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Production of Trichothecenes by the Apple Sooty Blotch Fungus *Microcyclospora tardicrescens*

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Supporting Information

ABSTRACT: The sooty blotch and flyspeck (SBFS) syndrome of apples and other fruits is caused by a complex consortium of epiphytic fungi that colonize the fruit cuticula. SBFS fungal strains isolated from apples were screened for growth inhibition of the phytopathogen *Colletotrichum fioriniae* in dual culture tests. Extracts of 11 isolates of SBFS fungi (*Microcyclospora malicola*, *Microcyclospora pomicola*, *Microcyclospora tardicrescens*, and *Microcyclosporella mali*) inhibited growth of the test strains and were studied for production of antibiotics. A strain of *Microcyclospora tardicrescens* strongly inhibited growth and was cultivated on a larger scale to characterize its secondary metabolites. Bioassay-guided fractionation and subsequent structure elucidation by spectroscopic and spectrometric methods (NMR, HRMS) yielded trichothecolone acetate (**1**) and its novel derivative (*S*)-7-hydroxytrichothecolone acetate (**2**) as active principles. *Microcyclospora tardicrescens* was thus identified as a producer of the hazardous trichothecene type mycotoxins for the first time, which should give reason to monitor these foodborne fungi more carefully in the future.

KEYWORDS: *sooty blotch and flyspeck, microbial interactions, trichothecolone, secondary metabolites, structure elucidation*

INTRODUCTION

Sooty blotch and flyspeck (SBFS) refers to dark blemishes and smudges that consist of mycelial mats, hyphae, or fruiting bodies formed by a complex of fungi that epiphytically colonize the epicuticular wax of fruits. SBFS fungi are mainly known to affect apples, but they can also affect pears, persimmons, bananas, papayas, vines, and gourds.[1,2] The dark blemishes can partly or almost entirely cover the surface of ripe apples and reduce the economic value of organically produced apples.[3] Even though they are not known to cause fruit rots, they are recognized as causal agents of the so-called apple summer disease.[4] Although different SBFS species can co-occur in the same orchard and even on the same apple, their interactions with one another, to host trees, and with other pathogens, as well as their potential to produce hazardous mycotoxins, are as yet little understood. During inventory studies for SBFS fungi in Slovenia, we observed that juxtaposed colonies of different SBFS fungi were clearly separated from each other. Our hypothesis follows that of Venkatasubbaiah et al.,[5] who stated that secondary metabolites produced by SBFS could have an inhibitory potential against other fungi. Since then, secondary metabolites do not seem to have been studied in other SBFS fungi and were only sporadically reported for other taxa of the order Capnodiales.[6] The aim of the current study was to isolate pairs of juxtaposing SBFS taxa from apples, to test their inhibitory capacity against plant pathogens in dual culture assays, and to identify secondary metabolites that could inhibit growth of other fungi in vitro and on fruit surfaces.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were determined with a model 241 MC polarimeter (PerkinElmer, Waltham, MA, USA), IR spectra were measured with a model Spectrum 100 FTIR spectrometer (PerkinElmer), and UV spectra were recorded with a model UV-2450 UV−vis spectrophotometer (Shimadzu, Kyoto, Japan). NMR spectra were recorded with a model Ascend 700 spectrometer (Bruker Biospin, Karlsruhe, Germany) equipped with a 5 mm TXI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz). ESI-MS spectra were obtained with an Amazon ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany), and HRESIMS spectra were obtained with a Maxis time-of-flight mass spectrometer (Bruker Daltonik), both combined with an 1200 series HPLC-UV−vis system (Agilent, Santa Clara, CA, USA), using the same parameters as recently described by Thongbai et al.[7]

Isolation of Fungal Strains. Procedures for the isolation of SFBS fungi and their morphological and molecular phylogenetic characterization were described previously.[8,9] The strains had been isolated from apples with SBFS symptoms in Slovenia during the harvest seasons in 2009−2011 and are preserved at the culture collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL), Slovenia, and at the Agricultural Institute of Slovenia (Table 1). Duplicates are also kept in the mycological collection of HZI, Braunschweig, Germany, and some representative isolates were sent to the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

