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ORIGINAL ARTICLE

# *Penicillium expansum* strain isolated from indoor building material was able to grow on gypsum board and emitted guttation droplets containing chaetoglobosins and communesins A, B and D

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**Keywords**
Cytotoxicity, fungal contamination, mycotoxins, *Penicillium*, toxins.

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## Abstract

**Aims:** Emission of toxic metabolites in guttation droplets of common indoor fungi is not well documented. The aims of this study were (i) to compare mycotoxins in biomass and guttation droplets from indoor fungi from a building following health complaints among occupants, (ii) to identify the most toxic strain and to test if mycotoxins in guttation liquids migrated trough air and (iii) to test if toxigenic *Penicillium expansum* strains grew on gypsum board.

**Methods and Results:** Biomass suspensions and guttation droplets from individual fungal colonies representing *Aspergillus*, *Chaetomium*, *Penicillium*, *Stachybotrys* and *Paecilomyces* were screened toxic to mammalian cells. The most toxic strain, RcP61 (CBS 145620), was identified as *Pen. expansum* Link by sequence analysis of the ITS region and a calmodulin gene fragment, and confirmed by the Westerdijk Institute based on ITS and beta-tubulin sequences. The strain was isolated from a cork liner, was able to grow on gypsum board and to produce toxic substances in biomass extracts and guttation droplets inhibiting proliferation of somatic cells (PK-15, MNA, FL) in up to 20 000-fold dilutions. Toxic compounds in biomass extracts and/or guttation droplets were determined by HPLC and LC-MS. Strain RcP61 produced communesins A, B and D, and chaetoglobosins in guttation droplets (the liquid emitted from them) and biomass extracts. The toxins of the guttation droplets migrated *c.* 1 cm through air and condensed on a cool surface.

**Conclusions:** The mycotoxin-containing guttation liquids emitted by *Pen. expansum* grown on laboratory medium exhibited airborne migration and were >100 times more toxic in bioassays than guttation droplets produced by indoor isolates of the genera *Aspergillus*, *Chaetomium*, *Stachybotrys* and *Paecilomyces*.

**Significance and Impact of the Study:** Toxic exudates produced by *Pen. expansum* containing communesins A, B and D, and chaetoglobosins were transferable by air. This may represent a novel mechanism of mycotoxin dispersal in indoor environment.

## Introduction

*Penicillium expansum* is a ubiquitous filamentous fungus causing the serious postharvest disease known as blue mould in harvested apples, peaches and hazels (Julca *et al.* 2015). It is known to produce the potent mycotoxins communesins, chaetoglobosins and patulin when growing on fruits (Andersen *et al.* 2004; Nielsen *et al.* 2004; Tannous *et al.* 2016). As *Penicillium expansum* results in fruit losses and is a public health issue (Tannous *et al.* 2016), its ecology as a fruit pathogen and its mycotoxin production on fruits have been extensively studied. On artificially contaminated apples, the optimal conditions for growth have been predicted to occur near 25°C, pH 5·1 and at a high water activity ($a_w$) value of 0·99. Growth of *Pen. expansum* strains occurred between 4–30°C and optimal patulin production was recorded at 16°C (Tannous *et al.* 2016).

It is likely that any cellulolytic, saprophytic or biodeteriogenic fungi can grow in indoor environment if finding suitable substrate and moisture (Li *et al.* 2015). *Penicillium expansum* has been shown to produce cellulolytic enzymes as β-glucanase, endoglucanases, cellobiohydrolases and β-glucosidase and has been isolated from surface-sterilized timber and deteriorating cedar wood in historical buildings (Duncan *et al.* 2006; Zayne *et al.* 2009). This indicates that *Pen. expansum* may be able to colonize indoor building materials in addition to fruits. However, growth of *Pen. expansum* on modern building materials and toxin production by indoor isolates identified by modern molecular methods as *Pen. expansum* have not been documented.

Moisture and indoor growth of ascomycetous fungi, considered together, are positively associated with respiratory illness according to studies performed in many geographical regions (Korkalainen *et al.* 2017; Mendell and Kumagai 2017; Caillaud *et al.* 2018; Tähtinen *et al.* 2019). Irrespective of definition, moisture and mould damage are internationally common and are estimated to occur in 18–50% of buildings (Mendell *et al.* 2011; Norbäck and Miller 2013). Moisture or dampness and mould damage are found in 2·5–26% of Finnish buildings, being most prevalent (12–26%) in public educational buildings and healthcare facilities (Annila *et al.* 2017).

Growth of filamentous fungi indoor depends on moisture enabling cell division, mycelial growth, sporulation, formation of membrane-surrounded liquid-containing organelles (vesicles, vacuoles and peroxisomes), synthesis of secondary metabolites and emission of volatile organic compounds (Kenne *et al.* 2014; Kistler and Broz 2015; Bennet and Inamdar 2015). Mould odour has been connected to unhealthy indoor air and is a possible indicator of active fungal growth (Mendell and Kumagai 2017). However, no guidelines for unhealthy levels of indoor mould exposure have been defined (Bennet and Inamdar 2015; Hurraß *et al.* 2016).

Biologically active fungal secondary metabolites may be toxic to the producer organism, transported to the cell surface and liberated to the exterior by membrane-surrounded organelles like vacuoles and vesicles (Kenne *et al.* 2014; Kistler and Broz 2015; Bennet and Inamdar 2015). Compared to conidia and hyphal fragments, membrane-surrounded organelles of indoor fungi have gained little attention and their impact on indoor air quality is not understood yet. Also, occurrence of *Pen. expansum* isolates in indoor environments has been of less concern compared to other indoor *Penicillium* species such as *Pen. chrysogenum*, and the toxigenic *Aspergillus* species (Nielsen 2003).

