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ARTICLE

Production of Fumonisin B_2 and B_4 by *Aspergillus niger* on Grapes and Raisins

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The recent discovery of fumonisin production in *Aspergillus niger*, raises concerns about the presence of these mycotoxins in grapes and raisins as well as other commodities where *A. niger* is a frequent contaminant. Here we investigate the potential production of fumonisins in *A. niger* cultured on grapes and raisins. Sixty-six *A. niger*, 4 *A. tubingensis*, and 16 *A. acidus* strains isolated from raisins were tested for fumonisin production on laboratory media. Neither *A. tubingensis* nor *A. acidus* strains produced fumonisins, but 77% of *A. niger* strains did. None of the strains produced ochratoxin A. Ten selected fumonisin producing *A. niger* strains were further able to produce fumonisin B_2 and fumonisin B_4 on grapes in the range 171−7841 μg fumonisin B_2/kg and 14−1157 μg fumonisin B_4/kg. Four selected strains were able to produce fumonisin B_2 (5−6476 μg/kg) and fumonisin B_4 (12−672 μg/kg) on raisins.

KEYWORDS: Fumonisins; *Aspergillus niger*; mycotoxins; grapes; raisins.

INTRODUCTION

Black Aspergilli are the most common fungi responsible for postharvest decay of fresh fruit (*1*). Of these *Aspergillus carbonarius* and *A. niger* are very important opportunistic pathogens of grapes causing bunch rot or berryrot and causing raisin mold (*2*). Since *A. carbonarius* is a much better ochratoxin A producer, the main focus concerning grapes and black Aspergilli has been on *A. carbonarius* and ochratoxin A (*3*). However, *A. niger* has also been reported to grow and damage a large number of crops and foods worldwide, including corn, peanuts, raisins, onions, mangoes, apples, and dried meat products (*1*).

A. niger is used for production of single cell protein for feed (*4*) and is extensively used for production of several organic acids and extracellular enzymes. Furthermore it is used as a transformation host in the biotechnological industry (*5*, *6*). The discovery of putative homologues to the *Fusarium verticillioides* fumonisin gene cluster in three *A. niger* genomes led to the discovery of a fumonisin B_2 production by all three full genome sequenced strains and the ex type culture of *A. niger* (*6*−*8*). It has also been shown that *A. niger* is able to produce the biosynthetic precursor fumonisin B_4, which lacks a hydroxyl group on the backbone (*9*). The structure of fumonisins B_1, B_2, and B_4 are shown in **Figure 1**.

Fumonisins are important mycotoxins because they are suspected to cause human and animal toxicoses by the consumption of contaminated corn-based food and feeds (*10*). The fumonisins are structurally similar to sphingolipids and have shown to inhibit the sphingolipid biosynthesis via the ceramide synthase pathway (*11*). Fumonisins have shown to induce outbreaks of leukoencephalomalacia in horses and pulmonary edema and hydrothorax in pigs (*11*, *12*). Fumonisin B_1 is hepatocarcinogenic, hepatotoxic, and nephrotoxic in rats and rat liver cancer can be promoted and initiated by fumonisin B_1 (*11*, *12*). Fumonisin B_1 and B_2 have been declared class 2B carcinogens, which are possible human carcinogens (*13*, *14*). The regulatory limit for fumonisins in corn is, according to the U.S. Food and Drug Administration, 2−4 μg/g total fumonisins (*15*). EEC has a regulatory limit of 0.2−2 μg/g (*16*). Fumonisins have been isolated from corn and corn-based products (*12*) such as tortillas (*17*) and beer (*12*), as well as rice (*12*), black tea leaves (*18*), asparagus (*19*), and pine nuts (*20*). Very few factors that affect the fumonisin production by *A. niger* have been investigated; so far only temperature and water activity and a limited amount of media have been investigated (*8*, *21*). Recently fumonisins B_2 and B_4 from *A. niger* were detected in green coffee beans, although the concentrations (up to 10 μg/kg) were well below the regulatory limit for other commodities (*9*). In a study performed on grape must, 2 out of 12 samples were found to be positive for fumonisin B_2 (0.01 and 0.4 μg/g)(*22*).

In this study, we investigate the presence of ochratoxin A and fumonisin producing black Aspergilli on raisins, as well as fumonisin production in grapes, dried grapes, and raisins.

