doi.org/10.1002/cbdv.202401747 Research Article CHEMISTRY & BIODIVERSITY

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Unveiling the Substrate-Dependent Dynamics of Mycotoxin Production in *Fusarium verticillioides* Using an OSMAC-Metabolomics Approach

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Fusarium verticillioides is a prevalent plant pathogenic fungus known to produce harmful mycotoxins, including fumonisins and emerging toxins. This study aimed to investigate the influence of substrate on the temporal patterns of mycotoxin biosynthesis by *F. verticillioides*, employing a combined OSMAC (One Strain-Many Compounds) strategy and metabolomics approach. The fungus was cultured under various media conditions, and samples were collected over time. LC–MS/MS analyses and a dereplicative workflow were used to profile the secondary metabolite production, focusing on mycotoxins. The results demonstrated that modifying the culture conditions led to significant variations in fungal growth and the nature and relative concentrations of mycotoxins produced. Corn meal agar (CMA) medium was favorable for fumonisins A_1 and B_1, while malt extract agar (MEA) favored fumonisins A_2 and B_2. The study also identified the production of other mycotoxins related compounds as fusarins, bikaverin derivatives and fumonisins analogs, under different growth conditions. This study highlights the potential of combining OSMAC and metabolomics to unravel the substrate-dependent and time-dependent variations in mycotoxin biosynthesis by *F. verticillioides*. The insights gained provide a better understanding of the ecophysiology of this fungus and the occurrence of its mycotoxins, which can inform targeted mitigation strategies to ensure food and feed safety.

Introduction

Fusarium verticillioides is a powerful phytopathogen, causing economic losses and producing an array of mycotoxins such as fumonisins or fusarins (Figures 1 and 2). Notably, Fumonisin B1 (FB_1) may increase the risk of cancer, in particular in liver and kidney and was classified by the International Agency of Research on Cancer (IARC) as potentially carcinogenic to humans (group 2B).[1] This fungus can also produce less studied and less regulated mycotoxins known as "emerging" mycotoxins like beauvericin and fusaproliferin, or masked mycotoxins which are derivatives of parent mycotoxins that have been chemically modified, usually by plants, fungi, or food processing. plant altered forms, in response to changing environmental conditions.[2]

In the context of global environmental changes, there is a potential for increased susceptibility to *F. verticillioides* infections, leading to a higher prevalence of contaminated crops and elevated mycotoxin exposure risks for human and animal populations.[3] Importantly, shifts in fungal growth conditions could also result in the production of new mycotoxins or modification in their occurrence patterns.

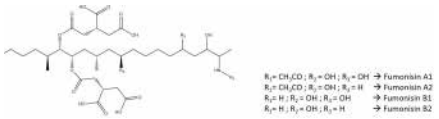

Figure 1. Structures of fumonisins A1, A2, B1 and B2.

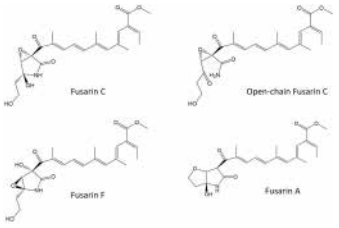

Figure 2. Structures of fusarin C, open-chain fusarin C, fusarin F, and fusarin A.

Metabolomics has emerged as a powerful tool for investigating the biosynthesis of secondary metabolites, including mycotoxins, in fungi. This approach allows for the simultaneous detection and identification of a wide range of metabolites, providing a comprehensive view of the organism's metabolic profile.[4,5] Additionally, the One Strain-Many Compounds (OSMAC) strategy, which involves cultivating a single fungal strain under diverse culture conditions, has proven effective for the discovery of new secondary metabolites.[6,7]

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.202401747

Previous studies on *Fusarium* species, including *F. verticillioides*, have explored the influence of various culture parameters on fungal growth, mycotoxins production, and the regulation of biosynthetic pathways.[4,8–17] However, most of these studies have focused on regulated mycotoxins, such as fumonisins, with limited attention to the production of emerging and modified mycotoxins. The latter are naturally occurring mycotoxins that have undergone chemical changes throughout metabolization in plants, fungi, animals or through food and feed processing.[18]

In this study, we aimed to expand our understanding of mycotoxin production by *F. verticillioides* in relation to the substrate over time, using a combined OSMAC and metabolomics approach. The insights gained from this investigation can inform the development of targeted strategies to mitigate mycotoxin contamination in agricultural commodities.

Results and Discussion

F. verticillioides was inoculated in 6-well plates containing MEA, PDA, CZA or CMA media, and its growth was observed over time. During the first 48 hours, the fungal colonies started to develop, and their macroscopic appearance varied depending on the culture medium (Figure 3). Despite the differences in substrate, *F. verticillioides* was able to grow on all four media, as it has low trophic requirements and can consume a broad range of carbon sources.[18]

Figure 3. Evolution of the mycelium morphology over time on the four culture media (CMA, CZA, MEA and PDA). Plates were incubated during 21 days in dark, at 27 °C.

