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ORIGINAL ARTICLE

Growth of indoor fungi on gypsum

F.J.J. Segers[1], K.A. van Laarhoven[2], H.A.B. Wösten[3] and J. Dijksterhuis[1]

1 Applied and Industrial Mycology, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands
2 Department of Applied Physics, Eindhoven University of Technology, Eindhoven, The Netherlands
3 Microbiology, Department of Biology, Utrecht University, Utrecht, The Netherlands

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Correspondence
Jan Dijksterhuis, Applied and Industrial Mycology, Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.
E-mail: j.dijksterhuis@westerdijkinstitute.nl

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Abstract

Aims: To have a better understanding of fungal growth on gypsum building materials to prevent indoor fungal growth.
Methods and Results: Gypsum is acquired by mining or as a by-product of flue-gas desulphurization or treatment of phosphate ore for the production of fertilizer. Natural gypsum, flue-gas gypsum and phosphogypsum therefore have different mineral compositions. Here, growth of fungi on these types of gypsum was assessed. Conidia of the indoor fungi *Aspergillus niger*, *Cladosporium halotolerans* and *Penicillium rubens* were inoculated and observed using microscopic techniques including low-temperature scanning electron microscopy. Elemental analysis of gypsum was done using inductively coupled plasma atomic emission spectroscopy and segmented flow analysis. Moisture content of the gypsum was determined using a dynamic vapour sorption apparatus. *Aspergillus niger*, *C. halotolerans* and *P. rubens* hardly germinated on natural gypsum and flue-gas gypsum. The latter two fungi did show germination, outgrowth, and conidiation on phosphogypsum, while *A. niger* hardly germinated on this substrate. Other experiments show that *C. halotolerans* and *P. rubens* can develop in pure water, but *A. niger* does not.
Conclusions: The observations show that the lack of germination of three indoor fungi is explained by the low amount of phosphor in natural, flue-gas and laboratory-grade gypsum. Additionally, *C. halotolerans* and *P. rubens* can develop in pure water, while conidia of *A. niger* do not show any germination, which is explained by the need for organic molecules of this species to induce germination.
Significance and Impact of the Study: Indoor fungal growth is a potential threat to human health and causes damage to building materials. This study possibly helps in the application of the right type of gypsum in buildings.

Introduction

Natural and industrial gypsum is widely used as a building material. A variety of fungal species including members of the genera *Aspergillus*, *Cladosporium* and *Penicillium* can grow on gypsum-building materials (Andersen *et al.* 2011). Fungal growth on indoor surfaces is undesirable because it may lead to adverse health effects in inhabitants (Adan *et al.* 2007; Bonnefoy 2007; Mitchell *et al.* 2007). Furthermore, as a result of growth, gypsum may be solubilized, disfiguring the surface of interior building material (Gharieb *et al.* 1998).

Growth of fungi on building material depends on nutrients and water availability. Relative humidity (RH) within the indoor environment is dynamic with an average of about 50% (Adan *et al.* 2007). Peak values ≥80% sustain indoor fungal growth (Adan and Samson 2011; Van Laarhoven *et al.* 2016). Growth of *Penicillium rubens* on laboratory-grade gypsum at RH ≥80% is negligible but is observed in the presence of nutrients (Adan 1994; Bekker *et al.* 2012). Moisture content of gypsum also impacts growth of *P. rubens*, promoting the accessibility of nutrients (Van Laarhoven *et al.* 2015).

Depending on its origin, gypsum may contain different traces of minerals. Natural gypsum is mined, while flue-gas gypsum and phosphogypsum are by-products from industrial processes. Flue-gas desulphurization is used to remove sulphur dioxide from flue-gas from fossil fuels in energy power plants. Calcium sulphite that is produced during this process oxidizes to calcium sulphate during storage. Phosphogypsum is a by-product of the production of phosphate fertilizer. During this process, phosphate ore is treated with sulphuric acid resulting in gypsum.

In the present work, it was studied whether the mineral composition of gypsum also impacts fungal growth. This was studied by growing the indoor fungi *Aspergillus niger* van Tieghem, *Cladosporium halotolerans* Zalar, de Hoog & Gunde-Cimerman and *P. rubens* Biourge on gypsum with different chemical compositions.

