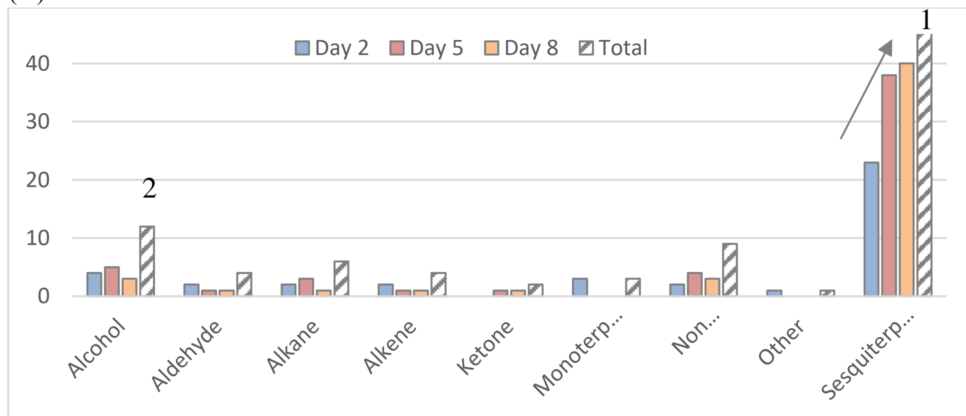


Figure 7-2. Distribution of the chemical classes emitted by non-toxigenic *A. flavus* on PDA at 23°C (A) and at 30°C (B) over a ten-day period. (1,2,3: order of predominance and arrow: significant trend)

In the case of the toxigenic *A. flavus* strain, temperature had no effect on the order of chemical classes detected. The sesquiterpenes followed by alcohols were the main classes. However, the evolution over time is divergent. At low temperature, sesquiterpenes emission increased, whereas at higher temperature, only a peak was established on the first day, followed by a significant decrease (**Figure 7-3**).

(A) …at 23°C on PDA



(B) …at 30°C on PDA

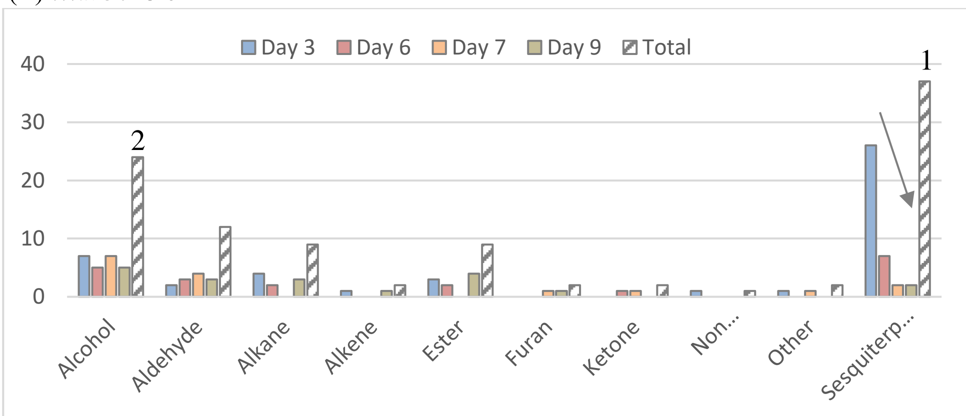


Figure 7-3. Distribution of the chemical classes emitted by toxigenic *A. flavus* on PDA at 23°C (A) and at 30°C (B) over a ten-day period. (1 and 2: order of predominance and arrow: significant trend)

1.1.3. Species VOC biomarkers

As shown in the previous sub-sections, VOCs vary with temperature and substrate. However, a common base of VOCs is emitted, as shown in **Figures 4-2** and **5-4**. The possible VOCs, underlined in the discussion of chapters 3 and 4, have been compared to confirm their possible use as fungal biomarkers.

Fungal contamination is reported by the emission of a pool of alcohols (2-methylbutan-1-ol, 2-methylpropan-1-ol, 3-methylbutan-1-ol), commonly emitted by all the strains under all the conditions studied including substrates, temperature and growth conditions. Additionally, these alcohols always occur with microorganism presence.

