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Analysis of *Fusarium avenaceum* Metabolites Produced during Wet Apple Core Rot

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Wet apple core rot (wACR) is a well-known disease of susceptible apple cultivars such as Gloster, Jona Gold, and Fuji. Investigations in apple orchards in Slovenia identified *Fusarium avenaceum*, a known producer of several mycotoxins, as the predominant causal agent of this disease. A LC-MS/MS method was developed for the simultaneous detection of thirteen *F. avenaceum* metabolites including moniliformin, acuminatopyrone, chrysogine, chlamydosporol, antibiotic Y, 2-amino-14,16-dimethyloctadecan-3-ol (2-AOD-3-ol), aurofusarin, and enniatins A, A1, B, B1, B2, and B3 from artificially and naturally infected apples. Levels of moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1 were quantitatively examined in artificially inoculated and naturally infected apples, whereas the remaining metabolites were qualitatively detected. Metabolite production was examined in artificially inoculated apples after 3, 7, 14, and 21 days of incubation. Most metabolites were detected after 3 or 7 days and reached significantly high levels within 14 or 21 days. The highest levels of moniliformin, antibiotic Y, aurofusarin, and the combined sum of enniatins A, A1, B, and B1 were 7.3, 5.7, 152, and 12.7 μg g^{-1}, respectively. Seventeen of twenty naturally infected apples with wACR symptoms contained one or more of the metabolites. Fourteen of these apples contained moniliformin, antibiotic Y, aurofusarin, and enniatins in levels up to 2.9, 51, 167, and 3.9 μg g^{-1}, respectively. Acuminatopyrone, chrysogine, chlamydosporol, and 2-AOD-3-ol were detected in 4, 11, 4, and 10 apples, respectively. During wet apple core rot, *F. avenaceum* produced high amounts of mycotoxins, which may pose a risk for consumers of apple or processed apple products.

KEYWORDS: Fusarium; mycotoxins; LC-MS-MS

INTRODUCTION

Fusarium avenaceum is one of the most commonly encountered *Fusarium* species in cereals and maize in northern Europe (*1*−*3*). During investigations in 2004−2006 in apple orchards in Slovenia, *F. avenaceum* was also identified as the causal agent of a wet apple core rot (wACR) (*4*). The disease is characterized by a white, rose, or reddish mycelium developing initially in the apple core and a light-brown wet rot spreading destructively into the surrounding cortex of infected apples (**Figure 1**). When harvesting the full yield of 21 Gloster apple trees in fall 2004, ca. 5% of the apples showed symptoms of wACR, and wACRs were also regularly encountered in other cultivars, such as Fuji and Jona Gold in 2004−2006 (*4*). The disease represents a potential economical problem for apple growers, who have to discard infected apples, and a safety problem for consumers due to potential production of mycotoxins during infections, especially for apple juice and cider production, since apples with infected cores can be removed from processes only with difficulties. *Fusarium avenaceum* is able to produce a wide range of chemically different bioactive secondary metabolites on artificial laboratory media (*5*), ranging from small polar compounds such as moniliformin and butenolide to larger apolar compounds such as enniatins (**Figure 2**). The toxicity of several *F. avenaceum* metabolites has been thoroughly investigated. The toxic mode of action of moniliformin is suggested to be linked with inhibition of enzyme systems and glucogenesis (*6*), and it can, like butenolide, induce myocardial damage (*7, 8*). Chlamydosporol and acuminatopyrone are related compounds of which the former has been shown to cause weight loss in rats and cytotoxicity in human and mouse cell lines (*9*). 2-Amino-14,16-dimethyloctadecan-3-ol (2-AOD-3-ol) is a sphingosine analogue recently isolated from *F. avenaceum* (*10*). The compound is cytotoxic because it disrupts sphingolipid biosynthesis according to hypothesized models (*10*). Chrysogine, whose toxicity is undetermined, is produced by various phylogenetically unrelated ascomycetous genera including *Alternaria*, *Aspergillus*, *Fusarium*, and *Penicillium* (*11*−*14*). Fusarin A and C are metabolites produced by several *Fusarium* species (*15, 16*) and show mutagenic properties (*17*). Antibiotic Y was originally

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Figure 1. Early (top) and late (bottom) symptoms of wet apple core rot.

isolated from *F. lateritium* (*18*) and called lateropyrone. The structure was later amended and named antibiotic Y (*19*). This compound is produced by several *Fusarium* species, including *F. avenaceum*, *F. lateritium*, *F. torulosum*, *F. acuminatum*, *F. flocciferum*, and *F. tricinctum* (*18*, *20*−*22*), and it has, as the name suggests, antibiotic properties (*18*). Aurofusarin is a red pigment belonging to the naphthoquinone group and is produced by several *Fusarium* species (*21*, *22*). Aurofusarin has been shown to have a negative effect on the antioxidant system of the quail egg yolk and causes alterations in fatty acid composition of the egg yolk (*23*). Enniatins are cyclic hexadepsipeptides consisting of three alternating *N*-methyl-L-amino acid and D-α-hydroxyisovaleric acid residues, which are able to form cation selective channels in cellular membranes (*24*). They are cytotoxic (*5*) and toxic to insects (*25*), bacteria (*24*), and fungi (*26*). *Fusarium avenaceum* is able to produce at least six enniatins, which often occur in the following successively decreasing amounts in the sequence B > B1 > A1 > A > B2 > B3 on laboratory media (*5*). The enniatins occur in similar proportional amounts in Scandinavian cereals and maize, where *F. avenaceum* is one of the most abundant species (*27*−*29*). The bioactivity and natural occurrence have only been examined for a few metabolites, but fractionated extracts indicated in cytotoxicity tests that 2-AOD-3-ol and enniatins were the most potent cytotoxic compounds produced by *F. avenaceum (5)*.