Materials and methods

Gypsum

Industrial phosphogypsum and a laboratory-grade gypsum (Sigma-Aldrich, Zwijndrecht, The Netherlands) were used as a hemihydrate form ($CaSO_4 \cdot 0{\cdot}5H_2O$) and flue-gas gypsum and natural mined gypsum were used in the dihydrate form ($CaSO_4 \cdot 2H_2O$). When mixed with demineralized water 3 : 2 (w/w) gypsum was casted in moulds with a diameter of 10 mm and a height of 3 mm. The gypsum pellets were left to dry at room temperature for a day before use.

Strains and growth conditions

Aspergillus niger N402 (Bos *et al.* 1988), *C. halotolerans* CBS 139586 (Segers *et al.* 2015) and *P. rubens* CBS 401.92 (Adan 1994), which was formerly known as *P. chrysogenum* (Houbraken *et al.* 2011), were grown on malt extract agar (MEA) and stored at −80°C in 30% glycerol, 0·01% Tween 80, 5 mmol l^{-1} *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), pH 6·8. Conidia were harvested from 1-week-old colonies using ice-cold ACES and washed twice with ice-cold demineralized water by centrifugation at 1500 ***g*** for 10 min. Conidia were taken up in ice-cold demineralized water at a final number of 10^6 conidia per ml. Gypsum pellets were inoculated in their centre with 5 μl conidia suspension and left to dry for 20 min within a flow cabinet at room temperature. The pellets were placed within a 6-l desiccator containing 250 ml of saturated K_2SO_4 solution, which creates an atmosphere of 97% RH (Winston and Bates 1960; Greenspan 1977). Growth was followed visually for 25 days and at the last day images were taken using a Zeiss Discovery V20 stereomicroscope (Zeiss, Sliedrecht, The Netherlands) equipped with a Nikon DS-Ri2 camera (Nikon instruments, Amsterdam, The Netherlands). The experiments were performed with at least three technical replicates and at least two biological replicates.

Germination of conidia in water was monitored using cavity slides and cover slides (VWR, The Netherlands, Amsterdam) that had been cleaned for 24 h using 1 mol l^{-1} hydrochloric acid and rinsed with ultrapure water. A hanging droplet of conidia in 10 μl of water was created within the cavity and the slide was incubated at 25°C within a 6-l desiccator containing 200 ml of saturated K_2SO_4 solution (97% RH). Germination was followed daily using a Zeiss Imager A2 microscope. Pictures were taken using a Nikon DS-Ri2 camera. The experiments were performed with at least three technical replicates and two biological replicates.

Dynamic vapour sorption

Moisture content of samples was determined at RH 97% at 21°C using a dynamic vapour sorption (DVS) apparatus (Q5000SA; TA instruments, New Castle, DE, USA). DVS gravimetrically monitors the amount of water contained in a sample that is exposed to a controlled RH. Samples (1 mm^3) were dried by exposure to RH 0% until the weight did not change more than 0·005% per h after which RH was gradually raised to RH 97%. The equilibrium moisture content at RH 97% was calculated based on the equilibrium weight at RH 97% and RH 0%.

Cryo-scanning electron microscopy

Gypsum pellets were transferred into the bottom a copper cup that contained a small droplet of frozen-tissue medium (KP-Cryoblock; Klinipath, Duiven, The Netherlands), snap-frozen in nitrogen slush and transferred into an Oxford CT1500 Cryostation connected to a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan). Samples were sputter-coated (3 × 1 min) using a gold target in the cryostation. Electron micrographs were taken at an acceleration voltage of 5 kV.

Elemental analysis

Elemental analysis of gypsum was performed by the Chemical Biological Laboratory Bodem (Wageningen, The Netherlands). Samples (400 mg) were dried, ground, taken up in 250 ml of ultrapure water, and filtered over a 0·45-μm filter to remove insoluble material. Ca, P and S were quantified using inductively coupled plasma atomic emission spectroscopy (Varian Vista PRO, Amstelveen, The Netherlands), while $N(NO_3 + NO_2)$, NH_4 and

nitrene were determined using segmented flow analysis (Skalar, Breda, The Netherlands). The amount of organic carbon was determined by oxidation with 98% (w/w) H_2SO_4 at 135°C in the presence of excess $K_2Cr_2O_7$. Conversion of Cr^{6+} into Cr^{3+} was monitored at 585 nm. Glucose was used as a standard to calculate the amount of organic carbon present in samples.