Dual Culture Tests. A total of 72 SBFS strains including *Microcyclospora malicola* (5 strains), *M. pomicola* (1), *M. tardicrescens* (1), *Microcyclosporella mali* (21), *Schizothyrium pomi* (31 strains), *Peltaster* spp. (12), and *Devriesia pseudoamericana* (1) were tested against *Colletotrichum fioriniae*, isolated from apple fruit anthracnose, in modified dual culture assays.[10] Colony plugs of 5 mm diameter were excised from the margin of 13-day-old cultures grown on potato dextrose agar (PDA) (Difco, USA) and placed upside down on the surface of Czapek yeast agar [35 g of Czapek-Dox Broth (Beckton-

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Table 1. Bioactivity of Crude Extracts of Fungal Strains HJS 1929−1942[a] against Selected Fungal and Bacterial Strains after 55 Days (Strain 1937) or 41 Days (All Others)[b]

	Colletotrichum fioriniae	*Pichia anomala*	*Saccharomyces cerevisiae*	*Bacillus subtilis*	*Escherichia coli*
Microcyclospora					
M. malicola 1929	6.25	300	>300	>300	>300
M. malicola 1930	25	>300	>300	150	>300
M. malicola 1931	25	>300	>300	>300	>300
M. malicola 1932	12.5	>300	>300	>300	>300
M. pomicola 1933	12.5	>300	>300	150	>300
M. pomicola 1934	6.25	300	>300	150	>300
M. tardicrescens 1935	12.5	>300	>300	300	>300
M. tardicrescens 1936	12.5	>300	>300	>300	>300
Microcyclosporella					
M. mali 1937	25	>300	>300	>300	>300
M. mali 1939	25	>300	>300	>300	>300
M. mali 1940	50	>300	>300	>300	>300
M. mali 1942	6.25	>300	>300	>300	>300

[a]Culture collection of H. J. Schroers, Agricultural Institute of Slovenia. [b]Values are given as minimal inhibitory concentration (MIC) of the crude extract.

Dickinson, Difco, USA), 5 g of yeast extract (Biolife, Italy), 15 g of technical agar (Biolife, Italy)]. Colony plugs of SBFS fungi were placed at a distance of 15 mm from the edge of a 90 mm diameter plastic Petri dish. Following incubation for 4 days in darkness at 23 °C, aqueous conidial suspensions of *C. fioriniae* were placed in the colony center by needle point inoculation. Negative control plates consisted of needle point inoculated *C. fioriniae* colonies. The dual culture plates were incubated at 23 °C in the dark. The inhibitory effect of *M. tardicrescens* was measured and described after 7, 8, 10, 12, and 14 days of incubation. The assay was replicated three times per SBFS strain. The percentage inhibition of mycelial growth (PIMG) was determined according to the method of Lahlali and Hijri:[11] PIMG = $100 \times (R_1 - R_2)/R_1$, where R_1 was the radial colony growth of *C. fioriniae* in control plates and R_2 the inhibited radial colony growth of *C. fioriniae* in dual culture plates toward the colony of *M. tardicrescens* (mm). Mean values were then calculated from three replicates.

Bioactivity-Guided Fractionation. The effect of methanolic SBFS fungal extracts (prepared by extraction of the agar plates with 20 mL of methanol for 20 min and subsequent filtration of the methanol and its evaporation in vacuo) was tested on the growth of *C. fioriniae* and *Mucor hiemalis* in agar diffusion assays using 10 mL of MYC medium [10 g/L phytone peptone; 10 g of glucose; 11.9 g of Hepes (Carl Roth, Germany), 18 g/L agar (BD) (Bacto, USA)] poured into 90 mm Petri dishes as the base layer. After solidification, 5 mL of MYC medium prepared with 9 g/L agar (BD) (Bacto, USA) together with 5 mL of conidial suspension from *C. fioriniae* and *M. hiemalis* (1×10^6 conidia/mL) was poured onto the base layer. Test substances were applied as methanolic solutions (50 μL, 1 mg/mL) on sterile filter disks of 10 mm diameter (Schleicher & Schuell), allowed to dry, and placed onto the surface of the agar plates. After incubation (25 °C for 48 h), the zone diameter (mm) of the growth inhibition was determined.

Cultivation of *Microcyclospora tardicrescens* Strain 1936 and Preparation of Crude Extracts for Preparative HPLC. Six colony plugs of *M. tardicrescens* strain 1936 were excised from 10-day-old PDA incubated at 23 °C, transferred to 100 mL of YMG medium[7] in 250 mL Erlenmeyer flasks, and incubated at 23 °C on a rotary shaker with 140 rpm for 10 days. Fungal mycelium and culture fluid were then transferred to CYA plates (30 replicates, ca. 3 mL of fungal culture per plate) and incubated for 10 days at 23 °C in darkness. The fungal biomass was lyophilized with the entire agar plates and extracted two times with 1300 mL of methanol for 30 min in an ultrasonic bath at 40 °C. The liquid extracts were filtered through a paper, combined, and evaporated to dryness. The crude extract was then partitioned between 300 mL of water and 300 mL of ethyl acetate (J. T. Baker, Germany). Whereas the aqueous phase was discarded because of lack of bioactivity, the bioactive organic phase was analyzed by ESIMS before fractionation by preparative HPLC.

