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Fumonisin B_2 Production by *Aspergillus niger*

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The carcinogenic mycotoxin fumonisin B_2 was detected for the first time in the industrially important *Aspergillus niger*. Fumonisin B_2, known from *Fusarium verticillioides* and other Fusaria, was detected in cultures of three full genome sequenced strains of *A. niger*, in the ex type culture and in a culture of *F. verticillioides* by electrospray LC-MS analysis of methanolic extracts from agar plugs of cultures grown on several substrates. Whereas *F. verticillioides* produced fumonisins B_1, B_2, and B_3 on agar media based on plant extracts, such as barley malt, oat, rice, potatoes, and carrots, *A. niger* produced fumonisin B_2 best on agar media with a low water activity, including Czapek yeast autolysate agar with 5% NaCl. Of the media tested, only rice corn steep agar supported fumonisin production by both *F. verticillioides* and *A. niger*. However, *A. niger* had a different regulation of fumonisin production and a different quantitative profile of fumonisins, producing only B_2 as compared to *F. verticillioides*. Fumonisin production by *A. niger*, which is a widely occurring species and an extremely important industrial organism, will have very important implications for biotechnology and especially food safety. *A. niger* is used for the production of citric acid and as producer of extracellular enzymes, and also as a transformation host for the expression of heterologous proteins. Certain strains of *A. niger* produce both ochratoxin A and fumonisins, so some foods and feeds may potentially contain two types of carcinogenic mycotoxins from this species.

KEYWORDS: Fumonisins; *Aspergillus niger*; *Fusarium verticillioides*; mycotoxin; growth media; food safety

INTRODUCTION

Fumonisins are carcinogenic mycotoxins produced by *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) and other Fusaria (*1–3*) and are among the most important toxins regarding food and feed safety. In a recent summary the authors propose 53 different fumonisins, but fumonisins B_1, B_2, and B_3 are all carcinogenic and regarded as the most important (*4, 5*). Fumonisin B_1 is the intensively examined fumonisin, but when it has been compared to fumonisin B_2, the latter is more cytotoxic than B_1 (*6*). Some reports indicate that the fumonisins may be involved in esophageal cancer in South Africa (*7, 8*), and they have been shown to be involved in leukoencephalomalacia in horses (*9*) and pulmonary edema in pigs (*10, 11*). Until recently the fumonisins had been reported from only *Fusarium*, except in one case when the tomato pathogen *Alternaria arborescens* (as *Alt. alternata* f.sp. *lycopersici*) was claimed to produce fumonisin B_1 (*12, 13*). Fumonisin production by *Alt. arborescens* has not been confirmed since 1996. The fumonisin gene cluster in *F. verticillioides* has been characterized by Proctor et al. (*14*) and Brown et al. (*15*) and consists of at least 15 genes. Recent independent reports by Baker (*16*) and Pel et al. (*17*) indicated that the phylogenetically very distantly related fungus *Aspergillus niger* has a putative gene cluster for fumonisins. Full genome sequencing of the *A. niger* isolates ATCC 1015 (*16*) and CBS 513.88 (*17*) concurred in showing that both strains had homologues to the *F. verticillioides* fumonisin genes. However, such gene clusters are not necessarily expressed phenotypically; for example, in *A. niger* the genes may be defective or silent.

If fumonisins can be produced by *A. niger*, however, this may have severe implications, both for the many biotechnological uses of the fungus and for potential fumonisin contamination of the large number of foods and feeds in which *A. niger* has been reported to grow. Furthermore, *A. niger* has often been used for the production of single-cell protein used for feed (*18–21*) and is extensively used as a producer of citric acid and extracellular enzymes and as transformation host in the biotechnological industry (*17, 22–25*). This species is also a very important opportunistic pathogen of grapes, causing bunch rot and berry rot, or grows in raisins (raisin mold) (*26*). This disease is important in warmer grape-growing areas such as Spain, Portugal, France, Italy, Greece, Morocco, and Egypt (*27–32*).

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A. niger has also been found to produce ochratoxin A on grapes (*33*). In a recent three-year study, more than 2114 black *Aspergillus* isolates from grapes were analyzed for ochratoxin A production (*34*). Whereas about 3% of isolates belonging to the *A. niger* species aggregate produced ochratoxin A, *A. carbonarius* was regarded as the most important toxinogenic fungus in *Aspergillus* section *Nigri* because most isolates of this species from grapes and coffee produce ochratoxin A in large amounts (*32*). On the other hand, *A. niger*, rather than *A. carbonarius*, has been reported to grow and damage a large number of crops worldwide, including corn, peanuts, onions, mango, and apples (*35*).