Results

Growth on gypsum

Gypsum tablets were studied by light and electron microscopy (Cryo-SEM). Stereo microscopy indicated that *A. niger* was not able to grow on any gypsum (Table 1), however, electron microscopy showed some germination on phosphogypsum (data not shown). Growth was also absent on natural gypsum, flue-gas gypsum and laboratory-grade gypsum in the case of *C. halotolerans* and *P. rubens*, but some germinating conidia were observed with Cryo-SEM on flue-gas gypsum (data not shown). The latter two species did grow well on phosphogypsum (Fig. 1). *Cladosporium halotolerans* had formed small conidiophore forming colonies (Fig. 2), while *P. rubens* had formed a mycelium consisting of long hyphae stretching out over the gypsum and conidiophores (Fig. 3). Some of the hyphae of *P. rubens* were collapsed (Fig. 3b).

Elemental analysis and water content of gypsum

DVS showed that natural gypsum at 21°C and 97% RH had 1·1% moisture content, while phosphogypsum, flue-gas gypsum and the laboratory-grade gypsum had 1·9, 1·5 and 1·6% moisture content respectively (Table 1). All gypsum types contained around 25% (w/w) Ca and 20% (w/w) S. P represented 0·22% of phosphogypsum, while it was barely detectable in the other gypsum types. The amount of nitrogen in gypsum was around 0·01% except for the laboratory-grade gypsum (0·00%). All gypsum types contained around 0·1% organic carbon (Table 2).

Germination in pure water

Conidia of *A. niger* did not germinate at 25°C in water within a 1-week period (Fig. 4c). In contrast, conidia of *C. halotolerans* germinated within a day and conidiophores with nonpigmented conidia had formed within 3 days (Fig. 4b). After a week, *C. halotolerans* had formed small colonies with melanized conidia (Fig. 4d). Conidia had formed on conidiophores that sprout from small hyphae, which was similar to what was seen on phosphogypsum (Fig. 2). Conidia of *P. rubens* also had germinated after 24 h and had formed long hyphae. Formation of simple conidiophores and lysis of the long hyphae was observed after 7 days (Fig. 4a). Growth and development resembled that on phosphogypsum (Fig. 3).

Discussion

Fungal growth was studied on natural gypsum, phosphogypsum, flue-gas gypsum and laboratory-grade gypsum. Germination of *A. niger* conidia was rare or even absent on all gypsum types at 97% RH. In contrast, *C. halotolerans* and *P. rubens* did show outgrowth and subsequent formation of conidiophores on phosphogypsum. Phosphogypsum has previously been shown to be susceptible to microbial growth (Shirakawa *et al.* 2002). Flue-gas gypsum showed only some germination of conidia of *C. halotolerans* and *P. rubens*, while germination was hardly observed in the case of natural and laboratory-grade gypsum. Almost no germination on laboratory-grade gypsum was also described previously (Adan 1994; Bekker *et al.* 2012).

All gypsum types were shown to contain 0·1% carbon, which is sufficient to support fungal growth. Fungi even show visible grow on a 10-fold lower amount of glucose (Van Diepeningen *et al.* 2010). The amount of Ca, S and N was also similar in natural gypsum, phosphogypsum and flue-gas gypsum. In contrast, the amount of P was higher in phosphogypsum, which likely explains why *C. halotolerans* and *P. rubens* showed growth on this type of gypsum but not on the other types. Germination could be explained by the presence of phosphates, as

Table 1 Absence of growth (–), growth of fungi on gypsum visible by stereomicroscopy (++), or occasional germination visible by cryo-SEM (+) at 25°C and 97% RH. Equilibrium moisture content of gypsum was determined at 21°C and 97% RH

		Aspergillus niger	*Cladosporium halotolerans*	*Penicillium rubens*	Equilibrium moisture content (w %, ± 0·1)
1	Natural gypsum	–	–	–	1·1
2	Phosphogypsum	+	++	++	1·9
3	Flue-gas gypsum	–	+	+	1·5
4	Laboratory-grade gypsum	–	–	–	1·6

sporangiospores of *Rhizopus oligosporus* from tempe starter, showed germ tube formation in a phosphate-containing buffer (Thanh *et al.* 2005). Incubation at 97% RH lead to different moisture content in the different types of gypsum, ranging from 1·1 to 1·9% (w/w). This low moisture content of gypsum samples could have an influence on the germination rate of conidia as water is not as readily available in comparison to the case of an aqueous environment surrounding conidia (Van Laarhoven *et al.* 2015). The Ca^{2+} ions in gypsum may also influence germination. It is essential for some fungi (Osherov and May 2001), while it inhibits germination of other

