Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Indoor *Trichoderma* strains emitting peptaibols in guttation droplets

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Keywords
ecotoxicity, environmental, fungi, moulds, mycotoxins.

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2018/0641: received 17 October 2017, revised 29 March 2018 and accepted 9 May 2018

doi:10.1111/jam.13920

Abstract

Aims: The production of peptaibols, toxic secondary metabolites of *Trichoderma*, in the indoor environment is not well-documented. Here, we investigated the toxicity of peptaibols in the guttation droplets and biomass of *Trichoderma* strains isolated from problematic buildings.
Methods and Results: Seven indoor-isolated strains of *T. atroviride, T. trixiae, T. paraviridescens* and *T. citrinoviride* were cultivated on malt extract agar, gypsum boards and paperboards. Their biomass extracts and guttation droplets were highly cytotoxic in resting and motile boar sperm cell assays and in inhibition of somatic cell proliferation assays. The toxins were identified with HPLC/ESI-MS/MS as trichorzianines, trilongins, trichostrigocins and trichostrigocin-like peptaibols. They exhibited toxicity profiles similar to the reference peptaibols alamethicin, trilongins, and trichorzianine TA IIIc purified from *T. atroviride* H1/226. Particular *Trichoderma* strains emitted the same peptaibols in both their biomasses and exudate droplets. The trilongin-producing *T. citrinoviride* SJ40 strain grew at 37°C.
Conclusions: To our knowledge, this is the first report of indoor-isolated *Trichoderma* strains producing toxic peptaibols in their guttation droplets.
Significance and Impact of the Study: This report proves that indoor isolates of *Trichoderma* release peptaibols in their guttation droplets. The presence of toxins in these types of exudates may serve as a mechanism of aerosol formation for nonvolatile toxins in the indoor air.

Introduction

The excessive moisture resulting from the water damage in buildings may change the typical diversity of indoor microbiota where *Penicillium* and *Aspergillus* are the usual dominant fungal genera (Nielsen 2003). Species like *Trichoderma* can grow on wet wooden materials and plywood colonized by other fungi, and therefore are indicators of high moisture content in buildings (Gravesen *et al.* 1999; Andersen *et al.* 2011; Druzhinina *et al.* 2011; Kubicek *et al.* 2011; Samson 2011; Mikkola *et al.* 2012; Mukherjee *et al.* 2013).

The presence of fungi indoors increases the risk of human infections due to inhalation of viable fungal fragments and small conidia (≤4 μm) moving from the building structure to the indoor air (Airaksinen *et al.* 2004; Straus 2009). Human pathogenic infections caused by *Trichoderma* have been increasingly reported in the literature (Mikkola *et al.* 2012; Hatvani *et al.* 2013) with *T. longibrachiatum* and *T. citrinoviride* the most frequently reported clinically relevant *Trichoderma* species.

Exudation is a well-known phenomenon of plants and fungi. Fungal exudation may occur during mycelial growth and is suggested to be a mean to expel waste products or an available water reservoir (Hutwimmer *et al.* 2009; Gareis and Gottschalk 2014). Fungal exudates contain proteins, mycotoxins (toxic secondary metabolites)

and exhibit enzymatic activities (Gareis and Gareis 2007). However, further studies are needed to determine the exact composition of exudates, the specific roles of exudation and to examine whether exudates are possible carriers of toxins in the indoor air.

Peptaibols form a group of bioactive secondary metabolites, mainly produced by *Trichoderma* species, with antibacterial, antiviral and antifungal activities (Panizel *et al.* 2013). They have a structure composed of peptides of 5–20 amino acids including α-amino-isobutyric acid, an acetylated N-terminus and an amino alcohol at the C-terminus (Leitgeb *et al.* 2007; Bohemen *et al.* 2016). A single *Trichoderma* species may produce up to five different types of peptaibols, while different *Trichoderma* species may produce the same peptaibols (Hermosa *et al.* 2014). Even though peptaibols are known for their specific effect in biomembranes, their roles remain unclear (Mukherjee *et al.* 2010). Trilongins produced by indoor *Trichoderma* strains were shown to form potassium- and sodium-selective channels in artificial biomembranes (Mikkola *et al.* 2012).

Boar semen bioassays are capable of detecting toxins which disrupt cation homeostasis by affecting the function of the plasma membrane (Vicente-Carrillo 2018). These bioassays have been used for screening the toxicity of indoor samples and exhibited high sensitivity for screening toxins like peptaibols (Peltola *et al.* 2004; Andersson *et al.* 2010). Marik *et al.* (2016) have shown that boar semen bioassays were more sensitive than lung cells when screening peptaibol toxicity.

The pathogenic potential, production of toxic metabolites and emission mechanisms of *Trichoderma* peptaibols in the indoor environment are poorly understood. To the best of our knowledge, the secretion of peptaibols in exudated guttation droplets of *Trichoderma* has not yet been reported in the literature. The aim of this study was to investigate the presence and toxicity of peptaibols in the extract of biomass and the exudates of *Trichoderma* strains isolated from buildings where occupants reported indoor air-related symptoms.

Materials and methods

Fungal strains

The *Trichoderma* strains were isolated from five buildings located in different Finnish cities where occupants reported indoor air-related symptoms and illnesses. Sampling details of the collected material, dust and air samples are shown in Table 1.

Material samples from exhaust air filter and mineral wool (between inner and outer ceiling) were collected in sterile plastic bags. Pieces of material samples (*c.* 1 cm × 1 cm) were spread on malt extract agar (MEA) plates (15 g malt extract from Sharlab, Barcelona, Spain, and 12 g of agar from Amresco, Dallas, USA, in 500 ml of H_2O). Dust samples were swept from surfaces (*c.* 30 × 30 cm^2) above floor level (1–2 m) with a sterile paper tissue. Floor dust was collected with a vacuum cleaner (Volta Equipt with Volta Equipt vacuum bags), the dust was removed from the vacuum cleaner bag with a sterile disposable spoon and placed into a sterile plastic bag. The dust (*c.* 10 mg) was spread with a sterile cotton swab on MEA plates. Air samples were collected with six-stage Andersen Impactor on MEA plates during 10 min at 1 m above the floor level, and with MEA fallout plates kept open 1 h at 1–1·5 m above the floor level (Andersen 1958).

Malt extract agar culture plates were inoculated, sealed and cultivated at 22°C for 4 weeks. Fungal colonies suspected to belong to the genus *Trichoderma* based on colony morphology and the characteristic conidiophores visible in the light microscope were rapidly screened for toxicity and the toxic colonies were pure cultured on MEA plates.

Extraction of ethanol-soluble compounds from biomass and collection of guttation droplets from MEA-cultured *Trichoderma* isolates

Fungal biomass (*c.* 100 mg wet wt) containing hyphae and conidia (no guttation droplets visible under UV light in stereomicroscope, 160× magnification) was extracted with ethanol, as described by Andersson *et al.* (2010), after 2 weeks of incubation at 22°C of the MEA plates.