Except these alcohols, the two species of studied fungi emitted different VOCs. Thus, the *F. verticillioides* contamination regardless the temperature and the substrate were associated to 3 terpenes, 4-epi-α-arocadinene, α-cedrene and β-acrorenol, and 2 esters, 2-methylbutyl acetate and 3-methylbutyl acetate, respectively the esters from the alcohols reported for a fungal contamination. These esters weren't detected in the *A. flavu*s species, suggesting a divergence of their metabolism. In addition, these 5 VOCs were continuously emitted also in the case of co-inoculation, making them robust constant biomarkers. Concerning the *A. flavus* species, the styrene was the permanent alkene in both toxigenic and non-toxigenic strains. The two strains of *A. flavus* can be distinguished by the emission of several sesquiterpenes such as epizonaren, δ-cadinene, β-selinene or even germacrene D. However, using the **Table 2-7**, reporting all the VOCs detected by each strain of *A. flavus*, as biomarkers for this species, the epizonaren and δ-cadinene should be added to the styrene, because they are known to be emitted by both toxigenic and non-toxigenic strain. On the other hand, β-selinene and germacrene D were only associated to toxigenic strains. As for the *F. verticillioides*, these compounds were permanently emitted even in the co-inoculation making them reliable biomarkers.

Additionally, the two species exhibited different predominant chemical families in their VOC emissions. Sesquiterpenes were primarily emitted by *A. flavus*, while esters were highly emitted by *F. verticillioides* (**Figure 5-3**). These data demonstrate that the two fungi have distinct metabolic pathways that result in the emission of different categories of compounds.

Indeed, the VOC biomarkers mainly reported in this section are the result of the experimental conditions applied in this study. However, it would be worthwhile to confirm their potential as reliable biomarkers for *A. flavus* and *F. verticillioides* by testing a larger number of strains, and using a wider range of substrate, temperature, and other parameters affecting the VOCs profile.

1.2. Do VOCs could be potential biomarkers for aflatoxin and fumonisin contamination?

As described in **Chapter 1**, *A. flavus* has the intrinsic property of containing two groups of strains linked to their aflatoxin production capacity by the presence or absence of the entire aflatoxin gene cluster. This makes it an ideal candidate for studying VOCs as potential biomarkers of aflatoxin contamination. For *F. verticillioides*, it was decided to use genetic mutants on the *fum* genes encoding fumonisin production from a wild-type strain fully capable of producing these mycotoxins. However, a comparison with further mutans including natural mutants,

whose same FUM genes are non-functional, is required to ensure that the VOCs highlighted in this study are indeed emitted and representative of this type of mutation in the fumonisin biosynthesis pathway.

It is fascinating that the absence of aflatoxins, for toxigenic *A. flavus* strains, or fumonisins for *F. verticillioides* strain, was always related to new emission of VOCs. Indeed, the loss of aflatoxin production was accompanied by a new emission of 6 terpenes (β-chamigrene, α.-dehydro-ar-himachalene, (7a-isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol), α-corocalene, τ-muurolol), and the loss of fumonisin was associated with a new emission of esters (ethyl butanoate, ethyl 3-methylbutanoate, ethyl 2-methylpropanoate and ethyl 2-methylbutanoate).

This suggests that the inactivation of these mycotoxin production forced the fungi to rearrange the precursors needed for their production to a directly or indirectly contribute to the emission of these VOCs. Another interesting pattern concerns the temporality of compound emission. The natural mutant of toxigenic *A. flavus*, which has lost its ability to produce aflatoxins, emitted daily certain terpene usually emitted punctually on a specific day (often day 3), and the esters generated by *F. verticillioides* mutants recorded the same daily emission. This constant emission may conceal a more complex role in fungi.

The best VOCs candidates are the most constantly emitted over the day and the most characterized ones. In this case, a mix of VOCs is required to better define the fungus species to be identified, but also the mycotoxin contamination. However, as shown in **Chapter 5**, where the correlation between VOCs and mycotoxins was reported, the VOCs correlated to aflatoxin were exclusively emitted by the toxigenic *A. flavus* strain and same for the fumonisin and *F. verticillioides*, except two VOCs. Indeed, these VOCs were commonly emitted by both species. The **Figure 5-7** allowed us to observe their detection in relation to production of both mycotoxins. Although no quantification has been carried out, the correspondence of the relative surface areas of each of the two VOCs and the production of mycotoxins has shown that these compounds may be good candidates, based on the amount emitted. Apparently, the combination of δ-cadinene and germacrene D may reflect the potential toxicity of a strain, independently of species. In fact, δ-cadinene is currently emitted by non-toxigenic and toxigenic *A. flavus* strain, but it is not the case for germacrene D that was never (as far as we know) detected in non-toxigenic strains (**Table 2-7**). This underlines the importance of combining different VOCs, which identification and quantification are both aspects to be considered before using them as biomarkers.