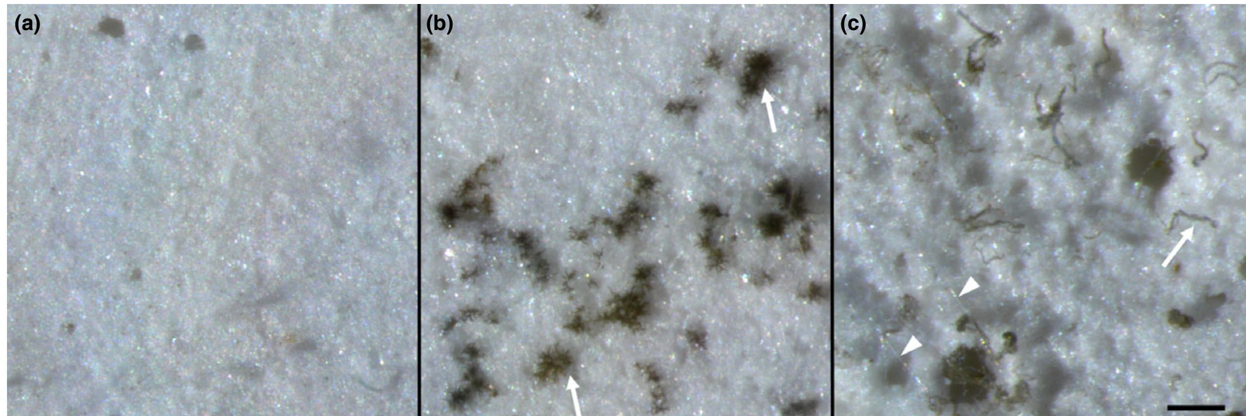

Figure 1 Light microscopy shows that conidia of *Aspergillus niger* (a) do not show outgrowth on phosphogypsum, while growth of *Cladosporium halotolerans* (b) and *Penicillium rubens* (c) is observed after 25 days at 25°C and 97% RH. Arrows indicate examples of microcolonies with conidiophores, while arrowheads represent examples of single hyphae. Scale bar represents 100 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

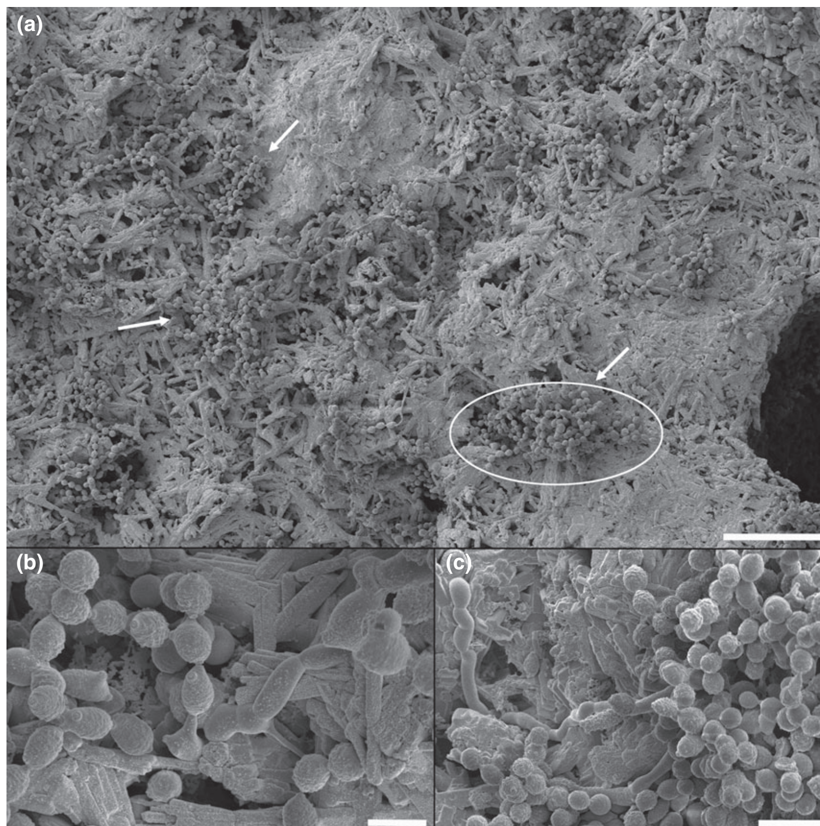

Figure 2 Growth and conidiophore formation of *Cladosporium halotolerans* on phosphogypsum (a–c) after 25 days at 25°C and 97% RH. Fungal growth is pointed out with arrows (a) and the condensed growth of the fungus is illustrated with a white oval. Scale bars represent 50 μm (a), 5 μm (b) and 10 μm (c).

