

Stachybotrys spp. and the guttation phenomenon

Manfred Gareis · Christoph Gottschalk

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Abstract The formation of guttation droplets is a long-known property of various fungi. However, their composition, biological function and metabolism in fungi have hardly attracted deeper research interest. The highly toxic mould *Stachybotrys* (*S.*) *chartarum* chemotype S is supposed to play—amongst other factors such as endotoxins and microbial volatile organic compounds (MVOCs)—an important role in indoor air toxicity, mainly after water damage. The way of toxins becoming airborne and leading to exposure via inhalation, however, is still under discussion. We hypothesised that guttation may be a factor for exudation of toxins into the environment. Therefore, selected isolates ($n=15$) of our own culture collection of *Stachybotrys* spp. (*S. chartarum* chemotype S, *S. chartarum* chemotype A, *S. chlorohalonta*) originating from various habitats were cultivated on malt extract agar for 3 weeks. All strains but one produced different amounts of guttation droplets, which were collected quantitatively and subjected to various independent analytical techniques like ELISA, effect-based bioassay (MTT cell culture test) and tandem mass spectrometry (LC-MS/MS). Actually, the toxigenic isolates ($n=5$) produced highly toxic guttation droplets, which was confirmed by all methods. The concentration of macrocyclic trichothecenes, such as satratoxin G and H, ranged between the LOD and 7,160 ng/ml exudate and 280 and 4,610 ng/ml as determined by LC-MS/MS, respectively. According to our knowledge, the ability of *S. chartarum* to produce toxic exudates is reported for the first time, which

possibly plays an important role regarding its toxic potential in indoor environments.

Keywords *Stachybotrys chartarum* · *Stachybotrys chlorohalonata* · Guttation · Exudate droplets · Mycotoxin · Macrocyclic trichothecenes · Satratoxin · Indoor air

Introduction

The genus *Stachybotrys* was first described in 1837 by Corda after isolation from a mouldy dwelling in Prague (Bisby 1943). Since 1930 it has attracted attention as the causative agent of stachybotryotoxicosis, a severe necrotic and haemorrhagic disease, especially observed in horses after feeding mouldy straw (Forgacs et al. 1958). Later, its highly toxic metabolites, the macrocyclic trichothecenes (Fig. 1), were found to be responsible for this disease (Bata et al. 1985; Harrach et al. 1981, 1983). In the 1990s, *Stachybotrys* was identified as a serious contaminant in indoor environments, being related to deaths of babies in Cleveland, Ohio, USA (Dearborn et al. 1999; Jarvis et al. 1998). Since then its involvement in adverse health effects for humans living or working in mouldy indoor environments is beyond all question (Etzet et al. 1998; Hodgson et al. 1998; Jarvis et al. 2000; Jarvis and Miller 2005; Johanning et al. 1996). Macrocyclic trichothecenes have been detected in mouldy materials such as wallpapers, gypsum boards and others (Gottschalk et al. 2006; Johanning et al. 1998; Nielsen et al. 1999) as well as in spores, house dust (Bloom et al. 2009; Sorenson et al. 1987; Wady and Larsson 2005) and indoor air (Brasel et al. 2005; Gottschalk et al. 2008). However, there is ambiguity about the way of the toxins becoming airborne and leading to inhalative exposure.

Guttation (originating from the Latin *gutta*=drop), the active exudation of water and water-soluble compounds, is

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M. Gareis · C. Gottschalk (✉)
Chair of Food Safety, Faculty of Veterinary Medicine,
Ludwig-Maximilians-University Munich (LMU), Schoenleutnerstr.
8, 85764 Oberschleissheim, Germany
e-mail: christoph.gottschalk@lmu.de