Analysis of naturally occurring *F. avenaceum* metabolites has mainly focused on moniliformin and enniatins A, A1, B, and B1 in cereal and maize based food and feeds. These compounds, together with antibiotic Y, were recently included in a LC-MS/MS screening method for 87 metabolites (*30*). In the present study, we developed a method for the simultaneous detection of thirteen *F. avenaceum* metabolites from apples collected in the field and showing a wet core rot and artificially induced apple core rots in healthy apples infected with spores of *F. avenaceum*.

MATERIALS AND METHODS

Chemicals and Standards. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Solvents were gradient grade, and other chemicals were analytical grade. Water was purified with a Milli-Q system (Millipore, Bedford, MA). Moniliformin was purchased as the sodium salt from Sigma-Aldrich, and a 73 μg/mL stock solution was made in 85% MeCN (*3*). An enniatin reference standard was kindly provided by Dr. Rainer Zocher, Technical University of Berlin, Germany, consisting of 17% enniatin A, 34% enniatin A1, 24% enniatin B, and 26% enniatin B1 (distribution determined by HPLC-UV at 200 nm and validated by LC-HRMS) (*28*). Of this solution, a 400 μg/mL stock solution was made in 100% MeCN and stored at −20 °C.

Antibiotic Y and chrysogine were available from previous studies (*31*), and a 250 μg/mL stock solution was made of the first in 100% MeCN and stored at −20 °C. 2-Amino-14,16-dimethyloctadecan-3-ol was a kind gift from Dr. Silvio Uhlig, National Veterinary Institute, Norway. A 250 μg/mL (100% MeCN) stock solution of aurofusarin was prepared from *F. graminearum* as described below and stored at −20 °C.

Aurofusarin Standard. Aurofusarin was extracted from *F. graminearum* (IBT 41393, available at the author's address) grown for 14 days at 25 °C in the dark on 9 cm (ID) millet agar plates (30 g of organic millet grains, autoclaved in 1000 mL of distilled H_2O, 15 g of agar, and 1 mL of trace metal solution consisting of 10 g/L $ZnSO_4 \cdot 7H_2O$ and 5 g/L $CuSO_4 \cdot 5H_2O$). The aerial mycelium of 400 plates were scraped off and extracted multiple times with ethyl acetate and finally acidic ethyl acetate while mixing using an Ultra Turrax T25 basic (IKA Werke GmbH & Co. KG, Staufen, Germany). The pooled extracts were evaporated to dryness *in vacuo*, dissolved in 50 mL of methanol, and filtered though a Whatman no. 4 filter (Brentford, U.K.). Primary separation was done on a Sephadex LH20 column (1000 mm × 50 mm, flow rate: 5 mL/min), eluting with methanol and collecting 15 mL fractions. The fractions were combined to 10 portions based on color. The fifth portion contained both aurofusarin and zearalenone and was subjected to multiple LC runs on a Gemini C_6-Phenyl (150 mm × 10 mm i.d., 5 μm) column (Phenomenex, Torrance, CA) using a H_2O−MeCN gradient system (20 mL/min) starting at 15% MeCN and increasing to 100% over 20 min, with aurofusarin eluting at 9.7 min. Analysis of a ^1H NMR spectrum in CD_3OD [Bruker 500 MHz] showed four singlets signals (methyl, methoxy, vinyl, and aromatic) matching data in ref *32*, and integrals showed a purity of >95%, which was also confirmed by LC-DAD-HRMS (*31*).

Fungal Strains, Media, and Growth Conditions. Six *F. avenaceum* strains isolated from maize and five strains from apples (**Table 1**) were grown on potato dextrose agar (PDA) (*21*), yeast extract agar (YES) (*21*), and apple agar (AA) to determine the metabolite profile of *F. avenaceum* on artificial growth media. AA with a pH value of 3.3 was made by autoclaving 300 g of homogenized Gloster apples (obtained from a local Danish supermarket) and distilled H_2O, 1 mL of a trace metal solution (10 g/L $ZnSO_4 \cdot 7H_2O$ and 5 g/L $CuSO_4 \cdot 5H_2O$ in a 1000 mL volume), and 30 g of agar. All media were cooled to 45 °C and poured into sterile 90 mm Petri dishes. The 11 *F. avenaceum* strains were transferred aseptically to the agar plates in three point inoculations and incubated 14 days at 25 °C in the dark.

Extraction of Metabolites from *F. avenaceum* Grown on PDA, YES, and AA. A modified version of the previously described microscale extraction method (*33*) was used. In brief, 9 agar plugs were taken from the colony center of cultures 14 days old and extracted ultrasonically for 45 min with 1.5 mL of ethyl acetate (0.5% formic acid) and subsequently with 1.5 mL of isopropanol. The solvent was removed *in vacuo*, redissolved in 400 μL of methanol, filtered through a 0.45 μm PTFE syringe filter (National Scientific, Rockwood, TN), and analyzed.

