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ORIGINAL PAPER

Production of fumonisins B_2 and B_4 in *Tolypocladium* species

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Abstract *Tolypocladium inflatum* is known primarily for its production of the cyclosporines that are used as an immunosuppressive drug. However, we report here the production of the carcinogenic fumonisins B_2 and B_4 by this biotechnologically relevant fungal genus. These mycotoxins were detected in 11 strains tested from three species: *Tolypocladium inflatum*, *T. cylindrosporum*, and *T. geodes*. Production of fumonisins by *Fusarium* spp. and *Aspergillus niger* is highly medium- and temperature-dependent, so the effect of these parameters on fumonisin production by three *T. inflatum* strains was studied. Maximum production was achieved on media with high sugar content incubated at 25–30°C. Since these results demonstrate that fumonisin production could be widespread within the genus *Tolypocladium*, the potential contamination of commercial cyclosporine preparations with fumonisins needs to be investigated.

Keywords Fumonisin · Cyclosporin · Mycotoxin · *Tolypocladium inflatum* · *Elaphocordyceps subsessilis*

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Introduction

Species in the fungal genus *Tolypocladium* are found worldwide as soil-borne insect pathogens and saprotrophs [6, 35]. *Tolypocladium inflatum* W. Gams (syn. *T. niveum* [Rostr.] Bissett) is the best-known species, because it produces the cyclosporines that are used worldwide as an immunosuppressive drug in organ transplant recipients. In addition, the species produces antifungal and insecticidal efrapeptins [4, 16, 18]. *T. inflatum*, an anamorph of *Elaphocordyceps subsessilis* [Petch] G. H. Sung, J. M. Sung and Spatafora), is currently classified within *Ascomycota, Sordariomycetes, Hypocreales*, *Ophiocordycipitaceae* [41].

Fumonisins (Fig. 1) are regulated mycotoxins that cause human and animal toxicoses when consumed via contaminated maize-based food and feeds [46]. Fumonisin B_1 (FB_1) has furthermore been implicated as a risk factor for neural tube defects in embryos [21, 45]. Fumonisins were first discovered in *Fusarium verticillioides* (Sacc.) Nirenberg [5] and are a significant problem in maize-based products [14, 36, 39, 42]. Fumonisins are also occasionally found in rice [31], black tea leaves [23], asparagus [19], pine nuts [22], and wine [25]. The US Food and Drug Administration recommends that maize should not be used for human consumption at levels above 2–4 ppm total fumonisin [43] while the European Union (EU) has a regulatory limit of 0.2–2 ppm in maize products [10]. *Fusarium*