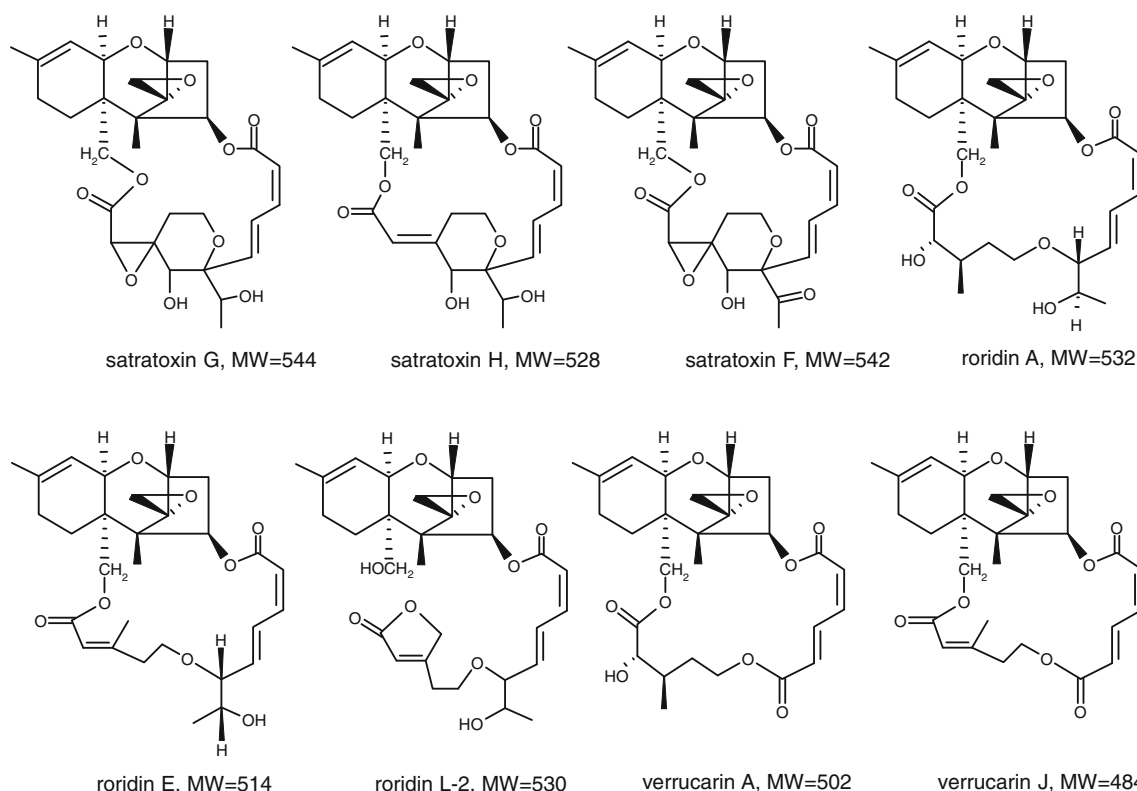


Fig. 1 Examples for macrocyclic trichothecenes. Satratoxins G, H, F and roridin E, L-2, and verrucarins J are produced by *S. chartarum* chemotype S. Roridin A and verrucarins A are metabolites of *Myrothecium verrucaria* or *Myrothecium roridum*

known from plants for nearly 350 years (Ivanoff 1963). In fungi this phenomenon has been observed for many decades (Colotelo 1978; Thom 1930), and the ability of certain species of *Penicillium* or *Aspergillus* to produce those exudate droplets is still used taxonomically (Samson et al. 2010). However, those droplets on fungal mycelia per se hardly attracted deeper research interest regarding their composition and biological function. In 1991, Jennings proposed guttation to be a sort of water reservoir, allowing growth of hyphae from afar of their primary substrate (Jennings 1991). A clear ecological function of fungal guttation droplets was described in two studies for *Fusarium culmorum* (McPhee and Colotelo 1977) and *Sclerotinia sclerotiorum* (Colotelo 1978). The exudates were shown to possess a degrading ability on plant tissues, demonstrating a high enzyme activity. However, the question of mycotoxins occurring in guttation droplets was only casually addressed: exudates of *Penicillium* were mentioned to contain penicillin as cited in Colotelo (1978). Grovel et al. (2003) conducted a study with a marine strain of *Aspergillus fumigatus*, which segregated gliotoxin into its exudate droplets, and showed these toxins to accumulate in mussels. However, a study aiming in the mycotoxinological characterisation of guttation droplets formed by certain species of *Penicillium* was conducted first in 2007 by our working group. Droplets were shown to contain the highest amounts of ochratoxins A (OTA) and B (OTB) compared with the corresponding

underlying mycelia and agar. Maximum concentrations of OTA of 8,670 ng/ml and of OTB of 2,940 ng/ml were detected by high performance liquid chromatography (HPLC) (Gareis and Gareis 2007). Recently, Hutwimmer et al. (2010) examined the formation of exudate droplets in *Metarhizium anisopliae* under various conditions and revealed the presence of destruxins in the guttation fluids.

The aim of this study was to examine whether *Stachybotrys* species are also able to produce guttation droplets and whether their highly toxic metabolites, such as the satratoxins, roridins or verrucarins, can be released into the exudates. For that purpose, 15 isolates of three different *Stachybotrys* (*S.*) species (*S. chartarum* chemotype S, *S. chartarum* chemotype A, and *S. chlorohalonata*) were investigated in this study by means of cultural and bioanalytical methods like MTT cytotoxicity test, ELISA and tandem mass spectrometry (LC-MS/MS).