MATERIALS AND METHODS

Unless otherwise is stated all solvents were HPLC grade, chemicals were analytical grade, and water was purified on a Milli-Q (Millipore, Billerica, MA). Media were prepared in 9 cm Petri dishes, each with 20 mL of medium. The fumonisin standard was a mixed certified standard containing both fumonisin B_1 and fumonisin B_2 with concentrations of 50.2 and 51.0 mg/L (Biopure, Tulln, Austria). The ochratoxin A standard contained 10 mg/L (Biopure). Fungal strains were three-point inoculated and all samples were incubated in microperforated plastic bags at 25 °C for 7 days in darkness. All water activity measurements were

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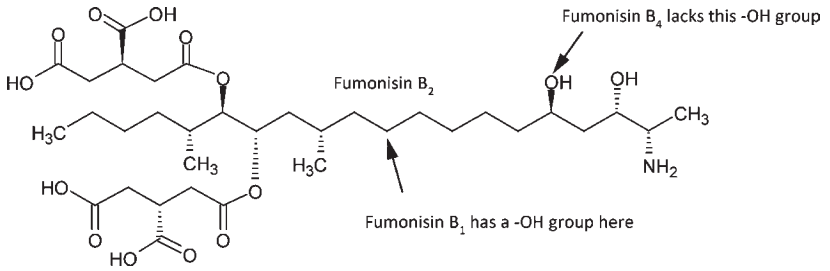

Figure 1. Fumonisin B_2 and the Difference to Fumonisin B_1 and B_4.

performed in triplicate and measured with an Aqualab (ADAB Analytical Devices, Stockholm, Sweden).

Isolation and Characterization of Black Aspergilli Isolated from Raisins. Seventeen different raisin samples were purchased from supermarkets and shops in Denmark, U.S.A., or Uganda. The raisins originated from California, Argentina, or Uganda. Six raisins were selected randomly from each sample, placed on a dichloran 18% glycerol agar (*23*) plate, and incubated at 25 °C for seven days. All black Aspergilli were transferred to Czapek yeast autolysate agar (CYA) and yeast extract sucrose agar (YES) (*24*) plates and afterward identified using a polyphasic approach (macro- and micromorphology and metabolite profile) according to previous reports (*25*−*27*). All strains were subsequently screened for a potential fumonisin production on three different media, CYA, YES, and Czapek yeast autolysate agar with 5% NaCl (CYAS) (*24*). Fumonisins and ochratoxin A were extracted using an agar plug extraction method (*8*) with small modifications. Six agar plugs (D = 6 mm) were transferred to a 2 mL vial and 800 μL of methanol/water (3:1) was added. The samples were extracted ultrasonically for 1 h, filtered through a PFTE 0.45 μm filter (National Scientific, Rockwood, TN), and used directly for LC-TOF-MS analysis. Representative isolates were preserved in the IBT collection (author's address).

Preparation of Grapes. Conidial suspensions for inoculation of grapes were obtained by harvesting spores of each isolate grown on CYA and suspending them in sterile distilled water containing 0.05% of Tween 80 (Merck, Hohenbrunn, Germany) and 0.05% agar (Bie & Berntsen, Rødovre, Denmark). The final concentration of the conidia was assessed by using a counting chamber and was adjusted to 10^6 conidia/mL.

Fresh grapes (Thompson seedless), cut in half, were placed in an empty Petri dish and inoculated with 10 μL of the *A. niger* conidia suspensions. The grape experiment was prepared in duplicate. Ten strains randomly selected from the before mentioned screening were selected for the grape experiment, IBT 28747, 28753, 28934, 28937, 28948, 28964, 28965, 28966, 28994, and 29019.

Preparation of Commercial Raisins. From one brand, two subsamples of 125 g raisins (California, Thompson seedless) were randomly selected and surface sterilized with hypochlorite (3%) for 15 min and then washed twice with sterile water (weight gain 7%). One of the two subsamples was covered with water and both subsamples were kept at 5 °C for 24 h (weight gain 50%). The raisins placed in water were transferred to an empty Petri dish; the other subsample was placed on a water agar separated by a filter (pore size 1 μm). Four *A. niger* strains with the highest production of fumonisins in grapes were chosen; these were IBT 28753, 28934, 28948, and 29019. The raisins were inoculated with 10 μL of the *A. niger* conidia suspensions in quadruplicates as described above.

