INDOOR *CHAETOMIUM*-LIKE ISOLATES: RESISTANCE TO CHEMICALS, FLUORESCENCE AND MYCOTOXIN PRODUCTION

Emmanuelle Castagnoli[1], Maria A. Andersson[1], Raimo Mikkola[1], László Kredics[2], Tamás Marik[2], Jarek Kurnitski[1,3], Heidi Salonen[1]

[1] Aalto University, Finland. [2] University of Szeged, Hungary. [3]Tallinn University of Technology, Estonia.

ABSTRACT

Chaetomium-like fungi growing on indoor building materials produce toxic substances. Fourteen toxigenic indoor *Chaetomium*-like isolates from buildings in Finland were investigated. Six *Ch. globosum*-like strains from indoor dusts were toxic with boar sperm assay and cytotoxic to porcine kidney cells (PK-15), emitted green fluorescence and produced chaetoglobosin inhibiting cellular glucose transport. OT7 and OT7b strains from indoor dust were cytotoxic with PK-15 cells, non-fluorescent and produced the extremely cytotoxic protein synthesis inhibitor, chaetomin. The six *Ch.globosum*-like strains were resistant to borax and very sensitive to the wetting agent genapol used in cleaning chemicals. This may indicate that indoor *Chaetomium*-like fungi occupy their own ecological niche in buildings.

INTRODUCTION

The genus *Chaetomium* - and specially the species *Ch. globosum* - is the most common representative of *Chaetomiaceae* in indoor environments. The genus *Chaetomium* represents ubiquitous cellulolytic fungi producing over 500 bioactive substances when growing on various indoor building materials. These 500 bioactive metabolites have potential for the medicinal industry, indoor growth of mycotoxin-producing *Chaetomium globosum* strains is an important condition connected to asthma in mold-infested buildings, furthermore, *Chaetomium globosum* is also known as an important human pathogen. Other *Chaetomium*-like species found indoor have also been isolated from clinical samples. Knowledge about the diversity of indoor *Chaetomium*-like fungi, their ecology and metabolite production is therefore of great importance (Wang et.al. 2016). Species diversity, toxicity towards mammalian cells, toxin production and biocide/chemical resistance of *Chaetomium*-like indoor isolates in Finland has not been investigated. The aim of the study was to screen indoor *Chaetomium*-like isolates from buildings in Finland with 2 toxicity assays.

METHODS

Isolation of indoor strains, rapid toxicity screening and ethanol extraction of fungal biomass

Settled dusts and inlet air filters samples were collected in urban and rural buildings where severe health problems were observed among the humans and piglets.

The *Chaetomium*-like isolates revealed toxic in a rapid screening assay (Mikkola et al. 2015) were suspected to belong to *Chaetomiaceae* after the morphological examination of their ascomata (Wang et al. 2016).

Isolation of the indoor microbial isolates and the ethanol-soluble compounds of biomasses of pure culture grown on malt extract agar (MEA) were performed as described in Mikkola et al. (2015).

Identification of fungal isolates and strains to the genus and species level

Isolates were identified to the genus level based on the colony morphology on MEA, conidiophore morphology, the size of conidia and fluorescence abilities.

Strains identified to species level (MTAV 35, MTAV 37) were identified in DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Indoor *Trichoderma atroviride* strains were identified according to their internal transcribed spacer (ITS) sequences by DNA barcoding (www.isth.info).

Outdoor *T. atroviride* strains derived from the Szeged Microbiology Collection (www.szmc.hu, University of Szeged, Hungary).

Toxicity tests

Toxicity of the ethanol-soluble compounds of biomasses were tested with sperm motility inhibition assay (BMSI), sperm membrane integrity disruption assay (SMID) and mammalian somatic cell toxicity assay (MSCT) as described in Bencsik et al. (2014), except that the exposure in BMSI was for 20 min at 37°C.

Continuous cell line PK-15 (porcine kidney cells) and a malignant cell line MNA (murine neuroblastoma cell line) provided by EVIRA (Helsinki, Finland) were used in the MSCT assay.

Analysis of mycotoxins

Analysis of the mycotoxins contained in the ethanol-soluble compounds of biomasses of the indoor *Chaetomium*-like strains was performed with LC-MS like described in Mikkola et al. (2014). Pure mycotoxins were purchased from Sigma-Aldrich Finland.