Surprisingly, ethyl 2-methylbutanoate was commonly detected by the natural toxigenic *A. flavus* mutant, no longer producing aflatoxins, and the non-toxigenic *A. flavus* strain, but was also reported as one of the new esters emitted by *F. verticillioides fum* mutants. Thus, ethyl 2-methylbutanoate is released when no aflatoxin or fumonisin is produced. Moreover, this VOC was also detected in co-inoculation only on day 8 in the case where non-toxigenic *A. flavus* and *F. verticillioides* were in contact condition. This VOC could be attributed to the non-toxigenic *A. flavus* strain with a delay due to the temperature of the experiment. The emission of ethyl 2-methylbutanoate suggests that the absence of mycotoxin

production leads to the activation of a metabolic mechanism leading to its and/or corresponding acid creation, as proposed in the discussion in **Chapter 4**, which could also be of interest. Could ethyl 2-methylbutanoate have been produced to compensate or play a similar role of mycotoxins not produced? This is an interesting question to be more deeply investigated.

As toxigenic *A. flavus* consistently produces aflatoxins, species detection in this case is useful and sufficient to associate strain precocity and aflatoxins. But for fungi that do not have a similar genetic separation between the toxigenic and non-toxigenic *A. flavus* strains, toxin production is much subtle, since within the same species there will be high mycotoxin variability among the strains with the respect to the production, but no clear separation between toxigenic and non-toxigenic strains. Moreover, this does not include other mycotoxins produced by fungi. Indeed, the same species can produce several types of mycotoxins at different times or simultaneously.

Finally, the **Figure 7-4** report the possible VOC biomarkers arisen in these studies. However, it is necessary to enrich the number of strains to better evaluate inter-species variability and the fluctuation affected by environmental factors, and compare the potential biomarkers to the global literature.