Exudate vesicles fluorescent under UV light appeared on MEA plates after 1 week of incubation, at the beginning of sporulation. Exudates with a volume of 1–5 μl were collected under UV light (360 nm), mixed with an equal volume of ethanol (96%, all the chemicals were purchased from local suppliers) and heated for 10 min at 80°C in a water bath. The exudates collected from MEA plates into glass ampules, 20–200 μl per plate, contained no hyphae or conidia when inspected with phase contrast microscope (Olympus CKX41, Tokyo, Japan; 400× magnification). The ethanol-soluble compounds from biomasses and exudate suspensions were used to expose the test cells in the toxicity assays.

Cultivation and extraction of *Trichoderma atroviride* colonies grown on gypsum boards and paperboards

Purchased pieces of gypsum boards and paperboards of 25 cm^2 were autoclaved, saturated with sterile water and inoculated with conidia of *T. atroviride* strains 14/AM, H1/226 and H3/226 (200 μl of phosphate buffered saline, PBS, containing *c.* 10^6 conidia per ml). The inoculated paperboards and gypsum boards were incubated at room

Table 1 Characterization of the *Trichoderma* strains isolated from five buildings in Finland

Species	Code	Location	Sampling description		Potentially pathogenic?*	ITS (GenBank)	*tef1α* (GenBank)
T. atroviride	H1/226	Office (Helsinki)	Fallout plate	1 m above floor level	–	KM853017	MH176994
T. atroviride	H3/226	Office (Helsinki)	Andersen impactor (plate 3)	1 m above floor level	–	–	MH176995
T. atroviride	8/AM	Office (Espoo)	Exhaust air filter	Attic	–	MH158553	MH176996
T. atroviride	14/AM	Office (Espoo)	Exhaust air filter	Attic	–	MH158554	MH176997
T. atroviride	Tri335	Office (Espoo)	Mineral wool	Opened ceiling	–	–	MH176998
T. atroviride	KIV10	School (Lahti)	Fallout plate	2 m above the floor	–	–	MH176999
T. paraviridescens	Sip335	Office (Espoo)	Settled dust	Vacuumed floor	–	MH158555	MH177000
T. trixiae	LB1	Apartment (Helsinki)	Settled dust	Bookshelf 1·5 m above floor	–	MH158556	MH177001
T. trixiae	NJ14	Ice rink (Nivala)	Settled dust	1·5 m above floor	–	MH158557	MH177002
T. trixiae	NJ22	Ice rink (Nivala)	Settled dust	1·5 m above floor level	–	MH158558	MH177003
T. citrinoviride	SJ40	Office (Espoo)	Settled dust	Bookshelf 1 m above floor	+	KP889007	MH177004
Reference strain							
T. longibrachiatum	SzMC Thg†	Apartment‡ (Oulu)	Insulation material	Bathroom floor	+	EU401573	EU401624

*Pathogenic potential was tested at 37°C. +: potentially pathogenic. –: nonpathogenic.
†The reference strain was identified in Druzhinina *et al.* (2008).
‡Mikkola *et al.* (2012).

temperature for 4 weeks inside Petri dishes sealed with gas-permeable tape. The Petri dishes were inspected weekly under the stereomicroscope and sterile water was added to maintain the moisture content of the gypsum boards and paperboards. Twenty to 50 mg (wet wt) of collected fungal material (including conidia, hyphae and guttation droplets) from the *T. atroviride* strains 14/AM, H1/226 and H3/226 cultivated on gypsum boards and paperboards for 2–4 weeks were extracted with ethanol as described by Andersson *et al.* (2010).

Identification of fungal strains

The suspected *Trichoderma* strains were deposited in the Szeged Microbiology Collection (http://www.szmc.hu). Total DNA was extracted from the strains' cultures grown on yeast extract—glucose agar medium (0·5 g l^{-1} yeast extract, 10 g l^{-1} glucose and 20 g l^{-1} agar) using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). A nuclear rDNA region containing the internal transcribed spacers 1 and 2 (ITS 1 and 2) and the 5·8S rRNA gene was amplified with primers ITS1 (5′-CCGTAGGTAACCTGCGG-3′) and ITS4 (5′-TCCTCCGC-TTATTGATATGC-3′) (White *et al.* 1990; Naeimi *et al.* 2011), while a fragment of the translation elongation factor 1 alpha (*tef1*) gene was amplified with primers EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and TEF-LLErev (5′-AACTTGCAGGCAATGTGG-3′) (Jaklitsch and Voglmayr 2015). PCR amplifications were carried out in a MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA) with the following temperature profiles: ITS—initial denaturation of 2 min at 94°C, 35 cycles of 30 s at 94°C, 40 s at 48°C, 40 s at 72°C and a final extension of 2 min at 72°C; *tef1*—initial denaturation of 1 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 59°C, 50 s at 74°C and a final extension at 74°C for 7 min. The ITS and *tef1* amplicons were sequenced by Sanger sequencing with the ITS4 and EF1-728F primers, respectively, on a 3500 Series Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was carried out with the aid of the programs *Trich*Okey 2.0 and *Tricho*MARK available online at http://www.isth.info/ (Druzhinina *et al.* 2005; Kopchinskiy *et al.* 2005).

Toxicity assays with resting and motile boar spermatozoa

Motility of boar sperm can be reversibly induced by warming to 37°C with oxygen availability (mimicking the

short-lasting behaviour of sperm cells during physiological condition inside the female)—or switched off by anoxia and cooling to room temperature (not induced to swim and rest, mimicking the long-lasting behaviour of sperm cells inside the male) (Kamp *et al.* 2003). Both resting (indicated with the subscript capital R) and motile (indicated with the subscript capital M) sperm cells were used in the toxicity assays.

Boar sperm motility inhibition assay with resting spermatozoa ($BSMI_R$)

The boar sperm motility inhibition assay with resting spermatozoa ($BSMI_R$) measuring motility inhibition, that is, inability to respond to induction of motility in resting sperm cells exposed for 1 day at room temperature, is described in Andersson *et al.* (1998). For testing the motility inhibition of the sperm cells, the test compounds were dissolved in ethanol. The ethanol solutions (0·5–10 μl) were dispensed in 2000 μl of extended boar semen (Figen Ltd, Tuomikylä, Finland; density of 27×10^6 sperms per ml) and motility of the sperms was inspected using the phase contrast microscope (400× magnification) with a heated stage as described by Andersson *et al.* (2004). The EC_{50} concentration for motility inhibition was concluded as the toxin concentration closest to that provoking a >50% decrease in the number of sperm cells exhibiting rapid tail beating, visible in microscope by the human eye as sperm cells with two tails, compared with the sperm cells in the solvent control as described in Bencsik *et al.* (2014). The EC_{50} was calculated from the equation of the straight line between $EC_{50\text{-}40}$ and $EC_{80\text{-}90}$: $Y = -\Delta Y/\Delta X \times X + C$ where Y is the motility closest to 50% of the motility of the solvent control, X is the EC_{50} concentration and C is a constant between 100 and 60%. All tests were run in triplicates and differences between replicate tests were within one dilution step (twofold). The sperm assays were calibrated with triclosan and valinomycin.