In our preliminary study we found that isolates of *Pen. expansum* are able to produce chaetoglobosins and communesins, which are secreted in membrane-surrounded vesicles and liberated as liquid exudates (Salo *et al.* 2015). Recently we showed that indoor *Trichoderma* strains produce toxic vesicles and guttation droplets containing peptaibols (Castagnoli *et al.* 2018b). The reported connection between exposure to toxins and weakened immune tolerance (Genius 2010; Tuuminen and Lohi 2019), as well as our preliminary findings aroused our interest in microbial toxigenesis and vesicle formation and guttation in indoor environments. In this study we investigated toxicities in vesicles and exudates from indoor *Penicillium* and *Aspergillus* strains properly identified by molecular methods.

## Materials and methods

### Sampling and microbiological protocols

Rooms in a large, mechanically ventilated office building (200 rooms) associated with severe adverse health effects of several occupants were investigated. The building has a concrete frame, mineral wool and cork board isolation and a tile façade. Office B23 was on the 1st floor, C35 on the 2nd and others on the ground floor of a building erected in 1959–1967 and renovated in 1997–2000. For cultivation, the samples (Table 1) were grown on 2% malt extract agar (35 ml per plate, Ø 9 cm); sealed with gas-permeable adhesive tape to slow moisture loss during the 2–4 weeks culturing at a relative humidity (RH) of 30–40% and a temperature of 22–24°C.

Fungal isolates of the *Aspergillus niger* complex, *Pen. expansum* MH 6, *Chaetomium* sp. MH52 and *Trichoderma* sp. were identified by microscopy (Samson *et al.* 2002), fluorescence emission of biomass suspensions, toxicity profiles in three toxicity assays and comparison to

 

**Table 1** Moulds from a university office building where several occupants reported severe, building-related adverse health symptoms

| Taxon found | Offices | Type of sampling |
|---|---|---|
| *Acrostalagmus luteoalbus*[a] | A46 | Cork insulation[bd] |
| *Acrostalagmus* sp.[a] | *A31b*[a] | Swab |
| *Aspergillus calidoustus*[a] | *A31b*[a] | Swab[bc] |
| *Aspergillus niger*[a] | *A45b*[a] | Swab |
| *Aspergillus versicolor*[a] | *A31b*[a], *C35*[a] | Swab, cork, dust |
| *Aspergillus westerdijkiae*[a] | *C35*[a] | Dust, mineral wool insulation |
| *Chaetomium globosum*[a] | *A31b*[a], *A45b*[a] | Swab, fallout |
| *Penicillium expansum*[a] | *A31b*[a], *A45b*[a] | Swab[bb], cork[ba], fallout, impactor |
| *Penicillium* sp.[a] | *A31a*[a], A34 | Swab |
| *Rhizopus* sp.[b] | A34, B23 | Swab |
| *Trichoderma* sp.[a] | *C35*[a] | Dust, mineral wool insulation |

Offices associated with building-related health complaints are marked in italics.

[a]Indicates the sample from which the strains were isolated. a*Pen. expansum* RcP61, b*Pen. expansum* MH6; c*Asp. calidoustus* MH34; d*Acr. luteoalbus* P0b8.

[b]Indicates that the majority (>60%) of the isolates/office produced toxic metabolites.

[c]Plates overgrown with *Rhizopus* may have contained other species.

strains identified by DSMZ (Deutche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany), and deposited in the culture collection HAMBI (University of Helsinki, www.helsinki.fi/hambi). The reference strains *Aspergillus versicolor* GAS226, *Aspergillus westerdijkiae* PP2, *Paecilomyces variotii* Paec 2 and *Pen. expansum* SE1 were identified to species level by DSMZ and deposited in the HAMBI culture collection.

Strains *Acrostalagmus luteoalbus* P0b8, *Aspergillus calidoustus* MH34, *Pen. expansum* RcP61 and SE1, as well as *Pen. chrysogenum* RUK2/3 were identified by sequence analysis of the ITS region (Andersson *et al.* 2009) and a calmodulin gene fragment (Hong *et al.* 2006; Pildain *et al.* 2008). Sequences were deposited in the GenBank Nucleotide database (http://www.ncbi.nlm.nih.gov) under the following accession numbers: *Acr. luteoalbus* P0b8: KM853014; *Asp. calidoustus* MH34: KM853016; *Pen. expansum* RcP61: KP889005, MK201596, *Pen. expansum* SE1: MK217414, MK201595, *Pen. chrysogenum* RUK2/3: KM853015, MK217415. These strains were deposited both in the Szeged Microbiology Collection (http://www.szmc.hu) in Hungary and the HAMBI (www.helsinki.fi/hambi/collection) in Finland.

In addition, the identity of the strain *Pen. expansum* RcP61 was confirmed by the Westerdijk Institute based on ITS and beta-tubulin sequences as *Pen. expansum* Link, and the strain was deposited in the Westerdijk Institute strain collection under the accession number CBS 145620.

### Analytical procedures

For initial toxicity screening, a loop (10 µl) containing 10–20 mg biomass (wet weight) of each colony on the primary culture plates was tested for toxicity. The fungal biomass was dispersed into 0·2 ml of ethanol, the vial sealed and incubated in a water bath for 10 min at 60 °C. The obtained ethanolic lysates were used to expose porcine spermatozoa (obtained from an artificial insemination station) and kidney tubular epithelial cells (PK-15). The lysate was considered toxic when 2·5 vol% (boar sperm) or 5 vol% (PK-15) of the lysate inhibited target cell functions: motility (sperm, within 30 min or 1 day) and proliferation (PK-15, 2 days) (Castagnoli *et al.* 2018a). The colonies that displayed toxicity were streaked pure and identified to genus or species level.

Exudate droplets harvested from mycelial surfaces with micropipettes and diluted (step = 2) in ethanol were incubated at 60 °C for 10 min and then serially diluted (step = 2) for toxicity testing. The *in vitro* toxicity test was performed using porcine cells (sperms, somatic cell lines) as indicators according to Bencsik *et al.* (2014) and Ajao *et al.* (2015). Fluorescence emission of the fungal secondary metabolites was photographed and illuminated at 360 nm. Mycotoxins were identified using LC-MS methods (Mikkola *et al.* 2012; Mikkola *et al.* 2015).