Toxicity of biocides and the wetting agent genapol towards fungi

Toxicity of biocides and chemicals towards fungi was tested with a germination inhibition (spore germination shown on fig. 1B) test of fungal spores, c. 10^6 fungal spores ml^{-1} as described by Chitarra (2003). The test was performed with a microtiter plate as described for the MSCT assay in Bencsik et al. (2014). Formation of germ tubes was inspected by phase contrast microscopy after 1 and 2 d of incubation at 28°C. EC_{50} concentration of the chemicals was defined as the concentration resulting in inhibition of 50% of the conidia compared to the ethanol control (1 %). The tests were run in triplicate and calibrated with triclosan giving a SD of mean of $\pm < 20$ %. The biocides and the wetting agent genapol were purchased from Sigma-Aldrich Finland, the commercial fungicide Boracol x10RH was from a local supplier.

RESULTS

Characterisation of the *Chaetomium*-like isolates

Table 1 shows the size of ascospores and the fluorescence profile of the ethanol-soluble compounds of biomass of the *Chaetomium*-like isolates. The morphology of their ascomatal hair (Fig. 1A) was also used for characterization. The isolates were categorized into green, blue, yellow or no emitted fluorescence.

Table 1. Characterization of the Chaetomium-like isolates.

Isolate	Isolated from	Substratum	Fluorescence	Size of ascospores (µm)
MTAV 35	Public building, Oulu	Settled dust	green	10.6 x 9*
MTAV 37	Public building, Oulu	Settled dust	green	10.5 x 9*
MH1	Public building, Espoo	Settled dust	green	8.5 x 7.6*
RUK 10	Home, Helsinki	Settled dust	green	10.4 x 8.8*
ABCD	Home, Helsinki	Settled dust	green	9 x 7.6*
MO9	Piggery, Orimattila	Bedding	green	10.3 x 7.6*
2c/MT	Home, Vantaa	Settled dust	green	9.5 x 7.3*
MO15	Piggery, Orimattila	Bedding	yellow	12 x 8**
Ch1/tu	Public building, Espoo	Inlet air filter	blue	5.7 x 4.1**
OT7	Office, Helsinki	Settled dust	none	8.9 x 7.8*
OT7b	Office, Helsinki	Settled dust	none	9 x 7.7*

*Ascomatal hair coiled, unbranched. ** Ascomatal hair dichotomously branched

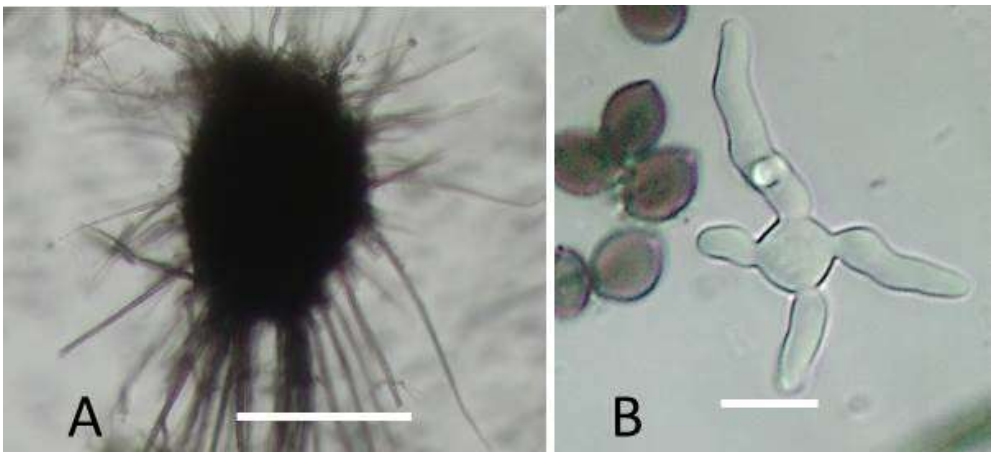

Figure 1. Phase contrast micrographs of an indoor ascomata-producing isolate. A) Formation of ascomata typical of Chaetomiaceae (bar 100 µm). B) Ascospores and formation of a germ tube indicating spore germination (bar 10µm).