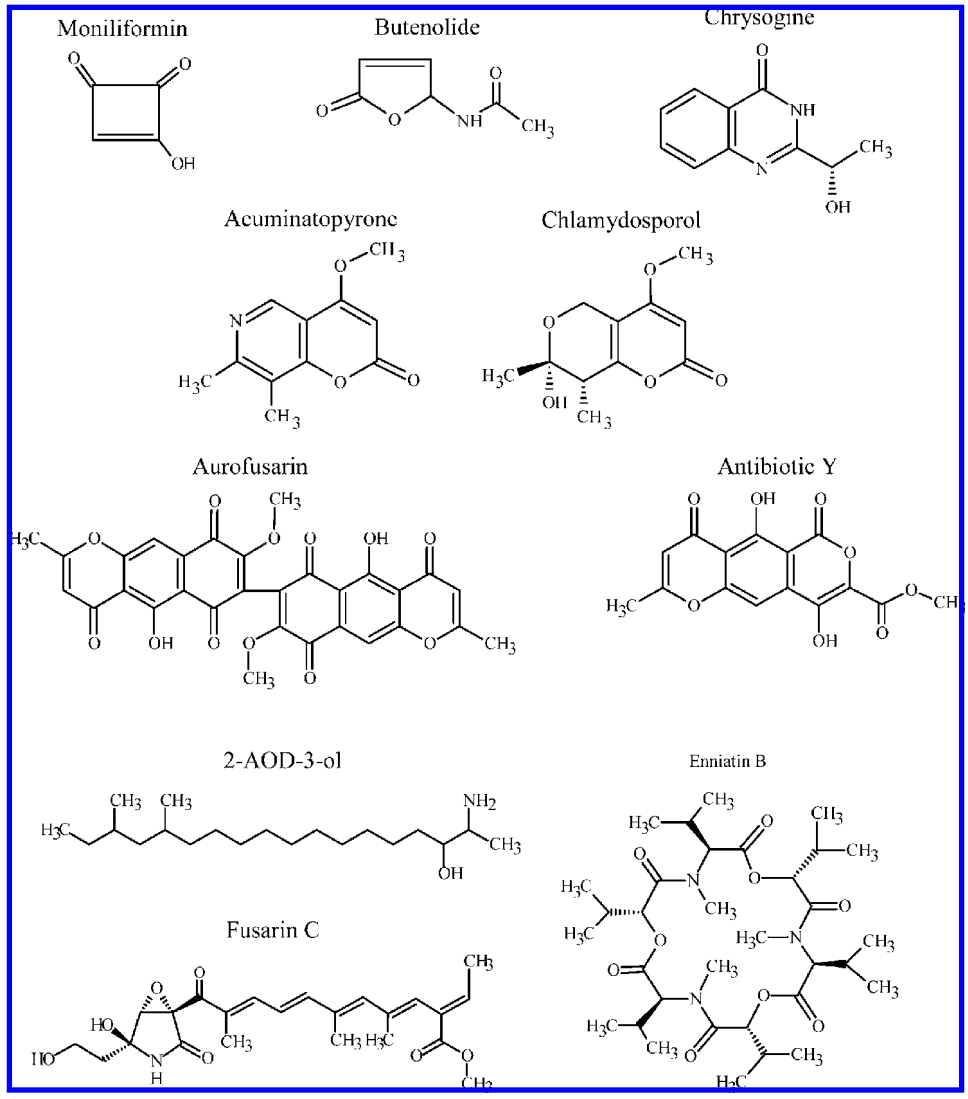

Figure 2. Metabolites potentially produced by *F. avenaceum*.

Table 1. *Fusarium avenaceum* Strains from Denmark (DK) and Slovenia (SI) Used in Experiments

strains[a]	origin	IBT collection[b]
JLS F1[c]	maize, DK	IBT 41177
JLS F2[c]	maize, DK	
JLS F3[c]	maize, DK	
JLS F4[c]	maize, DK	
JLS F29[c]	maize, DK	
JLS F35[c]	maize, DK	IBT 41180
HJS 346[c]	apple, SI	IBT 41123
HJS 846[c]	apple, SI	IBT 41117
HJS 848[c,d]	apple, SI	IBT 41118
HJS 849[c]	apple, SI	IBT 41122
HJS 851[c,d]	apple, SI	IBT 41119
HJS 288[d]	apple, SI	IBT 41125
HJS 911[d]	apple, SI	
HJS 912[d]	apple, SI	

[a] Number in personal collections of J. L. Sørensen (J.L.S.) and H.-J. Schroers (H.J.S.). [b] Number in IBT collection, Technical University of Denmark, Department of Systems Biology. [c] Strains used to determine metabolites from artificial culture media. [d] Strains used to artificially inoculate apples.

Spiked Apples. Material from a *Fusarium* free Golden Delicious apple, which did not contain any *Fusarium avenaceum* metabolites, was used to spike with moniliformin, antibiotic Y, aurofusarin, and enniatins. Freeze-dried apple material, equivalent to 30 g of fresh apple, was extracted ultrasonically for 60 min with 150 mL of MeCN−H_2O (85:15). One milliliter subextracts were spiked with moniliformin, antibiotic Y, aurofusarin, and enniatins to obtain the levels shown in **Table 2**.

Table 2. Spike Levels of Moniliformin, Antibiotic Y, Aurofusarin, and Enniatins (ng g^{-1})[a]

moniliformin	0	50	100	200	400	800	1600	3200	6400	12800
antibiotic Y	0	50	100	200	400	800	1600	3200	6400	12800
aurofusarin	0	50	100	200	400	800	1600	3200	6400	12800
enniatin A	0	17	34	67	134	268	537	1073	2146	4292
enniatin A1	0	34	67	134	269	537	1075	2149	4298	8596
enniatin B	0	24	48	95	190	380	760	1520	3040	6080
enniatin B1	0	26	52	104	207	414	829	1658	3316	6632

[a] Samples were spiked in triplicate.

