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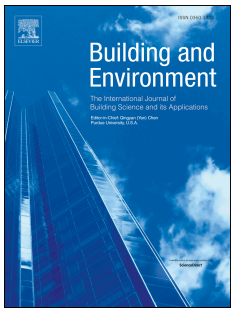

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ABSTRACT:

Stachybotrys chartarum is a fungal contaminant of damp indoor environments that can produce several toxins belonging to the family of macrocyclic trichothecenes. These toxins are suspected to be involved in different pathologies among residents of moldy indoor environments. However there are only few data on the capacity of *S. chartarum* to produce its toxins (type and proportion) while growing on different building materials. This study aimed to quantify by UPLC-MS/MS the production of four major macrocyclic trichothecenes (Satratoxins G and H, Roridin L2 and Verrucarin J) during colonization of different building materials (fiberglass, painted fiberglass wallpaper, wallpaper, vinyl wallpaper, fir) by *S. chartarum*. It showed that the four molecules were produced upon development of a toxin-producing strain of *S. chartarum* on the material. The nature of building material strongly influenced the levels of macrocyclic trichothecenes produced. Wallpaper appeared to be the most favorable to both fungal development and production of the four toxins. By contrast, no toxin production was observed on vinyl wallpaper, in agreement with lack of fungal growth. Satratoxin H was always the main toxin produced, on all tested substrates, and its concentration reached 14.2, 3, 1.8 and 1.1 mg/m^2 on wallpaper, fir, fiberglass and fiberglass wallpaper, respectively. This knowledge is important to define monitoring strategies and assess risk related to those contaminants.

KEY WORDS:

Stachybotrys chartarum, macrocyclic trichothecenes, building materials, wallpaper, UPLC-MS/MS

1. INTRODUCTION

In developed countries, people spend almost 80% of their time inside buildings (schools, offices, sports, homes…) and it is estimated that 20 to 40% of buildings in Northern Europe and North America, display macroscopically visible fungal development [1, 2]. In France, more than 600 000 homes have moldy surfaces of more than 1 m^2 [3]. Fungal growth in damp or water-damaged buildings is an important public health issue worldwide since it can lead to several adverse effects on the occupants and especially allergic and respiratory troubles [4-6]. Futhermore, during their development, some fungal species might also produce secondary metabolites toxic for humans, called mycotoxins. These toxins could play a role in the development of such disorders [7, 8].

Indeed, among fungal species frequently isolated from damp buildings, some are well known to be potent mycotoxins producers [9]. Thus, *Stachybotrys chartarum* is a frequent contaminant of indoor environments worldwide [9, 10] and may be isolated in about 50% of building material samples in USA [11, 12] and in 10 to 30% of samples in Europe depending on the surveys and history of buildings [13, 14]. Indeed, *S. chartarum* is mostly found in dwellings following water damage [11, 13, 15].

S. chartarum became the focus of attention following reports of its association with idiopathic pulmonary hemorrhage in infants in Cleveland, Ohio [16]. More recently, there has been increasing evidence of a possible relationship between the presence of *S. chartarum* in indoor environments and human illness such as sick building syndrome [17]. These deleterious effects could be related to either the allergic potential of spores [18] or mycotoxin production [8, 19].

This fungal species is able to produce several toxic compounds that belong to the family of macrocyclic trichothecenes such as satratoxins G and H, roridin L2, verrucarin J. These mycotoxins are known to be strong inhibitors of protein synthesis and their acute toxicity has been demonstrated following ingestion in horses [20], contact with skin [21] and also after inhalation in rodents [22]. In a recent study, Carey et al. [23] demonstrated that intranasal exposure to satratoxin G induces rhinitis, atrophy of the olfactory epithelium and apoptosis of olfactory sensory neurons in both mice and Rhesus monkeys.

Thus, the frequent isolation of *S. chartarum* in buildings where inhabitants display respiratory problems raises the question of a possible involvement of macrocyclic trichothecenes in these pathologies.

The demonstration of such a relationship and subsequent risk assessment first require the quantification of the different toxins that may be produced during *S. chartarum*'s development on indoor materials. A few analytical methods, based on LC-MS or ELISA, have been developed to evaluate mycotoxins production by this contaminant but most of these were devoted to the characterization of the toxigenic potential of *Stachybotrys* strains on culture medium [24] or were more qualitative than quantitative [15, 25, 26]. The few quantitative surveys carried out focused only on satratoxins [27]. Moreover, in these studies, no relationship was drawn between fungal development and toxinogenesis or between material and the amount of toxin produced.

Within this context, the aim of this study was to measure the production of four major macrocyclic trichothecenes (satratoxins G and H, roridin L2 and verrucarin J) by *S. chartarum* when colonizing different indoor building materials using an UPLC-MS/MS method.