The three full genome sequenced *A. niger* isolates were compared with a known efficient producer of fumonisins, *F. verticillioides* MRC 826, to find out whether any of the three *Aspergillus* isolates were able to produce fumonisins. The ex type culture of *A. niger* was also included in these investigations, even though that particular strain has not been full genome sequenced. A simple screening method for fumonisin production in pure culture was also developed.

MATERIALS AND METHODS

Fungal Strains and Media. *F. verticillioides* MRC 826 = IBT 9400, a well-known fumonisin producer (*36*), was used as a reference. *A. niger* strain NRRL 3122 = IBT 23538, from which the full genome sequenced strain CBS 513.88 (= ATCC 22343) is derived, has been used for the production of enzymes (*17*). *A. niger* NRRL 328 = IBT 27878 (= ATCC 1015 = CBS 113.46) is a classical strain used for citric acid production and is also being full genome sequenced (*16*). *A. niger* NRRL 3 = IBT 23539 (= ATCC 9029 = CBS 120.49) has also been used for citric acid production and several other biotechnological applications and is also being full genome sequenced (*16*). NRRL 326 = IBT 27876 (= ATCC 16888 = CBS 554.65^T) is from a tannin–gallic acid fermentation and is the culture ex type of *A. niger*. The original substrate, from which the three first *A. niger* strains were isolated, is unknown.

Acronyms for culture collections are as follows: ATCC, American Type Culture Collection, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IBT, BioCentrum-DTU, Kgs. Lyngby, Denmark; MRC, Medical Research Council, Tygerberg, South Africa; NRRL, ARS Culture Collection, Northern Regional Research Laboratory, National Center for Agricultural Utilization Research, Peoria, IL.

Because corn has been used for fumonisin production in most studies (*3*), we examined a range of agar media for the ability to support production of this class of mycotoxins. *F. verticillioides* MRC 826 and *A. niger* NRRL 3122 were inoculated in three points on the following media: Czapek yeast autolysate (CYA) agar (*37*), yeast extract sucrose (YES) agar (*37*), yeast extract agar (YEA) (YES agar without sucrose), CYA with 5% NaCl (CYAS) agar (*37*), CYA with 20% sucrose (CY20S) agar (*38*), dichloran 18% glycerol (DG18) agar (*39*), glucose minimal medium (GMM) agar (*40*), rice corn steep (RC) agar (*41*), three versions of malt extract agar (*38*) [MEA, with Bacto malt extract (Becton, Dickinson and Co., Sparks, MD); MEAG, with malt extract (Biokar Diagnostics, Beauvais, France); and MEAB, with malt extract (Merck, Darmstadt, Germany)], potato dextrose agar (PDA) (*42*), potato carrot agar (PCA) (*43*), and oatmeal (OAT) agar (*37*). All media were incubated for 7 days in darkness at 25 °C. The LC-MS analysis of the extracts was done in triplicate.

Subsequently, four *A. niger* strains, NRRL 3122, NRRL 328, NRRL 3, and NRRL 326, were inoculated in three points on CYA, YES, MEA, and CYAS (in triplicate) and incubated for 7 days in darkness at 25 °C and analyzed for fumonisins, in order to determine whether these isolates differed fundamentally in their production profiles of fumonisins and whether they produced fumonisins at all.

Analysis of Fumonisins in Pure Culture. A semiquantitative method based on agar plugs (*44*) was developed for the detection of fumonisins. Five agar plugs (diameter = 6 mm) were cut out of the colony from the center and in a radius toward the edge of the colony. The five agar plugs were placed in a 2 mL glass vial, 750 μL of 75% methanol in water (75:25, v/v) was added, and the plugs were extracted for fumonisins for 50 min using ultrasonication. The extracts were filtered using a PFTE 0.45 μm filter and used directly for analysis.