Toxicity of biocides and genapol towards indoor *Chaetomium*-like isolates

The resistance of the fluorescent isolates showed in Table 2 were tested with biocides, chemicals and genapol (wetting agent used indoors). The fungal isolates were more resistant to genapol and the biocides than were the mammalian cell lines PK-15 and MNA. The green-fluorescent isolates were 4-fold more resistant to borax than the *Trichoderma atroviride* reference strains. The green-fluorescent isolates were quite sensitive to the other biocides compare to the other reference strains. Only the green-and blue-fluorescent isolates were over 1 000 times more sensitive to genapol than the yellow-fluorescent isolate and the reference strains. The blue-fluorescent isolate was also sensitive to borax. On the contrary the yellow fluorescent isolate was resistant to both genapol and borax, like were the Aspergillus and Paecilomyces strains.

Table 2. Toxicities of biocides and the wetting agent genapol to Chaetomium-like strains compared with selected, in- and outdoor fungal strains and mammalian cell lines.

	EC_{50} µg.ml^{-1}						
	Borax	**Boracol**	**PHMB**	**Genapol**	**Fenoxy-ethanol**	**Chloramine**	**Triclosan**
Indoor Chaetomium-like strains							
Green-fluorescing strains[a]							
	≥5000	100	4-8	<50	700-1500	1200-2500	2-4
Blue-fluorescing strain[b]							
	1200	100	4	<50	1500	1200	2
Yellow-fluorescing strain[c]							
	5000	100	4	50000	1500	1200	2
Mammalian cell lines (MSCT assay)							
MNA	150	<50	4	25	400	80	4
PK-15	600	<50	15	25	1500	150	15
Reference strains							
Indoor *Trichoderma atroviride* strains [d]							
	1200	100	4	>50000	1500-3000	1200	16-30
Outdoor *Trichoderma atroviride* strains [e]							
	1200	100	4-16	>50000	1500-3000	1200-2500	8-16
Indoor *Aspergillus* strains [f]							
	≥5000	400-800	30-60	>50000	1500-3000	600-1200	8-16
Indoor *Aspergillus* strains [g]							
	5000	100	30-60	>50000	800-400	600-1200	16
Indoor *Paecilomyces* strains [h]							
	≥5000	400	30	>5000	3000	1200	4

[a] MTAV 35, MTAV 37, MH1, RUK 10, ABCD, MO9, 2c/MT; [b] Ch1/tu; [c] Mo15; [d] Ke14, Kiv10, Tri335, 14/AM; [e] SZMC: 12323, 12474, 12495, 12541, 1723, 207080; [f] growing at 37ºC: 32/skk, 33b/skk, 7D /skk, 1/skk, Asp21/skk; [g] not growing at 37ºC: *A. westerdijkiae* PP2, AW/KL, *A.versicolor* SL/3; [h] growing at 37 ºC: *P. variotii* Pac2/kop, Pac/skk, Pac /his.

Metabolite and toxicity profiling of *Chaetomium*-like isolates

The toxicity against mammalian cells and metabolite profiling of the green- and non-fluorescent strains is shown on Table 3. The green-fluorescent strains MTAV35, MH1, RUK 10 and ABCD containing 3-4 mg/mL of Chaetoglobosin A were more toxic in the BSMI than the MSCT but had 100-fold higher EC50 concentration in the SMID than pure Chaetoglobosin A. This indicate that Chaetoglobosin and Chaetoglobosin-containing extracts immobilized sperm cells but did not disrupt the sperm plasma membrane like the lethal toxin Alamethicin. The 2c/MT strain excreted Chaetoglobosin in the exudates. The non-fluorescent strains ethanol-soluble compounds of biomass were 100-fold more toxic in MSCT that the Chaetoglobosin-producing isolates. The non-fluorescent strains ethanol-soluble compounds of biomass were more toxic in the MSCT than in the BSMI and SMID assays like the protein synthesis inhibitor Sterigmatocystin. Besides the of protein synthesis inhibitor Chaetomin was detected in the ethanol-soluble compounds of biomass of the non-fluorescent strains.

Identification of *Chaetomium*-like isolates to the species level

The MTAV 35 and MTAV 37 were identified as *Chaetomium globosum*. The other green-fluorescent isolates MH1, RUK 10, ABCD and 2c/MT were probably same species as MTAV 35 and MTAV 37 because they also produced chaetoglobosin and Chaetoviridin, had similar ascomatal hair and size of ascospores. The difference between OT7 and OT7b strains and *Ch. globosum*-like isolates were the chaetomin production, extreme cytotoxicity and absence of fluorescence.