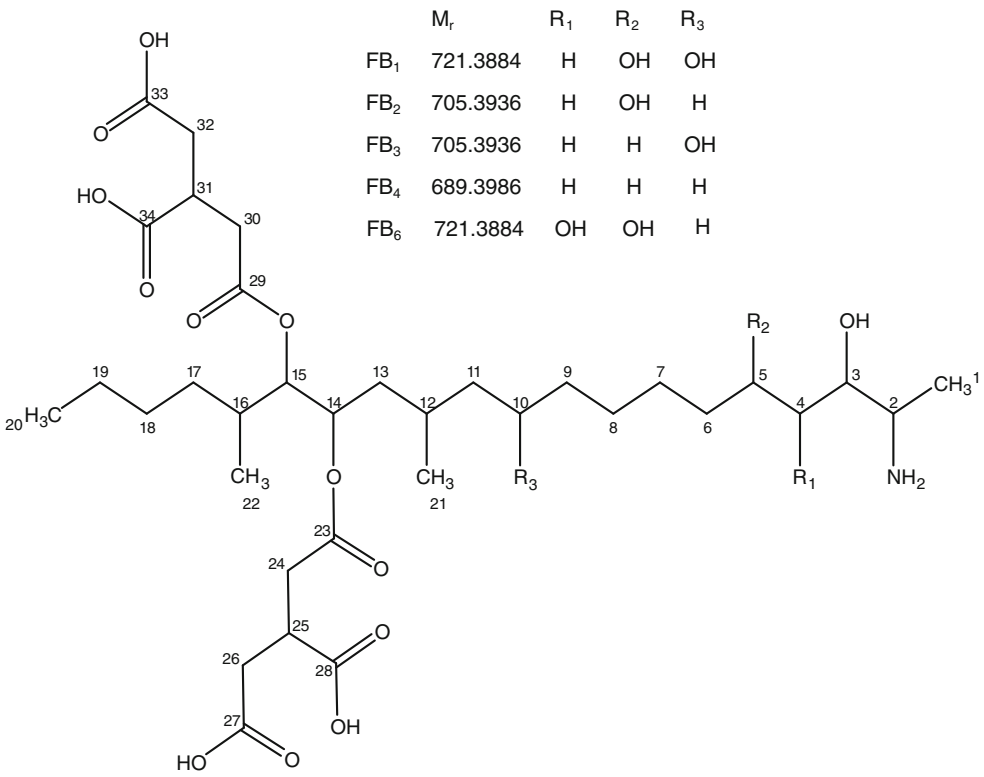

Fig. 1 Structure of fumonisins B_1, B_2, B_3, B_4 and B_6

is mainly the genus associated with fumonisin production, but a homolog to the *F. verticillioides* fumonisin gene cluster has been found in *Aspergillus niger* Tiegh. [1, 13, 20, 30, 32] and in a single strain of *Alternaria arborescens* E.G. Simmons (referred to as *Alternaria alternata* (Fr.) Kiessl. f. sp. *lycopersici* Grogan, Kimble & Misaghi in [47]). However, of the two only *A. niger* is able to produce fumonisins [13, 20, 30, 37].

During a routine screening of cyclosporine-producing fungi with the multi-detection method described by Vishwanath et al. [44], we were surprised to discover that a *Tolypocladium* strain was able to produce fumonisins. Since this has a potentially important impact on drug safety and genetic analysis, this study was initiated to survey the distribution of fumonisin production in *Tolypocladium* and to examine the influence of media and temperature on fumonisin production by species of this genus.

Materials and methods

Unless otherwise stated all solvents were HPLC grade, chemicals were analytical grade, and water was purified on a Milli-Q system (Millipore, Bedford, MA). Certified reference standards of fumonisin (B_1, B_2, and B_3) were from Romer Labs (Tulln, Austria), and FB_4 and FB_6 were purified from *A. niger* and validated by nuclear magnetic resonance (NMR) spectroscopy at the Technical University of Denmark [20].

Fungal strains, media, and incubation

Eleven *Tolypocladium* strains and one *Aspergillus niger* strain (IBT 28144 = CBS 101705, NRRL 567, ITEM 7097) were used in this study (see Table 1). To analyze the effect of media on the production of fumonisins ten media were used: Czapek yeast autolysate agar (CYA) [12], Czapek yeast autolysate agar + 5% NaCl (CYAS) [12], dichloran Rose bengal yeast extract sucrose agar (DRYES) [11], potato carrot agar (PCA) [37], malt extract agar (MEA according to Blakeslee) [34], oatmeal agar (OAT) [12], potato dextrose agar (PDA, Difco), V8-juice agar with antibiotics (V8) [37], yeast extract sucrose agar (YES) [12], and dichloran 18% glycerol agar (DG18) [15]. The composition of the media is given in Supplementary Table 1. Media were prepared in 9-cm Petri dishes, each with 17 mL medium, and strains were inoculated as three-point inoculations. Petri dishes were incubated in micro-perforated plastic bags at 25°C in the dark. All samples were analyzed in triplicate on three individual plates. Colony diameter measurements are given as averages of 9 colonies on 3 plates. To analyze the effect of temperature on the production of fumonisins in *T. inflatum*, three strains (IBT 41581, IBT 41582 and IBT 41583) were inoculated

Table 1 Production of fumonisins B_2 (FB_2) and B_4 (FB_4) by *Tolypocladium cylindrosporum*, *T. geodes*, and *T. inflatum*

Species	Strain number	FB_2	FB_4
T. cylindrosporum	CCF 1450	+	+
	CCF 2531	+	+
	CCF 3237	+	+
T. geodes	CCF 2548	+	−
	CCF 2579	+	+
	CCF 3299	+	−
T. inflatum	DSM 915	+	+
	DSM 63544	+	+
	IBT 41581	+	+
	IBT 41582	+	+
	IBT 41583	+	+

CCF Culture Collection of Fungi (Prague, Czech Republic); *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany); *IBT* Culture collection at Center for Microbial Biotechnology, DTU Systems Biology (Kgs. Lyngby, Denmark)

on YES and incubated at 15, 20, 25, 30, or 37°C in the dark for 7 days.

