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Separate effects of moisture content and water activity on the hyphal extension of Penicillium rubens on porous media

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Summary
To prevent indoor fungal growth, understanding the moisture relations of fungi is a key element. Indoor moisture is quantified by the relative humidity (RH). RH controls the water activity of the indoor materials that fungi grow on, a well-studied parameter known to limit fungal growth. RH, however, also controls the amount of water present in these materials, the moisture content. The significance of the moisture content of these materials to indoor fungal growth is currently overlooked. In the work reported here, growth experiments with the indoor fungus Penicillium rubens on gypsum substrates were performed to test whether the moisture content influences growth on porous materials. Second, we report the development of a video microscopy method that for the first time quantified hyphal growth on a porous material. It is found that a higher moisture content leads to earlier colonization and higher hyphal extension rates. This is a fundamental step in unravelling the effect of RH on indoor fungal growth. The real-time monitoring of colonization of gypsum provides a new view of growth on indoor surfaces.

Introduction
Indoor mould may discolour the surfaces it grows on and may excrete metabolites and particles that induce allergic reactions or asthma (Miller, 1992; Flannigan, 2001; Green et al., 2011). To avoid such aesthetical and medical problems, strategies to inhibit indoor fungal growth are needed. For this, a better understanding of the circumstances that cause indoor growth lays the foundation.

Fungal growth is influenced by many abiotic factors, such as moisture, temperature, nutrients, oxygen and pH. Of these, temperature and especially moisture are considered the most important factors influencing indoor growth (Grant et al., 1989; Clarke et al., 1999; Adan et al., 2011). Moisture therefore receives ample attention in prevention strategies and prediction models for indoor fungal growth (Vereecken and Roels, 2012). So far, studies on the moisture relations of indoor fungal growth have mostly focused on the dependence of growth on the indoor relative humidity (RH). (e.g. Grant et al., 1989; Pasanen et al., 1992; Adan, 1994; Chang et al., 1995; Chang and Foarde, 1996; Viitanen et al., 2010; Johansson et al., 2013; Bekker, 2014).

The impact of RH on growth is usually understood in the context of its equivalence to water activity (aw). Although RH is a property of water vapour whereas aw is a property of liquid water, both are expressions of the water’s chemical potential (Atkins and de Paula, 2006). When an indoor substrate and ambient RH are in equilibrium, the RH defines the substrate aw via aw = RH/100%. To a fungus, these represent the ease of osmotic water uptake. Fungal protoplasm has an aw of its own, and water transport occurs from high to low aw. Uptake and growth therefore occur when the internal aw is below the aw of the environment. The impact of aw on fungal growth has also been investigated in experiments on agar for many fungal species (e.g. Scott, 1957; Ayerst, 1969; Magan and Lacey, 1984).

However, the RH may influence indoor fungal growth in an additional way via the total amount of water present in the material and available to the fungus. Indoor fungi grow on surfaces that are typically porous (Viitanen et al., 2010). When porous materials are exposed to vapour, water will adsorb on the surfaces of the pores via adsorption and capillary condensation (Hunter, 1993). This condensation process can be described by Kelvin’s law (Atkins and de Paula, 2006). The amount of water absorbed by a material depends on the RH it is exposed to and is quantified by the gravimetric moisture content (u), the ratio of the mass of the water present to the dry weight of the material. In equilibrium, u is related to RH via

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the so-called moisture sorption isotherm curve, a relation that is defined by the pore geometry of the material. Note that in equilibrium also the pore water $a_w$ and $RH$ are equal ($a_w = RH/100\%$). In the absence of equilibrium, mass exchange occurs through condensation or evaporation in the direction of decreasing $a_w$. As this happens, the material's $u$ changes until equilibrium is reached. As such the $RH$ not only determines the $a_w$ but also the amount of water present.