2. MATERIALS AND METHODS

2.1. Mycotoxin standards

Among used, only Verrucarin A (VerA) is commercially available and was purchased from Sigma (Saint-Quentin Fallavier, France). Satratoxin G (SG) and roridin L2 (RL2) were purified as previously described by Islam et al. [22]. Satratoxin H (SH) was prepared from cultures of *S. chartarum* (ATCC 62765) as described by Jarvis et al. [28] and verrucarin J (VerJ) was isolated and characterized from *Myrothecium verrucaria*, another macrocyclic trichothecene producing species (ATCC 24571), as previously reported by Jarvis [29]. Standards were dissolved in methanol (MeOH) to obtain stock solutions that were stored at -20°C.

2.2. Solvents and reagents

All reagents including the solvents used for sample preparation were purchased from ICS (Lapeyrouse-Fossat, France) and were analytical grade. Acetonitrile (AcN) used for mobile phase was LC/MS grade and purchased from Thermo Fischer Scientific (Illkirch, France) and water was obtained from an ultrapure water (18.2MΩ) system (Elga Labwater Veolia, Anthony, France).

2.3. Fungal strains

The study was conducted using a strain of *S. chartarum,* previously isolated from contaminated straw by direct plating on Malt Extract Agar (MEA, BioKar, France), shown to produce SG, SH, RL2 and VerJ on MEA (ST82 strain). It was stored at 4°C and its viability checked regularly by culture on MEA.

2.4. Materials for toxinogenesis tests

Fiberglass (FG) (GF/B glass fiber filter, Whatman) was used as a reference material. Four other commercially available materials frequently encountered in indoor environments were tested. These materials were purchased in a specialized store and were as follows: painted fiberglass wallpaper (FWP) (Toile de verre, maille chevron, BATCH N°S2009061492 + Paint MS SAT LUXENS, Leroy Merlin, France), wallpaper (WP) (Papier Peint BLAN BLA 0 INSP, Leroy Merlin, France), vinyl wallpaper (VWP) (P.VINYL/INT BLAN BLA 0 INSP, Leroy Merlin, France), and fir (Leroy Merlin, France). They were cut into 2×5cm pieces and then sterilized (121°C, 20 min) before use as described by Gorny et al. [30] and Peitzsch et al. [31].

2.5. Characterization of macrocyclic trichothecenes production on materials

The ST82 strain of *S. chartarum* was grown on potato dextrose agar (PDA, BioKar, France) for 14 days at 25 °C to obtain highly sporulating cultures. Spore suspension was prepared from these fungal cultures by adding 10mL of Tween 80 (0.05%) to the Petri dish. The number of spores was quantified by direct counting on a Malassez cell. Spore suspension was then diluted to obtain the required concentration. Contamination was achieved by applying 100 µL of this suspension (10^7 spores/mL) to each material. Contaminated materials were placed in flasks, on a layer of 2 cm of glass beads with sterile water in order to maintain moisture level at a saturation level throughout the test. Samples were then cultured for 10 days at 25 °C in total darkness. After incubation, fungal development was assessed by examination of samples under stereo-microscope (magnification from 12 to 120) (Olympus SZX9) and evaluation of both hyphae development (density and colonized surface) and density of sporulated conidial heads on the whole sample (10 cm^2). In order to determine mycotoxin concentration in the initial inoculum (T0 value), fiberglass material was frozen immediately after spore deposition and without incubation to avoid fungal development. It was further analyzed similarly to other samples.

All analyses were done in triplicate and three independent experiments were carried out. Results are expressed as the mean with CV %.

2.6. Extraction procedure

Macrocyclic trichothecenes were extracted from building materials by gentle mechanical agitation on a horizontal shaker (Reciprocating Shaker, IKA HS501 Digital, Grosseron, France) in 20 ml of chloroform/methanol (2:1). After 4 hours, extracts were centrifuged for 5 min at 3,500 rpm and filtered through a phase separator filter (Whatman 1 PS). Four milliliters of the filtered extract were evaporated to dryness and then suspended in 1 mL of MeOH. For each of the macrocyclic trichothecene quantification assays on building material, VerA was added (100 µL of a 40 µg/mL solution in MeOH) as an Internal Standard (IS) before starting the extraction procedure. Indeed, this macrocyclic trichothecene is the only one that is commercially available as a pure standard, it displays a similar structure to other toxins of this family (presence of the typical multi-cyclic sesquiterpene structure) and is not produced by *S. chartarum* [24, 29].