Artificial Inoculation of Apples. Healthy apple fruits of cultivar Golden Delicious were obtained from a local supermarket in Slovenia. The calyx region and the upper parts of the apples were wetted with ethanol (96%) and flambéed for several seconds. The apples were then artificially infected with 100 μL of a conidial suspension (ca. 10^6/mL) of selected *F. avenaceum* strains (**Table 1**). The conidial suspensions were prepared from 21 day old SNA/C cultures (Spezieller Nährstoffarmer Agar (*34*) amended with 50 g/L of finely sliced carrot pieces and filtered through three layered cheesecloth). The conidial suspension was injected with a sterile syringe and injection needle (0.8 mm × 38 mm), which was moved through the calyx region into the apple cores. Apples were incubated for 3, 7, 14, and 21 days at 22−24 °C and

Table 3. Parameters Used in the Mass Spectrometries Including Ionization Mode (mode), Retention Time (RT/min), Scan Range (range/min), Transition Ions (ion/*m*/*z*), Cone (v), and Collision Energy (CE/V)

compd	mode	RT	range	ion[a]	ion[b]	ratio[c]	cone	CE
moniliformin	−	3.0	1.0−4.5	Qt	97.0 → 41.2		25	15
acuminatopyrone	+	5.3	3.5−6.5	Qt	206.1 → 106.2	1.5	40	35
				Ql	206.1 → 174.0			25
chrysogine	+	6.8	4.5−8.0	Qt	191.1 → 130.1	1.6	20	30
				Ql	191.1 → 155.1			30
chlamydosporol	+	7.5	6.0−9.0	Qt	227.1 → 167.1	17.8	30	35
				Ql	227.1 → 107.2			20
antibiotic Y	+	11.3	10.0−12.5	Qt	319.0 → 286.9	491	20	15
				Ql	319.0 → 175.1			15
2-AOD-3-ol	+	11.8	10.5−13.2	Qt	314.3 → 296.3	131	20	15
				Ql	314.3 → 111.3			15
aurofusarin	+	13.3	12.0−14.5	Qt	571.1 → 484.8	1.2	60	60
				Ql	571.1 → 456.8			50
enniatin B3	+	15.0	14.0−15.8	Qt	612.4 → 196.1	1.8	100	25
				Ql	612.4 → 214.1			25
enniatin B2	+	15.3	14.5−16.5	Qt	626.4 → 196.1	1.7	100	25
				Ql	626.4 → 214.1			25
enniatin B	+	15.8	15.0−17.0	Qt	640.4 → 196.1	15.8	100	25
				Ql	640.4 → 527.3			25
enniatin B1	+	16.2	15.4−17.4	Qt	654.4 → 196.1	2.6	100	25
				Ql	654.4 → 228.0			25
enniatin A1	+	16.8	15.8−17.8	Qt	668.4 → 210.2	4.0	100	25
				Ql	668.4 → 541.2			25
enniatin A	+	17.2	16.2−18.2	Qt	682.4 → 210.1	4.6	100	25
				Ql	682.4 → 555.0			25

[a] Qt: quantifier, Ql: qualifier. [b] All transitions were made from $[M + H]^+$ except for moniliformin, which was made from $[M - H]^-$. [c] Ratio of quantifier and qualifier ions was calculated from spiked samples and media extracts.

subsequently cut open longitudinally. Rotten parts of the core and cortex were harvested with a spoon, weighed, ground with a mortar and pestle in liquid nitrogen, and freeze-dried.

Naturally Infected Apples. Twenty naturally infected Gloster apples showing wet apple core rot symptoms were obtained from trees in the experimental orchard of the Agricultural Institute of Slovenia, Brdo pri Lukovici, near harvest time. Each apple was processed for metabolite detection individually as described above.

Extraction of Metabolites from Apples. Freeze-dried apple material, equivalent to 2 g of fresh weight, was extracted ultrasonically for 60 min with 10 mL of MeCN−H_2O (85:15). Subsamples (1 mL) were filtered through 0.45 μm PTFE syringe filters and analyzed.

LC-MS/MS. Liquid chromatography was performed on an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany). Eight microliters of extract was injected and separated on a Gemini C_6-Phenyl 3 μm 2-mm i.d. × 50-mm column (Phenomenex, Torrance, CA) using a constant flow of a 0.3 mL/min MeCN−water gradient, starting at 0% MeCN and going to 100% over 21 min, followed by a 2 min wash with 100% MeCN at 0.5 mL/min before reverting to 0% MeCN over 1 min and maintaining for a further for 5 min. The water and MeCN were buffered with 20 mM formic acid. The LC was coupled to a Quattro Ultima triple mass spectrometer (Waters-Micromass, Manchester, U.K.) with a Z-spray ESI source using a flow of 700 L/h nitrogen at 350 °C; hexapole 1 was held at 12 V. The system was controlled by MassLynx 4.1 (Waters-Micromass). Nitrogen was also used as collision gas, and the MS was operated in the multiple reaction monitoring (MRM) mode (dwell time 200 ms) with the parameters shown in **Table 3**. Chromatography and MS/MS were optimized on pure standards of moniliformin, chrysogine, antibiotic Y, 2-AOD-3-ol, aurofusarin, and enniatins A, A1, B, and B1. The settings for acuminatopyrone, chlamydosporol, and enniatins B2 and B3 were optimized from fungal extracts because standards were not available. The identities of these compounds in the fungal extracts were validated by LC-DAD-HRMS (*31*). All metabolites were analyzed in positive ionization mode with $[M + H]^+$ as the parent ion, except moniliformin, which was analyzed in negative ionization mode with $[M - H]^-$ as the parent ion. Collision energy was optimized for the two specific (no loss of H_2O, NH_3, CO_2) major fragment ions for each compound (**Table 3**). The limits of quantification (LOQ) for moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1 were set as the minimum spike level (**Table 2**), which for quantifier ions had an average signal-to-noise (S/N) ratio of 13, 142, 8, 62, 80, 94, and 50, respectively. The limits of detection (LOD) were estimated to be linear down to S/N ratios of 5, resulting in LOD for moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1 of 19, 2, 31, 1, 2, 1, and 3 ng g^{-1}, respectively. The LOQ for acuminatopyrone, chrysogine, chlamydosporol, and 2-AOD-3-ol was set to 0.1% of the maximum level detected.