On PDA medium, *F. verticillioides* invaded the well after 4 days of culture, presenting a white aerial mycelium with an orange tinge observed on the reverse side and a filamentous appearance. When grown on MEA, the fungus showed a white aerial mycelium with a pinkish tinge from the upper side, a dark center, and a purple to brown coloration on the reverse; it completely invaded the well after 5 days of culture. On CZA media, *F. verticillioides* invaded the well after 3 days, exhibiting abundant white to slightly yellow fluffy aerial mycelium and a yellow reverse with greenish spots. Finally, on CMA medium, the fungus displayed a pink aerial mycelium with a powdery appearance and a pink to slightly red reverse, fully occupying the well by day 5. The observed reddish coloration of some fungal colonies may be attributed to the presence of red or purple naphtoquinone pigments, such as bikaverin mycotoxins and its derivatives, produced by many *Fusarium* species[19,20] and detected in our *F. verticillioides* extracts. Bikaverin is a red polyketide pigment known to be produced by several *Fusarium* species, including *F. verticillioides*. Our LC–MS/MS analysis confirmed the presence of bikaverin and some bikaverin-related compounds in the extracts from colonies exhibiting reddish pigmentation. The identification of bikaverin was based on its exact mass, fragmentation pattern, and comparison with spectral libraries.[19,20] The detection and the annotation of fumonisins and other mycotoxins in the extracts were carried out by comparing high-resolution mass (MS1 and MS2 data) and retention time with those of standard solutions and the literature data. The identification of FB_1 and FB_2 was achieved by comparing the MS1 and MS2 data of the molecules present in the extracts with those of a standard solution. For other annotated fumonisins, the MS1 and MS2 data of the detected molecules were compared with literature data (Table 1).

Additionally, other mycotoxins such as norbikaverin, bikaverin, fumonisin AK_1, fusarin A, fusarin C, dihydrofusarin C, open-chain fusarin C, fusarin F, and fusarin X were also annotated. The structures of some of these mycotoxins were manually verified (Table 2). A targeted approach using the retention time (Rt) and mass-to-charge ratio (*m/z*) pair (Rt_*m/z*)

Table 1. Pseudomolecular ions and fragment ions of annotated mycotoxins detected in samples. In bold, fragment ions detected in the samples and verified by the literature. Fragments are displayed in order of their abundance.

Name	$[M+H]^+$	Fragment ions	References
Fumonisin A_1	764.4071	**746 ; 728 ; 376** ; **394** ; **334** ; 116 ; 60 ; 74 ; 159 ; 316 ; **570** ; **764** ; 710 ; **552** ; **412** ; 228 ; 358 ; 352 ; 236 ; **588** ; 278 ; 296 ; 534	[21]
Fumonisin A_2	748.4127	336 ; **318** ; **730** ; **378** ; 116 ; **396** ; 74 ; 159 ; **360** ; 60 ; **748** ; **712** ; **554** ; **572** ; 238 ; 354 ; 220 ; **536** ; 280	[21]
Fumonisin B_1	722.3966	722 ; **334** ; 352; 74 ; 159 ; **316** ; 186 ; **370** ; **528** ; **704** ; **510** ; **686** ; **254** ; **236** ; **546** ; **492** ; **272** ; **668**	[21]
	722.3966	704 ; 686 ; 528 ; 546 ; 352 ; 370 ; 334 ; 510 ; 564 ; 668 ; 388 ; 299 ; 254 ; 204; 232 ; 254 ; 272 ; 388 ; 412 ; 437 ; 448 ; 492 ; 615 ; 646	Standard
Fumonisin B_2	706.4016	**706** ; **336** ; **318** ; 74 ; 159 ; **354** ; **512** ; **688** ; **494** ; **530** ; 238 ; 220 ; 256 ; **670**	[21]
	706.4013	688 ; 512 ; 530 ; 670 ; 336 ; 354 ; 494 ; 548 ; 318 ; 301 ; 203 ; 238 ; 249 ; 274 ; 372 ; 396 ; 434 ; 458 ; 476 ; 652	Standard

Table 2. Summary of detected mycotoxins annotated thanks to MSFINDER, culture media on which they have been detected, their level of annotation and if they have been compared to literature or not. Mycotoxin standards matches = level 1, in silico matches = level 2 (2.1. Fusarium verticillioides metabolites, 2.2. Fusarium metabolites, 2.3. Mycotoxins and 2.4. MS-FINDER generic databases). *MS^2 not available.*

Annotated mycotoxins	Culture media	Annotation level
Fumonisin B_1	CMA, CZA, MEA, PDA	Level 1
Fumonisin B_2	CMA, CZA, MEA, PDA	Level 1
Fumonisin A_1	CMA, CZA, MEA, PDA	Level 2.1
Fumonisin A_2	CMA, CZA, MEA, PDA	Level 2.1
Open-chain Fusarin C	CMA, CZA, MEA, PDA	Level 2.1
Fusarin C	CMA, CZA, MEA, PDA	Level 2.1
Fusarin F*	CMA, CZA, MEA, PDA	Level 2.1
Fumonisin AK_1*	CMA, CZA, MEA, PDA	Level 2.1
Fusarin A	CZA, MEA, PDA	Level 2.2
Fusarin X*	CMA, MEA, PDA	Level 2.2
Bikaverin	CZA	Level 2.2
Norbikaverin	CMA, CZA, MEA, PDA	Level 2.4

of a standard solution of FB_1 and FB_2 was employed to detect these mycotoxins in all samples on all four media. Other mycotoxins were detected using online databases or an internal database.