Little is known about possible effects of $u$ on fungal growth; some studies suggest that $u$ might indeed influence growth on porous substrates (Adan, 1994; Li and Wadso, 2013). Since hyphae can draw resources from contact with liquid water, we hypothesize that a higher $u$ will be beneficial for fungal growth on porous substrates. Because $u$ is coupled to the $RH$ and substrate $a_w$, more knowledge of the potential effects of $u$ will be crucial for a sound interpretation of experiments relating $RH$ to growth on porous substrates.

To investigate fungal growth on porous materials, a method to quantify growth is needed. Studies of fungal moisture relations on building materials so far have mostly relied on macroscopic methods for this, chief among them the assessment of surface coverage with stereoscopy (Adan, 1994; Viitanen et al., 2010; Johansson et al., 2013) and digital images (Nielsen et al., 2004; Bekker, 2014). While these methods successfully quantify growth, these are unsuitable for measuring individual hyphae. Scanning electron microscopy (SEM) has been used to gain a microscopic view of hyphal development on building materials as a function of moisture (e.g. Adan, 1994; Bekker et al., 2012), but the method's destructive nature makes real-time measurements impossible. Measuring hyphal growth real time on porous materials would provide a new view on fungal colonization of such substrates. Moreover, this could aid in comparing studies of fungal moisture relations on building materials with similar studies on agar, as the hyphal growth rate is a popular measure for fungal growth in such studies, measured either microscopically (e.g. Christiansen et al., 1998; Judet et al., 2008; Nanguy et al., 2010; Gougouli and Koutsoumanis, 2013) or macroscopically via the colony edge (e.g. Ayerst, 1969; Sautour et al., 2003; Ponizovskaya et al., 2011; Bekker, 2014).

In this work, growth experiments are reported with *Penicillium rubens*, a typical indoor fungus (Samson et al., 2010; Andersen et al., 2011; Samson, 2011), on substrates made of gypsum, a common porous building material. This work had two objectives. The first objective was to develop a system suitable for the real-time monitoring of fungal colonization of porous substrates on the hyphal level and under controlled humidity conditions. This may help to bridge the gap between knowledge of fungal hyphal growth and macroscopic observations of fungal growth on porous substrates. The second objective was to test whether a higher moisture content $u$ in the gypsum leads to a faster growth rate of $P$ rubens, independent of the $a_w$. To this end, we controlled the $u$ and $a_w$ of the gypsum separately during the growth experiments. Since the relation between indoor $RH$ and $u$ is material specific, this may eventually lay the foundation for new strategies to control indoor fungal growth through the choosing or development of fungal resistant materials.

**Results**

**Observing hyphae growing on gypsum**

In order to quantify the extension rates of individual hyphae growing on gypsum, samples were monitored with video microscopy while stored under controlled moisture conditions. The resolution and contrast of the images were too low to identify spores or germ tubes, but growing hyphae could be recognized. Several time-labelled images of hyphae on gypsum are shown in (Fig. 1). The images consist of cropped frames from a movie of growth on an initially dry gypsum substrate supplemented with Czapek that was equilibrated with a $RH$ of 97%. This figure also illustrates typical events observed during measurements: some individual hyphae cease growth even though many other hyphae still grow and continue growing on the same substrate (Fig. 1A); many other hyphae displace or shift out of focus after some time (Fig. 1B and C).

In general, growing hyphae could be followed for a few hundreds of micrometres before they displaced or ceased growth. Branching of hyphae could be recognized only sporadically and often resulted in immediate displacement of the followed hypha. Not all hyphae in the field of view (FOV) could be followed. New hyphae often grew away from the surface, roughly perpendicular to the microscope’s working plane and grew out of focus quickly. Further, in cases of abundant growth, the FOV was eventually obscured, making it impossible to distinguish hyphae from each other after a certain time. Typically, 5 to 15 hyphae could be followed in one movie.