Table 3. Toxicity- and metabolic profiling of the ethanol-soluble compounds of biomasses of the non-fluorescent and some green-fluorescent isolates.

	EC_{50} µg ml^{-1} of the ethanol-soluble compounds of biomasses				
Strain	BMSI 20 min	SMID 2h	MSCT 2d	Identified metabolite	Concentration ($mg.ml^{-1}$)
Ch. globosum MTAV35	5	450	40	Chaetoglobosin A	3.4
				Chaetoviridin A	0.02
				Chaetoviridin C	0.2
Ch. globosum MTAV37	10	350	30	No data	
MH1	5	310	50	Chaetoglobosin A	3.9
				Chaetoviridin A	0.5
				Chaetoviridin C	0.2
RUK 10	5	300	20	Chaetoglobosin A	4.2
				Chaetoviridin A	0.04
				Chaetoviridin C	0.05
ABCD	5	450	30	Chaetoglobosin A	4.24
				Chaetoviridin A C	0.3
				Chaetoviridin C	0.05
2c/MT				Chaetoglobosin*	
OT7	10	480	0.5	Chaetomin	1.3
				Chaetoviridin A	0.13
				Chaetoviridin C	0.02
OT7b	10	480	0.8	Chaetomin	1.2
				Chaetoviridin A	0.3
				Chaetoviridin C	0.2
Commercial pure mycotoxins **				Biological activity	
Alamethicin	5	1	8	K^+ and Na^+ ion channel former	
Chaetoglobosin A	1	12	2	Inhibitor of glucose transport	
Citrinin	>100	50	10	Cytotoxic, nephrotoxic	
Sterigmatocystin	>20	>20	0.5	Inhibitor of protein synthesis	

*Chaetoglobosin found in exudates from MEA plates ** Signa-aldrich

DISCUSSION

The results indicate that the *Ch. globosum*-like isolates represented the most common toxigenic ascomata-producing fungi in Finnish buildings. Wang et al. (2016) reported that the most common isolated indoor *Chaetomium* species worldwide is *Ch. globosum*.

The blue-fluorescent CH1/tu isolate possibly originating from outdoor air was more sensitive to borax that the other indoor *Chaetomium*-like isolates indicating that indoor *Chaetomium*-like isolates may occupy their own ecological niche in buildings.

The chaetomin-producing indoor isolates OT7 and OT7b were not reported in Finland earlier.

The blue-(CH1/tu) and yellow-fluorescent (MO15) isolates had dichotomously branched ascomatal hair like the genus *Dichotomophilus* which is separated from the genus *Chaetomium* (Wang et al 2016).

Fluorescence, biocide/genapol resistance, as well as toxicity- and metabolite profiling may be useful in preliminary tracking of diversity of indoor ascomata-producing *Chaetomium*-like isolates.

The results demonstrates species variability in biocide/chemical resistance among indoor fungal species and genera. The indoor use of biocides and chemicals may influence proliferation and species diversity of the indoor microbiota.

ACKNOWLEDGEMENT

The work was supported by TEKES (grant 4098/31/2015) and Academy of Finland (grant 289161). The somatic cells from Riika Holopainen (Finnish Food Safety Authority EVIRA/Virology). LK and TM were supported by the project GINOP-2.3.3-15-2016-00006.

REFERENCES

Bencsik O, Papp T, Berta M, Zana A , Forgó P , Dombi G, Andersson M, Salkinoja-Salonen M, Vágvölgyi C and Szekeres A. Ophiobolin A from *Bipolaris oryzae* perturbs motility and membrane integrities of porcine sperm and induces cell death on mammalian somatic cell lines. *Toxins* **2014**, *6*, 2857-2871.

Chitarra G.S. 2003. Germination inhibitors of fungal spores: identification and mode of action. Thesis. Wageningen University, Wageningen, The Netherlands, ISBN90-5808-914-2.

Mikkola R, Andersson MA, Hautaniemi M, Salkinoja-Salonen MS. Toxic indole alkaloids avrainvillamide and stephacidin B produced by a biocide tolerant indoor mold *Aspergillus westerdijkiae*. *Toxicon* **2015**, *99*, 58-67.

Wang XW, Houbraken J, Groenewald JZ, Meijer M, Andersen B, Nielsen KF, Crous PW, Samson RA. Diversity and taxonomy of *Chaetomium* and chaetomium-like fungi from indoor environments. *Studies in Mycology* **2016**, *84*, 145-224.