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Aflatoxin biosynthetic pathway extrolites in airborne *Aspergilli* series *Versicolores*

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Abstract

The *Aspergilli* of the series *Versicolores* include airborne species that produce sterigmatocystin, a mycotoxin possibly carcinogenic to humans (IARC group 2B). Molecular identification of 116 fungal isolates by *benA* gene amplification and sequencing revealed 8 species belonging to this series in French bioaerosols. All these isolates and a reference strain of each species were extracted using ethyl acetate + acetic acid 1% (v/v). In each extract, 10 extrolites of the aflatoxin biosynthetic pathway were investigated by UPLC-HRMS: norsolorinic acid, versicolorin A, 6-demethylsterigmatocystin, sterigmatocystin, 8-O-methylsterigmatocystin, 5-methoxysterigmatocystin and aflatoxins (B₁/B₂/G₁/G₂). All extrolites except aflatoxins were found. Sterigmatocystin was found in extracts of these species, including *A. sydowii* and *A. tabacinus* for which the ability to produce sterigmatocystin was questioned or unknown, respectively.

Peer-review under the responsibility of the organizing committee of the ICMB23.

Keywords: *Aspergillus*, series *Versicolores*, bioaerosols, extrolites, aflatoxins

1. Introduction/Background

According to the World Health Organization, 30-50% of European homes show signs of mould contamination [1]. In France, 37% of homes are considered to be contaminated by moulds [2]. In bioaerosols collected indoors, the genus *Aspergillus* is predominantly represented by species belonging to the series *Versicolores*: mainly *Aspergillus creber* and *A. jensenii* but also by *A. amoenus*, *A. fructus*, *A. protuberus*, *A. puulaauensis*, *A. sydowii* and *A. tabacinus* [3]. These moulds are known to synthesise extrolites of the aflatoxin biosynthetic pathway such as sterigmatocystin, which is classified as possibly carcinogenic to humans (group 2B of the International Agency for Research on Cancer (IARC)) [4].

We therefore investigated 10 extrolites of the aflatoxin biosynthetic pathway in extracts of 124 fungal isolates belonging to eight airborne species of the series *Versicolores*: norsolorinic acid (NOR), versicolorin A (VERA), 6-demethylsterigmatocystin (6-DMSTC), sterigmatocystin (STC), 8-O-methylsterigmatocystin (8-OMSTC), 5-methoxysterigmatocystin (5-MOSTC) and aflatoxins B₁, B₂, G₁ and G₂ (AFB₁, AFB₂, AFG₁ and AFG₂). The objective of this study was to explore the presence of these extrolites in the series *Versicolores*.

2. Materials and methods

A total of 116 isolates mainly collected from indoor bioaerosols were identified at species level using partial beta-tubulin (*benA*) sequences [5]. Among these isolates, eight species were identified: *Aspergillus amoenus* (n=1), *A. creber* (n=45), *A. fructus* (n=2), *A. jensenii* (n=39), *A. protuberus* (n=6), *A. puulaauensis* (n=5), *A. sydowii* (n=15) and *A. tabacinus* (n=3). Reference strains of these eight species were purchased from Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). All isolates were cultured on Malt Extract Agar medium supplemented with chloramphenicol (0.02% (w/v)) at 25°C.

Extractions were performed after 21 days of culture. Four agar plugs were made in triplicate using a sterile cork-borer on each agar plate. The plugs were transferred to a 5 mL glass tube and 2 mL of ethyl acetate acidified with 1% acetic acid (v/v) was added. The tubes were vortexed for 30 sec and centrifuged for 15 min at 1500 rpm and the supernatant (V=1.5 mL) was transferred to a new clean 5 mL glass tube. The extracts were then completely evaporated. The dry extracts were dissolved in

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1 mL of ultrapure water/acetonitrile (90/10, v/v), ultrasonicated for 15 min and filtered through 0.2 µm PVDF filters. The samples were then diluted as necessary with ultrapure water/acetonitrile (90/10, v/v) and [¹³C₁₈]-sterigmatocystin internal standard to a final concentration at 5 µg/L.

Standard stock solutions of 5-MOSTC, AFB₁, AFB₂ and AFG₂ at a concentration of 10 µg/L were prepared in ultrapure water/acetonitrile (90/10, v/v). Standard stock solutions of AFG₁, STC and [¹³C₁₈]-STC were prepared at concentrations of 20 µg/L, 500 µg/L and 250 µg/L respectively with the same mixture of ultrapure water/acetonitrile. All solutions were filtered through 0.2 µm PVDF syringe filters.