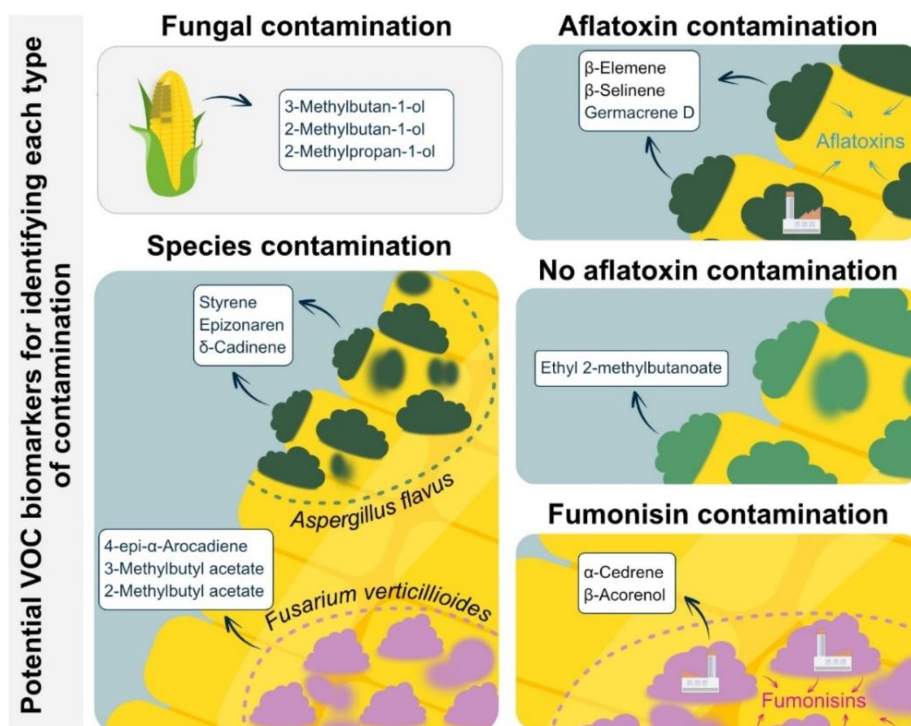


Figure 7-4. Potential VOCs reported in the Chapter 1.1 and 1.2

1.3. Does the type of interaction among the species involve different reactions?

Research based on a single fungal species is still widespread, enabling us to identify the inherent molecules secreted, constituted or emitted by fungi. However, the reality of nature forces us to make the biological model more complex in order to grasp the intricate network among multiple species sharing the same environment. In order to reproduce as close as possible the field conditions, we studied the co-inoculations bringing two fungal species together on the same substrate. Under these conditions, the species are able to communicate and interact not only with VOCs, but also with soluble molecules, including mycotoxins. This dual level of communication between the species concerned tends to increase competition between fungi. On the other hand, we also have mimed the presence of species in an area, avoiding competition between substrates. This is akin to have two species present in the same field or storage area, but whose primary metabolism is not endangered because no competition involves their substrate. This case allowed us to clearly observe the modification of VOCs only when another species is detected in its environment, without the stress caused by the diffusion of soluble molecules in the substrate. **Chapter 5** highlights this point. In another hand, the focus on two co-occurrent species in maize was integrated by the use of toxigenic and non-toxigenic *A. flavus* strains, helping to consider also this kind of interaction in the VOCs and mycotoxins dynamics.

The results underlined the importance of the type of interaction. Indeed, **Chapter 5** highlights 2 VOCs (junipene and α-neoclovene) specifically emitted in contact conditions between *A. flavus* and *F. verticillioides*, while another specific VOC (4-ethylbenzamine) was noted when the strains were in non-contact condition. Surprisingly, A. *flavus* strain toxicity also led to variations in VOCs, with the emission of benemethanol with *A. flavus* toxigenic strain and aristolochene for the interaction with non-toxigenic *A. flavus* strain. Finally, no common VOCs were found for all the conditions. However, VOCs specific to each strain combination did appear, suggesting that the metabolism involved in aflatoxin production in the toxigenic *A. flavus* strain versus the non-toxigenic *A. flavus* strain triggered a specific reaction by *F. verticillioides* that was responsible for this volatolome variation. However, not enough is known about the VOC biosynthesis pathway to propose a robust explanation, and so only assumptions can be made.

Interestingly, a synergy between mycotoxin production, at least under non-contact conditions, has led to an increase in their production. Currently, the use of non-toxigenic strains of *A. flavus* in the field is being standardized to avoid excessive colonization by aflatoxin-producing strains. The results of their efficacy have been proven by several articles, but the question that arises is whether this method does trigger the aggressiveness of other mycotoxin producers such as *F. verticillioides* and its production of fumonisins.

1.4. Single VOC can be used as potential antifungal or antimycotoxin agent?

The choice of a single VOC to be used as a potential biocontrol agent, acting on fungal growth and/or mycotoxin production was based on the observation made in the studies in the previous chapters. Indeed, the human strategy of observing and then mimicking nature, has been fully applied. In **Chapter 3**, for example, a natural mutant of the *A. flavus* strain was used to identify potential VOCs, namely terpenes, which were well known to have numerous properties, such as antifungal activity. But the complexity to obtain the standards of these terpenes hampered the extension of the study in the time allotted to this thesis. However, numerous studies using mixtures of VOCs such as volatolomes from other species, essential oils or even single VOCs against *A. flavus* and its main AFB_1 production were reported in Chapter 1. In addition, the anti-aflatoxigenic effect of applying VOCs has often been shown, and not always in conjunction with a reduction in growth. Some studies have even observed an increase in AFB_1 production under their applications.

On the other hand, **Chapter 4**, with the intriguing emission by *F. verticillioides fum* mutants, proposed 4 candidate esters. As the wild type of *F. verticillioides* did not produce them, the test concerning firstly their antifungal activities was carried out and confirmed its action over a short period, attributed to stunted growth.