Figure 2 shows data that were obtained from a typical movie of growth on gypsum equilibrated with a $RH$ of 97%, in which six individual hyphae could be followed (■). The hyphal length is plotted versus time, up to the moment where the individual hypha displaced or stopped extending, with $t = 0$ being the moment of inoculation. The hyphal length increases linear with time. For further analysis, individual hyphal growth rates and the moments in time at which hyphae became visible are therefore determined from linear fits of the data. In this movie, the growth rate of the hyphae is $-9 \pm 3 \mu m h^{-1}$, and the moments at which the individual hyphae started growing are $-60 \pm 10 h$.

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Figure 2 also shows the length of hyphae growing on malt extract agar (MEA, ▲) and customized Czapek agar (Δ and ○). Observation of the colonization of agar in the movies revealed differences in hyphal growth compared with that on gypsum. Young hyphae on agar did not displace but rather remained stably aligned to the surface. Further, hyphae on agar never ceased growing and could therefore be followed until they grew out of the FOV. Branching was clearly recognizable on agar. Figure 2 shows that hyphae on agar initially accelerate rather than maintaining a constant growth rate. Quantitatively, hyphae on agar with a length of ~100 μm have a growth rate of ~30 ± 5 μm h⁻¹, independent of the nutrient content of the medium. This is significantly higher than the growth rates on gypsum (unpaired Welch’s t-test, p < 0.001). Also the spread in starting times is markedly lower on agar media (~19 ± 2 h on MEA and ~25 ± 2 h on Czapek agar).

The moisture dependence of hyphal growth rates on gypsum

The relationship between hyphal growth rates on gypsum and RH was investigated as a benchmark for the video.
Although the differences in growth rates were not all statistically different, the used extension rates increase with the growth rates of individual hyphae. The data indicate that growth on equilibrated samples of the gypsum at certain RH conditions, meaning that growth on substrates that were equilibrated with water vapour. Growth rates on samples soaked with NaCl (Fig. 3, ■) and KCl (Fig. 3, ▲) solutions again also decrease with decreasing aw and overlap with the results for samples soaked with glycerol solutions. For aw = 0.97, growth rates on samples soaked with polyethylene glycol (PEG) solutions (Fig. 3, ★) overlap with growth rates on samples soaked with a glycerol solution. However, hyphae on the soaked samples always grow faster than those on equilibrated substrates at the same aw: a set of Welch’s unpaired t-tests shows that, at each individual aw, the growth rate on RH-equilibrated samples is significantly lower than those on soaked samples (p < 0.004 for the comparison at each activity). The highest aw at which no growth occurs on the soaked samples within 1440 h after inoculation is 0.79, which is lower than that for the samples equilibrated with water vapour. Growth rates on the samples soaked with NaCl (Fig. 3, ■) and KCl (Fig. 3, ▲) solutions again also decrease with decreasing aw and overlap with the results for samples soaked with glycerol solutions. For aw = 0.97, growth rates on samples soaked with polyethylene glycol (PEG) solutions (Fig. 3, ★) overlap with growth rates on samples soaked with a glycerol solution. No growth was detected for samples soaked with a PEG solution of any other aw.

Initial observation times of hyphal growth as a function of aw and u

Fitting of the raw data (e.g. Fig. 2, ■) also provided the times at which each hypha became visible in the used magnification. Figure 4 shows the starting times of hyphae that were followed per aw for both samples that were equilibrated and samples soaked with glycerol, NaCl, KCl and PEG solutions.

The data from the samples equilibrated with vapour show the general trend of the earliest occurrence of growth being delayed with decreasing aw down to aw = 0.9. Apart from that, also the spread in the times of the individual starting events increases with decreasing aw. On the samples soaked with aqueous solutions of glycerol and KCl, such a clear trend is absent, whereas on samples soaked with a NaCl solution, the delay with decreasing aw is pronounced very strongly. Also the spread in starting events on soaked samples seems to be largely unaffected by aw.

A set of Welch’s unpaired t-tests shows that, at each individual aw, growth on the RH-equilibrated samples starts significantly later than on samples soaked with glycerol solutions, except at aw = 1.0 (p = 0.02 for the comparison at aw = 0.97, p < 0.001 at aw = 0.93, aw = 0.90 and aw = 0.86).