Materials and methods

Fungal isolates and their cultivation

The isolates of *S. chartarum* chemotype S, *S. chartarum* chemotype A and *S. chlorohalonata* originated from various habitats (Table 1). They have been genetically characterised by polymerase chain reaction (PCR) and subsequent

Table 1 Origin and identity of the *Stachybotrys* isolates on study ($n=15$)

Isolate no.	Species	Origin
S1340	<i>S. chartarum</i> chemotype S	Wallpaper (Germany)
S1343		Plaster (Germany)
S1468		Gypsum board (USA)
S480W		Straw (Germany)
S1248		Carpet (USA)
S1380	<i>S. chartarum</i> chemotype A	Wallpaper (Poland)
S1470		Wood (Poland)
S1695		Plaster (USA)
S1091		Gypsum board (USA)
S1637	<i>S. chlorohalonata</i>	Wallpaper (USA)
SM6-2		Marjoram (Germany)
SM10-2		Marjoram (Germany)
S6		Straw (Germany)
S23St		Straw (Germany)
H47C		Hay (Germany)

sequencing of segments of the ITS region (White et al. 1990) and of the *tri5*, *tub2* and *chs1* genes (Cruse et al. 2002) in previous studies (unpublished data).

For examination of the guttation ability, all isolates were cultivated on standard malt extract agar (MEA) plates (21 ml per 9.4-cm diameter plate) as one-point and three-point cultures and on large MEA plates (70 ml per 14 cm diameter plate) as three-point and six-point inoculates. All plates were incubated at 25 °C for 3 weeks.

For all mycotoxin analyses which are described as follows the isolates were cultivated as three-point inoculates on malt extract agar plates (70 ml per 14 cm plate) at 25 °C for 3 weeks. This period proved to give the highest concentrations of macrocyclic trichothecenes in extracts of complete three-point cultures, as revealed in a previous study (unpublished data).

Collection of the guttation fluids

The cultures were visually examined for their ability to form exudate droplets. The droplets were collected after 3 weeks of cultivation with a pipette and transferred into Eppendorf caps. The volume was measured by means of a Hamilton syringe. Before subsequent analysis steps, the exudate was filtered using a 0.45- μ m Millipore 13-mm Millex HV13 syringe filter (Merck Millipore, Darmstadt, Germany) in order to remove spores.

Effect based bioassay (MTT test)

The cytotoxic effect of the exudate droplets was examined using the MTT cell culture test (effect-based bioassay) with

swine kidney target cells. This system proved to be especially sensitive towards mycotoxins (Gareis 2006; Hanelt et al. 1994). The test is based on the transformation of MTT, a yellow tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), to purple formazans by viable cells (Altman 1976).

The swine kidney cells were grown in minimum essential medium (MEM) Eagle for 4–5 days to obtain a total cell count of 3.5×10^5 cells/ml MEM. Afterwards cells were dissolved in special MEM (MEM+1.7 % ethanol+0.3 % dimethylsulphoxide (DMSO)+double concentrated foetal calf serum). An aliquot of the cell suspension (100 μ l) was placed in each well of a 96-well microtitre plate.

Ten microlitres of the filtered exudate was diluted with 490 μ l special MEM and serial 1:2 dilutions were prepared. An aliquot of each dilution (100 μ l) was transferred into the wells and the plates were incubated at 37 °C (5 % CO₂) for 48 h. After addition of 20 μ l MTT the plates were incubated under equal conditions for 4 h. Thus, the supernatant was removed and 100 μ l DMSO was added to each well in order to dissolve the formazan crystals. The optical density was measured at 510 nm (Hanelt et al. 1994) and the extinction values were compared with that of controls. The results are given as IC₅₀ values (inhibitory concentration 50), i.e. the respective exudate amount (μ l/ml medium) at which the formation of formazans is reduced by 50 % compared with the control cells (Gareis 2006).

ELISA

The presence of macrocyclic trichothecenes in the exudate droplets was measured with the Envirologix Quantitox™ Kit for Trichothecenes (Envirologix, Maine, USA), a competitive ELISA which is able to detect roridin A, E, H and L-2, satratoxin G and H, isosatratoxin F, verrucarol and verrucaridin A and J. However, a differentiation of the mycotoxins is not possible with this test system. Therefore, the results are calculated as a sum of macrocyclic trichothecenes in equivalents of roridin A (Märtlbauer et al. 1988). Due to different cross-reactivities, the results of this test are strongly influenced by the identity of the toxins and therefore should be regarded as semiquantitative.