RESULTS AND DISCUSSION

Method Development. Liquid chromatography was done with a Gemini C_6 phenyl column with a mobile solvent gradient starting at 100% H_2O in order to achieve satisfactory retention of moniliformin (RT: 3.0 min, $k' = 3.6$).

As a combined effect of the ability of the column to retain aromatic compounds and the low start concentration of organic solvent, chrysogine was also very well retained. The first target metabolite, moniliformin, eluted after 3.0 min, and the last, enniatin A, eluted after 17 min. In this window all other eleven metabolites eluted with little or no overlap (**Figure 3**). The good retention of moniliformin is also seen on a Gemini C_{18} column (*30*), indicating that the retention is probably caused by retention of the Gemini particles rather than the RP phase. When a Luna phenyl C_6 column (Phenomenex) with identical dimensions to the Gemini phenyl C_6 column was used with the same solvent gradient, moniliformin eluted after 1.7 min, further supporting that the good retention of moniliformin is due to the Gemini particles.

Butenolide could not be properly retained (RT: <1 min, $k' = 0.2$) and was therefore not included in the method, due to the risk of coelution with matrix components. Fusarins A and C were also not included in the method because they showed poor chromatography, resulting in multiple smaller peaks over 1−2 min.

Metabolite Production on Artificial Media. Eleven *F. avenaceum* strains were grown on PDA, YES, and AA to analyze the metabolite potential in a small scale experiment with the developed LC-MS/MS method (**Table 4**). All strains produced detectable amounts of moniliformin, chrysogine,