Discussion

Measuring hyphal growth rates on a gypsum substrate

The video microscopy set-up was used successfully to monitor hyphal growth real time. This allowed for the determination of hyphal growth rates, which have not been used to quantify growth on building material...
substrates before. Monitoring the colonization of the substrates real time during the steady-state experiments provided new insights on the hyphae’s attachment to these substrates. Further, the data match older studies of the relation between growth and steady-state RH well. The general trend of decreasing growth on building materials with decreasing RH has been described before for most common building materials and for many indoor fungal species (e.g. Grant et al., 1989; Pasanen et al., 1992; Chang et al., 1995; Chang and Foarde, 1996; Viitanen et al., 2010). A similar trend with aw, with an optimum in the growth rates just below aw = 1.0, has been observed for many fungi on agar before (e.g. Ayerst, 1969; Magan and Lacey, 1984). Further, while it is known that the critical RH (i.e. the RH below which no growth occurs) can vary with the substrate material and fungal species (Clarke et al., 1999; Kuenzel and Sedl Bauer, 2001), a critical RH of 85% has been reported for P. rubens on equilibrated gypsum specifically (Adan, 1994; Nielsen et al., 2004; Viitanen et al., 2010). This corresponds well to the critical RH of about 86% found here. Note that our substrate is different from the gypsum tested by Viitanen and Nielsen, as it has Czapek mixed in, but similar to the gypsum Adan used. Interestingly, the critical aw for P. rubens under ideal circumstances, i.e. on MEA, has been found to be 0.79 (Grant et al., 1989), which is also the critical aw we found for soaked gypsum samples.

It should be noted that the resolution and contrast of the images is not sufficient to resolve ungerminated conidia on the gypsum substrate. The hyphae observed on gypsum therefore likely are developed hyphae rather than germ tubes or primary hyphae. A SEM study of Bekker and colleagues (2012), who observed germination on similar substrates within 8 h after inoculation, affirms this. Further, the constant growth rate observed for hyphae on gypsum is typical for developed hyphae of sufficient length (Prosser and Tough, 1991; Gougouli and Koutsoumanis, 2013), although it is possible that hyphae were not followed long enough to detect changes in growth rate. It is not clear if the hyphae observed on gypsum should already be considered aerial mycelium. A plausible assumption is that the shifting and displacement of hyphae as illustrated in Fig. 1 happens due to hyphal tips colliding with or tipping over the rough surface. This would suggest that observed hyphae are still growing close and roughly parallel to the surface. Since the microscopy images provide a two-dimensional top view of a three-dimensional geometry, however, this remains uncertain. Contrast on agar is better due to its smoother surface, and there hyphae are observed much earlier after inoculation. Hyphae on agar also accelerate, which is typical for primary hyphae growing from germinated conidia (Prosser and Tough, 1991; Gougouli and Koutsoumanis, 2013). When comparing growth rates on agar with those on other substrates, this possible difference in developmental stage should be taken into account.

The influence of moisture content on growth on porous substrates

The increased growth rates and lowered critical aw on substrates soaked in glycerol solutions as compared with samples equilibrated with vapour (Fig. 3) indicate that the moisture content affects growth rates independently from aw. A similar conclusion might be drawn from the comparison of growth rates on agar and gypsum, as illustrated in Fig. 2, which showed that growth on high-u Czapek agar was significantly faster than growth on low-u gypsum even when aw and nutrient conditions were similar.
Further, the results in Fig. 4 suggest that a lower $u$ leads to a delay and greater spread in the germination and development of hyphae.

These results confirm the hypothesis that a higher $u$ in the gypsum leads to faster colonization by *P. rubens* independently of the $a_w$. This could be further tested in a number of ways. First, since branching could not be clearly recognized with the video microscopy set-up, the movies provide no complete measure for biomass increase on the substrate. Macroscopic methods such as the measurement of ergosterol production (Bjúrman, 1994; Nielsen *et al*., 2004), heat production (Wadso, 1997; Li and Wadso, 2013) or colony-forming unit production (Pasanen *et al*., 2000) could be used to measure biomass increase as a function of $u$. Second, measurements in this work did not include conidiation times. These could be measured macroscopically with an assessment of surface discolouration (e.g. Bekker, 2014). The video microscopy method presented here could also be used to detect the earliest formation of conidiophores.