Sperm membrane integrity disruption assay with resting spermatozoa ($SMID_R$)

The sperm membrane integrity disruption assay with resting spermatozoa ($SMID_R$), measuring intactness of the plasma membrane integrity in resting sperm cells, applies double staining with the DNA labelling stains propidium iodide (PI) and Hoechst 33342. PI cannot penetrate the intact plasma membrane of viable sperm cells but binds to dsDNA emitting red fluorescence in sperm cells with disrupted plasma membrane integrity. Hoechst 33342 penetrates living cells with intact plasma membrane integrity, binds to intact dsDNA and emits blue fluorescence. The staining protocol was as follows: 200 μl of extended boar semen containing 27×10^6 sperm cells per ml was mixed with 200 μl PBS containing 10 μg ml^{-1} PI and 10 μg ml^{-1} Hoechst 33342.

Mitochondrial membrane potential assay with resting spermatozoa ($D\Psi m_R$)

The mitochondrial membrane potential assay with resting spermatozoa ($\Delta\Psi m_R$) monitored the mitochondrial membrane potential changes ($\Delta\Psi m$) by staining with the lipophilic potentiometric stain JC-1 as described by Mikkola *et al.* (2015).

For the staining with PI plus Hoechst 33342 or JC-1, the sperm cells were incubated at 37°C for 15 min and 5 min, respectively, and inspected with the fluorescence microscope using 400× magnification (Nikon Eclipse E600; Nikon Corporation, Tokyo Japan) with filters BP 330–380 nm per LP400 nm and BP 450–490 nm per LP 520. The EC_{50} concentration in these microscopic assays was defined as the lowest concentration where the ratio of cells similar to those in the solvent control was <50%. This EC_{50} fitted between EC_{90} and EC_{10} observed in the microscope calculating *c.* 100–120 sperm cells from three microscopic fields. The maximal difference between four parallel tests in each of the two methods was one dilution step. The assays were calibrated with triclosan.

Boar sperm motility inhibition assay with motile spermatozoa ($BSMI_M$)

Boar sperm motility inhibition assay exposing motile sperm cells ($BSMI_M$) to dilutions of the biomass extracts and exudates at 37°C for 20 min was performed as follows: aliquots of 200 μl of extended boar semen were exposed to 0·5, 1 and 2 μl of ethanol-soluble compounds from 10-fold dilutions of biomass extracts or exudates. Estimation of the ratio of motile spermatozoa compared to the control and calculation of EC_{50} was done as in the $BSMI_R$ assay described above.

Sperm membrane integrity disruption assay with motile spermatozoa ($SMID_M$)

Disruption of sperm cells membrane integrity in motile sperm cells exposed at 37°C for 2 h was assessed by staining with PI as described by Bencsik *et al.* (2014) with modifications. Aliquots of 50 μl PBS were pipetted into a microtitre plate. Ethanol-soluble compounds from biomasses or guttation droplets (50 μl) of *Trichoderma* strains were added to the first column of the microtitre plate, serially diluted to 2^9, and extended boar cell aliquots (150 μl) were added to the wells. The possible

autofluorescence of the toxins was excluded by measuring no fluorescence emission of the crude extracts (50 μl of the crude extracts solved in 150 μl of PBS). PBS was used as a blank reagent. Three parallel dilutions were performed for each sample. Frozen-thawed semen only exposed to ethanol was used as a positive control (100% mortality) representing the maximal fluorescence emitted by the cells permeable to PI. Sperm cells only exposed to ethanol were used as a negative control (viable cells). The microtitre plate was pre-incubated for 2 h at 37°C on an orbital shaker (Innova 5000 New Brunswick Scientific, Enfield, CT) at 160 rev min^{-1}. A volume of 100 μl PI solution (10 μg ml^{-1}) was added to each well of the microtitre plate. The plate was incubated for 15 min at 37°C in the dark. Fluorescence was measured with a microplate reader (Fluoroskan Ascent; Thermo Scientific, Vantaa, Finland) at excitation and emission wavelengths of 544 and 590 nm respectively.

Loss of viability, that is, mortality (permeability to PI) in the samples was calculated as described by Alm *et al.* (2001) using the following equation:

$$\text{Loss of viability of sample (\%)} = \frac{\text{fluorescence of sample} - \text{background}}{\text{fluorescence of dead control} - \text{background}} \times 100$$

The toxicity reported as EC_{50} (the half maximal effective concentration) corresponded to the concentration causing a 50% decrease in mortality compared to the positive control (=100% mortality). The lower the EC_{50} value is, the more toxic is the substance. The assay was calibrated with triclosan in five parallel tests, the EC_{50} was 2 μg ml^{-1} (SD ±0·6).

Toxicity assay with somatic cell lines (ICP)

The inhibition of cell proliferation (ICP) assay with kidney tubular epithelial cells (PK-15) and feline fetus lung cells (FL) (FL and PK-15; Finnish Food Safety Authority, EVIRA, Finland) and the determination of EC_{50} concentrations followed the methods described by Bencsik *et al.* (2014).

Rapid toxicity screening of single colonies with boar sperm and somatic cell lines

For initial toxicity screening, 10–20 mg of biomass (wet wt) from each colony on the original culture plates was looped into 0·2 ml of ethanol and heated in a water bath for 10 min at 80°C (Andersson *et al.* 2004). Porcine spermatozoa ($BSMI_M$) and kidney tubular epithelial cells (ICP, PK-15) were exposed to the obtained ethanolic lysates, which were considered toxic when 2·5 vol% inhibited boar sperm motility or 5 vol% inhibited proliferation of PK-15 cells.

Identification and purification of peptaibols

The ethanol-soluble toxic compounds from biomass and guttation droplets of the *Trichoderma* isolates were identified with high-performance liquid chromatography/electrospray ionization—tandem mass spectrometry (HPLC/ESI-MS/MS) performed with an Esquire ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with ESI source and Agilent 1100 series liquid chromatography (Agilent Technologies, Wilmington, DE). The liquid chromatography column was SunFire C18, 2·5 μm × 2·1 mm × 50 mm (Waters, Milford, MA). Separation of the toxins was performed with gradient elution using eluents A (0·1% formic acid) and B (methanol). Gradient elution was from 60% A to 100% B in 30 min at a flow rate of 0·2 ml min^{-1}. Positive mode mass analyses were performed in the mass range of m/z 50–2000. Alamethicin was used as a reference compound. HPLC fractions of the ethanol extract of *T. atroviride* H1/226 were collected as described in Mikkola *et al.* (2012). The toxicity of the fractions was tested using boar sperm assays.

Results

Species diversity of *Trichoderma* in the sampled buildings

Trichoderma atroviride was the most frequently isolated *Trichoderma* species (6 out of 11 strains) in the five buildings sampled in Finland (Table 1). The other isolated *Trichoderma* species were *T. trixiae*, *T. paraviridescens* and *T. citrinoviride*. Strain *T. citrinoviride* SJ40 (and the reference strain *T. longibrachiatum*) grew at 37°C which suggests possible pathogenic potential.

Exudates and biomass extracts of MEA-cultured *Trichoderma* contained toxic metabolites

The presence of toxic metabolites in the biomass and exudate of selected MEA-cultured *Trichoderma* strains representing each species (Table 1) was tested by motility inhibition ($BSMI_M$ assay), disruption of sperm plasma membrane integrity ($SMID_M$ assay) of motile boar sperm and ICP with feline fetus lung cells (FL) and porcine kidney cells (PK-15).