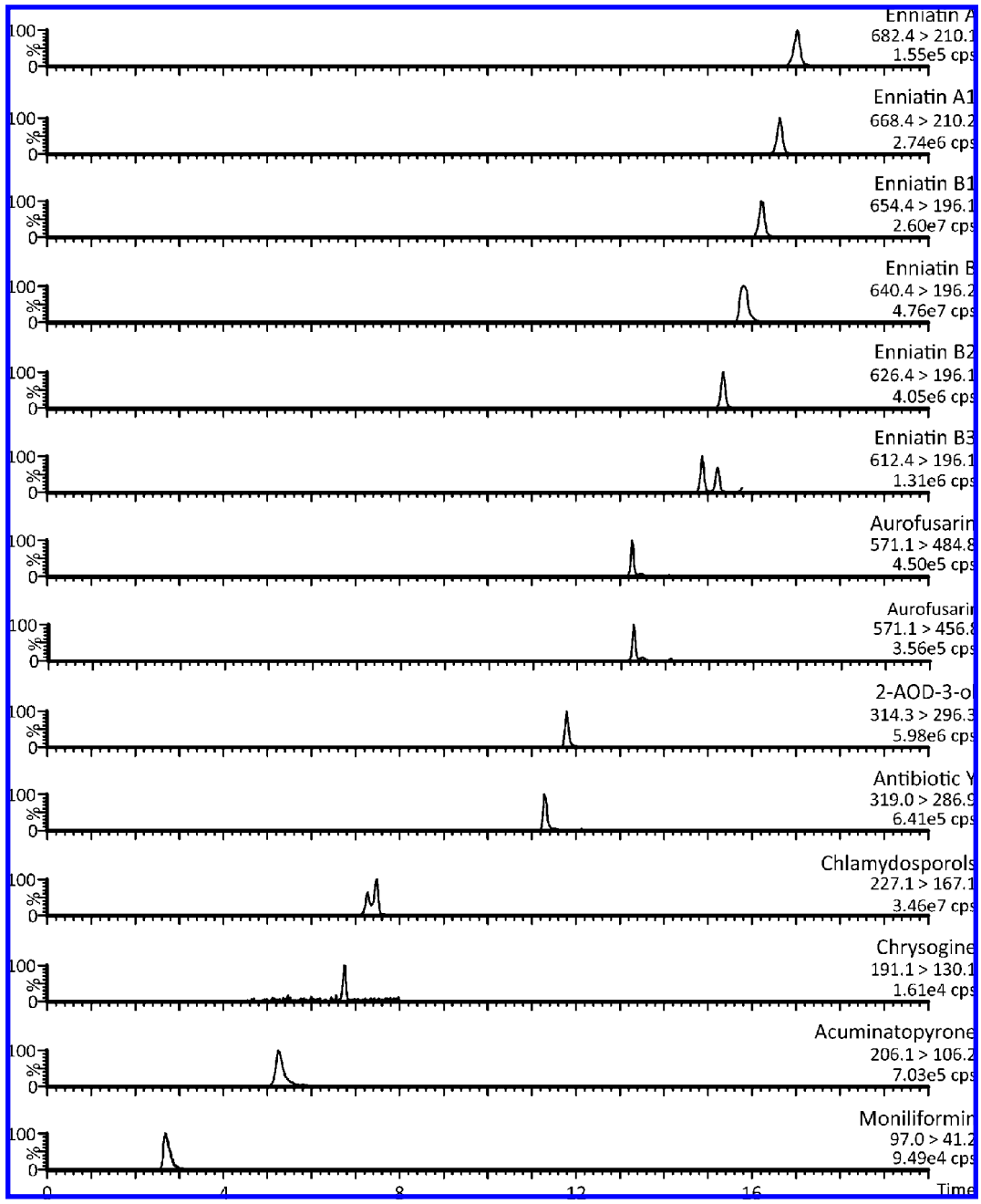

Figure 3. LC-MS/MS detection of quantifier ions of 13 metabolites from *F. avenaceum* strain JLS F29 on YES in counts per seconds (cps) and percent. Qualifier ion of aurofusarin is also included.

antibiotic Y, 2-AOD-3-ol, aurofusarin, and the six enniatins on all three media. Unexpectedly, two peaks were detected with the enniatin B3 settings. We suggest that the first peak is derived from enniatin B3, as this peak was from a compound eluting 0.4 min before enniatin B2, which is the same interval as between the other B type enniatins. The second peak might be from another enniatin with an identical mass and fragmentation pattern, which could be enniatin J1 (*35*). Two overlapping chlamydosporol peaks were detected from all strains on PDA and YES and from 6 strains on AA. This suggests the presence of chlamydosporol and one of its analogues (such as iso-chlamydosporol) having an identical mass and fragmentation pattern, which previously has also been observed in *F. tricinctum* and *F. chlamydosporum* (*36*). Distribution of acuminatopyrone production was sparse, as it was detected only in two strains on AA and PDA and four strains on YES. The distribution of the metabolites in this small study concurs with the large scale study made by Uhlig et al. (*5*). The acidic apple agar (pH 3.3) did not seem to inhibit production of any of the metabolites, and it is therefore likely that all metabolites can be present in apples infected with *F. avenaceum*.

Detection Limits and Recovery of Selected Metabolites from Spiked Apples. Apple samples were spiked with moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1 in triplicate to obtain the 10 levels shown in **Table 2**. All metabolites were recovered linearly with high R^2 values ranging from 0.987 to 0.999 (**Table 5**). Little or no matrix effects were observed for moniliformin, antibiotic Y, and aurofusarin; however, the apple matrix seemed to enhance the enniatin signals, resulting in apparent recoveries above 100%, which we also have observed previously with maize matrix (*28*). The LOD of moniliformin was 19 ng g^{-1}, which is comparable to other LC-MS/MS methods that had 10 ng g^{-1} (*27*) and 20 ng g^{-1} (*30*). The LOD for the four enniatins was also in the range of previously published LC-MS/

Table 4. Production of Metabolites by 11 *Fusarium avenaceum* Strains on AA, PDA, and YES

	AA	PDA	YES
moniliformin	11	11	11
acuminatopyrone[a]	2	2	4
chrysogine	11	11	11[c]
chlamydosporols[b]	6	11	11
antibiotic Y	11	11	11
2-AOD-3-ol	11	11	11
aurofusarin	11	11	11
enniatin B3	11	11	11
enniatin B2	11	11	11
enniatin B	11	11	11
enniatin B1	11	11	11
enniatin A1	11	11	11
enniatin A	11	11	11

[a] By JLS F29 and HJS 846 on all three media; JLS F3 and F35 also on YES. [b] By JLS F29, F35, HJS 346, 846, 848, and 849 on AA. [c] Trace amount by HJS 346.

Table 5. Recovery of Moniliformin, Antibiotic Y, Aurofusarin, and Enniatins from Spiked Apples

metabolite	n[a]	spike level (ng g^{-1})	recovery % (min−max)	st dev[b]	R^2
moniliformin	27	50−12800	100 (89−112)	6	0.999
antibiotic Y	27	50−12800	93 (75−112)	11	0.998
aurofusarin	27	50−12800	101 (70−156)	22	0.987
enniatin A	27	17−4352	115 (98−149)	13	0.999
enniatin A1	27	34−8704	113 (99−141)	10	0.999
enniatin B	27	24−6144	113 (98−166)	17	0.997
enniatin B1	27	26−6566	114 (98−145)	14	0.999

[a] Number of samples. [b] Standard deviation.

MS methods. For instance, the LOD of enniatin B was 1 ng g^{-1} in our method, which is lower than we had in a previous study of maize (12 ng g^{-1}) (*28*), but higher than others who had 0.4 ng g^{-1} (*27*) and 0.03 ng g^{-1} (*30*). The spiked apple samples were used to calculate the occurrence of moniliformin, antibiotic Y, aurofusarin, and enniatins A, A1, B, and B1.

Artificially Infected Apples. Five strains of *F. avenaceum* (**Table 1**) were used to artificially infect apples, and their metabolites after 3, 7, 14, and 21 days of incubation were determined. Strain HJS 912 did not produce detectable amounts of any of the target metabolites except for aurofusarin and 2-AOD-3-ol (data not shown). The remaining four strains produced detectable amounts of moniliformin, chrysogine, antibiotic Y, 2-AOD-3-ol, aurofusarin, and enniatins A, A1, B, and B1 except for strain HJS 911, which did not produce moniliformin (**Figure 4**).