Toxicity tests of ethanol-soluble substances extracted from plate-grown biomass and the identification of the toxins by LC-MS were described previously (Mikkola *et al.* 2012; Mikkola *et al.* 2015; Castagnoli *et al.* 2018a).

### Other protocols

Translocation of fungal metabolites by water vapour from one surface to another was measured using an experimental setup as shown and explained in Fig. 1.

To cultivate fungi, moisturized gypsum boards, 100 $cm^2$, thickness of 12 mm, were seeded with spores from a 20-day-old plate culture suspended in 0·1 vol% Tween 80. The seeded board was incubated in a steel chamber (12·5 l) and sealed with a glass lid at RH 95% and 20 ± 1 °C. Biomass (2 mg) was scraped with a microscopic slide from the surface of the gypsum liner and dispersed in 40 µl ethanol, incubated at 60 °C for 10 min and then tested for toxicity to PK-15 cells using inhibition of proliferation as endpoint and applying a fluorometric readout confirmed with microscopic examination as described in Bencsik *et al.* (2014). Concentrations of conidia (2 µm) and hyphal fragments (larger than 0·1 µm) in biomass lysates and guttation droplets were

 

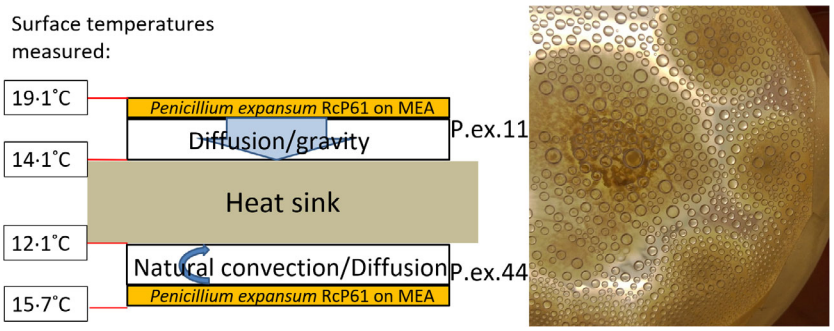


**Figure 1** Experimental set-up to study transit of toxicity of guttation droplets through air. Two malt agar plates were inoculated with *Penicillium expansum* RcP61 and sealed with adhesive tape to prevent drying out. A thermostatically controlled cooled steel plate was sandwiched between the lids of the culture plates, with the lid facing down (top plate) and lid facing up (bottom plate). The measured surface temperatures are shown. The right panel shows the droplets condensing in 14 days on the inner surface of the lid facing up. Condensates on both lids were harvested and analysed using LC-MS (Fig. 3, Table 4).

calculated as the average from 10 microscopic fields by phase contrast microscope (Olympus CKX41 Tokyo Japan) with 400 × magnification.

### HPLC-mass spectrometry analysis

HPLC-electrospray ionization ion trap mass spectrometry analysis (ESI-IT-MS) was performed using an MSD-Trap-XCT plus ion trap mass spectrometer equipped with an Agilent ESI source and Agilent 1100 series LC (Agilent Technologies, Wilmington, Del., USA) in positive mode with the mass range of *m/z* 50–2000. The column used was a SunFire C18, 2·1 × 50 mm, 2·5 µm (Waters, Milford, MA, USA). Separation of the compounds from exudate droplets and condensates of *Pen. expansum* RcP61 was performed using an isocratic method of solution A: $H_2O$ with 0·1% (v/v) formic acid and B: methanol in a ratio of 40/60 (v/v) for 15 min and a subsequent gradient of 100% B for 30 min at a flow rate of 0·2 ml $min^{-1}$.

### Chemicals and suppliers

Boar semen, 27 × $10^6$ sperms per ml in MRA extender, was purchased from Figen Ltd., Tuomikylä, Finland. The porcine kidney (PK-15), murine neuroblastoma (MNA) and feline lung (FFL) cells retrieved from EVIRA (Echard 1974; Andersson *et al.* 2009) were grown in a tissue culture facility as described in detail by Ajao *et al.* (2015). Malt extract agar media contained 15 g malt extract (Sharlab, Barcelona, Spain) and 12 g agar (Amresco, Dallas, USA) in 500 ml of $H_2O$. Tween 80 was from Sigma Aldrich, St Louis, Missouri, USA.

The UV illuminator was from UVA Finland Ltd., Kauniainen. The toxic fungal droplets were photographed using a Dino-Lite microscopic loupe (Taiwan), magnification 200 ×, connected by USB to a laptop computer.

## Results

### Toxic droplets emitted by the indoor fungus *Penicillium expansum*

Indoor dust and materials were collected from offices where occupants had complained of severe, building-associated health symptoms. Among the biomass suspensions of the 122 colonies from primary culture plates seeded with samples from the affected offices, 63–100% were toxic *ex vivo* towards porcine sperm cells and *in vitro* towards somatic PK-15, FFL and MNA cell lines (Table 1). The fungi corresponding to the toxic biomass suspensions found in the screening procedure were identified as *Pen. expansum*, *Acr. luteoalbus, Asp. calidoustus, Asp. niger, Asp. versicolor, Asp. westerdijkiae, Trichoderma* sp. and *Chaetomium* sp. (Table 1). Guttation droplets produced by the single colonies were screened for toxicity: toxic droplets were produced by *Acrostalagmus* sp., *Trichoderma* sp., *Chaetomium* sp. and the *Pen. expansum* isolate. For the *Pen. expansum* RcP61 isolate, concentrations of conidia and toxicity endpoints, as $EC_{100}$ against PK-15 cells, between biomass lysate and guttation liquid were compared. Biomass lysate and guttation liquid contained 2 × $10^7$ and 1 × $10^4$ conidia per ml, respectively, the toxicity titres were 160 and 640 respectively. The guttation droplets containing 1000 times less conidia were more toxic than the biomass lysate. Particles classified as hyphal fragments were detected in the biomass dispersal at an estimated concentration below $10^4$ particles per ml. Hyphal fragments were not detected in the guttation liquid (<1 particle in 10 microscopic fields). This indicated that the liquid of the guttation droplet contained toxic substances.