Bikaverin was detected only in CZA medium (Table 2). This red pigment was first isolated from *Gibberella fujikuroi* (teleomorph form of *F.verticillioides*) mycelium[22] and from *Fusarium oxysporum*,[23] while its demethylated form, the norbikaverin, detected in all four media was characterized by Kjaer et al.[24] Bikaverin had long been classified as a mycotoxin.[23] This molecule is known for having antibiotic activity against *Leishmania brasiliensis*[22,25] and an antioomycete activity against *Phytophtora infestans*.[23]

The biological function of these molecules for the producing fungi is still unknown, but its antibiotic effects could provide competitive edge in its natural environment.[25] Fusarins, such as fusarin A,[26] fusarin C and its analogs open-chain fusarin C and dihydrofusarin C,[27] fusarin F[28] and fusarin X,[29] were detected in the four different media (Table 2). These compounds are polyketide-non ribosomal peptide hybrids.[30] Fusarin C, a highly mutagenic molecule produced by *F. verticillioides*,[31] is an estrogenic agonist that stimulates breast cancer *in vitro*[32] and it might be the cause of esophageal cancer as documented in China and Africa.[33] Fusarin C undergoes rearrangements which produce fusarin C analogs such as open-chain fusarin C.[34] Fusarin C derivatives have shown antibacterial properties which could be a way for the fungi to defend its host[35] or which could be involved for niche competition with others microorganisms. Five different fumonisins were annotated in the extracts of the four culture media. These compounds are sphinganine analog mycotoxins that inhibit mammalian acyl CoA-dependent ceramide synthase, which is involved in the *de novo* biosynthesis of sphingolipids.[36] This disruption in the sphingolipid pathway can lead to various diseases, mainly observed in livestock. The specific role of fumonisins for the producing fungus, *F. verticillioides*, is not yet fully elucidated. Some studies have suggested that stressful conditions may enhance fumonisin production, but the production of these mycotoxins represents a metabolic cost for the fungus.[10] Fumonisins may have an ecological role, but this appears to vary based on the disease and the maize genotype. It is known that certain mycotoxins can participate in fungal pathogenicity and act as virulence factors.[37,38] In the case of corn seedling blight, fumonisins might increase the virulence of *F. verticillioides*, but they are not deemed necessary or sufficient for disease development.[38–40] For ear rot disease, it has been suggested that fumonisin production is not necessary for *F. verticillioides* to cause the disease.[41] Fumonisins might play a role in the fungus's competition with other microorganisms (fungi or bacteria) in its natural environment. Additionally, a fumonisin derivative, FAK_1, was detected in the samples, but no MS2 data were found in the literature to confirm the annotation. Further investigation is needed to understand the significance of this emerging mycotoxin. The relative quantification of fumonisin analogs FA_1, FA_2, FB_1 and FB_2 showed that the CMA medium was the most favorable for the production of FA_1 and FB_1, while the MEA medium was the most favorable for the production of FA_2 and FB_2 (Figure 4). In contrast, the production of fumonisins by *F. verticillioides* was relatively low on the CZA and PDA media. The kinetic study of the production of the four mains Fumonisin (FA_1, FA_2, FB_1 and FB_2) showed that on the CMA medium, FA_1 was first detected after 5 days of incubation and reached three local maxima at 10, 14, and 18 days, with the highest production observed at day 14. On the MEA medium, FA_1 was detected from day 7 of incubation, reaching its highest level after 16 days. FA_1 was detected at trace levels on PDA and was not detected on CZA. For FA_2 production, the MEA medium was the most favorable, with a maximum at 16 days of incubation. FA_2 was also detected on CMA, with two maxima at 14 and 18 days, while it was detected at low intensity on PDA and not detected on CZA.

FB_1 was not detected on CZA and PDA, barely detected on day 16 of incubation on MEA, and detected at low intensity on CMA, with three maxima at 10, 14, and 18 days. FB_2 was mainly

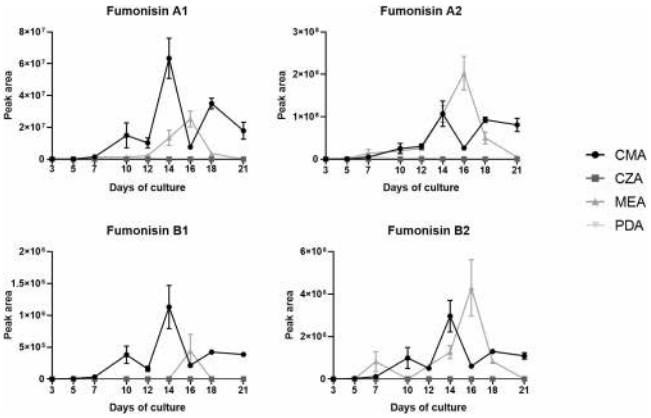

Figure 4. Overlay of peak areas of FA1, FA2, FB1 and FB2 under different incubation times on MEA, PDA, CZA and CMA media.

detected on MEA, with two maxima at 7 and 16 days, and on CMA, with three maxima at 10, 14, and 18 days. FB_2 was not detected on CZA and PDA. The four mycotoxins studied followed a similar trend in their production. On CMA medium, they were produced starting from day 5 of incubation, increased until day 10, decreased to reach their highest levels on day 14, decreased again until day 16, and then increased until day 18, presenting three local maxima. On MEA medium, they also followed a similar trend, with two local maxima on days 7 and 16. The optimal conditions to study fumonisins would be after 14 days of fungal growth on CMA medium and after 16 days on MEA medium while CZA and PDA media proved not to be a good candidate for the production of such metabolites. The relative abundance of the detected fumonisins generally followed the pattern: $FA_2 > FA_1 > FB_2 > FB_1$. In previous studies, it has been described that fumonisin B analogs are usually produced at relatively low levels (about 5% of the total fumonisins produced).[42] In this study, we showed that FB_1 and FB_2 were produced at lower levels than their A-series analogs. Therefore, the fumonisin production profile observed in our current study did not match the scheme described by Lazzaro et al.[11,43] for cornmeal medium, where $FB_1 > FB_2$ and total fumonisins (FBs) > total fumonisins (FAs), with $FA_2 + FA_3 > FA_1$. Although we did not search for FA_3 due to the lack of an analytical standard, our study revealed that the relative quantity of FA_2 was still greater than FA_1's. This discrepancy could be attributed to differences in the extraction efficiency or ionization properties of the various fumonisin analogs under the analytical conditions used in this study. Also, previous studies by Lazzaro *et al.*[11,43] have described a phenomenon wherein the production of FBs decreases while that of FAs increases, suggesting that FBs might undergo conversion into minor compounds.[5] This hypothesis could explain why FBs are not the major compounds observed at 14 days of incubation on CMA medium and at 16 days of incubation on MEA medium, as they may have been transformed or their production has shifted towards the synthesis of other derivatives. Despite the relatively low abundance of FB_1, which is considered the most toxic fumonisin, all derivatives and analogs within the fumonisin family contribute to the total fumonisin content present in the samples. Further investigation would be needed to fully understand the factors driving these differences in fumonisin production profiles.