Moniliformin was detected after 7 days, reaching maximum amounts after 14 days in strains HJS 288 (7.34 μg g^{-1}) and HJS 851 (1.29 μg g^{-1}) and after 21 days in HJS 848 (3.34 μg g^{-1}). Antibiotic Y was detected after 3 days in strain HJS 848, whereas it could be detected after 7 days in 288 and HJS 851 and after 21 days in strain HJS 912. Antibiotic Y levels were highest after 14 days in strain HJS 851 (0.21 μg g^{-1}) and 21 days in strains HJS 288 (0.26 μg g^{-1}), HJS 848 (5.7 μg g^{-1}), and HJS 912 (0.12 μg g^{-1}). Aurofusarin was detected after 3 days in all strains and reached a maximum after 14 days in strains HJS 848 (140 μg g^{-1}), HJS 851 (108 μg g^{-1}), and HJS 912 (152 μg g^{-1}) and after 21 days in strain HJS 288 (107 μg g^{-1}). These levels vastly exceeded the maximum spike level (12.8 μg g^{-1}), which is why accurate quantification in these samples is difficult. If present, the four enniatins, whose summed total is shown, occurred in successively decreasing amounts in the sequence B > B1 > A1 > A, as previously noticed (*5*, *28*). Strains HJS 288 and HJS 848 produced the highest amounts of enniatins (12 μg g^{-1} and 13 μg g^{-1}, respectively) after 14 days and were detected after 3 and 7 days, respectively. Enniatins could only be detected in strain HJS 851 after 14 days (0.47 μg g^{-1}) and after 3 days in strain HJS 912 (trace). Enniatin B2 was detected in the five samples with the highest amounts of enniatins A, A1, B, and B1, whereas B3 was only detected in the top three samples (data not shown).

2-AOD-3-ol was produced by all four strains and was detected after 3 days in strains HJS 848, HJS 851, and HJS 912 and after 7 days in strain HJS 288. The level of the metabolite was highest after 14 days in strains HJS 288, HJS 848, and HJS 851 and after 21 days in 912. Chrysogine was detected after 7 days in strain HJS 851 and after 14 days in strains HJS 288, HJS 848, and HJS 912. Strains HJS 288, HJS 851, and HJS 912 produced maximum levels after 14 days and strain HJS 848 after 21 days. Acuminatopyrone and chlamydosporols could only be detected in samples inoculated with strain HJS 288 (data not shown).

In general, all strains produced significantly accumulated amounts of various metabolites after either 14 or 21 days. Interestingly, highest amounts of moniliformin, aurofusarin, 2-AOD-3-ol, and chrysogine were measured in the majority of samples already after 14 days, which may indicate that the metabolism rate of the strains decreases or comes to a steady state after a certain amount of time.

Naturally Infected Samples. Twenty apples showing symptoms of wACR were collected and analyzed. One or several *F. avenaceum* metabolites were detected in 17 samples (**Table 6**). Antibiotic Y and aurofusarin were the most frequently observed metabolites occurring in 16 samples in levels up to 51 μg g^{-1} and 145 μg g^{-1}, respectively. Moniliformin was found in 14 samples ranging from trace amounts to 2.9 μg g^{-1}. The moniliformin levels were further validated by HILIC-UV-MS (*3*) (results not shown). The same 14 samples also contained at least one of the four enniatins A, A1, B, and B1, and the summed amounts of the enniatins ranged from trace amounts to 3.9 μg g^{-1}. Enniatin B2 was also present in these samples whereas B3 was present in 8 samples. Enniatins B2 and B3 were not quantified, but they will most likely only give a small contribution to the total enniatin amounts, given that these two analogues are normally produced in much smaller quantities than the other four major enniatins A, A1, B, and B1.

Chrysogine was found in 10 and 2-AOD-3-ol in 11 samples. To our knowledge, this is the first report of 2-AOD-3-ol detected in a naturally infected sample, while it has been observed only in artificially inoculated wheat (*10*). We were unable to quantify the levels of 2-AOD-3-ol, however, as we did not have sufficient amounts to spike apple samples for standards. LC-UV analyses of the raw extracts could not be used for estimating its quantity because 2-AOD-3-ol is not UV active. Chlamydosporols and acuminatopyrone co-occurred in four samples, suggesting that these two structurally related compounds share the same regulation mechanism. A clear correlation between the quantities of the various compounds, however, was not obvious, confirming the results obtained from the artificially infected apples.

Results of the artificially inoculated and naturally infected apples showed that antibiotic Y and aurofusarin are the most abundant metabolites, both occurring in relatively high amounts. To our knowledge, aurofusarin contamination has only been quantified once before, which was in wheat with levels up till 4.2 μg g^{-1} (*37*). We detected aurofusarin in much higher amounts, probably because the rots in our samples were well-developed. Biosynthesis of pigments, including aurofusarin, has been identified as a response of fungi to major stress factors such as low or high pH (*32*), and the relatively low pH in apples may have triggered