The filtered exudate was directly applied to the test and if necessary diluted in phosphate-buffered saline (PBS) in order to match the calibration curve of the ELISA. The test was conducted according to the manufacturers' specifications. Fifty microlitres of the roridin A standard (concentrations=0.2, 2.0, 18 ng/ml), of the negative control (PBS) and of the samples were transferred to the wells of a 96-well test plate. Subsequently, 50 μ l of enzyme linked roridin A were added (competitive ELISA). After an incubation time of 45 min, 100 μ l of the substrate were added. The optical density was measured at 450 nm after 15 min of incubation. The results

were evaluated using Ridasoft Win, Version 1.73 (r-biopharm, Darmstadt, Germany).

LC-MS/MS

The LC-MS/MS measurements were carried out on an API 3200 tandem mass spectrometer (ABSciex, Framingham, USA) and a Perkin Elmer PE200 HPLC system with a Gemini C18 analytical column (150 × 2.0 mm, 5 μm; Phenomenex, Aschaffenburg, Germany) based on previously published methods (Gottschalk et al. 2006, 2009).

For the chromatographic separation of the analytes, the eluents A (deionized water+5 mM NH₄ formate+0.1 % formic acid) and B (methanol+5 mM NH₄ formate+0.1 % formic acid) were applied using a binary linear gradient at a flow rate of 400 μl/min: 0 min 10 % B; 8 min 100 % B; 12 min 100 % B; 12.1 min 10 % B; 15 min 10 % B. The column oven temperature was maintained at 40 °C and the injection volume of the samples was 20 μl. Before analysis, the filtered extracts were diluted 1:10 with methanol/water 10/90 (v/v).

The MS measurements were carried out in ESI-positive mode with a source temperature of 300 °C and an ion spray voltage of 4,000 V. The curtain gas was set at a pressure of 20 psi, the nebulizer gas at 50 psi and the heating gas at 30 psi. The collision gas was used in medium mode. All analytes were measured as adduct ions of ammonia [M+NH₄]⁺ and the toxins were identified in multiple reaction monitoring mode (MRM). Table 2 shows the substance-specific parameters for the compounds on study.

The quantification of satratoxin G and H was done by external calibration (linear regression). Due to the limited availability of reference standards, satratoxin F, roridin E and L-2, and verrucarins J were semiquantitatively determined as equivalents of satratoxin G, roridin A, and verrucarins A, respectively. The limits of detection (LODs) were estimated using the signal-to-noise approach (S/N ratio=3). The LODs were 2.3 ng/ml for satratoxin G, 4.1 ng/ml for satratoxin H, 3.4 ng/ml for roridin A and 2.5 ng/ml for verrucarins A. Reference substances for tuning and qualitative identification of the other compounds were produced in our lab on rice cultures (Jarvis et al. 1986). Their identity was confirmed by comparison with typical elution profiles and fragmentation patterns of the respective compounds (Andersen et al. 2002; Hinkley and Jarvis 2001).

Results and discussion

Formation of guttation droplets

All isolates but one showed a formation of exudate droplets (Fig. 2). The main production of guttation fluids took place between day 7 and day 14 of cultivation, which corresponded to the main growth period of the fungal mycelia. A similar relationship was observed in former studies concerning fungal exudation properties (McPhee and Colotelo 1977).

The selected isolates of *Stachybotrys* (*n*=15) were grown on plates of different diameters and amounts of MEA agar. The effect of the colony density and agar disposability on the

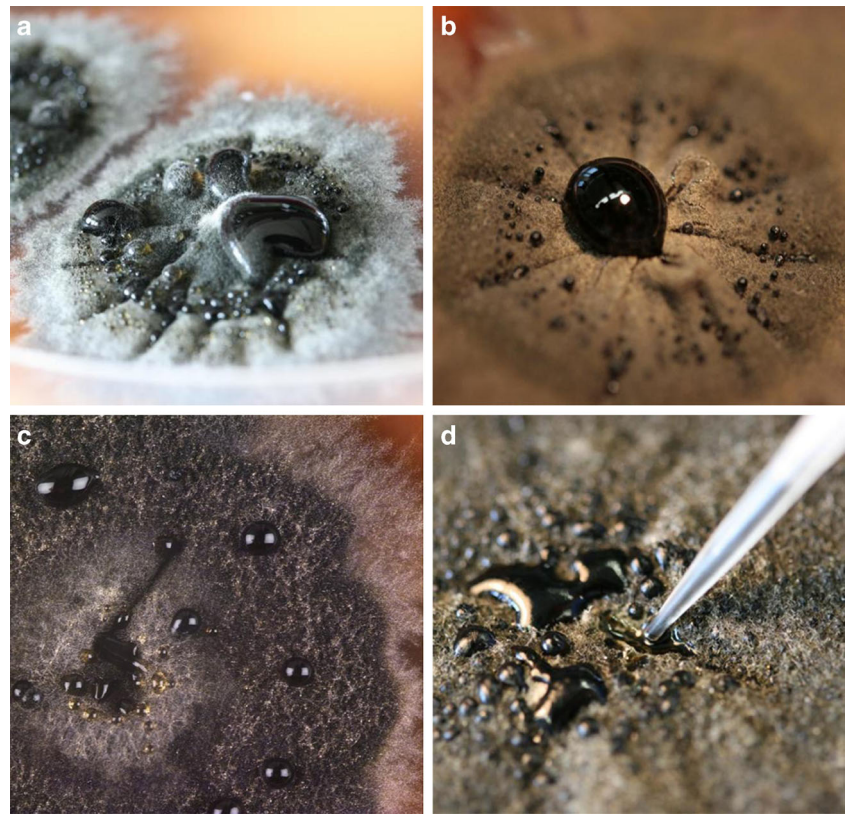
Table 2 MRM-transitions and substance-specific parameters