Preparation of Dried Grapes. Portions of 300 g grapes (size, 2−3 g) (Thompson seedless) were taken at random and dried in an oven at 75 °C for 4 h (weight loss, 32%, initial a_w 0.76 ± 0.04), 5 h (weight loss, 38%, initial a_w 0.69 ± 0.01), and 6.5 h (weight loss, 52%, initial a_w 0.54 ± 0.09). Subsequently, they were placed in an empty Petri dish and inoculated with *A. niger* as described above. All samples were as a minimum prepared in triplicates. Four *A. niger* strains with the highest production of fumonisins in grapes were chosen; these were IBT 28753, 28934, 28948, and 29019.

Extraction of Grapes and Raisins for Chemical Analysis. One grape or raisin was weighed and placed in a 5 mL cryo tube with 5−10 steel balls (D = 3 mm), 5 mL methanol/water (3:1) was added. The cryo tubes were shaken in a Mini Beadbeater 96 (Biospec Product Inc., Bartlesville, OK) for 5 min. The whole mixture was transferred to a 15 mL Falcon tube. Afterward it was placed on a shaking table (at an angle of 45°) for 60 min at 150 rpm. The Falcon tubes were centrifuged at 8000 g for 4 min and 200 μL extract was transferred to a 2 mL HPLC vial and used directly for LC-MS/MS analysis. Because of nonavailability of fumonisin B_4 as analytical standard, external quantification was performed with fumonisin B_2 and similar response factors were assumed for the two mycotoxins.

LC-TOF-MS Conditions. The screening for a fumonisin in culture extracts production was performed using a LC system coupled to an orthogonal TOF mass spectrometer (Micromass LCT, Manchester, U.K.) equipped with an electrospray source (*25*). The column used was a 50 × 2 mm i.d., 3 μm Gemini C_6-phenyl column (Phenomenex, Torrance, CA) with a linear gradient starting from 30% acetonitrile in water (both 20 mM formic acid) to 60% acetonitrile for 5 min at a flow rate of 300 μL/min, which was then increased to 100% acetonitrile in 1 min and a flow of 0.5 mL/min, keeping this for 3.5 min before returning to the start conditions in 6 min. The mass spectrometry was performed in ESI^+.

LC-MS/MS Conditions. LC-MS/MS analysis was performed as previously described (*9*) with an Agilent HP 1100 liquid chromatography system (Waldbronn, Germany) coupled to a Quattro Ultima triple mass spectrometer (Micromass, Manchester, UK) with ESI source. The gradient was as described before but with the gradient starting at 20% CH_3CN and going to 55% CH_3CN in 5 min and then to 100% in 30 s. Tandem mass spectrometry was performed in ESI^+ at a source flow at 700 L/h nitrogen at 350 °C. Nitrogen was also used as collision gas, and the MS operated in multiple reaction monitoring mode at the following transitions: fumonisin B_2 quantifier m/z 706 → 336 cone 50 V, collision 40 V, dwell time 50 ms, qualifier m/z 706 → 512, cone 50 V, collision 25 V, dwell time 100 ms (ion ratio, 1.7−2.1 (transition 1/2)); fumonisin B_4 quantifier m/z 690 → 320 cone 50 V, collision 35 V, dwell time 50 ms, qualifier m/z 690 → 514 a, cone 50 V, collision 30 V, dwell time 100 ms (ion ratio, 1.4−1.8 (transition 1/2)); ochratoxin A quantifier m/z 404→ 239, cone 30 V, collision 33 V, dwell time 100 ms, qualifier m/z 404 → 358, cone 30 V, collision 25 V, dwell time 100 ms (ion ratio, 1.4−1.7 (transition 1/2)).

Validation. The method was validated by spiking raisins and grapes with 100 μL of a mixture of fumonisin B_2 and ochratoxin A standards to the following six different final levels: fumonisin B_2, 15, 25, 50, 150, 200, and 300 μg/kg; ochratoxin A, 3, 5, 10, 30, 40, and 60 μg/kg in triplicate. The samples were left to dry for 2 h prior to extraction. Both the raisins and the grapes were extracted as described above.

RESULTS AND DISCUSSION

Preliminary testing (results not shown) of strong anion exchange purification (SAX) showed low or no recoveries of fumonisins from the grapes, presumably due to competition from high amounts organic acids from the grapes and/or from *A. niger* strains themselves. Furthermore immunoaffinity purification (results not shown) yielded viscous brown extracts from raisin extracts and was thus not suitable either. Consequently, it was decided to analyze the crude extracts.