The LC-MS analysis was done on a Waters (Micromass, Manchester, U.K.) LCT time-of-flight mass spectrometer connected to an Agilent 1100 HPLC (Agilent, Waldbronn, Germany) (*45*). The separation was done on a 50 × 2 mm i.d. Luna C-18 (II) column (Phenomenex, Torrance, CA) fitted with a 2 × 2 mm guard column and using a water/acetonitrile (both containing 20 mM formic acid) gradient at a flow rate of 0.3 mL/min. The gradient was started at 15% acetonitrile, which was increased to 100% acetonitrile in 20 min; 100% acetonitrile was maintained for 5 min before the gradient was returned to starting conditions. A 3 μL extract was injected for all samples. The mass spectrometer was operated in positive electrospray ionization mode and was tuned for maximal sensitivity on a solution of leucine enkephalin in 50% acetonitrile with 20 mM formic acid (protonated molecular mass *m/z* 556.2771) and a resolution of better than 5000 fwhm. The mass scale was calibrated on a solution of PEG in 50% acetonitrile with 20 mM formic acid. Spectra were collected at a rate of 0.4 s per spectrum with a 0.1 s interscan time. Every third second a reference spectrum was collected from the reference spray (LockSpray).

An approximate detection limit of fumonisins B_1 and B_2 was calculated on the basis of a certified standard of a mixture of 50 μg/mL of each of fumonisin B_1 and B_2 (Biopure, Tulln, Austria). With a signal to noise ratio of 5, this give an approximate detection limit of 15 pg/μL injected sample. Fumonisin B_3 was a gift from Dr. Michael Sulyok, Center for Analytical Chemistry (IFA-Tulln, Austria). The standards of fumonisins B_1, B_2, and B_3 eluted at 6.214, 7.531, and 7.090 min, respectively. Detection is based on a LC-MS screening analysis protocol where narrow ion traces (30 mDa width) around the protonated mass for fumonisin B_2 at *m/z* 706.4014 are used for detection. The method is only semiquantitative and is based on analysis of the same colony surface area (*44*). Due to different sensitivities in the two series of experiments, two different semiquantitative scales have been applied.

RESULTS AND DISCUSSION

Using a simple agar plug method, fumonisins were easily detected using selective ion monitoring electrospray LC-MS (**Figure 1**) in the known producer *F. verticillioides* MRC 826. The accurate mass and the retention times of fumonisins B_1, B_2, and B_3 from the extract of the *Fusarium* culture were the same as those of authentic standards. *A. niger* NRRL 3122 produced fumonisin B_2, whereas the production of fumonisins B_1 and B_3 in *A. niger* NRRL 3122 was not detectable (**Table 1**). The detection of fumonisin B_2 in *A. niger* NRRL 3122 was confirmed by comparison of its retention time and high-resolution mass spectrum with an those of an authentic standard (**Figure 2**). Further chemical investigations of *A. niger* derived fumonisin B_2, involving isolation and chemical characterization of the compound by NMR, X-ray, and optical methods, will conclusively reveal whether the nature and absolute stereochemistry of fumonisins from *Fusarium* species and *A. niger* are completely identical.

A. niger NRRL 3122 did produce fumonisin B_2 on RC, GMM, CYA, YES, CY20S, CYAS, and DG18, but did not produce any detectable fumonisin on most plant-based agar media, such as MEA, MEAB, MEAG, OAT, PCA, or PDA. The highest amount of fumonisin B_2 (+++ to ++++) was detected on CY20S, RC agar, and CYAS. Lower amounts (+ to ++) of fumonisin B_2 were produced on DG18, CYA, YES and GMM (**Table 1**).

F. verticillioides MRC 826 produced large amounts of fumonisin B_1 (+++ to ++++) on plant-based media such as malt extract media (MEA, MEAB, MEAG), RC, OAT, and PDA. Lower amounts (+) of fumonisin B_1 were produced on