The use of a VOC compound as a bioactive agent has several advantages and limitations, discussed in **Chapter 2**. Among the effective parameters for its application, continuous diffusion is often requested, as soon as the VOC is no longer present at a sufficient concentration, the antifungal or antimycotoxin effect is no longer observed. This is why the idea of better understanding fungal behavior under application of a VOC is important to target the one with the best bioactive potential by targeting both antifungal and antimycotoxin functions. The idea of proposing a process of degeneration or apoptosis is attractive, but also brings other kinds of advantages and limitations. The use of essential oils at various stages of the food chain is currently the subject of extensive integration. But it's important to note that storage offers the possibility of using stronger, more dangerous molecules, which can pose a problem in the field, where the diffusion of such molecules can contaminate the environment but also modify the dynamics of species in the ecosystem, or even worsen it, hence the importance of validating these compounds before using them.

In this study, ethyl 3-methylbutanoate has been identified as a potential bioactive VOC and was evaluated for its antifungal and antimycotoxin properties. While its antifungal activity remains uncertain, its effectiveness in inhibiting fumonisin production is noteworthy and efficient. Moreover, this ester is readily available, and its low cytotoxicity profile makes it as a strong candidate for mitigating fumonisins produced by *F. verticillioides*. However, further investigation is needed to confirm its efficacy against aflatoxins notably produced by *A. flavus* and other mycotoxins, as well as its potential applicability to all mycotoxin producing species in maize ecosystems and its impact on organoleptic properties.

1.5. What is the mode of action of the Ethyl 3-methylbutanoate ?

In this thesis, the application of ethyl 3-methylbutanoate was studied as a bioactive compound against *F. verticillioides* and its production of fumonisins. Following the convincing results in **Chapter 4** on its fungistatic effect and associated fumonisin reduction, its application was tested using two approaches. The first involved fumigating the VOC in the fungal growth environment by introducing a specific volume of its standard. The second involved incorporating the same volume into the synthetic medium on which the fungus was grown. These two approaches enabled us to compare the efficacy of each type of application. The fumonisins produced were analyzed together with the expression of *fum* genes and the variations in the volatolome emitted. As the link between fumonisin reduction and growth reduction was observed in **Chapter 4**, the mode of action triggered was still open.

Few modes of action have been reported to explain mycotoxin or fungal growth reduction (see **Chapter 2**), but among the strategies used, the study of mycotoxin pathway gene expression is one of them.

The results provided in **Chapter 6** have revealed some important information. Firstly, the type of application has an impact on the mechanism involved in fumonisin reduction. As the results show, the presence of ethyl 3-methylbutanoate in the fungal atmosphere resulted in down-regulation of the 6 genes studied (*fum* 1, *fum*3, *fum* 6, *fum*7, *fum* 8 and *fum* 14), easily explaining the drastic reduction in fumonisin production below 20 ppb. However, direct contact of ethyl 3-methylbutanoate with the fungus triggered a more complex reaction. In fact, although overexpression of these 6 *fum* genes was recorded, the reduction in fumonisin production remained significant. This suggests the involvement of other mechanisms at post-transcription level leading to these low levels of fumonisin production compared with the normal condition. Indeed, the addition of the stress generated by the physical contact of this condition implies additional stress in the fungal cell such as membrane alteration in addition to the perception of another potential competitor simulated by the presence of the ester.

2. Perspectives

2.1. Potential application of the VOCs as a biomarker

The creation of a VOC detector or sensor involves several stages, starting from the design and development phase to testing and calibration. Researchers work on developing and enhancing various types of sensors, such as semiconductor oxide sensors (MOS) and selective polymer sensors, among many others. However, this part will focus on the practical application of VOCs as detector.

Two functions could be associated with them: detection and monitoring.

Firstly, the VOC detector could contribute to the early detection of fungal contamination. Installed in fields of maize and storage facilities, may be useful tool to detect early signs of fungal contamination, caused by mycotoxin-producing fungi such as *Aspergillus* and *Fusarium*, contributing to food safety and quality control. VOC sensors can be strategically placed in storage facilities or silos where food and feed products are stored. In the field, a portable, remote-controlled detector can be moved from one location to another, enabling flexible, remote monitoring. This is particularly useful in agriculture, where crops may be spread over several fields or farms. In addition, the knowledge of the species involved could provide to target the analytical analysis and save time and money instead of screening using numerous standards and machine time. As suggested above, the focus on strain toxigenicity may be more relevant than mycotoxin type, paving the way for more general application of this type of detector.