It is stressed that a significant difference was seen between growth on soaked and equilibrated samples at $a_w = 1$. In this case, the high-$u$ samples were soaked with pure water. This confirms that $u$ influences hyphal growth even in the absence of the solutes that were used in the soaking solutions of lower $a_w$. While it is common practice to use glycerol as an agent to tune the $a_w$ of agar (Sautour *et al*., 2003; Judet *et al*., 2008; Ponizovskaya *et al*., 2011), it is also known that *P. rubens* and many other fungi use and produce glycerol to defend against osmotic stress (Luard, 1982; Blomberg and Adler, 1992). Further, Patriarca and colleagues (2011) suggest that glycerol could increase growth rates by serving as an additional carbon source. The growth on samples soaked with glycerol solutions might therefore have been accelerated by the additional glycerol in addition to acceleration due to a higher $u$. Similarly, $K^+$ and $Na^+$ ions too are known to serve as compatible solutes in the osmotic stress response (Luard, 1982), although the salts NaCl and KCl are generally considered to be inhibitory rather than stimulating when compared with glycerol as an osmolyte (Parra *et al*., 2004; Deacon, 2006). Although NaCl definitely inhibited growth by delaying germination and development of hyphae, hyphal extension rates on the KCl and NaCl samples were comparable with those on glycerol samples. This suggests that the glycerol did not significantly influence growth speeds by serving as an extra carbon source. We cannot, however, rule out the possibility of glycerol, NaCl or KCl influencing growth rates on soaked samples as compatible osmolytes to some extent.

The measurements on PEG samples gave no extra information. No growth occurred at $a_w$ below 0.97, even for $a_w$ at which growth was viable on gypsum in equilibrium with water vapour. This indicates that PEG had an inhibitory effect on growth that went beyond $a_w$ stress. PEG is considered an inert solute that is not taken up by fungi and used as such in experiments on agar (Hallsworth and Magan, 1999; Ramirez *et al*., 2004). It is, however, also known to change the properties of cell membranes during cell transformation protocols (Kawai *et al*., 2010). It is possible that PEG is fatal for fungi in environments where resources are not as abundant as on culture media, although the exact reason for PEG’s inhibitory effect is unclear.

The effect of growth rates increasing with increasing $u$ can be explained by an increase in resource availability for the fungus. As the $u$ of a gypsum sample increases, pores are gradually filled, improving fungal access to water and nutrients in several ways. First, water will become more abundant on the substrate surface, leading to an increase in hypha–water contact. Since contact with liquid water provides access to both nutrients and the water needed for hyphal extension, the availability and uptake of these resources will increase with $u$. Second, as $u$ increases, the water films that are stored in the pores become thicker and better interconnected. This allows for more or faster diffusion of nutrients through the substrate’s bulk towards its surface, where they become available for uptake. The flow of water towards hyphae could be limited by $u$ in a similar way, although this would presumably only limit growth when $u$ were so low that the volume of water in the vicinity of the hyphae became comparable with the volume of the hyphae. The above explanation can be extended to include the extreme case of agar, with a $u$ of ~100%, on which both hypha–water contact and nutrient diffusion would be even higher than on moisture saturated gypsum. Although we cannot distinguish from these experiments which of these effects dominate growth limitation, it is stressed that these effects are each fundamentally different from the osmotic influences of $a_w$ on growth.