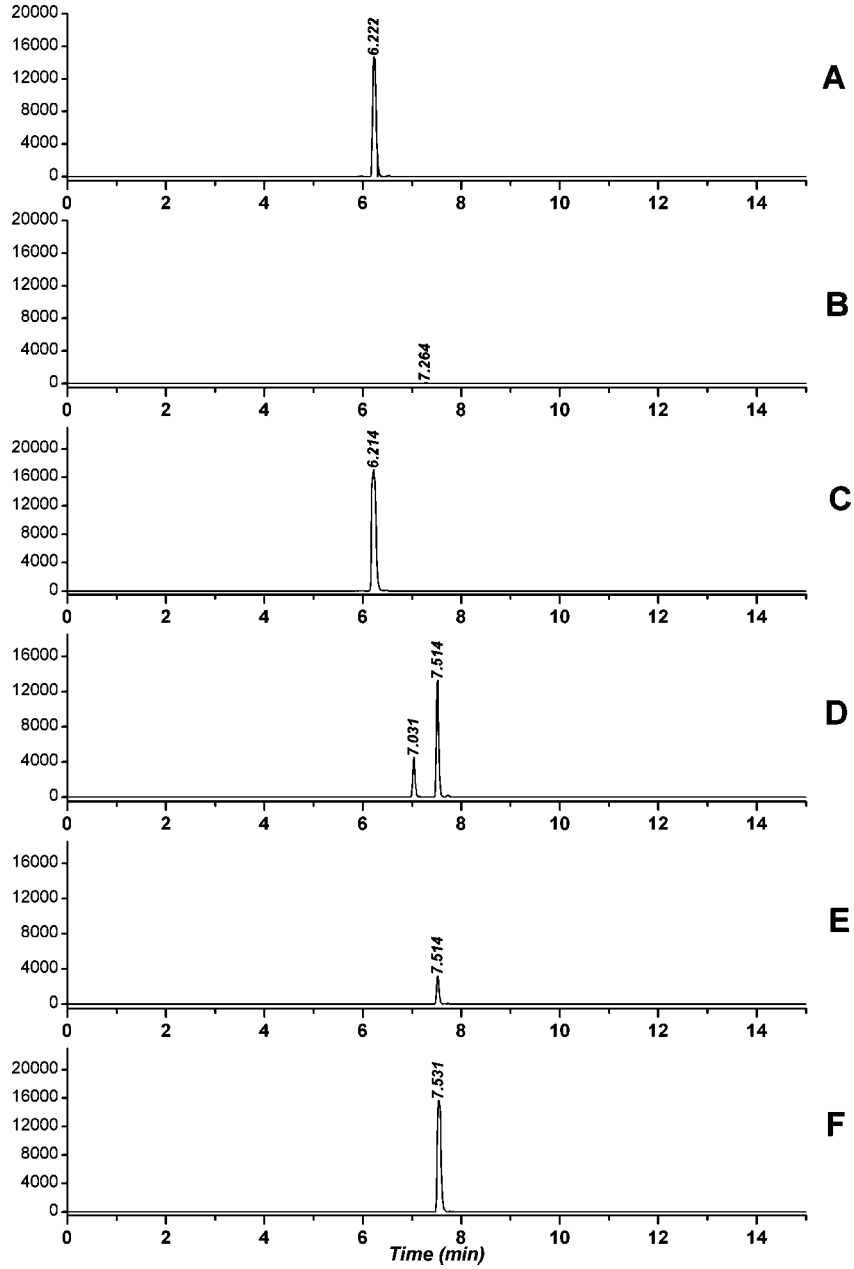

Figure 1. Selective narrow ion traces with a width of 30 mDa around the protonated molecular mass at *m*/*z* 722.3963 for fumonisin B_1 (**A**, **B**) and *m*/*z* 706.4014 for fumonisin B_2 (**D**, **E**); (**C**) fumonisin B_1 standard (retention time = 6.21 min) due to too high concentration of the ^{13}C isotopic trace at *m*/*z* 723.3996; similarly (**F**) is a fumonisin B_2 standard (retention time = 7.53 min). Traces from *F. verticillioides* MRC 826 cultivated on MEAG clearly show distinct peaks from fumonisin B_1 (**A**), fumonisin B_2, and also a peak from fumonisin B_3 (at 7.03 min), which has exactly the same mass as fumonisin B_2 (**D**). Traces from *A. niger* NRRL 3122 cultivated on CYAS agar show a distinct peak corresponding to fumonisin B_2 (**E**) confirmed by the mass spectrum, whereas fumonisin B_1 is not seen (**B**).

PCA. Fumonisin B_2 was produced in highest amounts (+++ to ++++) on malt extract media, PDA, and OAT. Lower amounts (+ to ++) of fumonisin B_2 were produced on RC agar and PCA (**Table 1**). For *F. verticillioides* MRC 826, fumonisin B_1 was produced in the highest amounts, and the amounts of fumonisin B_2 were higher than the amounts of fumonisin B_3 on most of the media used (**Table 1**). Some media, including those with chemically well-defined ingredients, yeast extract, or a low water activity based on sucrose, NaCl, or glycerol, did not support production of fumonisins by *F. verticillioides* at all, including GMM, CYA, YES, CY20S, CYAS, and DG18.

A major difference between fumonisin production by *A. niger* and *F. verticillioides* was that the former produced only fumonisin B_2 and no fumonisin B_1 or B_3 in detectable amounts. The only medium that supported fumonisin production in both *A. niger* and *F. verticillioides* was RC agar. Neither *A. niger* nor *F. verticillioides* produced fumonisins on YEA.