Toxin-producing *Pen. expansum* grew from cork insulation boards sampled from holes bored inside the walls

of rooms and settled dust from the offices named A31a, A31b, A45b (Table 1) sharing the health problem and the building history. A marked finding was that the colonies of *Pen. expansum* on plates seeded from samples from the offices associated with serious health concerns extruded amber-coloured guttation droplets of viscous liquid from the mycelium. The droplets emitted blue fluorescence under UV light.

These droplets emitting blue fluorescence were collected from surfaces of the cultured biomass and from the lids of the Petri dishes (Fig. 2). Interestingly the droplets proved to be highly toxic to porcine spermatozoa as well as PK-15, FFL and MNA cells. A pooled vesicle harvest (dry weight content of 8–9 mg $ml^{-1}$) was cytotoxic towards each of the three somatic cells up to dilutions of 5000–20 000-fold (Table 2). Motility of spermatozoa was lost by exposure to approximately 1 µl of the vesicle liquid of 50% of 27 × $10^6$ spermatozoa within 1 h, indicating that the vesicles contained compounds exerting immediate toxic action.

### Identification of the toxins in guttation droplets produced by *Penicillium expansum*

Considering the high toxicity of *Pen. expansum* vesicles towards mammalian cells (Table 2) and the scarcity of information about indoor isolates of this species, guttation droplets were analysed using HPLC-MS. Guttation droplets produced by indoor isolates from other buildings; *Asp. versicolor, Asp. calidoustus, Asp. westerdijkiae, Chaetomium* sp., *Pae. variotii, Pen. expansum*, *Pen. chrysogenum* and *Stachybotrys* sp. were analysed for reference. It is evident from Table 2, that the vesicles emitted by *Pen. expansum* RcP61 contained chaetoglobosins and communesins A, B and D (Table 3). The main mycotoxin identified in the droplet liquid was chaetoglobosin, representing 5·6% of the total ion intensity.

Interestingly, ethanol extracts from the biomass and vesicles of *Pen. expansum* RcP61 contained the same mycotoxins, chaetoglobosins and communesins A, B and D. Biomass extracts of *Asp.* sp. K20 and *Asp. calidoustus* MH34 were very toxic to the cells tested and contained mycotoxins (sterigmatocystin, ophiobolins H and K) (Table 3), but the contents of the guttation vesicles were not toxic to the tested cells (Table 2). Also, the guttation droplets from chaetoglobosin-producing *Chaetomium* sp. MH1, and melinacidin-producing *Acr. luteoalbus* POB8 exhibited very low toxicities compared to *Pen. expansum* RcP61 (Table 2).

### Mycotoxin-containing liquids are generated on hyphal surfaces and mobilize into the air

To test whether the toxin contents of *Pen. expansum* guttation droplets would mobilize from a mouldy surface into the air, we set-up a system (Figs 1 and 2) where the aerosolization of toxic exudates from a culture plate of *Pen. expansum* RcP61 was detected across a column of air. To generate natural air convection and to condense the humidity, the lids of the culture plates were set a few degrees cooler than the culture plates themselves (Fig. 1). To distinguish between the toxic droplets' translocation driven by natural air convection and diffusion driven by gravity, one culture plate was placed with its lid facing upwards and the other with its lid facing downwards.

After 8 days, the liquids condensed on the lids were decanted and subjected to mass spectrometric analysis. Boar sperm-toxic exudate droplets and condensates of *Pen. expansum* RcP61 upwards (P.ex.44) was analysed using HPLC-UV and electrospray ionization mass spectrometry (ESI-MS). Peak 1 (4·6 min) of *Pen. expansum* RcP61 exudate droplet (Fig. 3) contained protonated mass ion $[M + H]^+$ at *m/z* 457·5 of communesin A. Peak 2 (17·0 min) contained protonated mass ion $[M + H]^+$ at

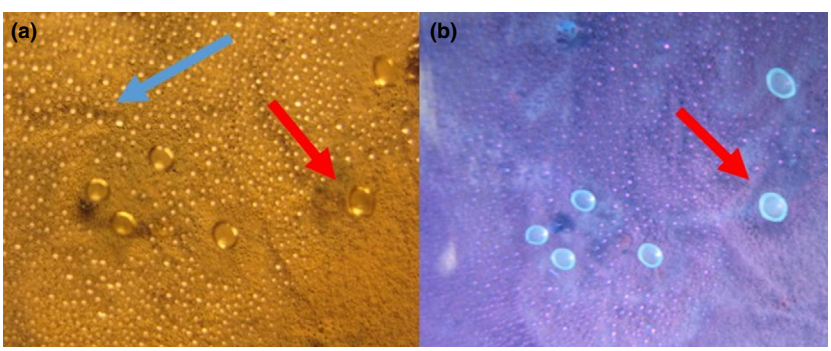


**Figure 2** Photographs of condensates on the lid of a Petri dish containing a 4-week-old culture of *Penicillium expansum* RcP61 inspected under visible light (a) and UV-light (b). Droplets emitting blue fluorescence (red arrows) were toxic in >100× dilutions compared with the non-fluorescent droplets (blue arrow, panel a) to boar sperm and porcine kidney cells PK-15.