The differences in mycotoxin production observed across the culture media can be attributed to the varying nature of the compounds present in each medium. According to Ochieng et al.,[17] the ability of fungi to produce mycotoxins is governed by genetic mechanisms and influenced by various environmental factors, including pH, temperature, light conditions, and relative humidity. These environmental factors may regulate the expression of genes involved in fumonisin production (*FUM*) or other genes located outside the FUM gene cluster. Additionally, post-transcriptional modifications must be considered when analyzing mycotoxin production.

Bluhm and Woloshuk[44] conducted a study that revealed the role of amylopectin, a glycan found in maize kernels, in inducing the production of FB_1 by *F. verticillioides* during colonization. This finding provides strong evidence for the influence of amylopectin on FB1 production, particularly in media containing cornmeal extract, such as CMA. The presence of amylopectin in the CMA medium offers a plausible explanation for the higher production of this mycotoxin in those conditions. It is noteworthy that both maize and barley, used in the CMA and MEA media, respectively, belong to the Poaceae family. This observation raises the possibility that these plants may contain not only amylopectin but also other molecules that promote FB_1 production by *F. verticillioides*, thereby contributing to the observed mycotoxin production in these media. However, more recent studies by Achimón et al. (2019)[15] have shown that glucose is a more powerful inducer of fumonisin production than amylopectin. The media preparation involved the addition of 20 g/L of glucose to MEA, PDA, and CMA media. The difference in carbon source composition and quantity is primarily due to the varying amounts of malt extract, potato extract, and cornmeal extract added. The CMA medium quantitatively contains more plant-based extract (60 g/L) compared to the MEA medium (40 g/L), while the MEA medium contains a higher concentration of plant-based extract than the PDA medium (4 g/L). This could explain the lower fumonisin production observed on the PDA medium.

These findings suggest that the influence of different carbon sources in the environment plays a crucial role in mycotoxin production. Previous studies have shown that the carbon-to-nitrogen (C:N) ratio is a factor influencing fumonisin production[10,45]. Increasing carbon sources, such as carbohydrates, has been shown to enhance fumonisin production, while decreasing nitrogen sources, like amino acids, decreases fumonisin production. Certain *F. verticillioides* strains have also been reported to produce more or less fumonisins depending on the type of sugar added to the medium.[14,45] The regulation of fumonisin production in *F. verticillioides* is a complex process involving multiple factors, including the activation and inhibition of certain genes through environmental signals, as well as the regulation of host-pathogen interactions. Previous studies have highlighted various factors that modulate fumonisin production, such as the role of amylopectin in decreasing pH and inducing fumonisin production,[46] the influence of the AREA gene regulating nitrogen metabolism, and the impact of an unfavorable C:N ratio and alkaline pH on fumonisin production.[10,46] Our team is actively investigating the relationship between amylopectin, glucose, or other molecules present in maize and their impact on FB_1 production by *F. verticillioides*. The ongoing experiments aim to provide valuable insights into the mechanisms underlying mycotoxin production in different media and cereals. By elucidating the specific factors that contribute to increased mycotoxin production, our research seeks to enhance the understanding of fungal colonization and develop effective strategies to mitigate mycotoxin contamination in agricultural settings.

Given that the CMA medium was the most favorable for fumonisin production, it was chosen to perform an untargeted metabolomic approach to compare the metabolite production of the *F. verticillioides* strain over time. The raw data were processed in both positive and negative ionization (PI and NI)

modes using MSDial, MSCleanR, and MSFinder software. The final data were then analyzed on the Metaboanalyst online platform to obtain multivariate analyses.

First, a principal component analysis (PCA) was applied to provide an overview of the impact of incubation times on the *F. verticillioides* metabolome (Figure 5).

This analysis clustered all independent biological replicates from the same condition. It explained for 55.4% of the total variance. Component 1 (X-axis) counted for 40.6% of the variability while component 2 (Y-axis) explained for 14.8% of the variability between groups. This visualization allowed to separate replicates from day 3 and those from day 5, from other extraction days. Then, a partial least squares-discriminant analysis (PLS-DA) (Figure 6A) was carried out, which explained 54.8% of the total variance. Component 1 (X-axis) explained 39.8% of the variability, allowing the discrimination of day 3 and day 5 from the other days. Component 2 explained 14.3% of the variability, discriminating a cluster composed of days 3 and 5, a cluster composed of days 7, 10, 12, 14, 16, and 18, and a cluster composed of day 21.