Metabolite extraction

Fumonisins were extracted by using the method previously described by Frisvad et al. [13]. Six plugs (6 mm diameter) were cut across the colony diameter through the center. If colony diameters were smaller than 20 mm, plugs were collected from two or three colonies from the plate. The six plugs were transferred to a 2-mL vial, 800 µL of methanol/water (3:1 v/v) was added, and vials were placed in an ultrasonication bath for 1 h. All extracts were filtered through a PTFE 0.45-µm syringe filter (National Scientific, Rockwood, Tennessee) into a new vial and used directly for analysis. Samples were made in triplicate from individual plates. This method was validated as in our previous reports with similar recovery of 75–85% [26, 28].

LC–MS/MS analysis of fumonisins

Fumonisins were analyzed by using liquid chromatography tandem mass spectrometry (LC–MS/MS) as previously described [24] with minor changes. The LC–MS/MS analyses were performed on a Quattro Ultima triple mass spectrometer (Micromass, Manchester, UK) with electrospray ionization (ESI) source, and separations performed on a 50 × 2 mm i.d., 3-µm Gemini C_6-phenyl column (Phenomenex, Torrance, California). A linear gradient was performed from 20% acetonitrile in water with 20 mM formic acid to 55% acetonitrile for 6 min at 0.3 mL/min, increasing to 100% acetonitrile in 30 s at 0.5 mL/min; this was maintained for 3.5 min before returning to the starting conditions in 6 min. Tandem MS was performed in ESI^+ with MS operated in multiple reaction monitoring (MRM) mode. Transitions were as previously described [24, 25]; fumonisins B_2 and B_3 quantifier *m/z* 706→336, qualifier *m/z* 706→512 a; fumonisin B_4 quantifier *m/z* 690→320, qualifier *m/z* 690→514 a; fumonisins B_1 and B_6 quantifier *m/z* 722→334, qualifier *m/z* 722→528 a. Reference standards of FB_1–FB_4 and FB_6 were co-analyzed with each analysis sequence. Strains of *T. cylindrosporum* and *T. geodes* (CCF and DSM) were analyzed according to Vishwanath et al. [44] on a similar instrument.

LC–TOF-MS screening

Selected extracts were additionally tested for other fumonisins, efrapeptins, and cyclosporines by liquid chromatography time-of-flight mass spectrometry (LC–TOF-MS) as described in Nielsen et al. [28]. This was performed on an LC system coupled to an orthogonal TOF mass spectrometer (Micromass LCT, Manchester, UK) equipped with an electrospray source [28]. The column was a 50 × 2 mm i.d., 3 µm Luna C_{18} (II) column (Phenomenex, Torrance, California) and a linear gradient was used with all solutions containing 20 mM formic acid, from 15% acetonitrile in water to 100% acetonitrile in 20 min at 300 µL/min; this was maintained for 3.5 min before returning to the starting conditions in 6 min. The MS operated in ESI^+ at a scan range of *m/z* 100–2,000. Peaks not matching the compounds in an internal database with approximately 850 reference standards [29] were matched against the 35,500 structures in Antibase 2009 (John Wiley and Sons, Inc, Hoboken, New Jersey). Extracted ion chromatograms (±0.02 amu) of the $[M + H]^+$ ions of the fumonisin series A, C, or P were constructed to search specifically for these compounds.

Results and discussion

A screening for fumonisins found that all eleven *Tolypocladium* strains used in this study produced fumonisins B_2 (FB_2) and nine (82%) also produced fumonisins B_4 (FB_4); however, no FB_1, FB_3 or FB_6 was detected in any of the strains (representative chromatograms in Fig. 2). All *T. cylindrosporum* and *T. inflatum* strains and one *T. geodes* strain (CCF 2579) produced both FB_2 and FB_4, while the other two *T. geodes* strains produced only FB_2 (Table 1).