Analyte	Precursor ion [M+NH ₄] ⁺	Product ions	DP [V]	EP [V]	CEP [V]	CE [eV]	CXP [V]
Satratoxin G	562.3	248.9	21	3.5	22	19	22
		231.0	21	3.5	22	21	22
Satratoxin H	546.3	245.2	21	4.5	20	25	22
		231.0	21	4.5	20	25	18
Satratoxin F ^a	560.3	249.1	41	4.0	26	21	12
		231.1	41	4.0	26	21	12
Roridin A	550.4	249.1	26	3.5	24	23	20
		133.0	26	3.5	24	41	10
Roridin E ^a	532.4	361.1	31	4.5	20	19	30
		231.1	31	4.5	20	25	20
Roridin L-2 ^a	548.3	249.1	31	4.0	24	21	22
		231.0	31	4.0	24	21	18
Verrucarins A	520.4	248.8	31	5.0	24	23	22
		230.9	31	5.0	24	27	20
Verrucarins J ^a	502.4	249.0	30	5.0	25	25	22
		231.0	30	5.0	25	25	22

DP declustering potential, EP entrance potential, CEP cell entrance potential, CE collision energy, CXP cell exit potential

^aNo reference standards available. The compounds were isolated from *S. chartarum* cultures and identified by mass spectra and elution profiles

Fig. 2 Typical appearance of exudate droplets formed by *Stachybotrys* spp. after 3 weeks of cultivation on MEA agar. The figures demonstrate the different size and colour of droplets. **a** Isolate of *S. chlorohalonata* with droplets overgrown by superficial hyphae. **b** Isolate of *S. chlorohalonata* with a centrally located big droplet, surrounded by a series of tiny droplets. **c** Isolate of *S. chartarum* with black and transparent droplets of different sizes. **d** Demonstration of harvesting droplets by using a pipette tip



exudate production is shown in Table 3. A three-point inoculation on 14-cm plates with 70 ml malt-extract agar proved to give the best results considering the amount of guttation fluid.

Depending on the species between 17 and 351 μl of exudate could be collected from 14 of 15 cultures. Therefore, this procedure of cultivation was used for collection of droplets

Table 3 Amounts of exudate formed by different isolates of *Stachybotrys* spp. ($n=15$) depending on plate diameter, amount of agar and colony density

Isolate	Species	Average amounts of exudate/colony (μl)			
		9.4-cm plate, 21 ml agar		14-cm plate, 70 ml agar	
		One-point culture	Three-point culture	Three-point culture	Six-point culture
S1340	<i>S. chartarum</i> chemotype S	0	9	63	50
S1343		10	10	67	60
S1468		0	0	63	40
S48OW		0	7	47	33
S1248		10	11	17	10
S1380	<i>S. chartarum</i> chemotype A	0	0	17	10
S1470		0	0	42	0
S1695		0	0	48	1.7
S1091		0	0	0	0
S1637		0	19	53	1.2
SM6-2	<i>S. chlorohalonata</i>	40	31	167	110
SM10-2		0	10	90	48
S6		152	107	351	193
S23St		11	8	320	258
H47C		123	54	188	70

for the following toxin or toxicity examinations. It was remarkable that one-point cultures on 9.4-cm diameter plates provided the least amount of guttation fluid, although these cultures had similar amounts of agar (and therefore of available water and nutrients) at disposal like the three-point inoculates on 14-cm plates. In comparison, the three-point inoculates on 9.4-cm plates and the six-point inoculates on 14-cm plates had less agar at disposal but formed more exudate than the one-point cultures. In that context, Sprecher (1959) observed an influence of the relative air humidity on the exudate production. Due to a higher overall metabolic activity on plates with three- and six-point cultures, the air humidity around these colonies was probably higher than on the plate with only one culture, which was considered to favour the formation of exudate droplets (Sprecher 1959).