The ethanol-soluble compounds from biomasses (Table 2) and the exudates (Table 3) were over 50 times more toxic than the exudates and extracts from the non-toxic reference strains representing the upper limits of nonspecific response in the assays. The lowest EC_{50} values

recorded in the $BSMI_M$ and the $SMID_M$ assays were two to 10 times smaller, respectively, than in ICP (FL, PK-15) assays. Thus, the toxic metabolites were more toxic to sperm cells than somatic cells, inducing visible motility inhibition after 20 min ($BSMI_M$ assay) and rapid necrotic cell death in sperm cells exposed for 2 h ($SMID_M$ assay). The different *Trichoderma* isolates exhibited uniform toxicity profiles in the three toxicity assays and similar responses were provoked by the ethanol-soluble compounds from biomasses and by the exudates. The toxicity profiles were comparable to the biomass extract of the trilongin-producing reference strain of *T. longibrachiatum*.

Toxigenic colonies of *T. atroviride* cultured on paperboards and gypsum boards emitted airborne exudate vesicles and conidia

Colonies of *T. atroviride* H1/226, H3/226 and 14/AM cultivated on building material substrates were visible after 2–4 weeks of incubation (e.g. *T. atroviride* 14/AM, Fig. 1). When cultured on paperboards and gypsum boards, the colonies of strain 14/AM contained big exudate vesicles compared to cellular biomass (Fig. 1b,c). The colonies emitted exudate vesicles and conidia capable to attach to the inner surface of the lid of the plastic Petri dish (Fig. 1d–f). Figure 1 shows that the potentially mycoparasitic *T. atroviride* 14/AM colonized paperboard without underlying fungal growth and colonies on paperboard were capable of airborne emission of exudate vesicles and conidia.

Biomass extracts of *Trichoderma* cultured on building materials and MEA revealed similar toxicity profiles

Toxicity of the ethanol extracts (from hyphae, conidia and guttation droplets) of H1/226, H3/226 and 14/AM cultured on building materials was tested towards somatic cells (ICP, PK-15) and resting boar spermatozoa (motility induction: $BSMI_R$, mitochondrial depolarization: $\Delta\Psi m_R$ and sperm plasma membrane integrity disruption: $SMID_R$ assays). The same protocol was applied for the MEA-cultured strains, the difference being that the extracts contained hyphae and conidia only (no exudate visible under stereomicroscope; Leica M25, Leica Microsystems, Mannheim, Germany; from 50 to 120× magnification).

Fluorescence micrographs of Fig. 2 illustrate the sperm cells exposed to ethanol control (Fig. 2a,b) and extracted compounds from *T. atroviride* 14/AM grown on gypsum board (Fig. 2c,d) in the resting boar sperm assays. The ethanol-exposed sperm cells capable of motility induction after 1 day of exposure in nonmotile resting conditions exhibited a high $\Delta\Psi m$ indicated by the orange fluorescence of the mitochondrial sheath in the midpiece of the sperm tail (Fig. 2a) and intact plasma membrane emitting blue fluorescence (impermeable to PI in the $SMID_R$ assay, Fig. 2b). At 4 $\mu g\ ml^{-1}$ the ethanol extract from biomass of *T. atroviride* 14/AM grown on paperboard inhibited motility induction and the immobilized sperm cells exhibited depolarized mitochondria as indicated by the green fluorescing mitochondrial sheath (Fig. 2c) and

Table 2 Toxicity of the ethanol-soluble compounds from the biomass of *Trichoderma* strains cultured on MEA

		EC_{50} ($\mu g\ ml^{-1}$)				
		Motile sperm cells (37°C)		Somatic cell lines (ICP)		
Species	Exposure time Code	2 h $SMID_M$	20 min $BSMI_M$	2 days FL	2 days PK-15	Identified peptaibol
T. atroviride	H3/226	2	50	60	n.d.	Trichorzianines
T. atroviride	14/AM	2	5	30	30	Trichorzianines
T. atroviride	Tri335	2	5	30	60	Trichorzianines
T. paraviridescens	Sip335	1	10	15	30	Trichostrigocins
T. trixiae	LB1	2	2·5	60	60	Trichostrigocin-like
T. citrinoviride	SJ40	1	5	15	15	Trilongins
Reference strain						
T. longibrachiatum	SzMC Thg	2	25	120	60	Trilongins
Reference toxin						
*Alamethicin**		0·6	5	8	8	
Nontoxic reference strain						
Penicillium sp.	TR	600	>100	500	n.d.	
Aspergillus sp.	Hk2	600	>100	500	n.d.	

n.d.—no data available.
*Forming potassium channel.

Table 3 Toxicity of the exudates of *Trichoderma* strains cultured on MEA

		EC_{50} (μl ml^{-1})				
		Motile sperm cells (37°C)		Somatic cell lines (ICP)		
Species	Exposure time Code	2 h $SMID_M$	20 min $BSMI_M$	2 days FL	2 days PK-15	Identified peptaibol
T. atroviride	H3/226	n.d.	10	>50	>50	Trichorzianines
T. atroviride	14/AM	1	2·5	>25	>50	Trichorzianines
T. atroviride	Tri335	2·5	<10	>25	>50	Trichorzianines
T. paraviridescens	Sip335	n.d.	n.d.	n.d.	>25	Trichostrigocins
T. trixiae	LB1	8	10	>25	n.d.	Trichostrigocin-like
T. citrinoviride	SJ40	0·5	2·5	>25	>50	Trilongins
Reference strain						
T. longibrachiatum	SzMC Thg	n.d.	n.d.	n.d.	n.d.	Trilongins
Nontoxic reference exudate						
Aspergillus calidoustus	MH34	>50	>50	>50	>50	
Aspergillus westerdijkiae	PP2	>50	>50	>50	>50	
Aspergillus versicolor	SL3	>50	>50	>100	>100	

n.d.—no data available.