 

**Table 2** Mammalian cell toxicities of vesicles emitted by indoor moulds isolated from offices listed in Table 1, associated with severe building-related ill health of the occupants, and of reference substances

| Isolate and culture age | Indicator cell and exposure time | | | |
|---|---|---|---|---|
| | Porcine sperm cell, 1 h | Porcine kidney, PK15, 2 days | Feline lung, FFL, 2 days | Murine neural, MNA 2 days |
| | Toxicity end-point | | | |
| | Motility inhibition | Inhibition of proliferation | | |
| *Penicillium expansum* RcP61[e] vesicle | Highest dilution causing maximal toxic effect, × | | | |
| | 1000× | ≥20 480× | ≥10 240× | 5120× |
| | Exposure concentration $EC_{50}$ µl vesicle liquid $ml^{-1}$ ($EC_{100}$, µg dry wt. $ml^{-1}$)[f] | | | |
| *Pen. expansum* RcP61 7 days | 2·5 | 0·08 | 0·08 | 0·16 |
| *Pen. expansum* RcP61 22 days | 2·5 (21) | 0·04 (≤0·4) | 0·08 (<0·8) | 0·16 (1·6) |
| *Pen. expansum* RcP61 35 days[e] | (42) | (1·6) | (1·6) | (1·6) |
| *Chaetomium* sp. MH52 22 days | | 50 | | 25 |
| *Acrostalagmus luteoalbus* POB8 14 days | 50 | 50 | | |
| *Aspergillus* sp. K20 13 days | >50 | >50 | >50 | >50 |
| *Asp. calidoustus* MH34 14 days | >50 | 50 | | >50 |
| Reference substances | | | | |
| *Asp. versicolor* GAS/226 14 days | >50 | >50 | >50 | >50 |
| *Asp. westerdijkiae* pp2 14 days | >50 | >50 | >50 | >50 |
| *Paecilomyces variotii* Paec 2 14 days | >50 | >50 | >50 | 5 |
| *Pen. chrysogenum* RUK2/3 exudate droplets 14 days | (160) | 50 (325) | 50 | 25 |
| *Pen. chrysogenum* RUK2/3 liquid squeezed from the mycelial biomass 14 days[g] | (210) | (210) | | |
| *Pen. expansum* SE1 | 2 | 0·1 | 0·1 | 2 |
| *Stachybotrys* sp. HJ5 14 days | >50 | 20 | 20 | |
| Triclosan (mitochondriotoxic reference) | (2) | (8) | (8) | (4) |

[a]Containing 86 µg $ml^{-1}$ of communesins A, B, D and 480 µg $ml^{-1}$ of chaetoglobosins.
[b]Numbers in brackets indicate dry weight of the liquid in the vesicle.
[c]22 days grown plate culture containing 4200 µg $ml^{-1}$ of meleagrin.

*m*/*z* 457·5, sodiated mass ion $[M + Na]^+$ at *m*/*z* 529·4, protonated mass ion at *m*/*z* 511·5 $[M + H\text{-}H_20]^+$ (representing loss of water from protonated mass ion in ESI source) and potassium adduct $[M + K]^+$ at *m*/*z* 567·3 of chaetoglobosin. Peak 3 (20·5 min) contained protonated mass ion $[M + H]^+$ at *m*/*z* 509·5 and sodiated mass ion $[M + Na]^+$ at *m*/*z* 531·5 of communesin B. Corresponding adduct ions of communesin A at *m*/*z* 457·5 (Fig. 3d), chaetoglobosin at *m*/*z* 511·5, 529·5, 550·4 and 567·4 (Fig. 3f) and communesin B at *m*/*z* 509·5 and 531·5 (Fig. 3g) in peaks 1 (4·4 min), 2 (17·0 min) and 3 (20·5 min), respectively, were found from a condensate of *Pen. expansum* RcP61.

The total ion chromatograms derived from HPLC-MS analysis of the exudate droplet (Fig. 3a) and condensate of *Pen. expansum* RcP61, experimental upwards set-up (P.ex.44) (Fig. 3a) were similar. Therefore, it was shown that toxic metabolites (chaetoglobosin and communesin A and B) of exudate droplets of *Pen. expansum* RcP61 are able to transfer by air. Similarly analysed, condensed water collected from experimental downwards set-up (P.ex.11) also showed that communesin A and chaetoglobosin were transferred by air.

It can be concluded, that the toxic contents of *Pen. expansum* RcP61 guttation droplets aerosolized and drifted through air (10 mm) both vertically upwards and downwards from the mycelial culture to the respective cooled lids.

Figure 4 shows micrographs of the guttation droplets, visualizing guttation droplet biomass (first left), trapped on the inner surface of the lid (second left), drying of the droplets (third left) and germination of the conidia (last right) on the lid of the Petri plate.

Gypsum board is a common indoor surface material of buildings in Finland. To test if *Pen. expansum* RcP61 produces droplets while growing on gypsum board, sections of moistened liner-covered gypsum board were seeded with

 

**Table 3** Toxicity and toxins identified from ethanol extracts from biomass of indoor moulds isolated from offices (Table 1) and of reference extracts

| | Indicator cell and exposure time | | | |
|---|---|---|---|---|
| | Porcine sperm cells 1 day | Porcine kidney cells, PK-15 2 days | Murine neural, MNA 2 days | |
| | Motility inhibition | Inhibition of proliferation | | |
| Taxon found | Exposure concentration $EC_{100}$, $\mu$g dry wt ml$^{-1}$ | | | Toxins identified |
| *Acrostalagmus luteoalbus* P0b8 | 10 | 10 | | Melinacidins II, III, IV |
| *Asp. calidoustus* MH4 | 1 | 1 | | Ophiobolins H, K |
| *Aspergillus* sp. K20 | 10 | 1 | 5 | Sterigmatocystin |
| *Chaetomium* sp. MH1 | 10 | 40 | 20 | Chaetoglobosin |
| *Pen. expansum* RcP61 | 1 | 0·7 | 0·7 | Communesins A, B, D and chaetoglobosin C |
| *Pen. expansum* MH6 | 1 | 0·8 | 1 | |
| Reference extracts | | | | |
| *Pen. expansum* SE1 | 1 | 1 | 0·5 | Communesins A, B, D and chaetoglobosin C |
| *Asp. versicolor* SL/3 | 10 | 1 | 5 | Sterigmatocystin, averufin |
| *Asp. westerdijkiae* pp2 | 5 | 15 | 15 | Stephacidin B, avrainvillamide ochratoxin A |
| *Paecilomyces variotii* Paec 2 | 5 | 500 | 500 | Viriditoxin |
| *Stachybotrys* sp. HJ5 | 20 | 5 | 5 | Not tested |
| *Penicillium* sp. TRP1 | 100[h] | 500[h] | 500[h] | None |
| *Trichoderma longibrachiatum* DSM768 | 100[h] | 500[h] | 500[h] | None[i] |