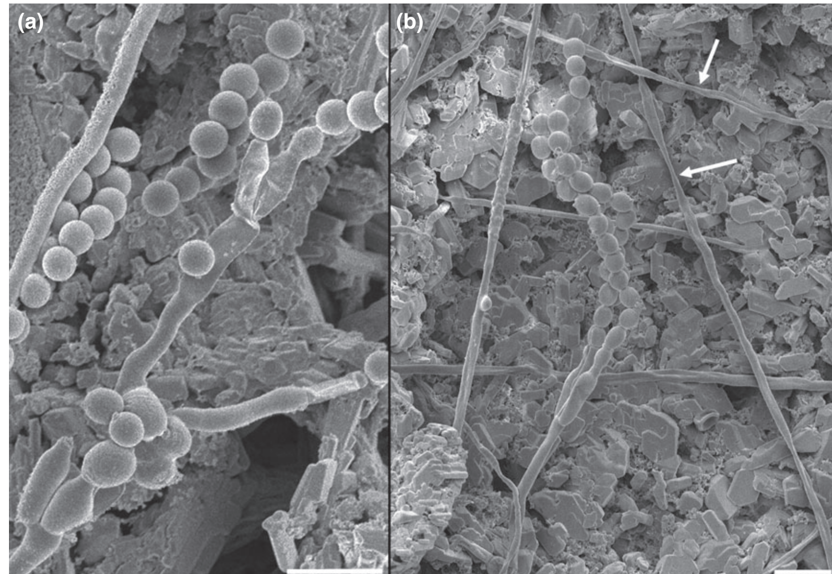

Figure 3 Growth and conidiation of *Penicillium rubens* after 25 days on phosphogypsum at 25°C and 97% RH. Scale bars represent 10 μm (a, b).

conidia (Biggs 1999). As the amount of Ca was similar in all gypsum types, any effect would likely be species dependent.

Table 2 Elements and minerals present in gypsum in w/w %

% in (w/w)	Ca	P	S	N-NH_4	N-(NO_3+NO_2)	Nts	C
Natural gypsum	25	0·01	17	0·00	0·00	0·01	0·1
Phosphogypsum	26	0·22	20	0·00	0·01	0·01	0·1
Flue-gas gypsum	27	0·00	212	0·00	0·01	0·01	0·1
Laboratory-grade gypsum	25	0·00	19	0·00	0·00	0·00	0·1

Values are expressed in percentages.

Cladosporium halotolerans and *P. rubens* showed different growth and development behaviour on phosphogypsum. *Cladosporium halotolerans* formed short hyphae with many conidiophores. This observation suggests a 'condensed growth' strategy, which is also seen on marble and rock surfaces where *Cladosporium* and other fungi inhabit small crevices (Sterflinger 1998; Gorbushina 2007). *Penicillium rubens* on the other hand, showed a more 'explorative growth' strategy by forming long hyphae stretching out over the gypsum in search for more favourable conditions and eventually forming simple conidiophores after lysis of older hyphae. These growth strategies of *C. halotolerans* and *P. rubens* were also observed in pure water and can also be observed on