2.7. Instrumental and analytical conditions

Macrocyclic trichothecenes quantification was performed with an Acquity ultra performance liquid chromatography (UPLC) system coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Macrocyclic trichothecenes and the internal standard (VerA) were separated on an Acquity BEH C18 column (2.1 x 100 mm; 1.7 µm; Waters). Samples were ionized in positive electrospray ionization mode (ESI^+). The capillary voltage and source temperature were set at 3.5 kV and 150°C, respectively. The desolvation temperature and nitrogen flow rate were set at 650°C and 800 L/h, respectively. Argon was used as the collision gas at a flow rate of 0.12 mL/min. Mycotoxins (5 µL of samples) were eluted on an Acquity BEH C18 column with an AcN/H_2O gradient (*t*(0-0.5 min): 10% AcN; *t*(0.5-4 min): 90% AcN) at a flow rate of 0.35 mL/min. Quantification was carried out by Multiple Reaction Monitoring (MRM) mode in positive electrospray ionization (ESI^+) and all the toxins gave the protonated parent $[M+H]^+$ or the sodium/potassium adduct $[M+Na]^+$ or $[M+K]^+$. The two MRM transitions generated for each macrocyclic trichothecene gave MS/MS fragments corresponding to the cleavage at the ester C4 and C15 (except for RL2) bonds between the 12,13-epoxy-trichothec-9-ene cycle and the macrocycle (or the aliphatic chain for RL2) (figure 1). The MRM transition with the highest signal to noise ratio and the highest intensity was selected for quantification and the second MRM transition was used for confirmation.

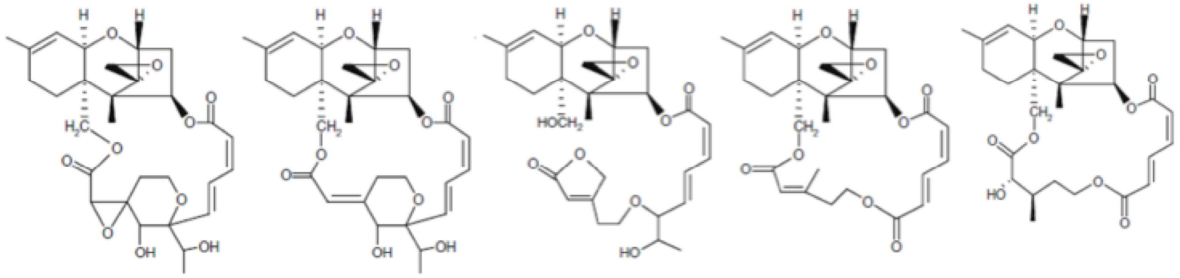

Figure 1: Structure of analyzed macrocyclic trichothecenes

MRM transitions, cone voltage and collision energies used for the different toxins are listed in table 1. Chromatographic data were monitored by Masslynx 4.1 software (Waters, Milford, MA, USA).

Table 1: MRM transitions, cone voltages and collision energies used for macrocyclic trichothecenes detection.

TCT	Molecular weight	Parent ions	MRM fragments	Cone voltage (V)	Collision energy (eV)
RL2	530	553	249	42	16
		553	305	42	26
SG	544	545	81	20	34
		545	231	20	16
SH	528	529	249	24	16
		551	303	48	28
VerJ	484	523	151	46	32
		523	293	46	34
VerA	502	503	249	22	14
		525	295	44	30

The chromatographic conditions used allowed the separation of the macrocyclic trichothecenes within 5 minutes with a relative standard deviation on the retention times of less than 1% for each analyte (figure 2).

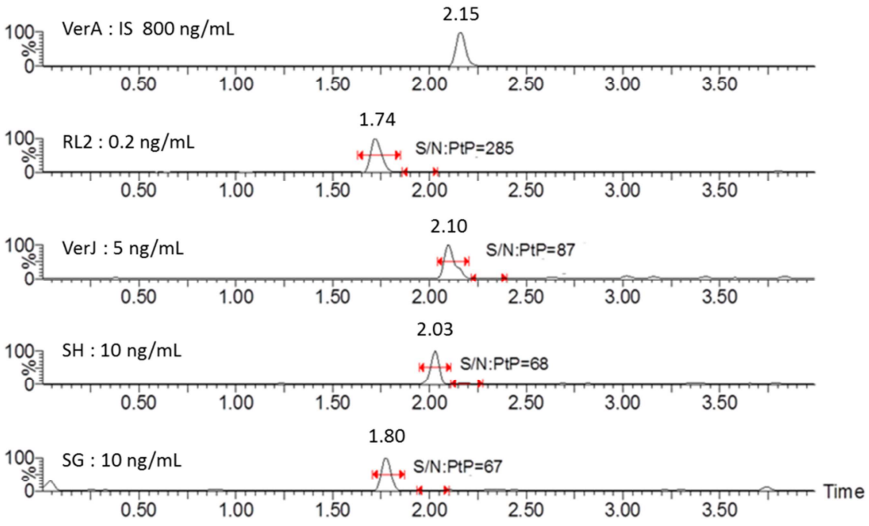

Figure 2: MRM chromatogram of each macrocyclic trichothecene at LOD with its respective signal to noise ratio and retention times (RT).