Isolation of 1 and 2. The crude extract was fractionated by preparative RP HPLC. A 250 × 21 mm i.d., 5 μm, VP Nucleodur C18 Gravity (Macherey-Nagel, Düren, Germany) column was utilized, and an acetonitrile−water gradient with 0.5% HCOOH, 20−100% MeCN in 30 min, and flow = 20 mL/min provided 4.2 mg of **1**, 1.1 mg of **2**, and 2.4 mg of a mixture of **1** and **2**, all with strong antifungal activity. **1** eluted at 16.8−17.8 min, and **2** eluted at 16.4−17.2 min. Silica gel HPLC with a 250 × 20 mm, 7 μm, Nucleosil 100 Å column (Knauer, Berlin, Germany) and isocratic conditions (97% dichloromethane, 3% methanol, flow = 20 mL/min) yielded 5.5 mg of **1** and 3.2 mg of **2**. **1** eluted at 3.8−5.8 min, and **2** eluted at 7.7−10.2 min.

Trichothecolone Acetate (1). ^{1}H (700 MHz, chloroform-*d*) and ^{13}C (175 MHz, chloroform-*d*) NMR spectroscopic data are shown in Table 3; ESI-MS, *m*/*z* 307.1 [M + H$^+$]; HRESIMS (*m*/*z*) found 307.1536 [M + H$^+$], calcd for $C_{17}H_{23}O_5$ 307.1540. Spectroscopic and spectrometric data are in good agreement with the literature.[12,13]

7-Hydroxytrichothecolone Acetate (2). **2** was obtained as a white amorphous powder; $[\alpha]^D_{25}$ = +34 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 226 (3.82); IR (KBr) ν_{max} 3450, 2963, 2926, 1737, 1685, 1261, 1243, 1081, 1034, 968, 802 cm^{-1}; ^{1}H (700 MHz, chloroform-*d*) and ^{13}C (175 MHz, chloroform-*d*) NMR spectroscopic data are shown in Table 3; ESIMS *m*/*z* 323.1 [M + H$^+$]; HRESIMS *m*/*z* 323.1481 [M + H$^+$] (calcd for $C_{17}H_{23}O_6$, 323.1489).

Biological Assays for Evaluation of Antimicrobial and Cytotoxic Activities. Biological assays were performed as previously described.[14] The compounds were tested against the bacteria *Bacillus subtilis* DSM 10, *Escherichia coli* DSM 1116, and *Staphylococcus aureus* DSM 346 and the fungi *Colletotrichum fioriniae* HJS 2018, *Pichia anomala* DSM 6766, *Candida albicans* DSM 1665, and *Saccharomyces cerevisiae* DSM 1333. The concentration of the purified compounds was tested at 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 μg/mL. The criterion of the minimal inhibitory concentration (MIC) was defined as the lowest concentration showing no visible bacterial or fungal growth after 24 h of incubation.

RESULTS AND DISCUSSION

Isolation of Strains and Co-Cultivation. Eleven strains (Table 1) were significantly antagonistic to fungal growth in dual culture and were chosen for further studies. Extracts of liquid cultures of all of these strains showed antifungal activity against *Colletotrichum fioriniae* with MIC values between 6.3 and 50 μg/mL (Table 1). Some of them were also active against *P. anomala* and *B. subtilis*. *Microcyclospora tardicrescens*

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strain 1936 inhibited the colony growth of *C. fioriniae* by 46% after 7 days of incubation and by 55% after 14 days (Table 2; Figure 1) and was selected for scale-up and preparative chromatography. An extract from cultivation of strain 1936 on 30 agar plates was processed by a bioassay-guided fractionation strategy using *C. fioriniae* as indicator organism. Fractionation of the crude extract by reverse phase HPLC resulted in an active fraction that consisted of a mixture of two bioactive metabolites according to HPLC-MS analyses. Subsequent silica gel HPLC resulted in the isolation of both metabolites (**1** and **2**) as white powders.