[a]Represented the nonspecific upper limit of the assays.
[b]Detection limit 0·01 mg ml$^{-1}$.

spore suspension, followed by incubation inside moistened climate chambers sealed with glass lids and rubber seals. After 6 days the gypsum boards appeared visibly mouldy, whereas after 10 days the guttation droplets were visible by naked eye (Fig. 5), having accumulated on the mouldy surface of the gypsum board, independently of whether the board was, or was not, autoclaved before inoculation. Biomass (2 mg) was collected from the surface of the gypsum liner (Fig. 5a) and the biomass lysates were found toxic to the PK-15 cells at a concentration of 0·5% (v/v).

### Toxins in guttation droplets of reference fungi from a culture collection of toxigenic fungi isolated from buildings

The currently studied office building had no major moisture-damage and was not visibly mouldy, however, the settled dust contained *Asp. versicolor*-like strains and *Asp. calidoustus*. These strains contained highly toxic sterigmatocystin and ophiobolins in their biomass extracts and produced visible guttation droplets (Table 3), but no toxicities were detected in the droplet liquids.

We also used the primary isolates of toxigenic fungi (*Pen. chrysogenum* RUK2/3 and *Pen. expansum* SE1, *Stachybotrys* sp. HJ5, *Asp. westerdijkiae* PP2, *Asp. versicolor* GAS226 and *Pae. variotii* Paec 2) isolated from indoor dusts and building materials, deposited in the HAMBI culture collection for testing toxicity and droplet formation (Table 2). Guttation droplets (dry weight content 6–8 mg ml$^{-1}$) were produced by isolate *Pen. chrysogenum* RUK2/3 and contained up to 4 mg ml$^{-1}$ of meleagrin. Despite the high concentration and substantial amount of the secondary metabolites (meleagrin) of the *Pen. chrysogenum* RUK2/3 guttation droplets, the toxic effects were modest: the droplets inhibited growth of PK-15 100–500-fold less effectively, whereas sperm motility and proliferation of feline pulmonary cells were 5–10-fold less inhibited (Table 2). The reference strain *Pen. expansum* SE1 produced chaetoglobosin and communesins A, B and D in the biomass and its guttation droplets were as toxic as those produced by the *Pen. expansum* strain

 