2.8. Validation procedures

The method was validated for each macrocyclic trichothecene according to the 2002/657/CE commission decision in terms of specificity, linearity, matrix effects, recovery, precision and accuracy, limit of quantitation and limit of detection [32].

2.8.1 Selectivity

The selectivity of the method was assessed by contaminating building materials with an *Aspergillus versicolor* strain, which does not produce macrocyclic trichothecenes. The chromatograms of contaminated samples were compared to those of macrocyclic trichothecenes at the lowest limit of quantitation. Retention times (RT) were checked for each toxin with a tolerable deviation of 1% from the expected RT value. This was done in triplicate.

2.8.2 Linearity

Calibration curves were prepared with the IS (VerA) in MeOH using a minimum of five data points. The calibration curve ranged from 0.01 µg/mL to 5 µg/mL for RL2 and VerJ and from 0.1 µg/mL to 5 µg/mL for SG and SH with a fixed IS concentration of 0.8 µg/mL, corresponding to the IS concentration obtained after the building materials extraction procedure. Each calibration standard was injected three times. Linear model (Y=aX+b) was tested with weightings of 1, 1/X and $1/X^2$ (X=nominal concentration) (supplementary figure). Three approaches were used to assess the linearity of the calibration curve: 1) calculation of the relative error between the nominal concentration and the concentration obtained with the model (RE%), which should be lower than ± 20%, 2) visual inspection of the residual distribution, and 3) application of a lack of fit test to check the goodness of fit of the model [33, 34]. All macrocyclic trichothecenes were validated with a linear model weighted by $1/X^2$ (with X = concentration) with RE lower than 20% for all calibration standards (Table 2). The weighted residuals obtained with this calibration curve were randomly distributed around the mean and the linearity of the curve was confirmed by a lack of fit test (supplementary table).

Table 2: Validation results of macrocyclic trichothecenes calibration standards used to assess linearity. Each calibration curve was validated with a linear model weighted by $1/X^2$ (X= concentration).

(n=3)	RL2		SG		SH		VerJ	
Concentration (µg/mL)	Measured mean (µg/mL)	RE %	Measured mean (µg/mL)	RE %	Measured mean (µg/mL)	RE %	Measured mean (µg/mL)	RE %
0.01	0.010	-2					0.010	-1
0.1	0.115	15	0.095	-5	0.103	3	0.107	7
0.2	0.262	5	0.284	13	0.231	-7	0.239	-4
1	1.001	0	1.027	3	0.957	-4	1.018	2
2	1.929	-4	1.956	-2	2.076	4	1.998	0
5	4.250	-15	4.571	-9	5.239	5	4.824	-4

2.8.3. Limits of detection and quantification

The lowest limit of detection (LOD) was defined as the lowest concentration that could be reliably differentiated from background noise (signal to noise > 3). LOD was determined from 3 injections of macrocyclic trichothecenes standards at the lowest concentration that could be detected with a signal to noise ≥ 3. The limit of quantification (LOQ) was determined and validated for the lowest concentration of the calibration curve chosen for its relevance to macrocyclic trichothecenes investigation in building materials.

The lowest concentrations of calibration standard that could be detected and differentiated from the baseline with S/N>3 were 0.2 ng/mL for RL2, 5 ng/mL for VerJ and 10 ng/mL for SH and SG: these values were considered as the LODs of each macrocyclic trichothecenes. The LOQs were set at 10 ng/mL for RL2 and VerJ and at 100 ng/mL for SG and SH which correspond to 0.05 mg/m^2 for RL2 and VerJ, and 0.5 mg/m^2 for SG and SH.

2.8.4. Precision and Accuracy

In order to preserve the macrocyclic trichothecenes standards, intra-day (repeatability) and inter-day (reproducibility) precisions and accuracy of the method were assessed using quality control (QC) samples of RL2 at a single concentration level of 0.022 µg/mL. Three replicates of this QC were injected three times at 4-day intervals. Accuracy was calculated as the percentage of the ratio between the mean calculated concentration and the theoretical nominal value. Precisions were expressed by the coefficient of variation (CV% = SD/mean*100). Intra/inter-day standard deviation was obtained from an analysis of variance (ANOVA) with a single factor.

2.8.5 Percent recovery and matrix effects

VerA was used as an internal standard for quantification experiments. MRM transitions were optimized for VerA to ensure its suitability. The two major MRM transitions were obtained in ESI+ with both the protonated parent $[M+H]^+$ and the sodium adduct $[M+Na]^+$. Furthermore, the highest MRM transitions gave the product ion at m/z 249 which was directly observed for RL2 and SH and corresponded to the sesquiterpenic cycle.