To what extent the interaction between the colonies plays a role in the exudation of guttation droplets is not yet known. Due to our observation of higher amounts of guttation fluids in more than one-point inoculations, we hypothesise that exudation may play a role in defence against other fungal colonies, especially if there is a threat of scarcity of nutrients and water for the growing fungal organism. This is also supported by the fact that mycotoxins were found to be present in guttation droplets in previous studies (Colotelo 1978; Gareis and Gareis

2007; Grovel et al. 2003; Hutwimmer et al. 2010). Gareis and Gareis (2007) have even shown that the concentration of ochratoxin A (OTA) was much higher in the fungal exudates than in the underlying mycelia of *Penicillium* spp. or their substrate. By means of stereo microscopy, we could notice that the mycelium of the *Stachybotrys* isolates investigated in this study harboured a lot of tiny droplets which could not be collected. It could be concluded that toxin concentrations in fungal mycelia are influenced by these toxic exudates. Additionally, the substrate itself was found to have an influence on the amount of produced guttation fluids: the *Penicillium* spp. cultivated on Czapek Yeast Agar (CYA) produced less exudate than the same fungi grown on MEA under equal conditions (Gareis and Gareis 2007).

As discussed earlier, the exudation droplets may also serve as a kind of water (Jennings 1991) or nutrient reservoir (Colotelo 1978; McPhee and Colotelo 1977; Sprecher 1959). Thus, the competition of fungal colonies against each other possibly triggers the building-up of reserves in terms of exudates.

The *S. chlorohalonata* isolates seemed to produce more exudate than *S. chartarum* (Table 3). On 14-cm diameter plates and three-point inoculation, the collected amount of guttation droplets of *S. chlorohalonata* ranged between 90

Table 4 Results of the MTT test, ELISA and LC-MS/MS analyses of the guttation droplets collected from isolates of *Stachybotrys* spp. ($n=15$)

Isolate no.	<i>Stachybotrys</i> spp.	MTT-cytotoxicity test IC ₅₀ value (μ l exudate/ml)	ELISA Roridin A-equivalent (ng/ml exudate)	LC-MS/MS (ng toxin/ml of exudate)							
				SG	SH	SF ^a	RA	RE ^b	RL-2 ^b	VA	VJ ^c
S1340	<i>S. chartarum</i> chemotype S	5.0	4,600	180	280	80	–	230	60	–	20
S1343		10.0	5,700	510	370	70	–	370	70	–	25
S1468		5.0	8,890	–	2,390	–	–	440	350	–	50
S48OW		1.25	393,900	7,160	4,610	690	–	740	850	–	70
S1248		10.0	13,900	–	2,960	–	–	320	270	–	30
S1380	<i>S. chartarum</i> chemotype A	0	40	–	–	–	–	–	–	–	–
S1470		0	30	–	–	–	–	–	–	–	–
S1695		0	45	–	–	–	–	–	–	–	–
S1091		n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
S1637		0	30	–	–	–	–	–	–	–	–
SM6-2	<i>S. chlorohalonata</i>	0	15	–	–	–	–	–	–	–	–
SM10-2		0	20	–	–	–	–	–	–	–	–
S6		0	5.0	–	–	–	–	–	–	–	–
S23St		0	30	–	–	–	–	–	–	–	–
H47C		0	15	–	–	–	–	–	–	–	–

IC₅₀ value inhibitory concentration 50 = toxin concentration which reduces the MTT cleavage activity by 50 % compared with the control cells; n.e. no formation of exudate; – not detected (LOD: SG=2.3 ng/ml, SH=4.1 ng/ml, RA=3.4 ng/ml, VA=2.5 ng/ml); SG satratoxin G, SH satratoxin H, SF satratoxin F, RA roridin A, RE roridin E, RL-2 roridin L-2, VA verrucaric acid, VJ verrucaric acid

^a Quantified as equivalents of satratoxin G

^b Quantified as equivalents of roridin A

^c Quantified as equivalents of verrucaric acid

and 351 μl , whereas the *S. chartarum* species produced 0–67 μl . However, a differentiation between the two chemotypes of *S. chartarum* was not unequivocally possible. Additionally, the number of examined isolates was too small to be able to infer any regularity.

Cytotoxicity and presence of macrocyclic trichothecenes

The collected exudates ($n=15$) were subjected to three independent (bio)analytical techniques for examination of their toxicity and of the occurrence of macrocyclic trichothecenes. The results of the effect-based bioassay (MTT test) revealed that only the exudates of the five *S. chartarum* chemotype S isolates caused cytotoxic effects. The IC_{50} values ranged from 10 μl exudate/ml medium (isolates S1343 and S1248) to 1.25 $\mu\text{l}/\text{ml}$ medium (isolate S48OW; Table 4), i.e. the cytotoxicity of the samples was still recorded down to an exudates'

dilution of 1:16. This was a clear hint for the presence of macrocyclic trichothecenes, as the test system is especially sensitive to such cytotoxic mycotoxins (Gareis 2006; Hanelt et al. 1994; Johanning et al. 1998).