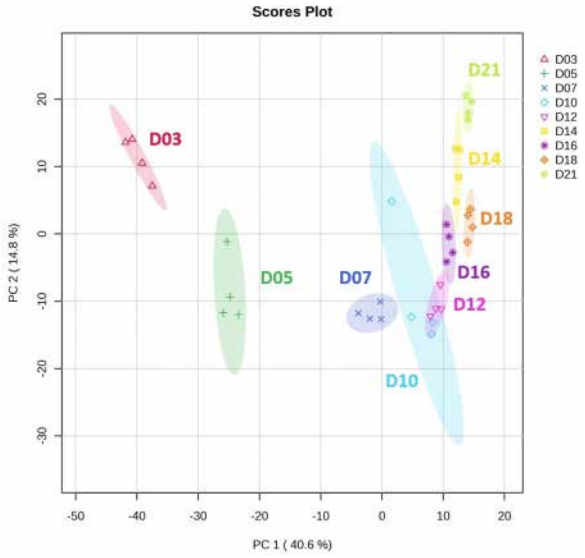

Figure 5. PCA corresponding to the extracts of *F. verticillioides* cultured on CMA medium during different incubation time based on features (*m/z* at Rt to its normalized peak area) detected in LC–MS chromatograms.

As seen in Figure 6A, the changes in the *F. verticillioides* metabolome follow a chronological order. Figure 6B shows the top 50 metabolites that most contributed to the differences observed in the PLS-DA score plot, focusing on component 1. Fumonisin analogs FA_1, FB_2, and FAK_1 were annotated among the VIP features. These compounds were mainly produced in the later days of incubation, from day 14 to day 21. Previously, it was observed that the fungus invaded the well after a maximum of five days, leading to a depletion of nutritional resources, which could induce a stressful situation resulting in the modification of the fungal metabolome over time.

To have an overview of the ability of *F. verticillioides* to produce mycotoxins, a t-SNE (t-distributed stochastic neighbor embedding) visualization was performed on the extracts from day 14 of incubation using the Metgem software (Figure 7).

The MS^2 data were visualized, allowing a better representation of the structural similarity between the detected peaks and mycotoxin-related molecules. A fumonisin cluster was defined, with two distinct groups corresponding to fumonisin A (and analogs) and fumonisin B (and analogs). The t-SNE network contained 602 nodes and 94 nodes were self-loops. The FB_1 and FB_2 were annotated by comparison to the standards while FB_3 was annotated through Metgem spectral libraries with a score of 0.80. Three nodes were annotated as FB_1 analogs and shared the same *m/z* as FA_1: 764.4072 at Rt 6.164 min; 764.4072 at Rt 6.008 min and 764.4074 at Rt 6.58 min, potentially corresponding to FA_1 or its positional isomers, iso-FA_{1a} or iso-FA_{1b}.[47] Three other nodes were annotated as FB_2 analogs and shared FA_2 *m/z*: 748.411 at Rt 6.437 min; 748.4119 at Rt 7.441 min and 748.4121 at Rt 6.789 min, potentially corresponding to FA_2 and FA3. Additionally, FAK_1 was identified within the fumonisin cluster.

The trend observed in the t-SNE visualization was consistent with the previous fumonisin-targeted detection, where most fumonisin or fumonisin-like compounds were detected in the *F. verticillioides* extracts grown on both CMA and MEA media, and

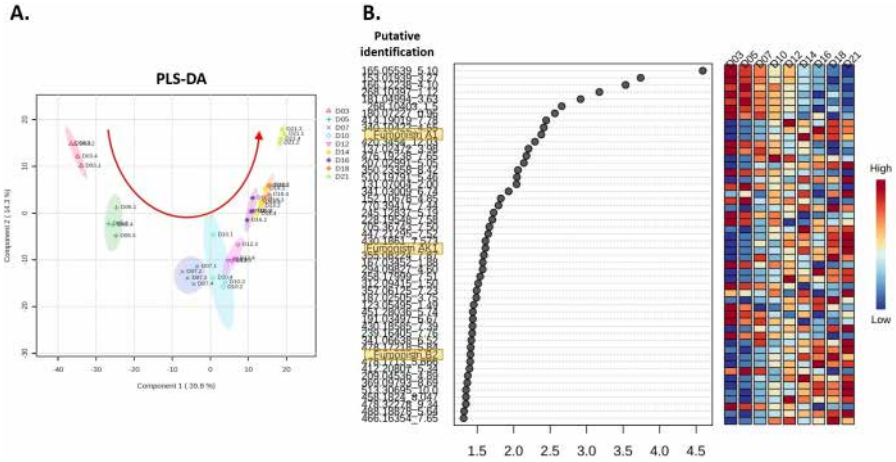

Figure 6. A. PLS-DA corresponding to the extracts of F. verticillioides cultured on CMA medium during different incubation time based on features (m/z at Rt to its normalized peak area) detected in LC–MS chromatograms. B. PLS-DA VIP projection for the top 50 features corresponding to each day, on component 1.