For validation, standard curves were prepared and shown to be linear. On grapes, the relative standard deviation of the lowest point (15 μg/kg) was 18% (fumonisin B_2 level); for raisins it was 7%. All R^2, level of detection (LOD), and level of quantification (LOQ) values are shown in **Table 1**. Because of the high fumonisin concentrations detected in the strains, LOD_{fum} were not

experimentally addressed, but estimation from the lowest point down to s/n indicates LOD of 5 μg/kg. Control analysis of conidial suspensions shows that in approximately 10 000 spores the concentration of fumonisin was in picogram levels and would be diluted 1:500 in the final extract thus not influencing the result.

The screening resulted in isolation of 86 black Aspergilli strains; of these 66 were *A. niger*, 4 *A. tubingensis*, and 16 *A. acidus* (= *A. foeditus* var. *acidus* (*28*)). None of the *A. tubingensis* and *A. acidus* strains produced fumonisins, but 77% of the *A. niger* produced fumonisin B_2 (LC-TOF). Similar results were found among a limited number of grape derived strains (*22*). A similar recent study performed on green coffee beans found the a similar fraction (76%) of *A. niger* as fumonisin B_2 producers (*9*). The percentage of ochratoxin A -producing *A. niger* is most often reported in the interval 0−30% (*3*, *29*−*31*), but in one case a percentage as high as 41% has been reported (*32*). The percentages of ochratoxigenic strains of *A. niger* are very low compared to our 77% fumonisin producing *A. niger*. Therefore this fumonisin production by *A. niger* could present an even larger food safety problem.

Table 1. R^2, Level of Determination (LOD), and Level of Quantification (LOQ) Determined from Spiked Raisin and Grape Samples

	fumonisin B_2			ochratoxin A		
matrix	R^2 (n = 18)	LOD[a]	LOQ	R^2 (n = 18)	LOD[b]	LOQ
grape	0.993	5 μg/kg	25 μg/kg	0.96	3 μg/kg	10 μg/kg
raisin	0.990	3 μg/kg	25 μg/kg			

[a] Estimated from the lowest point (15 μg/kg) to s/n (1:5). [b] The s/n is 1:5.

Table 2. Production of Fumonisins by *Aspergillus niger* in Grapes after 7 Days Growth at 25°C

isolate	fumonisin B_2 (μg/kg)		fumonisin B_4 (μg/kg)[a]		
IBT	sample 1	sample 2	sample 1	sample 2	ochratoxin A μg/kg[b]
28747	951	1283	75	189	nd
28753	7703	7979	772	1542	nd
28934	2098	5376	158	612	nd
28937	1045	1941	253	265	nd
28948	3380	4772	349	509	nd
28964	91	251	5	23	nd
28965	1074	2414	148	344	nd
28966	1261	5123	220	634	nd
28994	432	2422	36	158	nd
29019	4303	11189	143	405	nd

[a] Assuming same response factor as fumonisin B_2. LOD: 5 μg/kg. [b] LOD: 3 μg/kg. nd: not detected.

The 10 *A. niger* strains with the highest measured fumonisin production were selected for further experiments, IBT 28747, 28753, 28934, 28937, 28948, 28964, 28965, 28966, 28994, and 29019. All 10 strains were able to produce fumonisins in grapes (**Table 2**). Fumonisin B_2 was produced as the major component, followed by fumonisin B_4. The mean production of fumonisin B_2 varied almost 50 fold from 171 μg/kg (IBT 28964) to 7841 μg/kg (IBT 28753) and fumonisin B_4 varied almost 70 fold from 14 μg/kg (IBT 28964) to 1157 μg/kg (IBT 28753). Selected transitions from the LC-MS/MS analysis of infected and spiked grapes are shown in **Figure 2**. The measured fumonisin concentrations are similar, compared to those previously described by Logrieco et al. (200−2500 μg/kg) (*22*).

The four strains with the highest production of fumonisins on grapes were selected for the work with dried grapes and raisins. On the dried grapes, fumonisin B_2 and fumonisin B_4 were also produced (**Table 3**). The concentration of fumonisin B_2 was in the range of 91−1747 μg/kg and fumonisin B_4 9−69 μg/kg. The fumonisin production was highest in the grapes with the lowest weight losses and the lowest production was in all coincidences found in the grapes with the lowest water content.