The ELISA results reflected the findings of the cell culture test. Only the exudates of the five *S. chartarum* chemotype S isolates contained macrocyclic trichothecenes (Table 4). The content of roridin A equivalents in exudates of the isolates S1340, S1343, S1468, and S1248 ranged between 4.6 and 13.9 $\mu\text{g}/\text{ml}$. In the isolate S48OW, a much higher concentration of 393.9 $\mu\text{g}/\text{ml}$ was determined, which was in line with the result of the cytotoxicity measurement by the effect-based bioassay. The low values which were determined in the other isolates (5.0–45 ng/ml) may have been caused by non-specific matrix effects in the ELISA test system.

To get a deeper insight in the identity of the trichothecene compounds contained in the droplets and for confirmation of

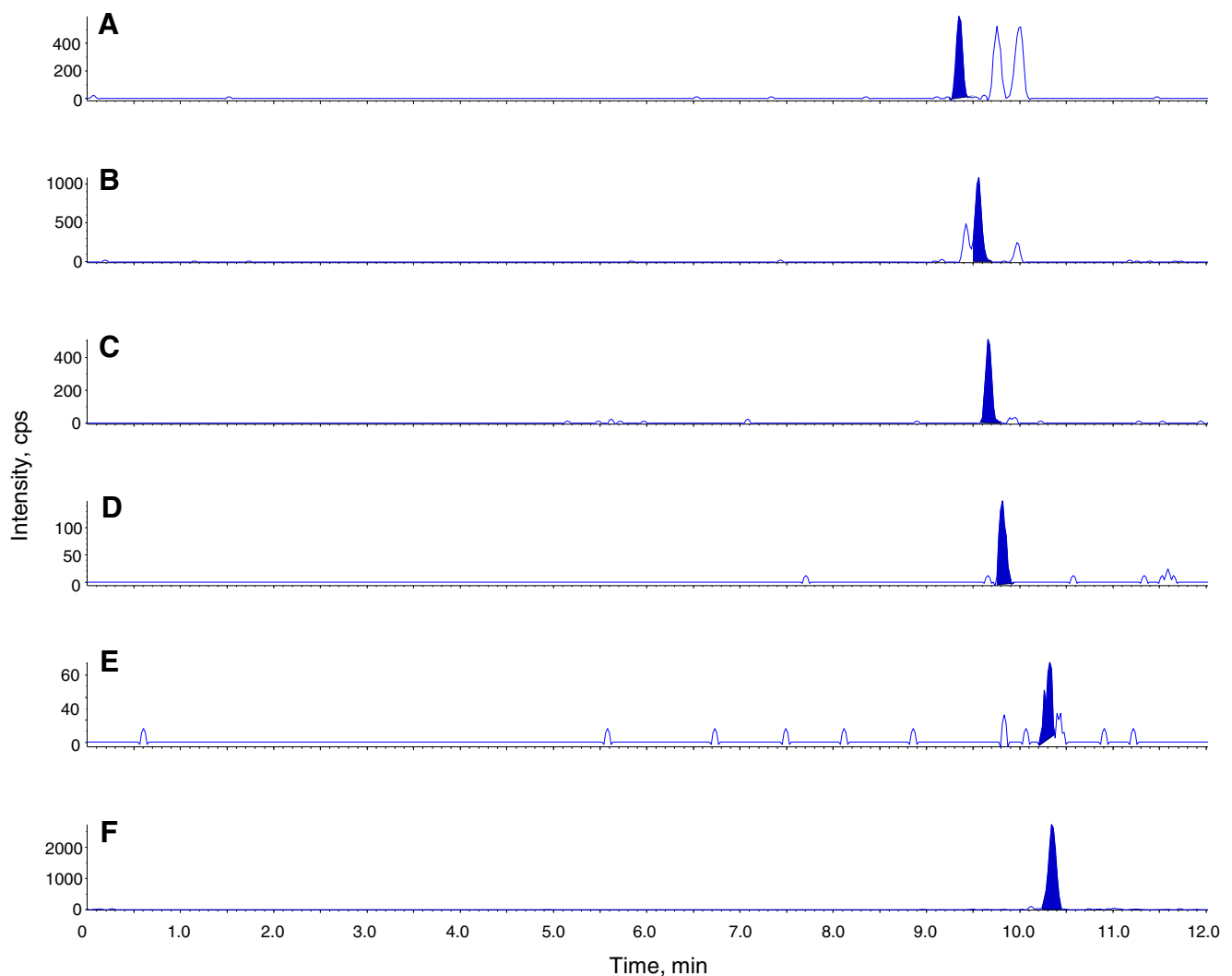


Fig. 3 LC-MS/MS extracted ion chromatogram of exudate (1:10) of *Stachybotrys chartarum* chemotype S isolate S1343: **a** roridin L-2, retention time (RT)=9.4 min; **b** saratroxin G, RT=9.6 min; **c** saratroxin H,