Table 2. In Vitro Inhibition of Colony Growth of *Colletotrichum fioriniae* in Dual Culture with Antagonistic *Microcyclospora tardicrescens* Strain 1936 on Czapek Yeast Agar

incubation time (days)	inhibited radial colony growth[a] (mm)	radial colony growth in control plate[a] (mm)	growth inhibition percentage of *C. fioriniae*[a]
7	11.7	21.7	46.1
8	12.3	22.7	45.3
10	12.7	27.0	53.0
12	15.3	33.7	54.6
14	17.0	38.0	55.3

[a]Means of three replicates.

Figure 1. *Microcyclospora tardicrescens* strain 1936 (left) inhibiting the colony growth of *Colletotrichum fioriniae* (right) after 10 days of dual culture incubation on Czapek yeast agar at 23 °C.

HRESIMS revealed the molecular formula of $C_{17}H_{22}O_5$ for compound **1**. The ^{1}H and ^{13}C NMR spectra (Table 3) indicated the presence of four methyl groups, one sp^2 olefinic carbon, one quaternary sp^2 carbon, three sp^3 quaternary carbons, one of which connected to oxygen, three oxygen-bearing methines, three methylenes, and two carbonyls. COSY and HMBC NMR data provided the characteristic structure of 12,13-epoxytrichothec-9-en-8-ones and identified the metabolite as the known trichothecolone acetate (Figure 2).[12,13]

Table 3. ^{13}C NMR (175 MHz) and ^{1}H NMR (700 MHz) Data of Metabolites 1 and 2 in $CDCl_3$

	1		2	
position[a]	δ_C	δ_H (J_{HH} in Hz)	δ_C	δ_H (J_{HH} in Hz)
2	79.5	3.93 (m)	80.1	3.99 (d, 4.8)
3	36.8	2.61 (dd, 15.6, 1.4)	36.6	2.55 (dd, 15.4; 7.9)
		2.08 (m)		2.14 (m)
4	73.9	5.52 (dd, 7.9; 3.7)	73.9	5.48 (dd, 7.9; 4.1)
5	48.9		49.7	
6	43.7		48.2	
7	42.1	2.88 (dd, 15.3; 1.0)	74.4	4.81 (s)
		2.30 (dd, 15.3; 1.4)		
8	198.6		200.1	
9	138.2		135.5	
10	137.0	6.48 (dd, 5.9; 1.5)	137.7	6.51 (d, 5.8)
11	70.1	3.93 (m)	70.8	4.06 (d, 5.8)
12	65.4		65.0	
13	47.5	3,15 (d, 4.0)	46.6	3.17 (d, 4.0)
		2.86 (d, 4.0)		3.15 (d, 4.0)
14	5.6	0.72 (s)	6.9	1.00 (s)
15	18.4	1.06 (s)	11.9	0.95 (s)
16	15.4	1.83 (s)	14.7	1.89 (s)
17	170.9		170.6	
18	21.1	2.10 (s)	20.7	2.11 (s)
7-OH				3.72 (s)

[a]Assignments confirmed by 2D COSY, HSQC, and HMBC experiments.

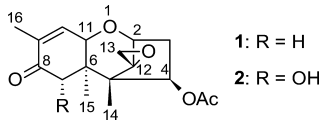

Figure 2. Chemical structures of **1** and **2** isolated from *M. tardicrescens* strain 1936.

For compound **2**, HRESIMS gave the molecular formula $C_{17}H_{22}O_6$. The molecular formula, together with an absorption at 3450 cm^{-1} in the IR spectrum, indicated that **2** is a hydroxylated derivative of **1**. Furthermore, the ^{1}H and ^{13}C NMR spectra of **2** (Table 3) resembled those of **1** with the exception of the absence of a methylene signal for 7-H$_2$ (δ_C 42.1; δ_H 2.88 and 2.30). Instead of the aforementioned methylene signal, an oxygenated methine (δ_C 74.4, δ_H 4.81) was observed together with a broad singlet at 3.72 ppm; the absence of an HSQC connection of this signal revealed it to be connected to an oxygen atom. The methine gave HMBC correlations (Figure 3) to C-5, C-6, C-8, C-9, C-11, and C-15 and the hydroxyl group to C-6, C-7 and C-8. Thus, the more polar metabolite **2** was identified as 7-hydroxytrichothecolone acetate. The stereochemistry (Figure 4) of the additional chiral center of **2** was assigned by interpretation of the ROESY spectrum in conjunction with the known stereochemistry of trichothecolone acetate (**1**).[15] In the ROESY spectrum 7-OH correlated with 13-H$_2$, whereas 7-H showed correlations to 14-H$_3$ and 15-H$_3$. These observations were confirmed by a 1D NOESY experiment irradiating at the shifts of 7-H and 7-OH. Thus, an *S*-configuration can be deduced for C-7.