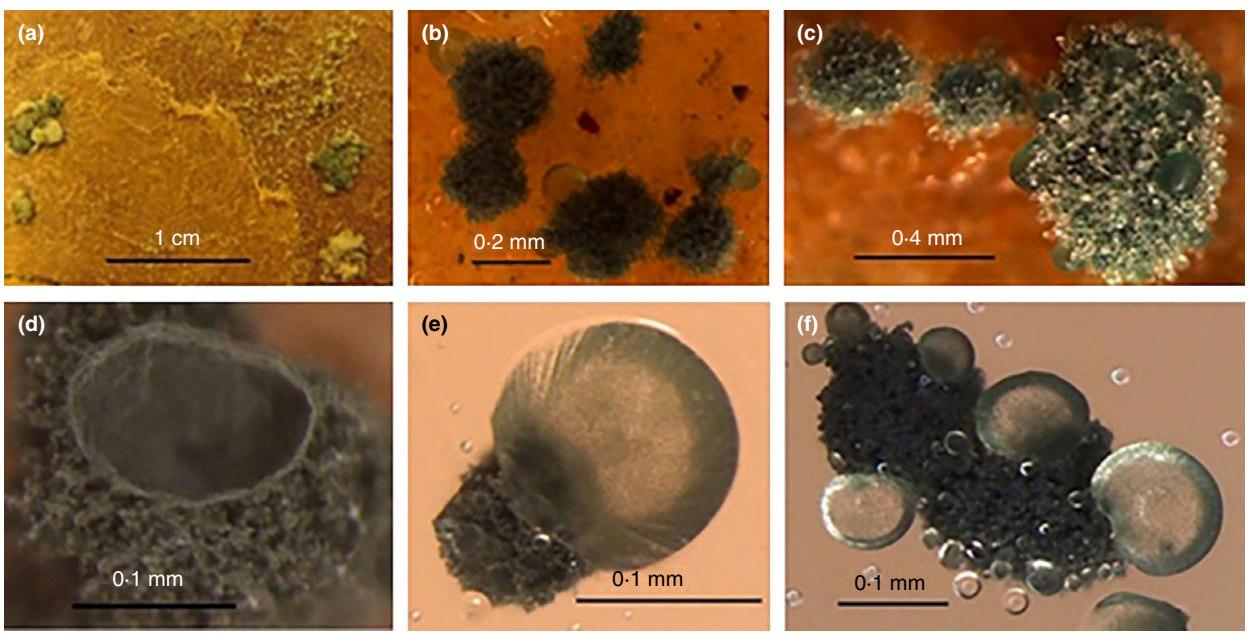

Figure 1 *Trichoderma atroviride* 14/AM (a) colonies were visible after 4 weeks of cultivation on paperboard. Stereomicroscope showed visible exudate (b) after 2 weeks of cultivation on gypsum board and (c) 3 weeks of cultivation on paperboard. (d) Empty dry membrane structures were frequently observed after 4 weeks of cultivation on gypsum board substrate. Stereomicroscopy revealed colonies with large exudates (e) on gypsum board and (f) on the inner surface of the lid of the Petri dish.

disrupted plasma membrane integrity permeable to PI (red fluorescence, Fig. 2d).

The toxicity endpoints obtained in the ICP assay (PK-15) and the three resting sperm assays ($BSMI_R$, $SMID_R$ and Ψm_R) are summarized in Table 4. Sperm cells were still capable of motility induction, that is, exhibited motility and showed high $\Delta\Psi m$ and intact plasma membrane integrity after exposure to 50 μg ml^{-1} ethanol-extracted substances from biomass of the reference strain *Penicillium* sp. TR grown on gypsum board, representing the upper limits of nontoxic responses.

The EC_{50} values of the *Trichoderma* crude extracts from colonies grown on building materials and MEA were 10 times lower in the three resting sperm assays

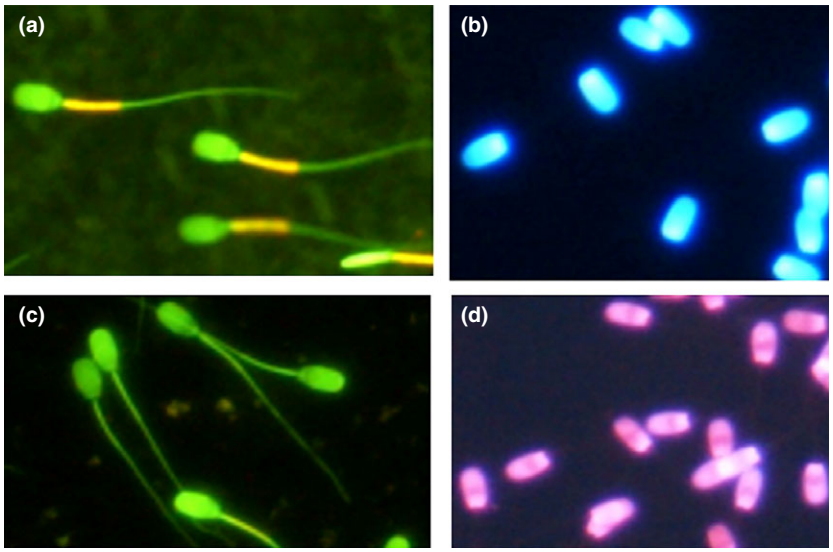

Figure 2 Toxicity of boar sperm cells exposed one day to the ethanol extracts of *T. atroviride* 14/AM cultivated on paper board (c and d, 5 μg ml^{-1}) compared to the ethanol control (a,b). After exposure, the cells were stained with the membrane potential responsive stain JC-1 (a,c) and DNA vital staining with PI and Hoechst 33342 (b,d). In the ethanol control, motile sperms exhibited (a) high mitochondrial membrane potential, orange fluorescent mitochondrial sheath in the mid piece of the sperm tail and (b) intact plasma membrane of sperm cells, impermeable to red fluorescent PI. Ethanol extract of *T. atroviride* 14/AM cultivated on paperboard showed depolarised mitochondria (c) and disrupted plasma membrane integrity (d). The size of the sperm head is 8 × 4 μm.

($BSMI_R$, $SMID_R$ and Ψm_R) than in the ICP assay and 10 times lower than for the reference strain TR. Thus, boar sperm cells were 10 times more sensitive to the toxins present in the extracted biomasses of *T. atroviride* H1/226, H3/226 and 14/AM than the somatic cell lines (ICP, PK-15) (Table 4).

The compounds extracted from biomasses grown on MEA and building materials exhibited similar toxicity profiles in the ICP (PK-15) and resting boar sperm assays ($BSMI_R$, $SMID_R$ and Ψm_R) as the reference toxins trilongin and alamethicin, concentrations inhibiting sperm motility also depolarized mitochondria and disrupted the integrity barrier of the plasma membrane (Table 4).

The toxic metabolites were identified as peptaibols

The toxic metabolites produced by the indoor-isolated *Trichoderma* strains were identified as peptaibols with HPLC/ESI-MS/MS analysis (Fig. 3). The peptaibols of strains H1/226, H3/226, 14/AM and Tri335 present in the ethanol-soluble compounds from biomasses and exudated guttation droplets were identified as trichorzianines (Tables 2–4). Strain SJ40 produced trilongins in the ethanol-soluble compounds from biomass and guttation droplets (Tables 2 and 3). Ethanol-soluble compounds from biomass and exudate of strain LB1 contained trichostrigocin-like peptaibols which resembled trichostrigocins of strain Sip335 except that the C-terminus of the trichostrigocin-like peptaibols contained phenylalaninol, whereas the C-terminus of trichostrigocins contained leucinol (Tables 2 and 3).

HPLC/ESI-MS/MS analyses showed that peptaibols were present in the ethanol-soluble compounds from biomasses and exudates of the indoor-isolated *Trichoderma* strains. Moreover, the *Trichoderma* isolates produced the same peptaibols in the ethanol-soluble compounds from biomass, as in the corresponding exudate. Results in Tables 2 and 3 show that the crude extracts and the exudates, containing trilongins, trichorzianines and trichostrigocins were more toxic in the boar sperm assays $BSMI_M$ and $SMID_M$ than in the ICP assays, exhibiting the same toxicity profile as the commercial peptaibol alamethicin.

Trichorzianine TA IIIc purified from biomass extract of *T. atroviride* H1/226 exhibited similar toxicity profile as purified trilongin and alamethicin

Over 10 sperm-toxic HPLC fractions, identified as trichorzianine peptaibols, were found in the ethanol-soluble compounds from the biomass of *T. atroviride* H1/226. The trichorzianines identified with MS/MS analysis were TA IIIb (MW = 1948), TA IIIc (MW = 1948), TA VII (MW = 1923), TA IVb (MW = 1962), TA VIb (MW = 1909) and TA VIa (MW = 1937), similar to the ones described earlier for *T. atroviride* by Stoppacher *et al.* (2007). The fraction containing the known voltage-dependent channel producer trichorzianine TA IIIc (MW = 1948) reported by Molle *et al.* (1987) was selected for further toxicity assays (Table 4).