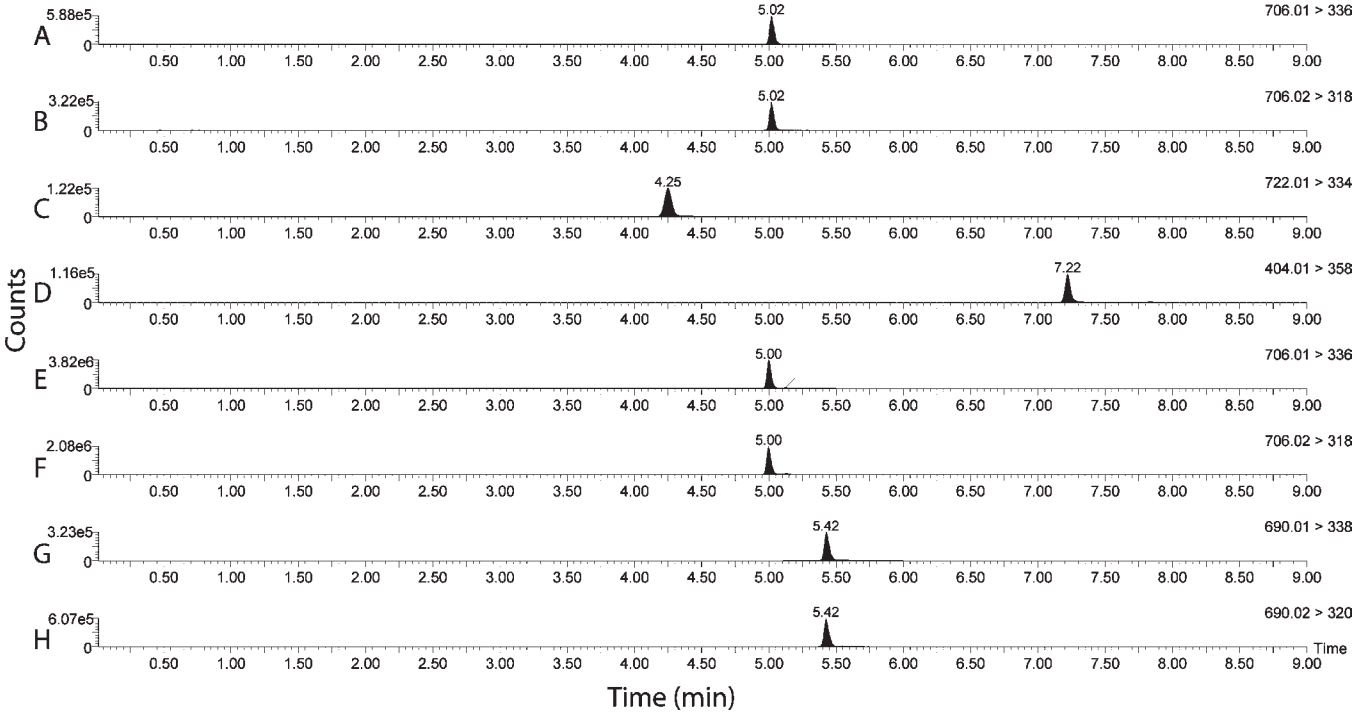

Figure 2. Results from the LC-MS/MS analysis. Transitions of Ochratoxin A. Fumonisin B_1. B_2 and B_4 in Spiked Grape and Infected Grape. (**A**) Quantifier of Fumonisin B_2 from a Spiked Grape (150 μg/kg). (**B**) Qualifier of Fumonisin B_2 from a Spiked Grape (150 μg/kg). (**C**) Quantifier of Fumonisin B_1 from a Spiked Grape (150 μg/kg). (**D**) Quantifier of Ochratoxin A from a Spiked Grape (30 μg/kg). (**E**) Quantifier of Fumonisin B_2 from an Infected Grape (7841 μg/kg IBT 28753). (**F**) Qualifier of Fumonisin B_2 from an Infected Grape (7841 μg/kg IBT 28753). (**G**) Quantifier of Fumonisin B_4 from an Infected Grape (1157 μg/kg IBT 28753). (**H**) Qualifier of Fumonisin B_4 from an Infected Grape (1157 μg/kg IBT 28753).