Percent recovery was then evaluated by comparing the peak areas of VerA (100 µL at 20 µg/mL in MeOH) and RL2 (100 µL at 1.1 µg/mL in MeOH) standards spiked on fiberglass before extraction and obtained after the extraction procedure with the peak area of

VerA and RL2 obtained after direct injection into the UPLC/MS system. Results were 107 +/- 20% and 119 +/- 20% for VerA and RL2 respectively. Matrix effects were quantitatively estimated with the matrix factor (MF) defined as the percentage ratio of the analyte peak area extracted from the matrix to the analyte peak area extracted without matrix [35]. The matrix effect was investigated for each building material with VerA (IS). Respective MFs for fiberglass, wallpaper, fir, vinyl wallpaper and painted fiberglass wallpaper were at 97%, 99%, 52%, 35% and 21%.

2.9. Statistical analysis.

Data were analyzed by applying the GLM option of the ANOVA program in the MiniTab software version v13.0. Significant differences between levels of toxins observed on the different materials tested in this study were evaluated by Tukey's test for each individual macrocyclic trichothecene. P value was set at 0.05.

3. RESULTS

3.1. Development of *S. chartarum* ST82 strain on different building materials

The development of *S. chartarum* ST82 strain on different building materials was estimated by macro- and microscopic examination of hyphae development and density of sporulated conidial heads before mycotoxins extraction and analysis. *S. chartarum* growth varied according to the substrate (figure 3). The vinyl wallpaper did not allow the development of *S. chartarum* ST82 strain and thus no active sporulation was observed on this material, with the exception of the edge of the sample, where paper was no longer protected by the polyvinyl chloride layer (data not shown). On fiberglass, hyphae development was located on circular zones of 3-5 mm diameter surrounding the inoculation site. Density of conidial heads and sporulation were moderate. The dark color on the material resulted both from *S. chartarum*'s development and the initial color of the inoculum. On fir and wallpaper, development was intense and regular with abundant hyphae colonizing the whole sample's surface with many sporulated heads. On the painted fiberglass wallpaper, development was moderate and irregular, and sporulation was weak. Examination under stereomicroscope showed that development mostly occurred in zones that were unequally covered by paint due to the irregular surface of the material.

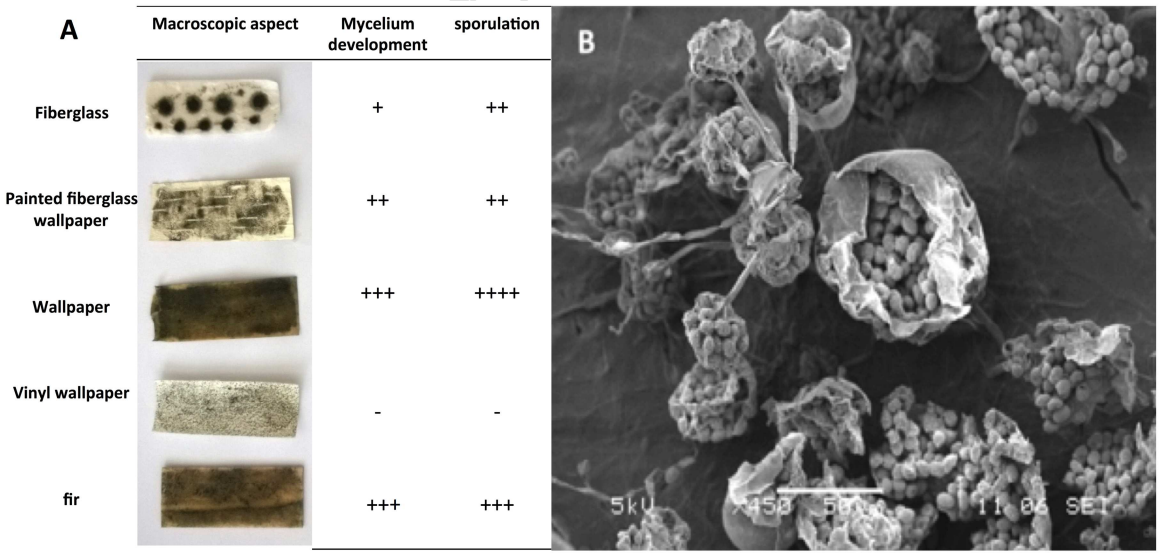

Figure 3: Development of *S. chartarum* ST82 strain on different building materials.
A: Macroscopic aspect of the different materials after development of ST 82 strain and evaluation of mycelium development and sporulation under stereomicroscope.
B: Observation by Scanning Electron Microscopy of ST82 growth on wallpaper.