In the ICP (PK-15) and resting boar sperm assays ($BSMI_R$, $SMID_R$ and Ψm_R) the toxicity of the purified trichorzianine TA IIIc was 20- and 100-fold, respectively, of the toxicity of ochratoxin A (upper limit of nonspecific response), thus resting boar sperm assays were the most sensitive to detect the toxic trichorzianine TA IIIc. Concentrations of 0·2, 0·4 and 0·5 μg ml^{-1} of alamethicin, trilongin and trichorzianine TA IIIC, respectively, inhibited sperm motility, depolarized mitochondria and depleted the

Table 4 Toxicity of the ethanol extracts from biomasses (including hyphae, conidia and exudate) of *Trichoderma* strains cultured on building material substrates, and of the purified trichorzianine TA IIIc

		EC_{50} (μg ml^{-1})			
			Resting boar sperm cells exposed at RT		
		ICP (PK-15)	$BSMI_R$	$\Delta\Psi m_R$	$SMID_R$
	Exposure time	2 days	1 day	1 day	1 day
Biomass grown on MEA					
T. atroviride	H1/226	50	3	3	3
T. atroviride	H3/226	60	6	6	6
T. atroviride	14/AM	50	5	5	5
Purified trichorzianine (TA IIIc) from *T. atroviride* H1/226		5	0·5	0·5	0·5
Biomass grown on paperboard					
T. atroviride	H1/226	>30	5	5	5
T. atroviride	14/AM	>20	5	5	5
Biomass grown on gypsum board					
T. atroviride	H3/226	>30	4	4	4
Reference strain grown on gypsum board					
Penicillium sp.	TR	500	>50	>50	>50
Reference toxins					
Trilongins BI-BIV*,†		5	0·4	0·4	0·4
Alamethicin*,†		8	0·2	0·2	0·2
Enniatin B*,‡		60	5	5	>50
Acrebol*,§		≥10	0·1	0·8	>4
Sterigmatocystin¶		0·1	>20	>20	>100
Ochratoxin A**		>100	50	50	>50

*Bencsik *et al.* (2014).
†Forming potassium channel.
‡Potassium carrier ionophore and mitochondrial toxins.
§Blocking respiratory chain in mitochondria.
¶Inhibitor of protein synthesis.
**Upper limit of nonspecific response.

plasma membrane integrity. These toxic responses differ from those provoked by the mitochondrial toxins enniatin and acrebol which had no effect on plasma membrane at motility-inhibiting concentrations. The toxic response of TA IIIc also differed from that exhibited by sterigmatocystin which was 1000 times more toxic in the ICP (PK-15) assay than in the resting sperm assays ($BSMI_R$, $SMID_R$ and Ψm_R). The toxicity actions of the purified trichorzianine TA IIIc were similar and comparable to the potassium channel-forming peptaibols trilongins and alamethicin, indicating that trichorzianine TA IIIc induces the same toxicity mechanism.

Discussion

According to our information, this is the first report of indoor-isolated *Trichoderma* strains producing peptaibols in their exudates. They were identified by HPLC/ESI-MS/MS as trichorzianines, trilongins, trichostrigocins and trichostrigocin-like peptaibols. Moreover, the same peptaibol was present in the biomass extract (hyphae and conidia) and the exudate of the corresponding *Trichoderma* isolate (MEA-cultivated).

The trichorzianines produced by the *T. atroviride* strains were previously described from *T. atroviride* and *T. harzianum* (Stoppacher *et al.* 2007; Panizel *et al.* 2013). From a forest soil isolate of *T. strigosum*, Degenkolb *et al.* (2008) isolated and identified trichostrigocins similar to the ones detected in this study from *T. paraviridescens* and *T. trixiae*. Mikkola *et al.* (2012) showed that trilongins were also produced by clinical and indoor isolates of *T. longibrachiatum*.

Only a few studies have reported the presence of toxins in fungal exudates. Gareis and Gareis (2007) described the secretion of a high concentration of mycoxotins in the exudates of *Penicillium expansum* (from a culture collection). Toxic trichothecenes were detected in the exudates of indoor *Stachybotrys chartarum* isolates, it was suggested that these toxins might be easily released into the environment due to the aerosolization of toxic guttation droplets favoured by ventilation or air-conditioning systems (Gareis and Gottschalk 2014). Recently, Salo *et al.* (2015) showed that indoor-isolated *Penicillium expansum* produced exudates containing toxic chaetoglobosins and communesins.

The exudates and biomass extracts (hyphae + conidia) of the *Trichoderma* isolates cultivated on MEA substrates were highly cytotoxic (Tables 2 and 3). Their toxicity patterns were similar to the biomass extract of *T. longibrachiatum* SzMC Thg producing channel-forming trilongins and to alamethicin produced by *T. arundinaceum* (Degenkolb *et al.* 2008; Mikkola *et al.* 2012). Moreover, the biomass extract (hyphae + conidia + exudate) of the *Trichoderma* cultivated on building materials contained substances exhibiting the same toxicity profile as the *Trichoderma* cultivated on laboratory medium MEA (hyphae + conidia) and as the purified peptaibol trichorzianine TA IIIc (Table 4). As expected, the peptaibol trichorzianine TA IIIc purified from strain H1/226 exhibited the same toxicity pattern as its peptaibol relative's alamethicin and trilongins and new peptaibols recently detected in forest-derived *Trichoderma* isolates from section *Longibrachiatum* (Mikkola *et al.* 2012; Marik *et al.* 2017). The resting and motile boar sperm assays were more sensitive for the screening of *Trichoderma* peptaibols than the ICP assay with somatic cells. The disruption of sperm cell membrane integrity assay ($SMID_M$) is very sensitive to detect and assess the exposure risk of mammalian cells to *Trichoderma* peptaibols (Peltola *et al.* 2004; Mikkola *et al.* 2012; Marik *et al.*