Secondly, they can help with crop management. When fungal contamination is detected and the species characterized, a fungicide application will be more effective by precisely targeting the contaminating species concerned. What's more, VOC detectors have the advantage of speed: immediately detected, immediately treated. This early and rapid action could help optimizing and targeting the most appropriate application of fungicides, which can be applied only when necessary, reducing overall chemical use and environmental impact.

Thirdly, as a monitor, it is a tool for monitoring crop health. Beyond fungal VOCs, changes in VOC profiles can indicate stress conditions, disease development or pest infestations, enabling farmers to implement appropriate interventions. Appropriately configured alarms or alerts, if detected VOC levels exceed predefined thresholds, indicating the presence of fungal contamination or some other problems, would contribute to immediate actions.

Fourthly, as an aid to harvest and storage decisions, to determine the optimum time to harvest maize based on VOC emissions.

Finally, VOC detectors can be used as research and development tools, to study the interactions between maize plants and various species of fungi in real-agronomic conditions, as well as the whole ecological sphere.

2.2. Potential application of VOCs as a biocontrol

This perspective was well introduced in **Chapter 2**, with key points on which action could be taken. All the results compiled over the chapters of this thesis, VOC fumigation as biocontrol is a very good option offering a good mode of action with significant effects, but it needs to be improved. Indeed, the main associated advantages are their wide distribution and non-contact with the substrate. Logistics in the field could be more complex, but its application in storage areas could be easy to set up. Indeed, the management of maize storage controls the parameters that favor fungal development, such as humidity and temperature. It could be easier to add to this existing control the diffusion of a VOC inside, which would ensure complete inhibition of growth and therefore of mycotoxin production.

Another interesting application, but still only a hypothesis in the current state of knowledge, would be to use a VOC that induces mycotoxin degradation in contaminated grains by favorizing specific microorganisms who degrade them or conjugated them. If mycotoxins could be eliminated without destroying the raw material, this would improve economics without affecting maize quality. Indeed, even if temperature is not our ally in eliminating them, mycotoxins are sensitive to other factors. So, VOC as factor to be combined with other factors, such as UV rays, to mitigate mycotoxins occurrence, could it be a possible option to consider. Could we also suggest making them visible by using a molecule or nanoparticles to react with the mycotoxin present and directly control a specific compound by the VOCs emitted?

2.3. Small Reflection

Thus, this work has been based on a desire to act on the fungi, leaving the host and its reaction to one side. But it may be useful to help the host by triggering its natural methods for decontaminating mycotoxin accumulation, using glycosylation for example, or other micro-organisms that could help the host.

Another remark is that we tend to direct projects towards adding VOCs (or other compounds) to regulate fungal and mycotoxin contamination, but why not direct research towards capturing the VOCs responsible for the production message or triggering mycotoxin production?

Nevertheless, we need to be careful with biocontrol of all living organisms in our environment. Indeed, nature ensures a balance between communities. As humans, we target species that appear to us as our enemies because they have a negative impact on us, but let's remember that nature has no harmful intent; it is life across the spectrum. What's more, altering the balance to exclude one species from an ecosystem automatically means the emergence of another species to take the place of the previous one. This is why acting in the field requires wisdom and expertise.

2.4. The Future Acquisition of Essential Knowledge

Obviously, the proposed perspectives provide a basis for reflection, but to hope for a solution, further knowledge is required in the coming years. To reach it, two aspects deserve particular attention for investigation. The first aspect pertains to biology. In our society, driven by economic considerations, there is often a tendency to prioritize short-term results and immediate applications. However, it is important to remember that fundamental science forms the foundation of the pyramid, leading us towards the necessary mastery and knowledge for long-term efficiency without creating future issues. Maintaining a long-term view is crucial, as innovation requires strong roots to reach greater heights. This is why understanding the biosynthesis pathways of VOCs and the metabolism of fungus are essential. VOCs represent a significant aspect of living organisms to consider as equal to soluble molecules. These secondary metabolites have sometimes been overlooked due to constraints in their detection and analysis, but technological advancements have progressed, leading to the second

aspect, the technology. Technology is closely intertwined with biology and other scientific domains. The desire to explore and understand drives advancements in machine design and capabilities. The new perspectives and results obtained through these machines generate fresh questions and curiosity within the field of biology and beyond. Science is the amalgamation of various scientific disciplines, working together to address complex questions and uncover knowledge. It is through this collaboration that we can gain a deeper understanding of the world around us.