Trichothecenes are a family of over 200 toxins with a common tricyclic 12,13-epoxytrichothec-9-ene core structure.[16,17] The trichothecenes are agriculturally important mycotoxins that present a potential threat to animal and

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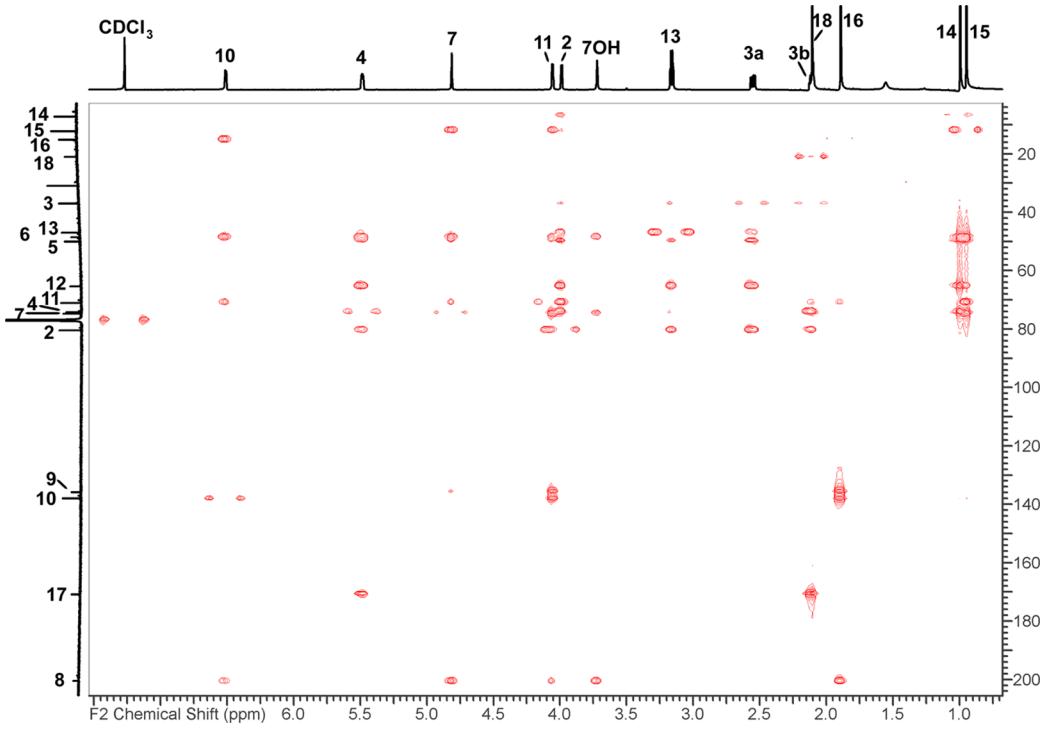

Figure 3. HMBC NMR (700 MHz, $CDCl_3$) spectrum of (*S*)-7-hydroxytrichothecolone acetate (**2**).

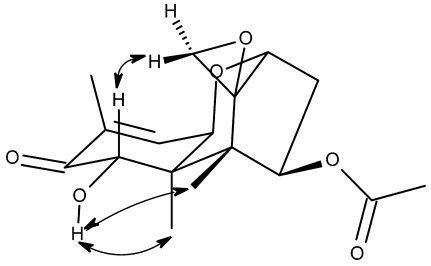

Figure 4. Selected NOE correlations indicating the configuration of **2**.

human health throughout the world.[17] This large family of toxins is produced by fungi in genera, such as *Fusarium*, *Myrothecium*, *Trichoderma*, *Trichothecium*, and *Stachybotrys* (and the compounds we have isolated are actually most similar to some metabolites from *Trichothecium*).[18] Those genera all belong to the order Hypocreales, whereas the SBFS fungi studied belong to the Capnodiales. It should be interesting to compare the biosynthesis gene clusters for trichothecenes in *M. tardicrescens* with those of the above-mentioned fungi. However, this was beyond the scope of the present study and would afford extensive work using methods of molecular biology. The only capnodialean fungus previously reported to produce trichothecenes is *Peltaster fructicola*. Among other compounds, this species produced trichothecolone acetate (**1**), which showed antifungal properties in vitro against *Botryosphaeria* causing bitter rot.[5] The here-reported rediscovery of **1** from *M. tardicrescens* strain 1936 indicates that trichothecenes might occur more widely in the Capnodiales or other related SBFS fungi. However, retrospective analysis of the extracts of strain 1935, another representative of *M. tardicrescens*, and of the other 70 isolated strains by HPLC-MS using pure compounds of **1** and **2** as internal standards did not yet result in the discovery of another trichothecene producer.