Three strains of *T. inflatum* (IBT 41581, IBT 41582, IBT 41583) were selected for quantitative analysis of fumonisin production on a variety of different media and at different conditions. We tested 10 different media, and found that on each medium at least one *T. inflatum* strain produced FB_2

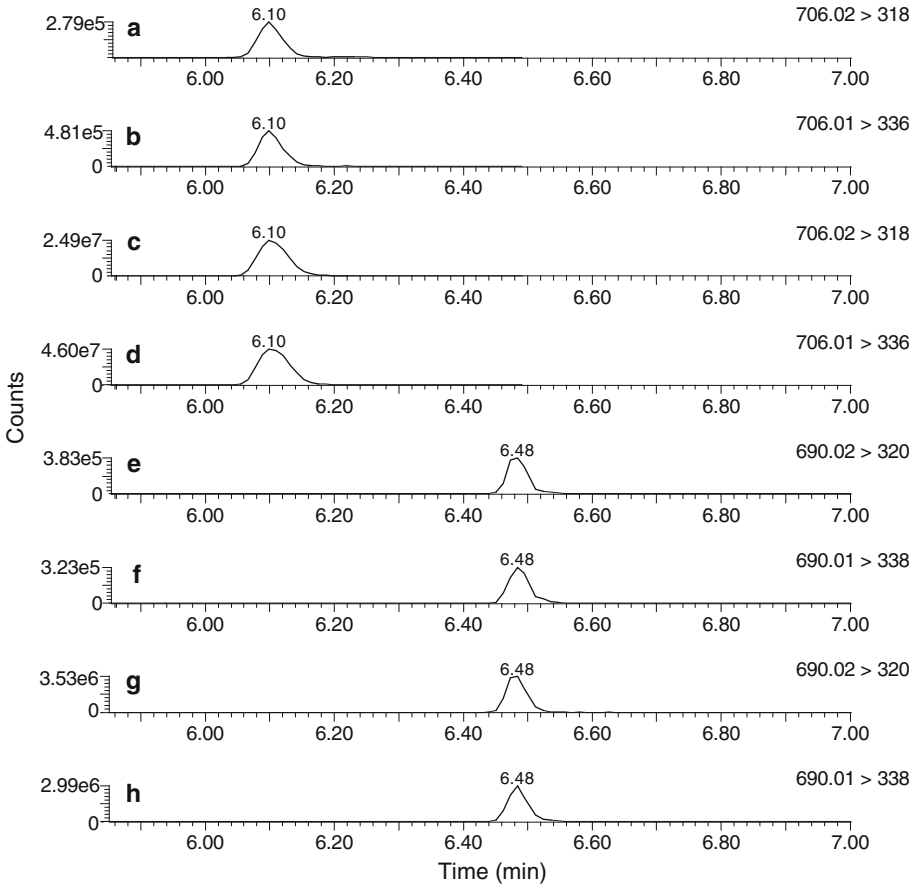

Fig. 2 LC–MS/MS analyses of fumonisin B_2 and B_4 in *Aspergillus niger* IBT 28144 and *Tolypocladium inflatum* IBT 41581 extracts from cultures incubated at 25°C for 7 days on YES. **a** Qualifier of a diluted certified FB_2 standard. **b** Quantifier of a diluted certified FB_2 standard. **c** Qualifier of FB_2 in a *T. inflatum* extract. **d** Quantifier of FB_2 in a *T. inflatum* extract. **e** Qualifier of FB_4 in an *A. niger* extract. **f** Quantifier of FB_4 in an *A. niger* extract. **g** Qualifier of FB_4 in a *T. inflatum* extract. **h** Quantifier of FB_4 in a *T. inflatum* extract

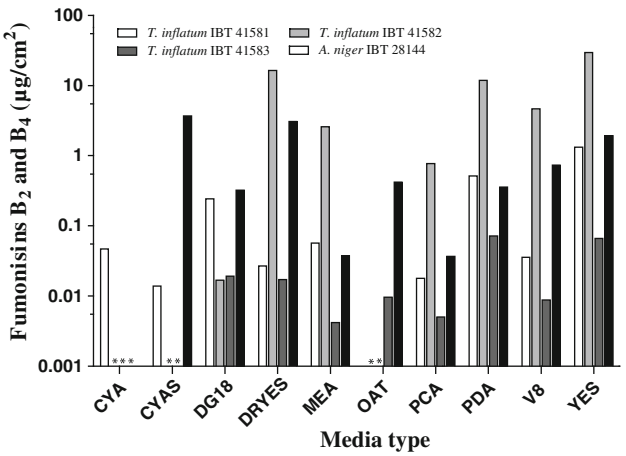

Fig. 3 Production of fumonisins B_2 and B_4 by three *Tolypocladium inflatum* strains (IBT 41581, IBT 41582, IBT 41583) and one *Aspergillus niger* (IBT 28144) on different media at 25°C. *Not detected