3. Conclusion

Mycotoxins are a major health and economic problem. A multi-faceted approach is needed to tackle them, and new tools are emerging to prevent and control mycotoxin contamination. In addition to combining chemical, biological and physical methods, it is possible to act at several levels of the food chain, to make it easier and quicker to detect mycotoxin-producing fungi, to avoid contamination in the field, after harvest or during storage, and if mycotoxins are already produced, to eliminate, degrade or trap them to avoid their assimilation into the body and therefore their toxic effects. Although many methods are already in use, we need to improve them and find natural alternatives to chemical fungicides and pesticides.

This thesis focused on VOCs as indicators of fungal and mycotoxin contamination, as well as the application of a VOC as an antifungal and antimycotoxin compound. The research explored the VOCs emitted by *A. flavus* and *F. verticillioides*, the two co-occurring species in maize, and the main producer of two families of mycotoxins associated with fatal human diseases, aflatoxins and fumonisins. These studies provided a comprehensive overview of the VOCs of the fungi under investigation, and for the first time, a compilation of all the VOCs emitted by *A. flavus* in the literature until 2021 was established (**Table 2-6**).

The use of mutants to elucidate the relationship between the two secondary metabolites, namely VOCs and mycotoxins, led to the identification of specific VOCs indicating the presence of both fungi and their ability to produce their respective mycotoxins. In fact, the antifungal and anti-fumonisin properties of ethyl 3-methylbutanoate on *F. verticillioides* were studied, demonstrating a delay in fungal growth and fumonisin production in maize.

To explore its mechanism and confirm its antifungal and antifumonisin properties, the genetic expression of the fumonisin pathway was monitored in addition to the production of VOCs and fumonisins. Analysis of the *fum* genes revealed that the introduction of this ester directly in the fungal atmosphere acts by downregulating certain genes in the fumonisin biosynthesis pathway, explaining the reduction of fumonisins under its application. In another hand, the physical contact application of the bioactive ester has triggered not only an overexpression of the *fum* genes, but also other mechanisms not defined here, that conduced to the fumonisin reduction observed.

In parallel, for the first time, the study of co-inoculation of these co-occurring species, monitoring changes in VOCs and the respective reactions of fungal mycotoxins, under two types of interactions between the species, has revealed remarkable VOC profiles for each interaction type. Indeed, the mode of interaction between the fungal species is an important factor to consider.

In conclusion, we can assert that VOCs reflect the metabolism of fungi. In our work, it has always been possible to distinguish mycotoxin-producing strains from non-producing strains based on the emitted VOCs. Furthermore, the action of VOCs on fungi has been highlighted in the study dedicated to co-inoculation and the application of an individual VOC. The relationship between VOCs and mycotoxins is not easy to establish, but it is evident that VOCs play a crucial role in species characterization and actively contribute to the optimal functioning and regulation of fungi in response to external factors and internal stimuli. This objective has underscored the major importance of VOCs and the need to enhance our understanding of these long-neglected secondary metabolites.

Moving forward, by closely collaborating with available and upcoming technologies, the elucidation of the mystery surrounding the production, regulation, and functioning of VOCs can be illuminated. Humans will always seek to conceptualize and apprehend the mysteries of nature, so let us keep an open mind and remember that we ourselves are participants in the vastness of our universe.

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List of the scientific communication

Publications (First author)

Volatile Organic Compounds Emitted by *Aspergillus Flavus* Strains Producing or Not Aflatoxin B_1, Josselin, L., De Clerck, C., De Boevre, M., Moretti, A., Jijakli, M-H., Soyeurt, H. and Fauconnier, M-L. (2021) *Toxins* 13 (10). https://doi.org/10.3390/toxins13100705. (Published)

Impact of Volatile Organic Compounds on the Growth of *Aspergillus flavus* and Related Aflatoxin B_1 Production: A Review, Josselin, L., De Clerck, C., De Boevre, M., Moretti, A., Fauconnier, M.- L. (2022) "*International Journal of Molecular Sciences*, 23(24), pp. 1–24. doi: 10.3390/ijms232415557. (Published)