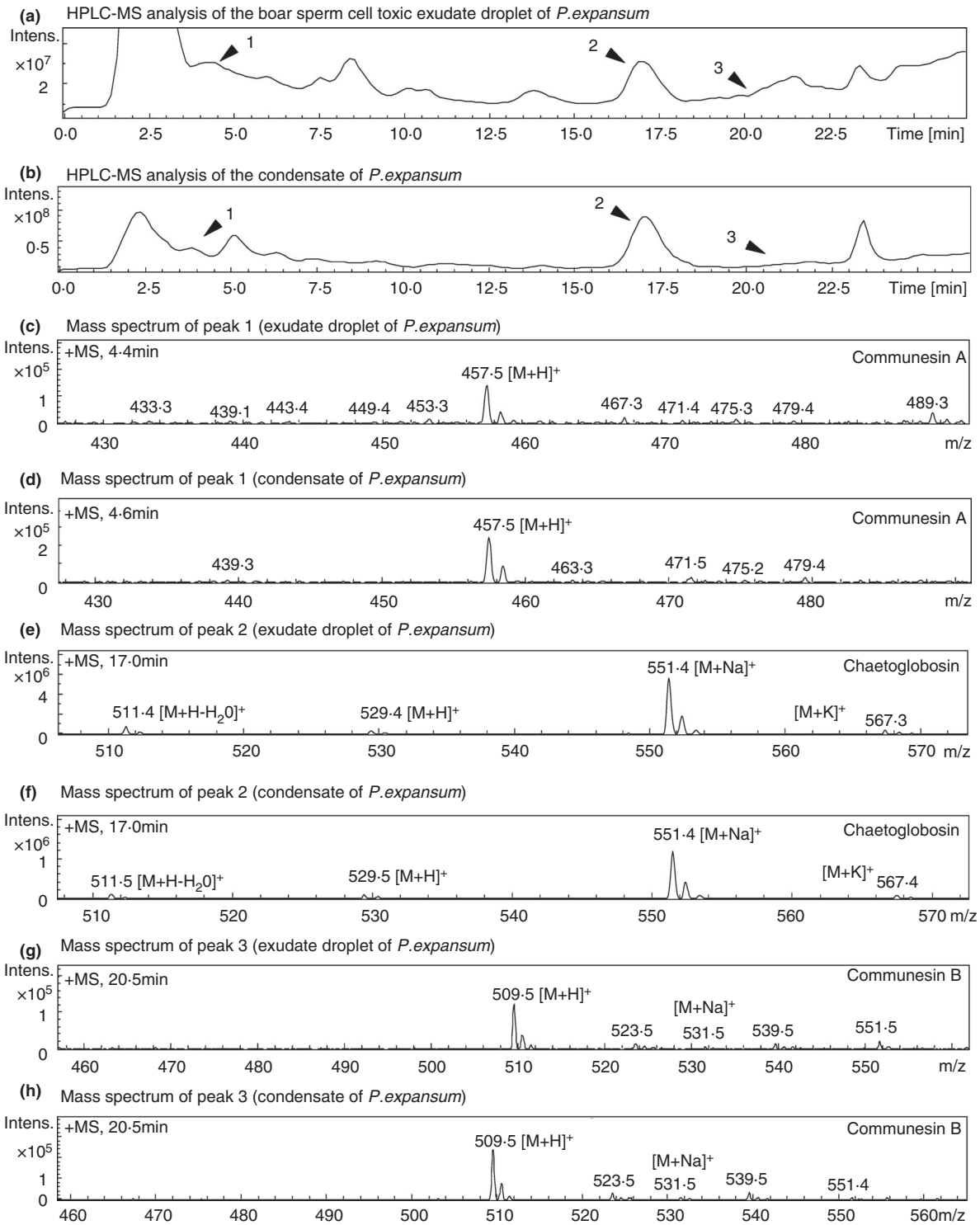


**Figure 3** Comparison of HPLC-ESI-MS analyses of the exudates in guttation droplets and vapour condensates of *Penicillium expansum* RcP61 from Fig. 2 (P.ex.44). HPLC chromatograms of the exudate droplets (a) and of the vapour condensates (b). The peaks 1, 2, 3 in panels a and b were identified as communesin A (peak 1), chaetoglobosin (peak 2) and communesin B (peak 3). Patulin was not measured. Molecular ion $[M + H]^+$ of peak 1 is *m/z* = 457·5 (c, d), of peak 2 is *m/z* = 551·4 (e, f) and of peak 3 is *m/z* 509·5 (g, h).

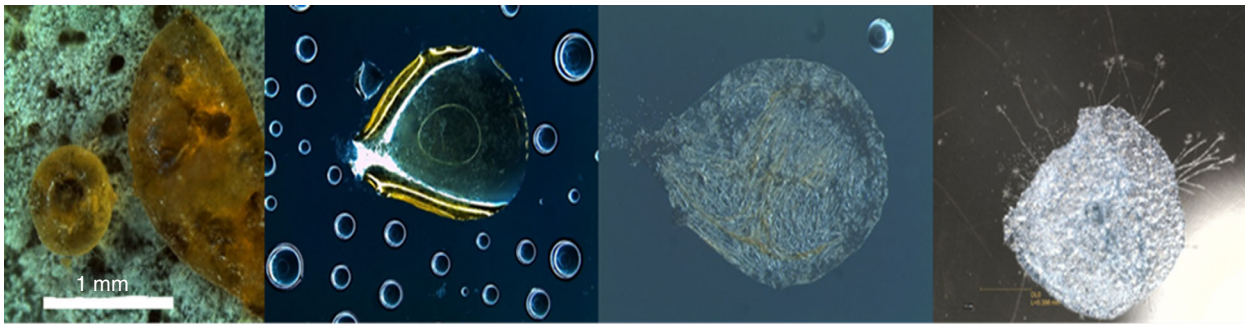


**Figure 4** Life-cycle of toxin-containing vesicles from *Penicillium expansum* RcP61. From left to right: a large, amber-coloured vesicle extrudes from the mycelial biomass of *Pen. expansum* RcP61. The vesicle meets air convection, becomes airborne, hits the polypropylene lid of the culture plate, among tiny droplets of airborne moisture (2nd from left). Air moisture is low, RH 30%, the droplet empties and desiccates (3rd from left). Nine days later, the vesicle has propagated a new generation of *Pen. expansum* conidiophores (last right).

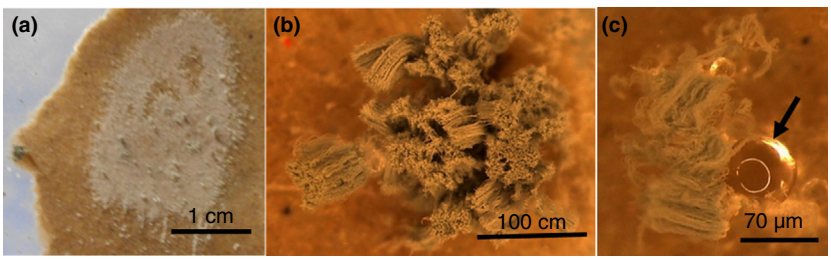


**Figure 5** *Penicillium expansum* RcP61 mycelium grown on gypsum board emits guttation droplets. (a) Visible mould growth, 3 weeks, on the gypsum plate; (b) stereo micrographs of the *Pen. expansum* conidiophores; (c) amber-coloured guttation droplet (arrow) extruding from the mycelial biomass grown on the gypsum board (a).

RcP61. The guttation droplets from other reference strains of toxigenic indoor isolates, viriditoxin-producing *Pae. variotii* Paec 2, and *Stachybotrys* sp. HJ5 producing yet unidentified toxins were 100 times less toxic than the guttation droplets of the *Pen. expansum* reference strains SE1 and *Pen. expansum* strain RcP61. Summarizing the results, we established that an indoor strain of *Pen. expansum* emitted substantial amounts of toxins in guttation droplets, furthermore, the toxins migrated aerially.

## Discussion

*Penicillium expansum* is known to produce some of the most potent mycotoxins within the genus *Penicillium*, the communesins and chaetoglobosins, which are produced when growing on fruits (Andersen *et al.* 2004; Nielsen *et al.* 2004). We report here for the first time that *Pen. expansum* strains isolated from building material and dust from an office associated with health complaints also produced communesins and chaetoglobosins. Furthermore, we demonstrated that these toxins migrated through the air (Figs 1 and 2, Table 4). Grown on laboratory medium, the *in vitro*- and *ex vivo*-measured specific toxicity of *Pen. expansum* emitted in guttation droplets was 100 times higher than those guttation droplets of toxic indoor isolates of *Aspergillus, Chaetomium, Acrostalagmus, Paecilomyces* and *Stachybotrys,* and is, to our knowledge, the highest among the indoor moulds reported to date. We also show that the strain produced guttation droplets when growing on gypsum liner, and that lysates of biomass grown on gypsum liner, containing guttation droplets and conidia were toxic *in vitro*. The toxicity value was five times lower than the threshold value for toxicity defined for the screening test for microbial biomass lysates.

The term guttation has long been known as a virulence mechanism of phytopathogenic and entomopathogenic micro-organisms (Hutwimmer *et al.* 2010; Singh and Singh 2013; Singh 2014).