3.2. Toxinogenesis of *S. chartarum* ST82 strain on different materials

3.2.1. Levels of macrocyclic trichothecenes produced on building materials.

Macrocyclic trichothecenes production during the development of *S. chartarum* ST82 strain on the different building materials was measured by UPLC-MS/MS (figure 4A). The four toxins were detected at different levels on tested materials. Initial levels of contamination (T0 value corresponding to inoculum deposit on the substrate) were 0.3 ± 0.01; 0.08 ± 0.02 and 1.3 ± 0.33 mg/m^2 of substrate for RL2, VerJ and SH, respectively. No SG was detected before incubation. It has to be noted that while fiberglass and wallpaper displayed only a mild matrix effect (MF near 100%), the interaction was more pronounced for the other substrates, thus demonstrating the importance of using an internal standard to quantify mycotoxins by UPLC-MS/MS on building materials.

All four quantified mycotoxins were produced at their highest level on wallpaper ($p<0.05$) (Figure 4A). By contrast, the levels of toxins found on vinyl wallpaper were not significantly different from the T0 value, indicating no active production of macrocyclic trichothecenes in agreement with the lack of fungal development observed on that substrate. Levels of SG, SH and RL2 measured on fiberglass, fir and painted fiberglass wallpaper were not significantly different despite the differences in growth and sporulation observed on these materials. Level of VerJ was significantly higher on fiberglass than on vinyl wallpaper (Figure 4A).

3.2.2. Comparison of toxinogenesis profiles

Since *S. chartarum* can produce several toxins simultaneously, an analysis of their relative proportions on different building material is of importance for risk evaluations. Figure 4B shows the relative proportion of each toxin as a function of the material used. SH was the predominant toxin, whatever the substrate, representing 51 to 64% of the total amount of macrocyclic trichothecenes produced. The proportions of the three other toxins varied with the substrate. Satratoxin G represented 26% of the total macrocyclic trichothecenes produced on wallpaper and fir, 16% on fiberglass and 10% on painted fiberglass wallpaper. RL2 was present on all substrates, and represented 11 to 21% of the total macrocyclic trichothecenes. VerJ was also found, on all substrates with the exception of vinyl wallpaper. However, it represented a lower percentage of total toxin load (2 to 7%) compared to SG, SH and RL2.