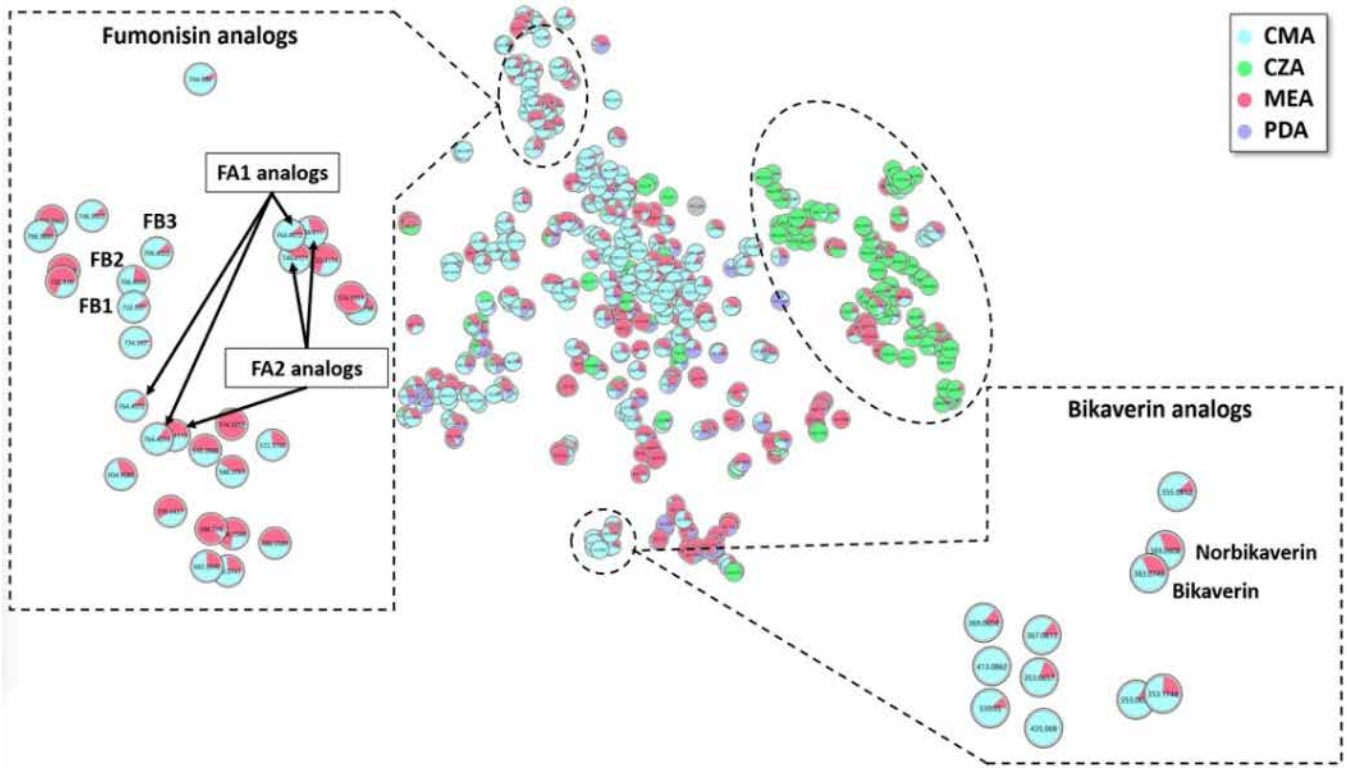

Figure 7. t-SNE network corresponding to extracts of *Fusarium verticillioides* cultivated on CMA, CZA, MEA and PDA for 14 days based on features (m/z at Rt to its normalized peak area) detected in LC–MS chromatograms.

less on PDA, but never on CZA. A similar trend was observed for another cluster, in which bikaverin and norbikaverin were annotated using in-house databases and literature data. In this cluster, we were able to annotate bikaverin-related compounds by comparison with literature data retrieved from Arndt et al.[48] The MS^2 data from closely related mycotoxins standards or annotated mycotoxins were compared to data from the literature and Metgem's annotation proposals. These features either matched MS^2 data with the putative annotation or shared enough spectral similarities to conclude it was a derivative of the annotated mycotoxins. Results were summarized in Table 3 and shows the distribution of annotated fumonisin and bikaverin-related compounds.

These results suggested a potential correlation between the genes involved in fumonisin (FUM) and bikaverin (BIK) production, as previously reported by Lazzaro et al.[49] This could imply that both fumonisin and bikaverin synthesis could be connected by a genetic regulatory system. Interestingly, the t-SNE visualization also highlighted the presence of a cluster of molecules mainly detected in the extracts of *F. verticillioides* grown on the CZA medium, but these molecules could not be annotated.

This study provides insights into the metabolomic profile of *Fusarium verticillioides*. Our results emphasizes the importance of investigating emerging and masked mycotoxins, as these compounds present additional risks for food safety. The findings underscore the need for further research on factors like substrate type, incubation conditions, and interspecies interactions to fully understand the complex dynamics of mycotoxin production by *F. verticillioides*.

Conclusions

This study demonstrates the value of metabolomics approaches in investigating the production of mycotoxins by *F. verticillioides* and highlights the importance of considering various factors, such as substrate type, incubation time, and interspecies interactions, to elucidate the complex dynamics of mycotoxin production. Further research is needed to fully understand the ecological and toxicological implications of the diverse mycotoxins produced by this economically and agriculturally important fungus.

Experimental Section

All solvents used for chromatography were HPLC grade (Fisher Chemical, Hampton, NH, USA). Milli-Q RG system (Millipore, France) was used to produce high purity water of 18.2 MΩ cm resistivity. Formic acid (FA) was >98% for LC–MS (Sigma-Aldrich, St. Louis, MO, USA). Mycotoxin standards [i.e., aflatoxin B_1 (AFB_1), ochratoxin A (OTA), and patulin (PAT)] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), diacetoxyscirpenol (DAS), fusarenon X (FUS-X), T-2 toxin (T-2) and HT-2 toxin (HT-2) mixture, fumonisin B1 (FB_1) and fumonisin B2 (FB_2) mixture, enniatins (A, A1, B, B1) and beauvericin (BEA) mixture,

Table 3. Summary of annotated fumonisins thank to Metgem's database libraries, in-house database and data from the literature.