The two strains of *A. niger* that had the putative fumonisin gene cluster (*16, 17*), both produced fumonisin B_2, but so did NRRL 3 and the ex type culture of *A. niger* CBS 554.65. For NRRL 3122 and NRRL 326 the highest production of fumonisin B_2 was on CYAS, followed by CYA and YES. We could not detect any fumonisin produced by *A. niger* on MEA (**Table 2**).

Fumonisin B_2 was detected in all independent examinations of cultures of the four isolates of *A. niger*, both in repeated experiments, on different media (CYAS, CYA, and YES) and in cultures derived from the same original isolate. The results did not vary on the semiquantitative scale used.

Table 1. Fumonisin Production by *Aspergillus niger* NRRL 3122 and *Fusarium verticillioides* MRC 826 on Different Agar Media[a]

	Aspergillus niger NRRL 3122			*Fusarium verticillioides* MRC 826		
	fumonisin B_1	fumonisin B_2	fumonisin B_3	fumonisin B_1	fumonisin B_2	fumonisin B_3
RC	nd	+++	nd	+++	++	+
DG18	nd	++	nd	nd	nd	nd
CYAS	nd	+++	nd	nd	nd	nd
GMM	nd	+	nd	nd	nd	nd
CY20S	nd	++++	nd	nd	nd	nd
CYA	nd	++	nd	nd	nd	nd
YES	nd	+	nd	nd	nd	nd
MEA	nd	nd	nd	++++	+++	++
MEAB	nd	nd	nd	++++	++++	++
MEAG	nd	nd	nd	+++	++++	+
PCA	nd	nd	nd	+	+	nd
PDA	nd	nd	nd	++++	+++	+
OAT	nd	nd	nd	++++	+++	+

[a] Abbreviations: CYA, Czapek yeast autolysate agar; YES, yeast extract sucrose agar; CYAS, CYA with 5% NaCl; CY20S, CYA with 20% sucrose; DG18, dichloran 18% glycerol agar; GMM, glucose minimal medium agar; RC, rice corn steep agar; MEA, malt extract agar with Bacto malt extract; MEAG, malt extract agar with Biokar malt extract; MEAB, malt extract agar with Merck malt extract; PDA, potato dextrose agar; PCA, potato carrot agar; OAT, oatmeal agar; +, 100–1000 ion counts; ++, 1000–5000 ion counts; +++, 5000–10000 ion counts; ++++, >10000 ion counts; nd, not detected.

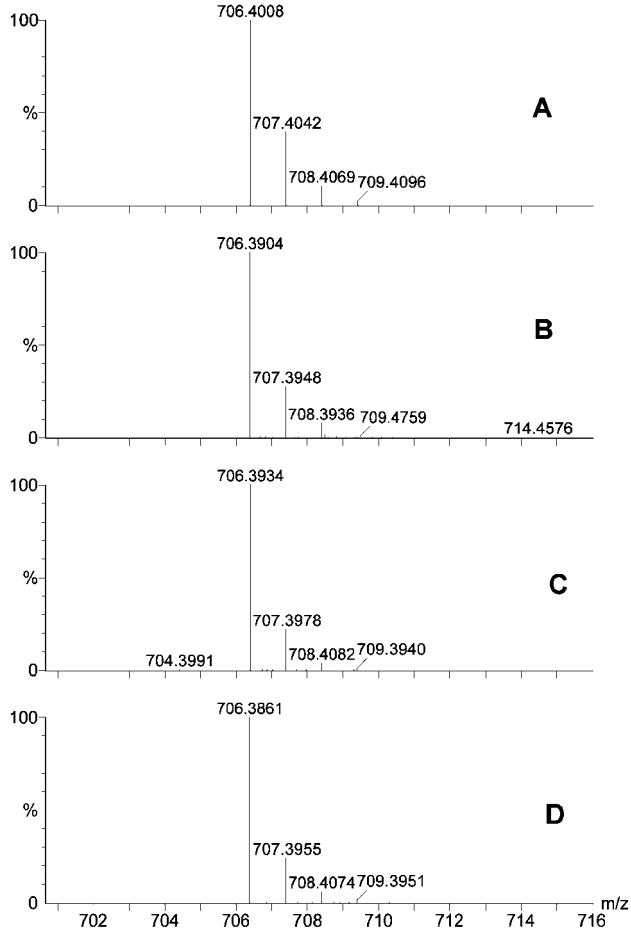

Figure 2. Mass spectra of fumonisin B_2: (**A**) theoretical isotope model of protonated fumonisin B_2, $C_{34}H_{59}NO_{14}$; (**B**) fumonisin B_2 from *A. niger* NRRL 3122 cultivated on CYAS agar; (**C**) fumonisin B_2 from *F. verticillioides* MRC 826 cultivated on MEAG; (**D**) standard of fumonisin B_2.