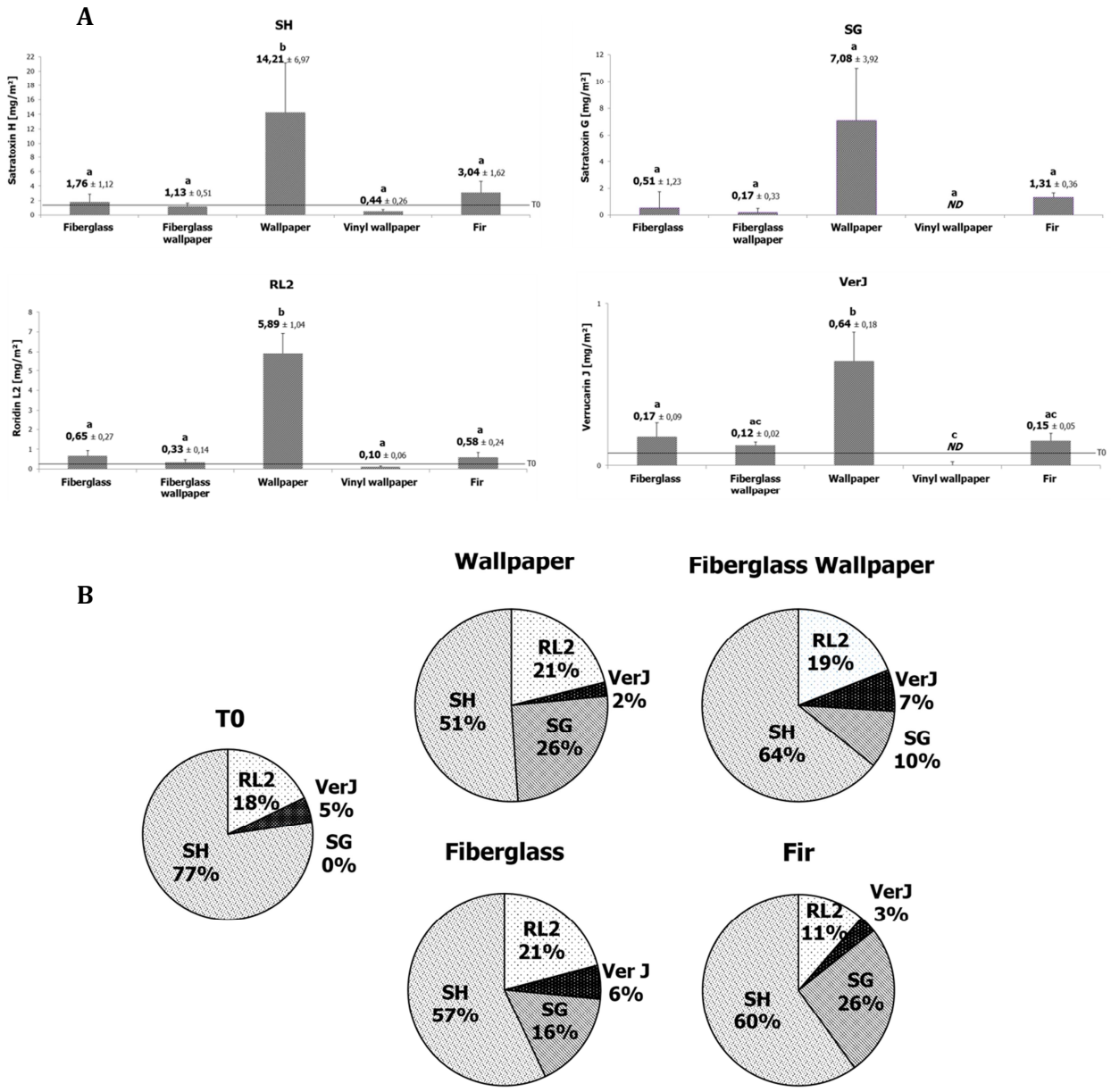

Figure 4: Macrocyclic trichothecenes productions with ST82 strain on the different materials measured by UPLC-MS/MS.

A: Results in mg/m² are expressed as mean ± SD of three distinct experiments (each in triplicate). Different letters indicate significant differences for one toxin as a function of the substrate. Horizontal line indicates T0 value for each toxin (no SG was detected at T0).

B: Relative proportion (%) of each macrocyclic trichothecene detected in the initial inoculum (T0) and produced on the different materials.

4. DISCUSSION

S. chartarum is a frequent contaminant of damp indoor environments where cellulosic substrates may allow its development. It occurs especially following a water damage that increases water activity to the levels required for *S. chartarum* development [17, 36, 37]. Despite the numerous data on its prevalence in indoor environments, the differential tropism of this fungal genus for the materials commonly used in habitations is poorly documented. Indeed most studies focused on airborne contamination. Nevertheless, Andersen et al. [14] highlighted the frequent presence of *S. chartarum* on both fiberglass and wallpaper. These materials are good substrates for the development of this fungus. This is not the case for other building materials such as linoleum or chipboard where other species were observed [14]. This is also in agreement with a previous study where *S. chartarum* was isolated on several wallpaper samples [27]. Our study demonstrated that wallpaper is the best substrate for both hyphal development and sporulation. Yet, we observed only a mild fungal development on painted fiberglass wallpaper. Although the paint used in the study had no antimicrobial activity, the paint layer might limit access to nutrients and slow down *S. chartarum*'s growth [38, 39]. The development on fiberglass was different with a limited development of mycelium and a sporulation comparable to that observed on painted fiberglass wallpaper. Once again, this observation could be related to a lack of nutrients accessibility that is essential for hyphal growth. Finally, the vinyl wallpaper appeared to be resistant to *S. chartarum*'s development for it did not allow its development or its sporulation. However, the observed development of *S. chartarum* on the edge of the sample, where the paper is no longer protected by the polyvinyl chloride layer, suggests that, despite an initial resistance to colonization, such material could become sensitive with time and aging, after protective layer becomes porous. All samples in this study were analyzed after a common incubation period of 10 days in order to compare them. It is then possible that the extension of incubation would have allowed larger a colonization of this material.

In the present study, we quantified four major macrocyclic trichothecenes produced during the development of a toxigenic strain of *S. chartarum* on wallpaper, fir, fiberglass and painted fiberglass wallpaper. Toxin production appeared to be higher on wallpaper, which is a substrate that displays the highest and easiest access to cellulose. Whereas, for other materials, the limited access to nutrients (protected by PVC or paint) could explain the differences in toxin production. As an illustration, toxin production was

lower on painted fiberglass wallpaper than on fiberglass. Moreover, our results demonstrate that there is no strict relationship between toxin production and fungal development. Indeed, on fir, development and sporulation of the *S. chartarum* strain were important and comparable to what was observed on wallpaper. However, the levels of toxins produced on fir were significantly lower than on wallpaper. The presence in fir of several terpenoid compounds, which were previously identified as possible inhibitors of mycotoxin production [40], could explain that observation.

Only few studies have investigated the production of macrocyclic trichothecenes on materials following *S. chartarum*'s development. Most of them were not quantitative but demonstrated the possible co-occurrence of several macrocyclic trichothecenes [41] and especially SG and SH [25]. In a study by Gottschalk et al. [27], SG and SH were quantified and the levels observed on wallpaper samples were in agreement with the present study. RL2 and VerJ were also previously detected but not quantified [27].