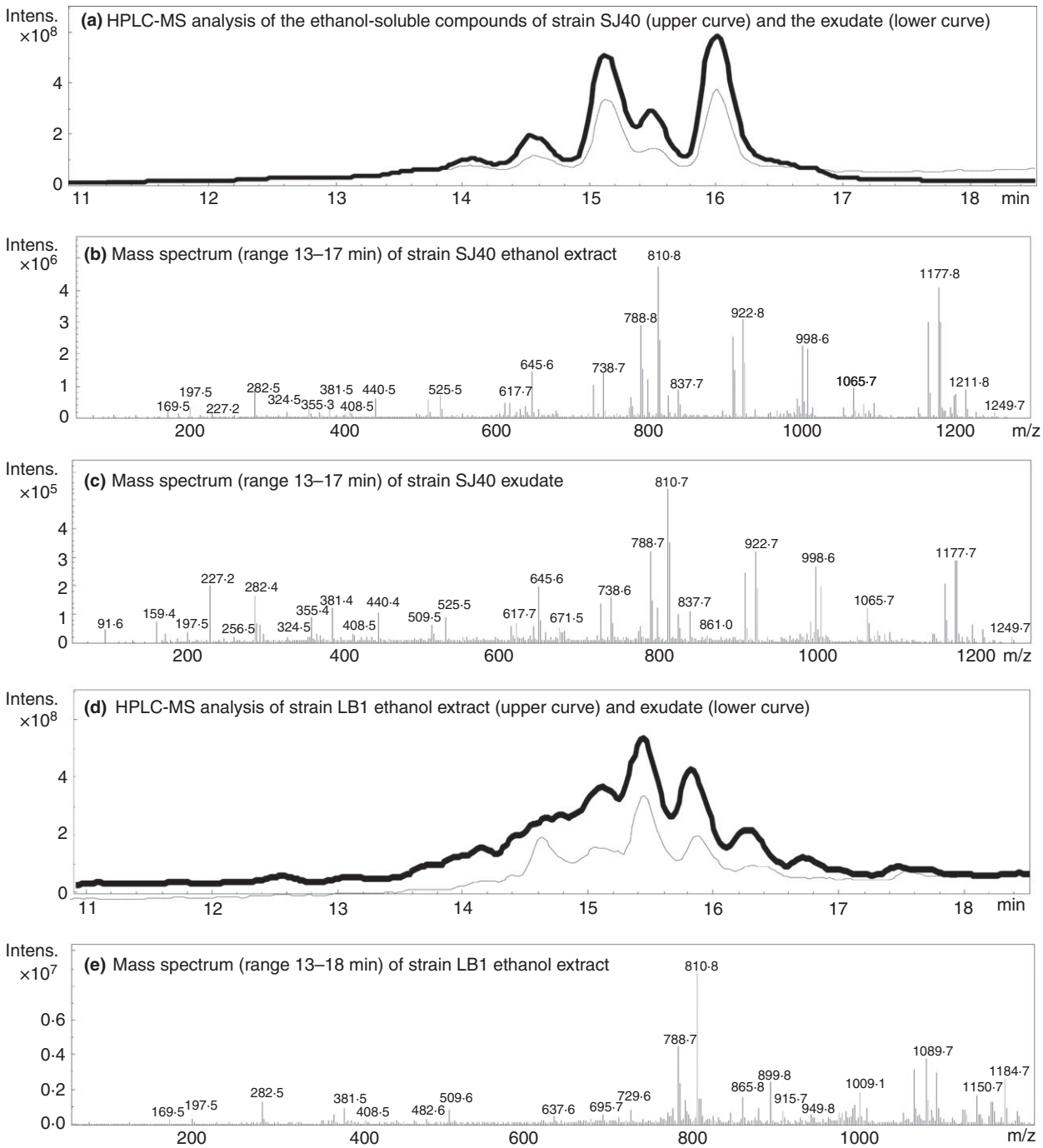

Figure 3 HPLC/ESI-MS/MS analyses of the ethanol-soluble compounds from biomass and exudates of the indoor-isolated *Trichoderma* species. a, d,g,j and m: total ion chromatograms of the ethanol-soluble compounds from biomass and exudate of strains SJ40, LB1, Tri335, 14/AM, and Sip335, respectively. Mass spectra (range 13-18 min) of the ethanol-soluble compounds from biomass (b,e,h,k,n) and exudate (c,f,i,l,o) of strains SJ40, LB1, Tri335, 14/AM and Sip335, respectively.

2017). McMullin *et al.* (2017) also reported membrane disruption of *Fusarium sambucinum* spores by trichorzianine-like peptaibols isolated from indoor *T. atroviride*.

In Nordic countries, negative pressure is commonly used to prevent moisture damage of buildings. Airaksinen *et al.* (2004) reported that an indoor negative pressure of 5–20 Pa enables fungal spores below 4 μm to cross

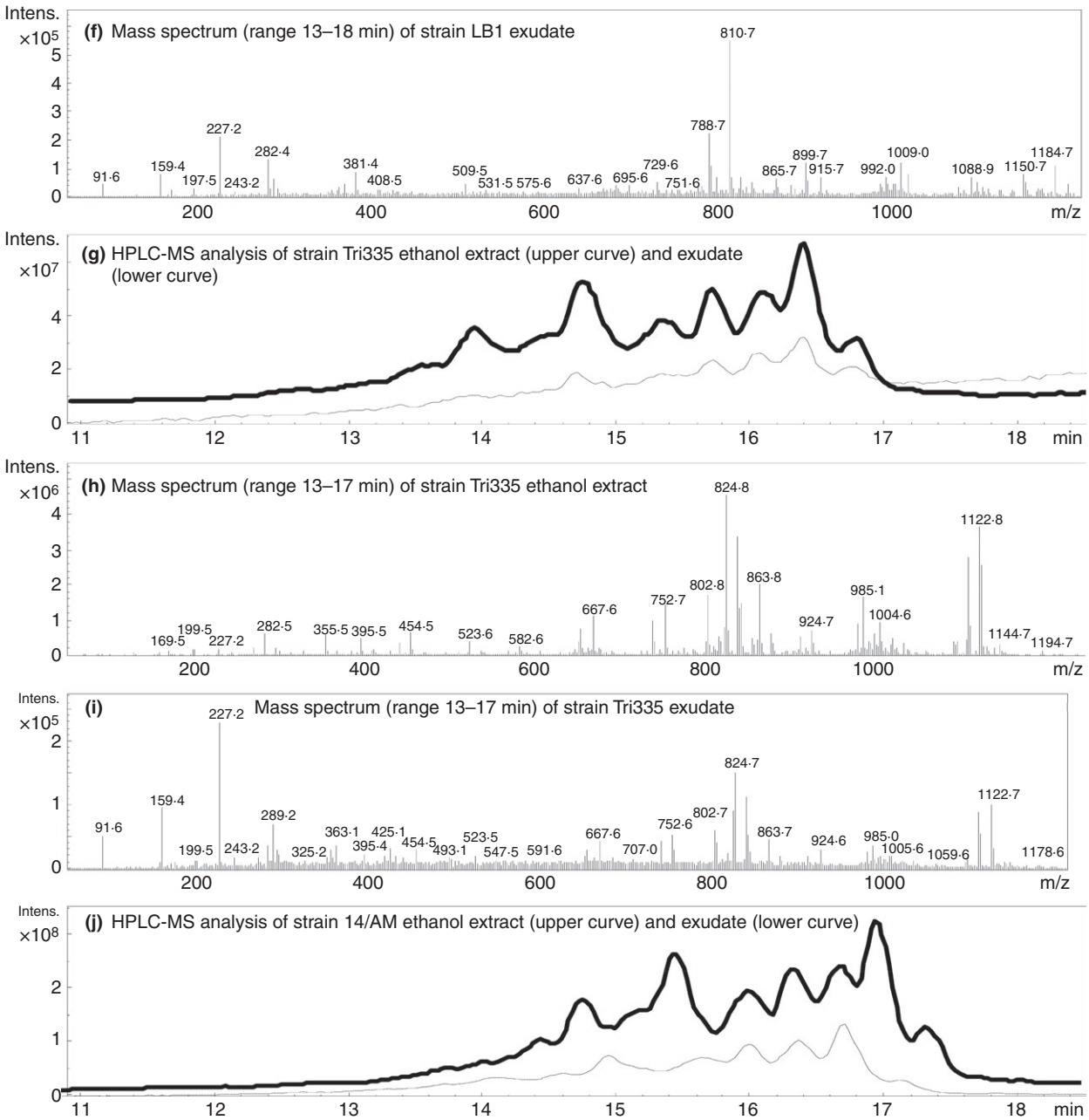

Figure 3 Continued.

structures. Thus, the small-sized conidia of *Trichoderma* may more easily spread in the indoor air. In this study, we observed that *T. atroviride* grown on building materials was capable of emitting conidia and exudate vesicles as airborne.