ID	Rt (min)	*m/z*	Adduct	Putative annotations	Distribution in the different media
584	5.813	722.3970	$[M+H]^+$	FB_1	CMA > MEA
579	6.443	706.4009	$[M+H]^+$	FB_2	CMA > MEA
580	6.200	706.4022	$[M+H]^+$	FB_3	CMA > MEA
592	6.164	764.4072	$[M+H]^+$	iso- $FA_{1a/b}$ or FA_1 analog	CMA > MEA
593	6.008	764.4072	$[M+H]^+$	iso- $FA_{1a/b}$ or FA_1 analog	CMA > MEA
594	6.58	764.4074	$[M+H]^+$	FA_1	CMA > MEA
589	6.437	748.411	$[M+H]^+$	FA_2 analog or FA_3	CMA > MEA
590	7.441	748.4119	$[M+H]^+$	FA_2	CMA > MEA
591	6.789	748.4121	$[M+H]^+$	FA_2 analog or FA_3	CMA > MEA
554	7.026	604.3686	$[M+H]^+$	FAK_1	CMA > MEA > PDA
363	8.872	383.0749	$[M+H]^+$	Bikaverin	CMA > MEA
339	7.263	369.0608	$[M+H]^+$	Norbikaverin	CMA > MEA
337	7.894	369.0604	$[M+H]^+$	Norbikaverin analog	CMA > MEA
336	9.245	367.0813	$[M+H]^+$	Dehydroxy-bikaverin	CMA > MEA
299	8.277	353.0657	$[M+H]^+$	Me-oxo-pre-bikaverin or analog	CMA > MEA
298	7.0509	353.0656	$[M+H]^+$	Me-oxo-pre-bikaverin or analog	CMA > MEA
267	6.795	339.0500	$[M+H]^+$	Oxo-pre-bikaverin	CMA > MEA

zearalenone (ZEA), moniliformin (MON) were purchased from Libios (Vindry sur Turdine, France).

Fungal Material

The Fusarium verticillioides strain INRA63 was isolated and identified by the INRAE MycSA group, in Bordeaux, Nouvelle-Aquitaine, France. The strain was received as small fragments in cryotubes containing 30% glycerol. Several fragments were cultivated on a Petri dish containing malt extract agar (MEA) medium to prepare the inoculum.

Culture Media and Conditions

Four culture media were used for the experiments: malt extract agar (MEA), potato dextrose agar (PDA, Difco™), Czapek agar (CZA, Difco™) and cornmeal dextrose agar (CMA). The precise composition of each medium and the preparation steps are described in supplementary information 1. F. verticillioides was incubated in 6-well plates (diameter of the well, 3.5 cm) containing 4 mL of MEA, PDA, CZA or CMA during 21 days at 27 °C, in dark conditions and with 80% of relative humidity. At days 3, 5, 7, 10, 12, 14, 16, 18 and 21 a culture plate and a control plate of each medium were removed from the incubator, photographed for the fungal growth comparison and then placed at −80 °C prior extraction.

Extraction and Preparation of Crude Extracts

For each condition, a generic extraction protocol using 8 mL of ethyl acetate was performed in quadruplicate.[58,59] The tubes were agitated for 1 hour and then sonicated for 30 minutes. The extracts were filtered on a Whatman paper filter and the filtrates were dehydrated with anhydrous magnesium sulfate. Finally, the supernatants were transferred into 2 mL Eppendorf® tubes and dried at 30 °C using a vacuum concentrator. The residues were then resuspended using methanol at the concentration of 2 mg/mL and stored at 4 °C prior analyses. A quality control (QC) sample was prepared by pooling a 20 µL aliquot of all the extracts.

LC–Mass Spectrometry Analysis

Ultra-high-performance liquid chromatography high-resolution mass spectrometry (UHPLC – HRMS) analyses were performed on a UHPLC Ultimate 3000 system (Dionex) coupled with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). The separation method was slightly modified from Pacheco-Tapia et al.[59] Briefly, a Zorbax Eclipse XDB–C18 HPLC Column, 2.1×150 mm, 3.5 µm (Agilent Technologies, Santa Clara, CA, USA) was used. The mobile phase gradient was made up with Milli-Q water acidified with 0.1% formic acid (A) and acetonitrile HPLC grade acidified with 0.1% FA (B). A gradient method at a constant flow rate of 0.3 mL/min was applied, starting at 95% A to 95% B from 0 to 10 minutes, held at 95% B from 10 to 12.5 min and a decrease from 95% B to 95% A from 12.5 to 15 minutes. The column temperature was set at 40 °C, the autosampler temperature was set at 15 °C and the injection volume was fixed at 5 µL. Mass detection was performed using an electrospray source (ESI) in positive (PI) and negative ionization (NI) modes at 15 000 resolving power [full width at half maximum (FWHM) at 400 m/z]. The mass scanning range was m/z 100–2000 for all samples. Ionization spray voltage was set to 3.5 kV and the capillarity temperature was set to 300 °C. Each full MS scan was followed by data-dependent acquisition of MS/MS spectra for the three most intense ions using stepped collision-induced dissociation (CID) at 35 arbitrary energy units. Exclusion time was set to 6 seconds.