This is the first report on fumonisin production by *A. niger*. On the basis of the examination of four *A. niger* strains, it appears that fumonisin B_2 is consistently produced by this species (**Table 2**). Small peaks with the retention time and mass of fumonisin B_1 were found in *A. niger* NRRL 3122, but they were below the approximate detection limit of our semiquantitative method. Further studies are needed to establish if

Table 2. Fumonisin B_2 Production by the ex Type Culture and Three Full Genome Sequenced Strains of *Aspergillus niger*[a]

	fumonisin B_2 production			
	CYAS	CYA	YES	MEA
NRRL 3122	++	+	+	nd
NRRL 328	++	++	++	nd
NRRL 3	++	++	++	nd
NRRL 326	+++	++	+	nd

[a] Abbreviations: CYA, Czapek yeast autolysate agar; YES, yeast extract sucrose agar; CYAS, CYA with 5% NaCl; MEA, malt extract agar with Bacto malt extract; +, 25–300 ion counts; ++, 300–1000 ion counts; +++, >1000 ion counts; nd, not detected.

fumonisin B_1 is produced by *A. niger*. Even though the putative fumonisin gene cluster from *F. verticillioides* (*14, 15*) was found in a relatively similar sequence in *A. niger* ATCC 9015 (*16*) and NRRL 3122 (*17*), it is never given that such a similar gene cluster is phenotypically expressed. It is obvious that both the genetic regulation and the production profile are very different in *A. niger* and *F. verticillioides*, even though fumonisin was actually produced by *A. niger*. These two fungal species are very distantly related, so it is difficult to explain the occurrence of fumonisins in these two widely different fungal species by vertical gene transfer. There appear to be so many differences in the gene sequence (*16, 17*) and in the regulation of fumonisin production that horizontal gene transfer of the whole gene cluster from a fumonisin-producing isolate of *Fusarium* to *A. niger* is also less likely. One reason for this may be that *Fusarium* species, which are mainly field fungi, rarely co-occur with storage fungi such as *Aspergillus* species. This is also reflected in the expression of fumonisins. In agreement with *Fusarium* being a plant-associated field fungus, it produces fumonisins on media made from plant materials and with high water activities. Also in agreement with *A. niger* being predominantly a storage fungus, growing on media with high sugar or salt contents (*35*), this fungus actually produces fumonisin B_2 on media with large amounts of sugar, glycerol, or salt. Furthermore, *Fusarium* species grow in media with higher pH than those of *A. niger*. A thorough study of the production of the same secondary metabolites by phylogenetically very different fungi could add to our understanding of the phylogeny and raison d'etre of secondary metabolite production.

The ability of at least some isolates of *A. niger* to produce the carcinogenic mycotoxin fumonisin B_2 has many conse-

quences. Strains of *A. niger* are used in biotechnology, and some of these strains may produce the mycotoxin ochratoxin A (*22, 42*) in addition to fumonisin B_2. For production strains of citric acid and other small chemicals, it should be investigated whether the two mycotoxins can end up in the final product. However, it can also be problematic that the fumonisin B_2 or ochratoxin A may be present in the waste from biotechnology companies producing citric acid or even in waste being used as feed for domestic animals. In fungal transformations involving *A. niger* and in extracellular enzyme production, or when the fungus is used as a transformation host, it may likewise be a problem that fumonisin or ochratoxin A may be present in one of the production steps or even the final product.

It is probably a much larger problem that *A. niger* is one of the most common contaminants of food, especially fruits and certain vegetables. *A. niger* is a common fungus growing in grapes, green coffee beans, onions, mango, corn and other cereals, peanuts, dried fruits, and many other products (*35*). The results reported here indicate that substrates with low water activity may be more prone to support fumonisin B_2 production. Further studies with numerous *Aspergillus* isolates of the section *Nigri* are underway to examine the occurrence of fumonisin production in *A. niger* and related taxa as well as determinations of the natural occurrence of fumonisins in *Aspergillus*-infected feed and food.

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