**Concluding remarks**

We successfully measured hyphal lengths with video microscopy to quantify fungal growth on gypsum. This revealed clear differences between growth on building material substrates and growth on the ideal substrates that are usually used in the lab. Specifically, the real-time nature of the images revealed that hyphae on gypsum are less adhered and aligned to the surface than hyphae on agar. More importantly, in a comparison of hyphal growth rates on equilibrated and soaked gypsum substrates, we confirmed our hypothesis that the growth rates of hyphae on gypsum decrease not only with decreasing $a_w$ but with decreasing moisture content as well. $a_w$ and moisture content have distinct effects and play a different role.
A few studies have previously suggested that small increases in $u$ induced by sorption hysteresis will lead to an increase in growth (Adan, 1994; Li and Wadso, 2013). The results in this work are in line with such suggestions. Up till now, however, the effects of $u$ on growth have been mostly overlooked, possibly because $u$ is by definition constant and close to 100% in agar substrates. The confirmation that $u$ affects growth is important for the study of indoor fungal growth, as $u$ inherently varies with RH in a porous building material system. This also underlines the importance of performing growth experiments on ‘real’ materials when studying indoor fungal growth.

Further research is needed to determine the principles behind the limiting effects of $u$. Effort should be made to support explanations with a microscopic model of the interactions between hyphae, water and porous substrate. Future experiments trying to link growth speeds to the state of water in porous substrates would greatly benefit from methods allowing a more refined control over the distribution of water on the substrate surface, which is a challenge in itself. Thanks to its non-destructive and real-time nature, the video microscopy method used in this work is well suited to investigate the response of hyphae to more realistic, dynamic indoor moisture conditions.

**Experimental procedures**

**Gypsum substrates**

Gypsum samples with added nutrients served as substrates for the growth experiments. These were produced by mixing gypsum [(Ca$_2$SO$_4$)$_2$·H$_2$O, Sigma-Aldrich] with an aqueous Czapek solution. The solution contained Czapek Dox Borth (Oxoid, 8.76 gl$^{-1}$) and trace metals ZnSO$_4$·7H$_2$O (2.5·10$^{-3}$ gl$^{-1}$) and CuSO$_4$·5H$_2$O (1.25·10$^{-3}$ gl$^{-1}$). The solution was autoclaved before use. The calcium sulfate hemihydrate and solution were mixed at a mass ratio of 3:2 and cast into 3 mm thick pellets. The samples were dried for 48 h at room temperature in a laminar flow cabinet (LFC) (CleanAir, Class II – EF/B) to remove excess water.

To provide sufficient contrast for microscopy, a thin, black layer of Fe$_3$O$_4$ (Metzger Black) was applied to the surface of each sample. The layer was deposited by pipetting 5 μl of a Fe$_3$O$_4$ suspension in water (33.3 g l$^{-1}$) on to the substrate. The suspension water was then evaporated out in the LFC, leaving a Fe$_3$O$_4$ layer with a thickness of about 20 μm, as determined with a profilometer. Inspection with cryo-SEM showed that the resulting layer was porous with pore sizes in the order of 0.1–1 μm (Fig. 5).

Adding nutrients to the gypsum samples changes the sorption behaviour of the material. A dynamic vapor sorption (DVS) apparatus (TA Instruments, Q5000SA) was used to find the effect of the added nutrients on the sorption curve of gypsum. DVS monitors the moisture content of a sample gravimetrically while exposing it to a controlled RH. The measured sorption curves of gypsum with and without the Czapek are shown in Fig. 6. Comparison shows that gypsum with added Czapek takes up significantly more water at higher RHs. The nutrients lower the pore water $a_w$ leading to increased sorption at lower RH values.

DVS was also used to investigate the times required for samples to reach equilibrium with their ambient RH. Figure 6 shows the water uptake and subsequent water release of the gypsum supplemented with nutrients as a function of time following a step increase in ambient RH from 30% (representing approximate room conditions) to 97% (the highest RH used in the experiments) and a second step back to 30%. It can be seen that most of the water is taken up within ~4 h, whereas release takes less than 0.5 h. Since a higher RH leads to a larger water uptake, this gives an upper limit for the timescales involved in equilibration during the experiments.