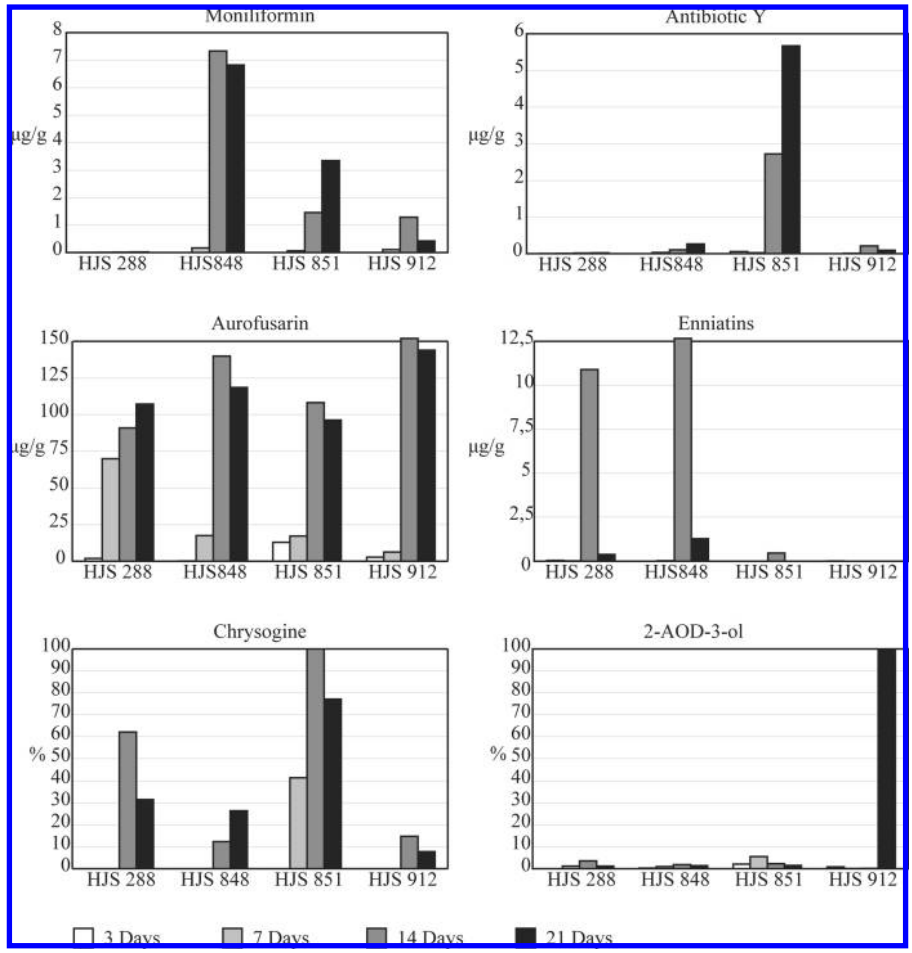

Figure 4. Occurrence of moniliformin, antibiotic Y, aurofusarin, and the sum of the four enniatins A, A1, B, and B1 (ng g^{-1}) in apples artificially inoculated with four *F. avenaceum* strains after 3, 7, 14, and 21 days of incubation. For 2-AOD-3-ol and chrysogine, the relative occurrence is given with the highest encountered amount set to 100.

Table 6. Occurrence of Moniliformin (mon), Antibiotic Y (anti Y), Aurofusarin (auro), and Combined Sum of Enniatins (enn) (μg/g) and 2-AOD-3-ol, Chrysogine (chrys), Chlamydosporols (chlam), and Acuminatopyrone (acumi) (%) in 20 Naturally Infected Apples Sampled from Trees[a]

	μg g^{-1}				%[b]			
#	mon	anti Y	auro	enn	2-AOD-3-ol	chrys	chlam	acumi
1	1.28		13.2	0.13	0.4	45.2	27.4	63.4
2								
3								
4	trace	2.57	0.64	0.34	0.4			
5	0.25	9.68	74.4	1.63	24.7	44.7		
6	2.87	trace	80.7	trace		12.9		
7	0.20	trace	145.1		4.3	11.3		
8	trace	0.29	34.4	0.12	2.1			
9		trace						
10	2.53	1.55	128.1	3.89	0.6	17.5		
11	2.74	4.17	166.6	0.54	24.0	3.0		
12	0.34	7.60	63.8	0.16	7.1	2.8	25.8	89.8
13		trace	0.18					
14	0.16	17.1	95.4	1.65	11.3	29.5	100	100
15	1.53	0.11	91.6	0.31	1.5	100		
16	0.33	0.08	1.20	0.20		25.4	62.6	3.2
17	1.32	51.3	103.6	2.60	100	21.0		
18	2.33	0.32	132.5	0.16	4.8			
19								
20								

[a] LOD and LOQ, see Material and Methods; trace, below LOQ (see Materials and Methods). [b] Highest peak area of each metabolite set to 100%.

production of high levels of aurofusarin in wet core rot samples. The toxicity of aurofusarin, however, is insufficiently known and it is therefore not possible to estimate its risk for apple consumers.

It should be noted that LC-MS/MS, due to is high selectivity, is blind for everything else and would thus overlook compounds not screened for. However, LC-DAD-HRMS (both ESI^+ and ESI^-) analysis of diluted extracts did not indicate other compounds in as high amounts as the selected target compounds. But clearly we would have overlooked compounds produced in low amounts and not expected from *F. avenaceum*.

The occurrence of several different types of mycotoxins produced by *F. avenaceum* is also a risk factor due to possible synergistic effects. As data on the cytotoxicity and mode of action of many of the mycotoxins produced by *F. avenaceum* are almost completely lacking, more effort in this area is needed for proper risk assessment (*38*). Because the infections start inside the apples where they cannot be seen, there is a high risk that mycotoxin contaminated apples would end up in food products such as apple juice, which again would have unacceptably high concentrations of mycotoxins. Thus, this disease could have devastating economic implications for apple orchards.

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