Data Processing and Statistical Analysis

LC–MS/MS data were processed according to previous metabolomic studies on mycotoxins by our group[60] using MS-CleanR workflow. Briefly, batches in PI and NI were processed separately with MS-DIAL version 4.90. MS1 and MS2 tolerance were set to 0.01 and 0.05 Da respectively, in centroid mode. Data were collected between 0 and 15 min and from 100 to 1500 Da. The minimum

peak height was set to 1e5. Peaks were aligned on a QC reference file with a retention time tolerance of 0.1 min and a MS1 tolerance of 0.025 Da. MS-DIAL data were cleaned with MS-CleanR using default parameters except for the maximum relative standard deviation (RSD) which was set to 40: all the filters were ticked; the minimum blank ratio was set to 0.8 and the relative mass defect (RMD) was included between 50 and 3000. The maximum mass difference was set at 0.005 Da and maximum RT difference at 0.025 min. Pearson correlation was used to compute clusters with a minimum correlation set at 0.8 and $\alpha = 0.05$. Two peaks were kept by clusters, the most intense and the most connected to other ions. Data retrieved at the workflow output were then annotated with MS-FINDER version 3.52. MS1 and MS2 tolerance were set at 5 and 15 ppm respectively and O, N and Cl atoms were selected in formula finder. Annotation was carried out by comparing with a in-house.MSP database of mycotoxin standards (including DON, AFB_1, 3-AcDON, DAS, FUS–X, NIV, OTA, ZEN, HT-2 and T-2 toxins, enniatins (B, B_1, A, A_1), FB_1, FB_2, PAT and BEA) (metabolite annotation level 1). In silico matches (annotation level 2) were performed using internal databases from literature: 2.1. F. verticillioides metabolites, 2.2. Fusarium metabolites, 2.3. Mycotoxins and 2.4. MS-FINDER generic databases (ChEBI, NPA, NANPDB, COCONUT, KNApSAcK, PubChem, UNPD and T3DB). Data were exported as.csv files for metadata information (RT, m/z, annotation results, peak area, etc.). Only mycotoxin annotations were kept and manually checked comparing high-resolution mass and MS/MS fragmentation to literature data. The RT_m/z pairs of annotated mycotoxins were then integrated into an internal database saved in.csv.

Relative Quantification of Fumonisins A1, A2, B1 and B2

Raw data from the experiments were processed in PI mode with MZmine version 2.53 using the targeted feature detection tab. Focusing on mycotoxins, FA_1, FA_2, FB_1 and FB_2 were detected in all raw files using the internal database as a feature list file, the intensity tolerance was set at 100%, noise level was set at 1.0e5. m/z tolerance and RT tolerance were set at 0.005 m/z (or 20 ppm) and 0.2 minutes respectively. Data files containing detected peaks were then aligned with a m/z tolerance set at 0.005 m/z (or 15 ppm), weight for m/z and RT were both set at 1, RT tolerance was set at 0.15 minutes. Isotope patterns were compared with an isotope m/z tolerance set at 0.005 m/z (or 10 ppm), minimum absolute intensity was set at 1.0e1 and minimum score at 90%. Gap filling has been applied using the peak finder tool. Intensity tolerance was set to 10%, m/z tolerance was set to 0.005 m/z (or 15 ppm) and RT tolerance was set at 0.2 min. Analyzed results were exported to.csv file.

Peak areas retrieved with Mzmine in PI mode were imported in GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California, USA). Outliers were removed and only 3 out of 4 values were kept. An XY table was generated for each molecule in the different media. Using "points and connecting lines with error bars", trend curves were created. Mean and error and SEM were selected to generate the graph. On the y-axis are found peak areas of each molecule and on the x-axis, days of culture are found.

Statistical Analyses

For the multivariate analyses,.csv files from MSFinder were uploaded on Metaboanalyst online platform version 5.0 (https://www.metaboanalyst.ca/). Features were filtered based on QC samples if their RSDs were greater than 20%. A statistical filter based on interquartile range (IQR) was applied, filtering out 40% of the features. Samples were first normalized by sum and variables were weighted by Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). A Principal Component Analysis (PCA) was carried out to show the sample distribution in groups. To get a better visualization, a Partial Least Squares Discriminant Analysis (PLS-DA) was carried out to show the impact of incubation time on F. verticillioides metabolome. Variable importance in projection (VIP) scores of the 50 most important features were presented.

t-SNE Visualization

The.MGF file from MSDial/MSCleanR/MSFinder workflow and quantification table in PI mode were imported into Metgem software 1.4.3 version. t-distributed Stochastic Neighbor Embedding network (t-SNE network), nodes were kept if there was at least one cosine score above 0.7 with others. The number of iterations, perplexity, learning-rate and early exaggeration parameters were set to 1000, 6, 200 and 12, respectively. The Barnes-Hut approximation was activated using an angle of 0.5. The spectra in the network were then searched for analogues against the spectral libraries available. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 4 matched peaks. m/z tolerance for the search of analogues was set to 100.

Author Contributions

E.G. performed formal analysis, investigated the study, curated the data, wrote the original draft. V.C. and M.V. investigated and visualized the study. M.H. and A.G. conceptualized and supervised the study, proposed the methodology and performed formal analysis, investigated the study, curated the data, wrote the original draft, reviewed and edited the article. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The authors wish to thank Nadia Pons (MycSA, INRAE Bordeaux) for providing the fungal strain studied and Margaux Fontaine for her help with extraction. The authors are grateful to Pascal Pineau (Institut Pasteur), Olivier Puel, Sophie Lorber-Pascal (Toxalim, INRAE) for the provision of some mycotoxin standards.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.11065062, reference number 11065061.

Keywords: fumonisins · *Fusarium verticillioides* · metabolomics · one strain many compounds approach (OSMAC) · emerging mycotoxins

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Manuscript received: July 17, 2024
Version of record online: October 31, 2024