**Fungal strain, conidial suspension and inoculation**

*Penicillium rubens* (strain CBS 401.92; CBS Fungal Biodiversity Centre, Utrecht, the Netherlands) was used as test organism for this work. The strain was formerly known as *Penicillium chrysogenum*, but was reclassified in 2011 (Houbren et al., 2011). *Penicillium rubens* is considered a common indoor mould (Samson et al., 2010; Andersen et al., 2011; Samson, 2011).

A stock of conidial suspension was harvested from well-sporulating, 1 week old *P. rubens* cultures on MEA. The conidia were collected by wetting the colonies with autoclaved, demineralized water that was supplemented with 0.05% Tween80 and then gently scraping the surface of the colonies. The conidial suspension was then filtered with sterile glass wool to remove mycelium fragments.
Subsequently, the suspension was three times pelleted by centrifugation followed by washing with autoclaved demineralized water + 0.05% Tween 80. Finally, 30 vols% of sterile glycerol was added to the suspension. The final concentration of the stock was counted using a hemocytometer and found to be $1.6 \times 10^8$ ml$^{-1}$. The stock was subsequently distributed over 0.5 ml Eppendorf tubes and stored at $-30^\circ$C.

For the inoculation of a gypsum sample, an amount of stock was unfrozen and diluted to a concentration of $10^6$ ml$^{-1}$ before use. The area on the substrate covered by the Fe$_3$O$_4$ layer on the substrate, growing cells could be observed. The thickness of the microscope working plane wherein hyphae were in focus was determined to be $\sim 75 \mu$m with a micromanipulator (Leica). A magnification of 470 was used, corresponding to a FOV of $(0.84 \text{ mm} \times 0.63 \text{ mm})$, with pixels sized 0.6 $\mu$m $\times$ 0.6 $\mu$m.

Growth was monitored at 1 h intervals with time-lapse recording for 60 days or until colonization was so advanced that hyphae overlapped extensively, whichever came first. The recorded movies were post-processed to quantify the observed hyphal growth. A custom MATLAB script was used to extract the location of hyphal tips in every frame. Hyphal length as a function of time could be determined accordingly, and growth rates and germination times were determined subsequently.

**Set-up for growth experiments**

The inoculated samples were stored in small incubation chambers (Fig. 7). Inside these chambers, RH was controlled by putting an aqueous glycerol solution of known concentration and $a_w$ on the bottom, below the samples (Forney and Brandl, 1992). An $a_w$ metre (Labtouch-aw Basic, Novasina) was used to verify the $a_w$ of the solutions. Pure water ($a_w = 1$) was used to generate a RH of 100%. The containers were airtight to ensure a homogeneous RH throughout the whole container. The set-ups were kept in a climate room and cameras were water cooled so that the set-ups had a uniform, constant temperature ($23.3 \pm 0.1^\circ$C). This again ensured a constant and homogeneous RH throughout the whole container.

During all experiments, samples were observed through the transparent lid of the container with a USB microscope (Dino-Lite 7013MZT, numeric aperture 0.22, optical resolution $\sim 1.5 \mu$m). Due to the contrast provided by the Fe$_3$O$_4$ layer on the substrate, growing cells could be observed. The thickness of the microscope working plane wherein hyphae were in focus was determined to be $\sim 75 \mu$m with a micromanipulator (Leica). A magnification of 470 was used, corresponding to a FOV of $(0.84 \text{ mm} \times 0.63 \text{ mm})$, with pixels sized 0.6 $\mu$m $\times$ 0.6 $\mu$m.

Growth was monitored at 1 h intervals with time-lapse recording for 60 days or until colonization was so advanced that hyphae overlapped extensively, whichever came first. The recorded movies were post-processed to quantify the observed hyphal growth. A custom MATLAB script was used to extract the location of hyphal tips in every frame. Hyphal length as a function of time could be determined accordingly, and growth rates and germination times were determined subsequently.

**Fig. 6.** Adsorption behaviour of the gypsum samples.

A. The equilibrium moisture content as a function of RH for a small piece (about 15 mg) of respectively gypsum (closed symbols) and gypsum supplemented with nutrients (open symbols) at 21°C. Squares mark the equilibrium moisture contents during wetting of the sample, whereas circles represent the equilibrium moisture content during drying.