Culture collection isolates of *Pen. verrucosum* and *Pen. nordicum* were reported to emit droplets containing 0·01–9 µg $ml^{-1}$ of ochratoxins A and B, which is 7–10-fold higher than the concentrations in the mycelial mass of the producer fungus (Gareis and Gareis 2007). For indoor fungi, toxic emission of guttation droplets have been hitherto sparsely reported. *Stachybotrys* sp. chemotype S culture collection strains originating from various indoor habitats were reported to produce droplets containing 3–5 µg $ml^{-1}$ satratoxin G and H per ml (Gareis

 

and Gottschalk 2014). Gareis and Gottschalk (2014) first observed that guttation droplets of *Stachybotrys chartarum* chemotype S contained roridins and satratoxins and produced low (0·2–0·4 ng $m^{-3}$) but measurable airborne concentrations of satratoxins G and H75.

It is impossible to directly predict *in vivo* mammalian toxicity based on *in vitro* toxicity results. In this study we used *in vitro* and *ex vivo* toxicity tests to compare toxicities of biomass extracts and guttation droplets produced by selected fungal isolates. Consistently high toxicity was obtained with a continuous lung cell line, FFL, a continuous kidney epithelial cell line, PK-15, a malignant cell line, MNA, as well as with an *ex vivo* assay using boar sperm for *Pen. expansum* strains RcP61 (CBS 145620) and SE1. Using bioassays in combination with chemical analysis we were able to identify the toxins as chaetoglobosins and communesins. The cork liner used as isolation material in the office building may have been the source of *Pen. expansum*. *Penicillium* spp. are reported as common contaminants in both moisture-damaged and non-damaged indoor spaces (Pasanen *et al.* 1992; Gravesen *et al.* 1995; Kaarakainen *et al.* 2009; Salonen 2009; Adan *et al.* 2011; Andersen *et al.* 2011; Nielsen and Frisvad 2011; Samson 2011), but little attention has been paid to *Pen. expansum* and emissions of chaetoglobosins and communesins in indoor air and their potential association with adverse health effects in moisture damaged buildings.

Using chicken tracheas, Piecková and Wilkins (2004) have demonstrated that indoor *Chaetomium* sp. produced very potent, ciliostatically active metabolites. Mycotoxins from indoor *Chaetomium globosum* strains and pure chaetoglobosin A from *C. globosum* also inhibited sperm motility by a sublethal ciliostatic mechanism, very likely by inhibiting sugar transport affecting glycolytic and mitochondrial energy production (Vicente-Carrillo *et al.* 2015; Castagnoli *et al.* 2017; Castagnoli *et al.* 2018b). The chaetoglobosin-containing guttation droplets inhibited sperm motility, possibly indicating ciliostatic activity. Thus, the risk of respiratory toxicity represented by airborne chaetoglobosin shown toxic to the primary lung cell line FFL and exhibiting ciliostatic activity is highly speculative, but cannot be excluded. The risk of airborne respiratory toxicity can be directly evidenced by *in vivo* experiments exposing laboratory animals to known airborne concentrations of chaetoglobosins and communesisns, which was out of scope for this article.

Chaetoglobosins known as cytochalasins (McMullin *et al.* 2013) exert their cytotoxicity by capping the growing end of actin microfilaments, thereby destroying the cytoskeleton of mammalian cells (Ueno 1985; Scherlach *et al.* 2010). Communesins are neurotoxic, insecticidal indole alkaloids (Hayashi *et al.* 2004; Kerzaon *et al.* 2009). Chaetoglobosin B was reported to be toxic to human erythrocyte membranes by competitively inhibiting glucose transport activity (Scherlach *et al.* 2010). Inhibition of glucose transport was also reported for chaetoglobosin A (Nielsen and Frisvad 2011). Thus, it is possible that the observed complete blocking of proliferation of the somatic cells PK-15, FFL and MNA (Table 2), as well as the motility inhibition of boar sperm by exposure to the *Pen. expansum* RcP61 vesicle fluid was caused by blocked glucose transport depriving the cells of energy.

This study is, to our knowledge, the first where a *Pen. expansum* strain, RcP61 (CBS 145620), growing on building material inside the construction in a building with reported health complaints, was shown to produce toxic guttation droplets on laboratory media and on gypsum liner. Toxin concentrations in guttation droplets emitted by *Pen. expansum* grown on laboratory medium, in the current work, appear 100–1000-fold higher than previously demonstrated from any fungus.

The weakened immune tolerance reported from urban environments caused by depleted and poor outdoor microbial diversity (Moore 2015; Schuijs *et al.* 2015; von Hertzen *et al.* 2015; Haahtela *et al.* 2015; Adams *et al.* 2016; Mhuireach *et al.* 2016; Stein *et al.* 2016; Li *et al.* 2018) may be attenuated by exposure to indoor microbes producing toxins inducing loss of tolerance (TILT; Miller 1997; Genius 2010). We were tempted to speculate that the absence of a diverse protective microbiome in combination with exposure to microbial TILT may be a potential factor to contribute to the diverse indoor air-related

**Table 4** The airborne transit of mycotoxins contained in exudate droplets of *Penicillium expansum* RcP61 from the mycelial mass towards a cool surface in the experimental set-up shown in Fig. 4

| Direction of transfer | Toxin identified | The precursor ions used for identification by MS/MS |
|---|---|---|
| Downwards (P.ex.11) | Chaetoglobosin | 529 $[M + H]^+$, 551 $[M + H]^+$ |
| | Communesin A | 509 $[M + H]^+$ |
| Upwards (P.ex.44) | Chaetoglobosin | 529 $[M + H]^+$, 551 $[M + H]^+$ |
| | Communesin A | 509 $[M + H]^+$ |
| | Communesin B | 457 $[M + H]^+$ |

health symptoms experienced in mould-damaged urban buildings.

Results of this study call for continued research on how mycotoxin-containing guttation droplets can be spread in indoor air. The toxicity and migration of guttation droplets in the air were shown in this study, but their spreading in the rooms needs to be studied in future research.

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## Conflict of Interest

No conflict of interest declared.

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