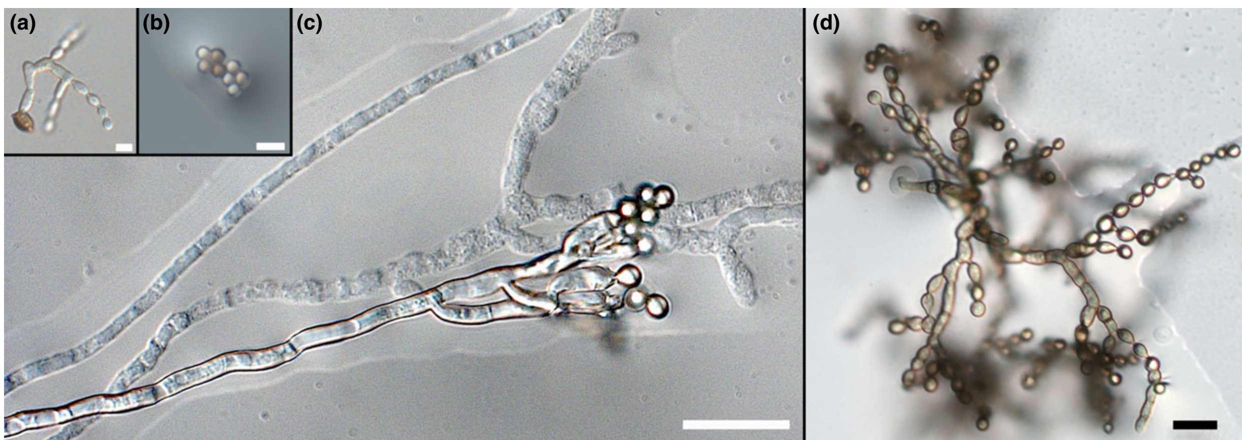

Figure 4 Incubation of conidia in ultrapure water at 25°C. Conidiophores had developed in the case of *Cladosporium halotolerans* with transparent and pigmented conidia after 72 h (a) and 7 days (d) respectively. Several long hyphae and a simple conidiophore with new conidia had also formed after 7 days in the case of *Penicillium rubens* (c). Germination and outgrowth of *Aspergillus niger* conidia were not observed (b). Scale bar represents 20 μm (c) and 5 μm (a, b and d). [Colour figure can be viewed at wileyonlinelibrary.com]

complete media such as MEA (data not shown). While conidiophores of *P. rubens* were relatively simple under oligotrophic conditions they show a much more complex branching pattern (terverticillate to quarterverticillate, Samson *et al.* 2010; Houbraken *et al.* 2012), when nutrients are not limiting. The environmental conditions may not only impact conidiophore morphology but also viability and stress resistance of the conidia.

Germination of conidia is often induced by the presence of particular molecules in the environment. For instance, *A. niger*, *Aspergillus nidulans* and *Fusarium graminearum* require L-amino acids and sugars for germination (Beyer *et al.* 2004; Hayer *et al.* 2013, 2014; De Assis *et al.* 2015). In contrast to conidia of *A. niger*, those of *C. halotolerans* and *P. rubens* did germinate in ultrapure water. This shows that these fungi follow a different strategy to respond to the environment. The spores of *A. niger* need an external trigger for swelling and germ tube formation as an *a priori* evaluation of favourable growth conditions. In contrast, according to our observations conidia of *C. halotolerans* and *P. rubens* follow an *a posteriori* strategy by germinating irrespective of the presence of (certain) nutrients.

Fungi may be primary, secondary or tertiary colonizers of indoor environments at low water activity (a_w) (Grant *et al.* 1989; Flannigan and Miller 2011). Fungi that are able to germinate in oligotrophic conditions might be the primary colonizers when the a_w is sufficient for growth. The resulting small colonies may lyse, providing resources for other fungi. As such, dark fungal spots on a gypsum wall can become larger over time containing primary, secondary or even tertiary colonizers. *Aspergillus niger* in this view would be a secondary or tertiary colonizer. The fact that an infection of *Aspergillus sydowii* was observed on phosphogypsum during this study implies that within the genus *Aspergillus*, different colonizing strategies are adopted.

Phosphogypsum was susceptible to the growth of *C. halotolerans* and *P. rubens*, but unsusceptible to *A. niger*. Flue-gas gypsum, natural gypsum and laboratory-grade gypsum were all unsusceptible to fungal growth and only showed some germination of *C. halotolerans* and *P. rubens*, but no substantial growth. Micro-colonies of *A. niger* and *P. rubens* are unable to survive dynamics in water availability from 0·97 to 0·75 a_w, while micro-colonies of *C. halotolerans* (Segers *et al.* 2016), and widely established colonies of *P. rubens* (Van Laarhoven *et al.* 2016) do survive. In addition, hyphae of *A. niger* and *P. rubens* disrupt after transfer to a medium with a higher a_w, while those of *Cladosporium* were hardly affected. These data and the condensed growth strategy under oligotrophic conditions with the formation of many conidia may explain why *C. halotolerans* is such a successful fungus in the indoor environment (Segers *et al.* 2015).

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Conflict of Interest

No conflict of interest declared.

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