B. The mass as a function of time after a sudden step in ambient RH from 30% to 97% at $t_1$ and back from 97% to 30% at $t_2$.
Growth experiments on gypsum equilibrated with vapour

Two sets of experiments were performed with the gypsum substrates. A first measurement series consisted of growth experiments on substrates exposed to a constant RH. Dry samples were put in containers with different steady-state RHs with values of 79%, 82%, 86%, 90%, 93%, 97% or 100%. Upon exposure of the samples to a certain RH, an amount of water condensed into the samples through equilibration. This resulted in the pore water of the samples attaining the corresponding $a_w$ (0.79, 0.82, 0.86, 0.90, 0.93, 0.97 or 1) and $u$, as described by the sorption curve (Fig. 6). The hyphal growth sustained by that water was then monitored. Experiments were mostly performed fourfold, but at least duplicate.

Growth experiments on gypsum soaked with aqueous solutions

In a second measurement series, the inoculated substrates were soaked with aqueous solutions of controlled $a_w$ to their maximum $u$ of ~35%. In a given measurement, glycerol, KCl, NaCl or PEG was used as solute. Every time, the $a_w$ of the solution was chosen to match the $a_w$ of the glycerol solution on the bottom of the container (0.79, 0.82, 0.86, 0.90, 0.93, 0.97 or 1) and its corresponding equilibrium RH. This meant that equilibrium set in immediately and that the sample would maintain its maximum $u$ rather than the equilibrium $u$ prescribed by the sorption curve. In the case of experiments with $a_w = 1$ (corresponding to RH = 100%), the samples were soaked with pure water. An $a_w$ metre (Labtouch-aw Basic, Novasina) was used to verify the $a_w$ of the solutions. The $a_w$ of soaked samples was measured too and was compared with the $a_w$ of the solutions. No difference in $a_w$ was found, which indicates that the concentration of Czapek in the gypsum was too low to affect the $a_w$ of the soaking solutions significantly.

Statistical analysis. A set of one-tailed, unpaired Welch’s t-tests was used to compare the hyphal growth rates on samples equilibrated with vapour with the growth rates on samples soaked with a glycerol solution. Only the growth rates on substrates with the same $a_w$ (but different $u$) were compared, since the goal was to exclude the effect of $a_w$ on growth rates from the test. The same procedure was used to test the effect of $u$ on hypha starting times.

Further, the general trend in the growth rates as a function of $a_w$ was investigated for experiments with the gypsum samples. Since only trends were tested and no regression was attempted, $a_w$ was treated as a categorical variable. Sample sizes were generally unequal and homogeneity of variances was not assumed, so a Welch’s one-way analysis of variance was used, followed by a Games-Howell post hoc analysis. This procedure was used each time to analyse the results from experiments with one specific substrate type, i.e. equilibrated with vapour or soaked with the aqueous solution of one particular solute. The general trends of hypha starting times with $a_w$ were investigated in the same way.

All statistical analyses were performed in Microsoft Excel 2010. We used an alpha level of 0.05 for all statistical tests.

Growth experiments on agar

Additional growth experiments on agar culture media were performed for comparison with growth on gypsum. MEA (Oxoid) and plates of agarized Czapek solutions were used. To mimic the nutrient concentrations of the gypsum substrates, two concentrations of Czapek were used: 0.001 g ml$^{-1}$ and 1 g ml$^{-1}$. These equalled, respectively, the bulk concentration of Czapek in the gypsum samples, and the concentration of Czapek in the pore water of a sample equilibrated with an RH of 80% (calculated from the bulk concentration and the volume of pore water based on the sorption data of Fig. 2). All agar plates were created by pouring 9 ml of agar solution into 10 ml Petri dishes. Two hundred micrometre thin glass panes were used as a cover to prevent the agar from drying out while observed with the camera set-up.

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References