and FB_4 (Fig. 3). The maximal production of total fumonisins for the three strains was 1.3 µg/cm^2 for IBT 41581, 30 µg/cm^2 for IBT 41582 and 0.072 µg/cm^2 for IBT 41583. These levels were comparable to both *Fusarium* spp. and *A. niger* in a similar study under similar conditions, which found fumonisin production of 0.006–22 µg/cm^2 for *Fusarium* spp. and 0.27–21 µg/cm^2 for *A. niger* [26]. Relative levels of FB_4 to FB_2 were in the range 0–95%, with most being in the 10–30% range. The media yielding the highest amounts of fumonisins were DRYES, PDA, and YES, while lower amounts were detected in cultures grown on MEA, OAT, CYA, CYAS, PCA, V8, and DG18. *Aspergillus niger* (IBT 28144) produced high amounts of fumonisins on media with high amounts of sugar and salt, e.g., CYAS, DRYES, and YES, with detectable production of fumonisins on the other seven media as shown in Fig. 3. This is in agreement with the results of Frisvad et al. [13]. Fumonisin production by the *T. inflatum* strains was similar to that by both *Fusarium* and *A. niger*, with PDA and YES also supporting substantial fumonisin production [13, 26]. The media used for cyclosporine production often consists of 2–8% carbon source (e.g., sorbose, glucose, or similar) and 1–6% nitrogen source (e.g., peptone, malt extract, or casein acid hydrolysate) with added trace metals [2, 3, 17]. The media most comparable to these in our study are CYA, CYAS, and MEA. On CYA and CYAS, only one strain produced fumonisin, whereas all three strains produced fumonisins on MEA. This suggests that the media used for cyclosporine production might support the simultaneous

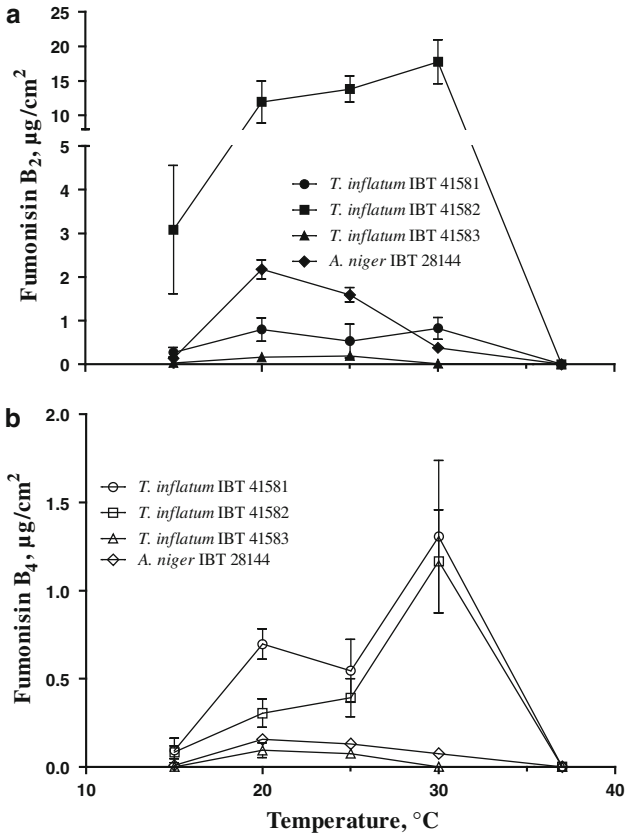

Fig. 4 Production of fumonisins B_2 (**a**) and B_4 (**b**) by three strains of *Tolypocladium inflatum* (IBT 41581, IBT 41582 and IBT 41583) and *Aspergillus niger* IBT 28144 incubated for 7 days at 15, 20, 25, 30, and 37°C on YES. The standard deviation of triplicates is shown by the error bars. Fumonisin concentration was 0 µg/cm^2 at 37°C because no growth occurred at that temperature