Immunocompromised patients exposed to fungal pathogens via—for example, their conidia, can develop peritonitis or systemic infections (Kuhls *et al.* 1999; Kredics *et al.* 2004; Kubicek *et al.* 2008; Druzhinina *et al.* 2011; Naeimi *et al.* 2011). Thus, humans may experience pulmonary mycoses or pathogenic infections triggered when exposed to potentially pathogenic strains like *T. citrinoviride* SJ40, isolated from an indoor settled dust sample (Hoog 1996).

If *Trichoderma* grows inside a building structure, the risks of respiratory exposure due to the air leaks caused by negative pressure are larger. Although most of the *Trichoderma* isolates identified in this study were either *T. atroviride* or *T. trixiae*, the total number of isolates

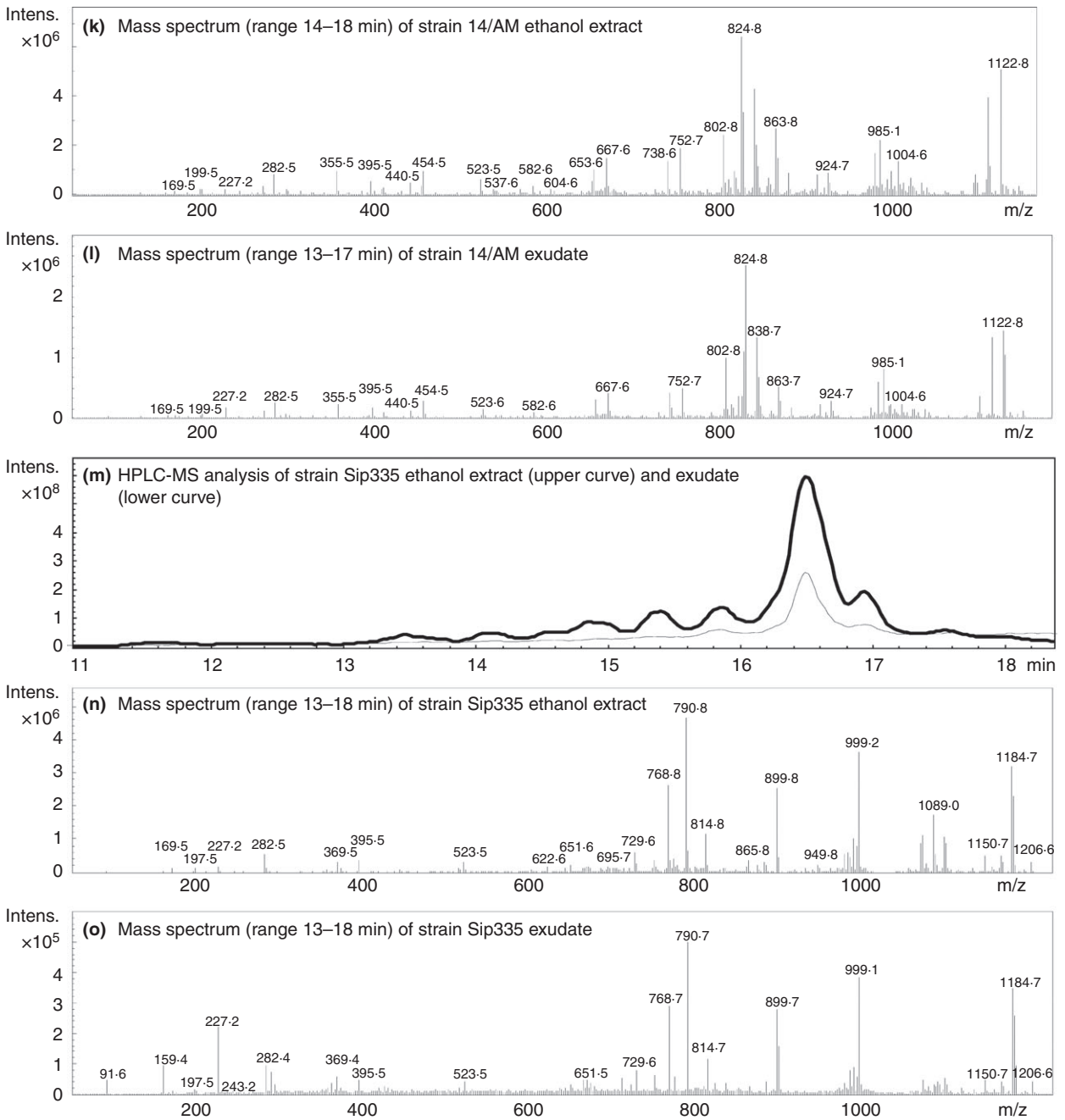

Figure 3 Continued.

was insufficient to conclude on species predominance in problematic buildings in Finland. Isolates identified as *T. atroviride* and *T. citrinoviride* have also earlier been reported from water-damaged buildings in Denmark and Canada (Lübeck *et al.* 2000; McMullin *et al.* 2017). The species *T. paraviridescens* and *T. trixiae* were described during the recent revision of the *T. viridescens* species complex (Jaklitsch *et al.* 2013), thus, even though these species are widely distributed, they have rarely been reported under their new names and only from outdoor samples (Błaszczyk *et al.* 2016; Braithwaite *et al.* 2017).

This is the first report of indoor *Trichoderma* isolates emitting toxic metabolites (peptaibols) in their exudated guttation droplets when growing on building materials or laboratory medium. Moreover, the same peptaibols

were detected in the ethanol-soluble compounds from biomass and the exudate of the same cytotoxic *Trichoderma* strain. Based on the results of this study we speculate that the toxin productions of indoor fungi in guttation droplets may serve as a mechanism of aerosol formation from nonvolatile toxins in the indoor air. Further studies are needed to determine the chemical composition and structure of the exudates, to examine their behaviour and to determine the possible indoor transport mechanisms.

Acknowledgements

The authors thank Riikka Holopainen at the Finnish Food Safety Authority (EVIRA) for providing the feline fetus lung and porcine kidney cells. Henri Gustavson, Liisa Hakamies-Blomqvist, Lauri Sipilä and Johanna Salo are thanked for providing some of the indoor samples.

The authors warmly thank the Academy of Finland (TOXICPM 289161), Business Finland (grant 4098/31/2015) and the National Research, Development and Innovation Office, Hungary (grant NKFI K-105972) for funding this research. LK is grantee of the János Bolyai Research Scholarship (Hungarian Academy of Sciences).

Conflict of Interest

No conflict of interest declared.

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