When related to an equivalent surface of 1 square meter of material, the concentrations of the different macrocyclic trichothecenes produced after *S. chartarum*'s development could reach several mg/m^2. For example, on wallpaper, mean concentrations of RL2, SG and SH reached 5.9, 7 and 14.2 mg/m^2, respectively. Such contamination levels of materials could be responsible for the reported detection of these mycotoxins in the air of water-damaged buildings with *Stachybotrys* contamination [15, 26]. Although no clear dose-effect relationship has been established for these mycotoxins, it has been recently demonstrated that an intranasal exposure of Rhesus monkeys to 5 µg SG for 4 days led to a widespread apoptosis of olfactory sensory neurons and to epithelial and olfactory nerve atrophy as well as an acute neutrophilic rhinitis [23]. This dose corresponds to the quantity of SG that was measured on only 10 cm^2 of contaminated wallpaper in our study. However, for such an exposure, toxins have to be aerosolized from contaminated building materials. Due to their microscopic structure (production in clusters and covered with dry slime) (figure 3B), the spores of *Stachybotrys* are not easily aerosolized and are consequently found at a lower frequency in the air than the xerophilic spores of *Aspergillus* or *Penicillium* [17]. Nevertheless, a recent publication reported the possible aerosolization of *S. chartarum* spores from contaminated gypsum board [42]. Moreover, macrocyclic trichothecenes have also been observed in particles smaller than conidia [43]. This could be due to the excretion of the toxins in droplets and their subsequent

adsorption on small dust particles that can then be aerosolized [44] or to the unhooking of small-contaminated particles of support following fungal degradation.

The presence of large quantities of macrocyclic trichothecenes on contaminated materials also raises the question on the impact of an occupational exposure to these mycotoxins during building remediation. Indeed both cutaneous toxicity and toxicity after inhalation have been clearly demonstrated for these compounds in animals [21, 23]. Moreover, no current remediation treatment is able to completely eliminate toxins from supports [31, 45]. Our study was conducted using a highly toxigenic strain of *S. chartarum* placed in environmental conditions favorable for toxin production. Although the conditions used in this study are not far from those that can be observed in homes with *S. chartarum* contamination following water damages (25°C, high humidity, darkness since *S. chartarum* can develop behind furniture), results shall now be confirmed by testing samples from naturally contaminated indoor environments.

The substrate appeared to have a strong influence on the total level of toxins produced and modulates the proportions of different toxins. SH appeared to be the main toxin produced on building materials. This predominance was also previously reported on rice [46]. It has to be noted that SG was not detected in the initial inoculum but was present, at different proportions, on all materials after incubation and fungal development. Our results are consistent with the recent genome sequencing of *Stachybotrys* that revealed the presence of several gene clusters involved in macrocyclic trichothecenes synthesis with locations that are compatible with a co-regulation [47]. However, since levels of toxin production observed on fir, fiberglass and painted fiberglass wallpaper were moderate, it would be now interesting to analyze the proportion of the toxins produced on other materials or substrates (gypsum board, straw) favorable for *S. chartarum* development and toxin production to see if toxins are produced in the same relative proportions.

Thus, such data are of interest for risk assessment and the monitoring of the exposure of inhabitants to these toxins. Indeed, it suggests that SH alone, the most toxic macrocyclic trichothecene [22, 48], could be used as a marker for contamination by the whole family. For that, rapid tests could probably be developed to allow direct monitoring in contaminated houses. Nevertheless, it would be interesting to analyze the toxigenic profile of other mycotoxin-producing strains of *S. chartarum* to assess possible inter-strain differences.

CONCLUSION

Characterization of the growth and toxinogenesis of a *S. chartarum* strain on different building materials showed that the nature of the material influenced the levels of macrocyclic trichothecenes produced by *S. chartarum*. The observed differences mainly concerned the total level of toxins produced, with no strict relation to fungal growth. Satratoxin H, the most toxic macrocyclic trichothecenes, was the main mycotoxin produced on all tested substrates. Such results are of importance for both risk assessment and monitoring of indoor exposure to those toxic compounds.

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Production of four macrocyclic trichothecenes by *Stachybotrys chartarum* during its development on different building materials as measured by UPLC-MS/MS.

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Highlights :

- *Stachybotrys chartarum* produce simultaneously SG, SH, RL2 and VerJ on building materials
- The total level of toxins rather than their proportion are strongly influenced by the building material
- The toxin production is not directly related with fungal development
- SH, the most toxic compound, can reach several mg/m^2 on wallpaper and fir
- Satratoxin H could serve as biomarker of macrocyclic trichothecenes in indoor environments