Does Alteration of Fumonisin Production in *Fusarium verticillioides* Lead to Volatolome Variation?, Josselin, L., Proctor R.H., Lippolis V., Cervellieri S., Hoylaerts J., De Clerck C., Fauconnier, M-L and Moretti A. (2023) **"***Food chemistry* (Submitted)

Interplay of VOCs and Mycotoxin Production in *Aspergillus flavus* and *Fusarium verticillioides*: Exploring Individual and Co-inoculation Strategies in Contact and Non-contact, Josselin, L., Howa-Lopez F., Proctor R.H., De Clerck C., De Boevre M., Fauconnier, M-L and Moretti A. (Revision of the co-authors)

Publications (Co-first author)

Weapons against Themselves: Identification and Use of Quorum Sensing Volatile Molecules to Control Plant Pathogenic Fungi Growth, De Clerck, C.; Josselin, L.; Vangoethem, V.; Lassois, L.; Fauconnier, M.L.; Jijakli, H. (2022) **"***Microorganisms*, 10(12), doi:10.3390/microorganisms10122459. (Published)

Orals (As a speaker)

Study of volatile organic compounds emitted by mycotoxin-producing fungi to develop specific sensors, November 14, 2019, ILSI Europe seminar on Process-Related Compound and Natural Toxins, Josselin L., De Boevre M., De Clerck C., Moretti A., Soyeurt H. and Fauconnier M-L.

Fungal volatile organic compounds, can be used to develop aflatoxin-specific sensors? November 9 to 12, 2021, MycoTWIN-MycoKey International conference, Josselin L., De Boevre M., De Clerck C., Moretti A., Soyeurt H. and Fauconnier M-L.

A qualitative and semi-quantitative study of volatile organic compounds released by mycotoxin-producing strains of *Aspergillus flavus*, October 29, 2021, 3 rd International Webinar on Chemistry and Pharmaceutical Chemistry, Josselin L., De Clerck C., De Boevre M., Moretti A., Soyeurt H. and Fauconnier M-L.

Several species in a similar environment, how can this impact volatile organic compounds' emission and subsequent mycotoxin production? May 30 to June 1, 2022, 43rd Mycotoxin Workshop – Society of Mycotoxin Research, Josselin L., De Boevre M., De Clerck C., Moretti A., Soyeurt H. and Fauconnier M-L.

Fungal volatile organic compounds: new tools to reduce mycotoxin contamination in crops? June 12 to 15, 2023, 16th European Fusarium Seminar, Josselin L., Proctor R.H., Lippolis V., Cervellieri S., De Boevre M., De Clerck C., Fauconnier M-L. and Moretti A.

Volatile organic compounds of *Aspergillus flavus*: Messagers and Regulators? February 1, 2022, UMRT, Josselin L.

Orals (Collaboration)

Biodiversity of and diagnostic tools for toxigenic *Aspergillus* and *Fusarium* species on maize, May 30 to June 2, 2022, Welcome to the XXVth EUCARPIA Maize and Sorghum Conference Belgrade – Current Challenges and New Methods for Maize and Sorghum Breeding, Susca A., Villani A., Haidukowski M., Josselin L., Fauconnier M. Laure, Logreico F. A., Moretti A.

Posters

Etude de composés organiques volatils (COVs) émis lors de l'interaction hôte – pathogène par des souches produisant des mycotoxines, May 30 to April 1, 2019, Journées Jeunes Chercheurs Condorcet, Josselin L., De Boevre M., De Clerck C., Moretti A., Soyeurt H. and Fauconnier M-L.

Can fungal volatile organic compounds be used to develop aflatoxin-specific sensors? January 31, 2020, 25th National Symposium for Applied Biological Sciences, Josselin L., De Boevre M., De Clerck C., Moretti A., Soyeurt H. and Fauconnier M-L.

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Fungal volatile organic compounds: new targets for the detection of fungal contamination and mycotoxin production, June 28 to 29, 2023, 2nd Advances in Separation Science: from extraction to chromatographic separation, Josselin L., De Boevre M., De Clerck C., Moretti A., Soyeurt H. and Fauconnier M-L.