The bioactivity of **1** and **2** was evaluated by determining their MIC against a broad test panel of bacteria and fungi (Table 4).

Table 4. Antifungal Activity of Compounds 1 and 2 from *M. tardicrescens* Strain 1936 against Selected Fungal and Bacterial Strains (as Minimum Inhibitory Concentration, MIC, in μg/mL after 24 h)[a]

	strain			
compound	*Colletotrichum fioriniae*	*Pichia anomala*	*Candida albicans*	*Saccharomyces cerevisiae*
trichothecolone acetate (**1**)	6.25	50	50	6.25
7-hydroxy-trichothecolone acetate (**2**)	12.5	50	100	12.5

[a]The bacteria *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* were also tested but proved insensitive up to 100 μg/mL.

Compounds **1** and **2** showed no activity against the bacteria *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* but moderate activity against *Colletotrichum fioriniae* and *Saccharomyces cerevisiae* besides weak activity against *Pichia anomala* and *Candida albicans*. In a proliferation assay against the mouse fibroblast cell line L-929, strong cytotoxic effects were determined for both **1** (IC_{50} = 0.08 μg/mL) and **2** (IC_{50} = 0.17 μg/mL). All of these effects can be explained by the well-known inhibitory activity of eukaryotic protein synthesis.[16,17] However, the biological role of the observed trichothecenes for SBFS fungi in nature remains elusive. They could inhibit competitor SBFS fungi occupying the same ecological niche or plant pathogenic microorganisms that colonize the exocarps before fruit invasion. However, trichothecenes are known to act also as phytotoxins and may present plant pathogen virulence factors in the SBFS/fruit system.

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To demonstrate a possible role of **1** and **2** in vivo, we cultivated *M. tardicrescens* 1936 on apple skin. Unfortunately, we could not detect either **1** or **2** due to strong contamination by wax in the extracts, which precluded their straightforward detection by analytical HPLC. A scale-up procedure and semipreparative HPLC separation prior to detection seems necessary to obtain more conclusive results. Therefore, we cannot assess the extension of a potential hazard resulting from trichothecene mycotoxins produced by SBFS fungi. However, the fact that juxtaposing colonies of different SBFS fungi are frequently separated from each other indicates that bioactive metabolites may be released on or into the fruit exocarp. SBFS are obviously well-adapted to epiphytically colonize fruit exocarps, whereas they are not known as fruit-destroying disease agents. Plants may profit mutualistically from the release of small amounts of toxic components, tolerated by the plant but inhibiting the development of other pathogenic agents.

Our results indicate that SBFS fungi are able to produce secondary metabolites with a wide spectrum of antibacterial and antifungal activities. The exploration of these substances might be suited as pharmaceuticals or in agriculture. Certainly, they will help us to understand the complex relationship between the different SBFS fungi and their plant hosts. Evaluation of the other strains listed in Table 1 is presently ongoing, and we expect to isolate further non-trichothecene bioactive compounds that may have some practical utility.

■ ASSOCIATED CONTENT

Supporting Information

HPLC-ESIMS and ^{1}H and ^{13}C NMR spectra of compounds **1** and **2**, and COSY, ROESY, HSQC, and HMBC spectra of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Structure determination of chemical compounds and manuscript preparation was done by F.S. Underlying hypotheses of the study are those of A.M., who found the bioactivity in the first place and helped isolate the active principles as a guest Ph.D. student in the laboratories of M.S.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

SBFS, sooty blotch flyspeck; PDA, potato dextrose agar; CYA, Czapek yeast agar; PIMG, percentage inhibition of mycelial growth; RP HPLC, reverse phase high-performance liquid chromatography; UPLC-DAD, ultraperformance liquid chromatography coupled with diode array detector; HR ESI-TOF-MS, high-resolution electrospray ionization coupled time-of-flight mass spectrometry; ITMS, ion trap mass spectrometry; MIC, minimum inhibitory concentration

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