The extracts were analysed using UPLC-HRMS approach. Data acquisition and processing was performed with UNIFI version 1.9.4. Extrolites were determined by precursor mass ions [M+H]⁺ in the first step. Retention time and collision cross-section (CCS) values were extracted from UNIFI data. STC, 5-MOSTC and aflatoxins (B₁, B₂, G₁, G₂) were confirmed by standard injections. Identifications and quantifications were processed with the retention time (tolerance ±0.50 min), CSS (tolerance ±2%), precursor and fragment ion mass (≤ 5 ppm for m/z ≥ 200; ≤ 1 ppm for m/z ≤ 200) and ion ratio within ±30% (relative) compared to standards from the same batch, as recommended [6]. Ion counts were used to compare the production levels of the extrolites.

3. Results

All 124 samples were successfully analysed. The ion counts obtained are shown in Table 1. Molecules with the same retention time and CCS as the AFB₁ and AFG₂ standards were detected in an extract of *A. creber* (3.20 log₁₀) and *A. jensenii* (4.28 log₁₀) respectively but the correspondence with the fragmentation of the standards does not allow the identification of these molecules according to the Guidance document on identification of mycotoxins in food and feed SANTE/12089/2016.

		NOR	VERA	6-DMSTC	STC	8-OMSTC	5-MOSTC
<i>Aspergillus amoenus</i>	Producing isolates (n/N; %)	0/2; 0.00	0/2; 0.00	0/2; 0.00	1/2; 50.00	0/2; 0.00	0/2; 0.00
	Ion count mean (log ₁₀)	-	-	-	4.95	-	-
<i>Aspergillus creber</i>	Producing isolates (n/N; %)	22/46; 47.83	7/46; 15.22	4/46; 8.70	46/46; 100.00	3/46; 6.52	45/46; 97.83
	Ion count mean (log ₁₀)	3.09	2.57	2.76	4.77	2.76	4.59
<i>Aspergillus fructus</i>	Producing isolates (n/N; %)	0/3; 0.00	0/3; 0.00	0/3; 0.00	1/3; 33.33	0/3; 0.00	0/3; 0.00
	Ion count mean (log ₁₀)	-	-	-	5.03	-	-
<i>Aspergillus jensenii</i>	Producing isolates (n/N; %)	29/40; 72.50	12/40; 30.00	4/40; 10.00	28/40; 70.00	5/40; 12.50	27/40; 67.50
	Ion count mean (log ₁₀)	3.14	2.87	2.86	4.21	2.92	3.74
<i>Aspergillus protuberus</i>	Producing isolates (n/N; %)	2/7; 28.57	1/7; 14.29	0/7; 0.00	2/7; 28.57	0/7; 0.00	0/7; 0.00
	Ion count mean (log ₁₀)	3.23	2.96	-	4.51	-	-
<i>Aspergillus puulaauensis</i>	Producing isolates (n/N; %)	1/6; 16.67	4/6; 66.67	0/6; 0.00	6/6; 100.00	0/6; 0.00	6/6; 100.00
	Ion count mean (log ₁₀)	2.81	3.15	-	4.66	-	4.33
<i>Aspergillus sydowii</i>	Producing isolates (n/N; %)	0/16; 0.00	0/16; 0.00	0/16; 0.00	1/16; 6.25	0/16; 0.00	1/16; 6.25
	Ion count mean (log ₁₀)	-	-	-	3.00	-	2.59
<i>Aspergillus tabacinus</i>	Producing isolates (n/N; %)	2/4; 50.00	0/4; 0.00	2/4; 50.00	3/4; 75.00	0/4; 0.00	0/4; 0.00
	Ion count mean (log ₁₀)	2.72	-	2.68	4.88	-	-

AFB₂ and AFG₁ were not found in any of our extracts.

Table 1. Aflatoxin biosynthesis pathway extrolites produced by *Aspergilli* from the section *Nidulantes* series *Versicolores*.

NOR: norsolorinic acid; VERA: versicolorin A; 6-DMSTC: 6-demethylsterigmatocystin; STC: sterigmatocystin; 8-OMSTC: 8-O-methylsterigmatocystin; 5-MOSTC: 5-methoxysterigmatocystin.

4. Discussion and conclusion

For the first time, sterigmatocystin was found in extracts of *A. tabacinus* [7] and we were able to report the production of sterigmatocystin by *A. sydowii* whereas this capacity was questioned by several studies [9-10].

The greatest diversity of extrolites and the highest ion counts were found in *A. creber*, *A. puulaauensis* and *A. jensenii* suggesting a possible link with the cytotoxicity previously observed for these extracts [10]. Furthermore, studies have shown

the ability of sterigmatocystin to be aerosolised and thus be inhaled by inhabitants of mould-damaged homes [11]. In addition, this mycotoxin has also shown an inhalation toxicity [12] showing the need to monitor these mould species and these mycotoxins in bioaerosols of mold-damaged homes. Our first data contribute to a better understanding of the exposure to fungal airborne contaminants of the inhabitants.

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