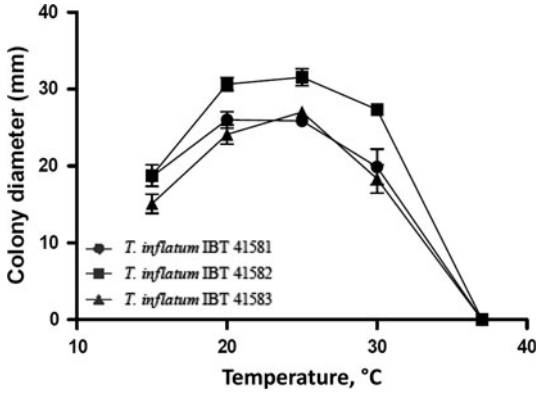

Fig. 5 Colony diameters of three strains of *Tolypocladium inflatum* (IBT 41581, IBT 41582, IBT 41583) after incubation for 7 days at 15, 20, 25, 30, and 37°C on YES. The standard deviation of triplicates is shown by the error bars

production of fumonisins, although the use of liquid medium compared to solid has to be accounted for.

The three *T. inflatum* strains produced fumonisins in a temperature range of 15–30°C (Fig. 4). The effect of temperature on fumonisin production was strain-dependent, with two strains giving maximal production at 30°C and one strain (IBT 41583) showing minimal production at 30°C, and maximum production at 25°C. This is similar to previous observations for *A. niger*, which showed peak fumonisin production at 25–30°C [26]. Except for one set of experiments in which IBT 41582 produced more FB_4 than FB_2 at 30°C, FB_2 was consistently produced at higher levels than FB_4 (data not shown). All three strains of *T. inflatum* were able to grow at temperatures from 15 to 30°C, with maximal colony diameters after 7 days of incubation at 20 or 25°C (Fig. 5). No growth was observed at 37°C.

In the three selected *T. inflatum* strains, no traces of fumonisin series A, C, or P were detected by LC–TOF-MS. However, FB_2 and FB_4 were detected in all samples of all cultures of the three *T. inflatum* strains in repeated experiments, and by the two different techniques of LC–MS/MS and LC–TOF-MS. Fumonisins were detected from cultures grown on different media (PDA, DRYES, and YES), and in separate cultures derived from the same original strain. In addition to FB_2 and FB_4, the three *T. inflatum* strains produced cyclosporines and efrapeptins with the fumonisins (data not shown).

This is the first report describing the production of FB_2 and FB_4 in the genus *Tolypocladium*. These findings are relevant because *T. inflatum* is used in the pharmaceutical industry as a producer of cyclosporin A. We recommend that the production strains of cyclosporines are analyzed for production of fumonisins under industrial production conditions to determine if these mycotoxins end up in the final product. Furthermore *Tolypocladium* spp. have been suggested as a possible fungal biological control agent, because of the entomopathogenicity of its production of efrapeptins [40]. With the discovery of fumonisin production by *Tolypocladium* spp., however, this raises concerns that these toxic metabolites may enter the environment and the food chain and pose a risk to humans and animals.

To date more than 20 species within the three genera *Fusarium*, *Aspergillus,* and *Tolypocladium* have been shown to produce fumonisins [13, 33]. *Alternaria arborescens* (as *Alternaria alternata* f. sp. *lycopersici*) was reported to produce fumonisins, but the results were later questioned and found to be due to only the chemically similar AAL toxin and not fumonisin [8, 9, 38]. So far, *Fusarium* spp. produce the greatest diversity of fumonisins, namely FB_1–FB_5 and the A, C, and P series [5, 7, 27], whereas *A. niger* produces only FB_2, FB_4, and FB_6 [13, 20, 30]. The discovery reported here shows that *Tolypocladium* spp. have a fumonisin profile similar to *A. niger*, but quite different from *Fusarium* spp. Because of these similar profiles, *Tolypocladium* spp. and *A. niger* could have a

higher degree of similarity in their fumonisin gene cluster compared to *Fusarium* spp., but this needs to be established by genome sequencing of the strains. This may help to determine if the genes were horizontally transferred and, if so, between which species. The discovery of fumonisin production in three distantly related fungal genera shows that the production of fumonisins may be more widespread in the fungal kingdom than previously believed.

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