RT=9.7 min; d saratroxin F, RT=9.8 min; **e** verrucarin J, RT=10.3 min; **f** roridin E, RT=10.4 min

previous results, the samples were also analysed for eight macrocyclic trichothecenes (satratoxin G, H and F, roridin A, E, L-2, verrucarins A and J) by LC-MS/MS (Table 4). At this, roridin A and verrucarins A served only for semiquantitative determination of roridin E and L-2, as well as verrucarins J, for which no reference standards are available. These two compounds themselves are not produced by *Stachybotrys* spp. Also the results of satratoxin F were only semiquantitative, expressed as equivalents of satratoxin G. Therefore, results of analytical measurements remain estimative to some extent unless certified reference materials for macrocyclic trichothecenes become commercially available. However, all exudates of *S. chartarum* chemotype S showed typical patterns of up to six macrocyclic trichothecenes (Table 4, Fig. 3), which are known from previous studies (Andersen et al. 2003; Gottschalk et al. 2006; Hinkley and Jarvis 2001). The levels of satratoxin G ranged between 180 ng/ml exudate and 7,160 ng/ml; the isolates S1468 and S1248, however, did not produce satratoxins G and F. The satratoxin H levels were between 280 and 4,610 ng/ml exudate in all five *S. chartarum* chemotype S isolates, in which roridin E, roridin L-2 and verrucarins J always occurred simultaneously. None of the *S. chlorohalonata* and the *S. chartarum* chemotype A isolates showed any cytotoxicity or a presence of macrocyclic trichothecenes in their exudate (Table 4).

The reasons for the production of toxic guttation droplets are not yet clarified. As mentioned before it is most probable that the active excretion of mycotoxins maybe a consequence of the fungal organisms competing against each other and other organisms for nutrient resources. Another hypothesis is that the fungus excreted its toxins into the guttation droplets to regulate their concentration which aims—at certain levels—in getting rid of these compounds to avoid self-poisoning. The droplets may have—like for water and nutrients (Colotelo 1978; Jennings 1991; McPhee and Colotelo 1977)—a kind of storage function. The fact that exudate amounts per colony were higher when more than one colony grew on a plate (Table 3), however, supports the hypothesis that guttation might be part of a defence strategy against competitors, at least under in vitro conditions.

Whether toxic guttation droplets are produced also under in vivo conditions remains putative. In any case, regarding the involvement of *Stachybotrys* in adverse health effects in indoor environments, its ability to produce toxic droplets deserves special attention. Many studies have reported the presence of macrocyclic trichothecenes in mouldy materials such as wallpapers and gypsum boards (Gottschalk et al. 2006; Johannings et al. 1998; Nielsen et al. 1999) and some authors even described their detection in dusts and indoor air (Bloom et al. 2009; Brasel et al. 2005; Gottschalk et al. 2008; Sorenson et al. 1987; Wady and Larsson 2005). However, it was questionable by which route the toxins actually could be transferred into the environment. If they are excreted in

guttation fluids, it is conceivable that they are easily released into the environment or air in terms of aerosols or bound to small dust particles. This will be particularly favoured by ventilation or air-conditioning systems, which are known to be a mould reservoir and responsible for the distribution of conidia and mycotoxins (Ahearn et al. 2004; Kuhn and Ghannoum 2003; Mahmoudi and Gershwin 2000).

Finally, all results obtained with the three different methods are in good accordance regarding variations in biological test systems compared with physicochemical analyses.

Conclusions

Our results revealed for the first time that *Stachybotrys* spp. were able to produce guttation droplets, which, in the case of toxigenic species, contained their highly toxic metabolites, the macrocyclic trichothecenes. These findings most likely contribute to the toxic potential of *Stachybotrys chartarum* and also may have an impact on the assessment of risks arising from inhalative exposure. However, the reason for the active segregation of the toxins into the guttation fluids remains speculative, as well as the question of whether the exudates are actually produced under in vivo conditions in mouldy indoor environments. This will be part of further research on this topic.

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Conflicts of interest The authors declare that there are no conflicts of interest.

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