Table 3. Production of Fumonisins by *Aspergillus niger* in Dried Grapes after 7 Days Growth at 25°C

isolate IBT	weight loss %	initial a_w	fumonisin B_2 μg/kg[a]	fumonisin B_4 μg/kg[a,b]	ochratoxin A μg/kg[c]
28753	32	0.76 ± 0.04	1747 ± 316	48 ± 22	nd
	38	0.69 ± 0.01	1128 ± 329	48 ± 16	nd
	52	0.54 ± 0.09	91 ± 73	9 ± 4	nd
28934	32	0.76 ± 0.04	1363 ± 954	69 ± 51	nd
	38	0.69 ± 0.01	1033 ± 460	50 ± 23	nd
	52	0.54 ± 0.09	636 ± 397	24 ± 16	nd
28948	32	0.76 ± 0.04	313 ± 323	54 ± 47	nd
	38	0.69 ± 0.01	154 ± 71	27 ± 11	nd
	52	0.54 ± 0.09	144 ± 104	29 ± 19	nd
29019	32	0.76 ± 0.04	203 ± 106	25 ± 11	nd
	38	0.69 ± 0.01	214 ± 137	28 ± 13	nd
	52	0.54 ± 0.09	102 ± 76	10 ± 5	nd

[a] The values are means of the quadruplicates, plus/minus the standard deviation. [b] Assuming same response factor as Fumonisin B_2. LOD: 5 μg/kg. [c] LOD: 3 μg/kg. nd: not detected.

Table 4. Production of Fumonisins by *Aspergillus niger* in Raisins after 7 Days Growth at 25°C

isolate IBT	weight gain	initial a_w	fumonisin B_2 μg/kg[a]	fumonisin B_4 μg/kg[a,b]	ochratoxin A μg/kg[c]
28753	7% (agar)	0.77 ± 0.05	1160 ± 686	105 ± 56	nd
	50% (water)	0.95 ± 0.001	407 ± 249	47 ± 25	nd
28934	7% (agar)	0.77 ± 0.05	229 ± 58	27 ± 7	nd
	50% (water)	0.95 ± 0.001	5 ± 5	nd	nd
28948	7% (agar)	0.77 ± 0.05	459 ± 430	45 ± 31	nd
	50% (water)	0.95 ± 0.001	112 ± 51	12 ± 4	nd
29019	7% (agar)	0.77 ± 0.05	6476 ± 1139	356 ± 46	nd
	50% (water)	0.95 ± 0.001	784 ± 636	672 ± 422	nd

[a] The values are means of the quadruplicates, plus/minus the standard deviation. [b] Assuming same response factor as Fumonisin B2. LOD: 5 μg/kg [c] LOD: 3 μg/kg. nd: not detected.

Two experiments were carried out with the raisins, one where the water activity (a_w) initially was low and over time increased toward 1, and another where the water activity started high and decreased over time. All of the tested *A. niger* strains were found to produce fumonisin B_2 and fumonisin B_4 on both types of raisins (**Table 4**). In all cases the production of fumonisins was highest when the raisins were kept moist. Fumonisin B_2 and fumonisin B_4 were produced in the range of 229−6476 and 27−356 μg/kg. The raisins with a decreasing water activity had a fumonisin B_2 concentration of 5−784 μg/kg and fumonisin B_4 of 12−672 μg/kg.

In the dried grapes the production of fumonisins decreased as the water activity was lowered. This correlates with earlier results that the lower a_w, the more reduced fumonisin production by *A. niger* (*21*). The reason why the raisins with the lowest a_w supported fumonisin production could be because the wet grapes dried quickly and the raisins on the water agar were kept moist with a high a_w on the surface. For the four tested strains, in the three experiments all had the highest production of fumonisins in grapes.

This is the first report describing the production of fumonisins B_2 and B_4 by *A. niger* in dried grapes and raisins. All the *A. niger* produced more fumonisin B_2 than fumonisin B_4. No production of ochratoxin A (LOD: 3 μg/kg) was detected in either the grape or raisin experiments. This is supported by earlier studies that have shown *A. carbonarius* and not *A. niger* as the most important source of ochratoxin A in raisins (*29*, *33*, *34*).

The presence of a fumonisin production in grapes and a detectable content of fumonisin in grape must (*22*) indicates that fumonisins may be found in wine unless degraded during the fermentation or storage. Consequently, a larger number of wines as well as grape musts need to be investigated to track the fate of fumonisins in all production steps from grapes to wine.

Fumonisin produced by *A. niger* may be an overlooked health risk in a broad variety of foods, because *A. niger* is a common food spoilage organism (*1*). New knowledge within this field is important, particularly because corn and derived products are currently the only major products that are monitored for fumonisins.

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