



Hyphal growth of *Penicillium rubens* in changing relative humidity

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Abstract

When considering mold prevention strategies, the environmental conditions in which fungi grow need to be taken into consideration. This environment is often characterized by a time-dependent relative humidity, and porous substrate. Growth has mainly been investigated in steady-state experiments. Therefore, the goal of this study is to understand the hyphal growth of *Penicillium rubens* on porous gypsum, under dynamic humidity conditions. Spores of *P. rubens* were inoculated on porous gypsum containing nutrients, and placed in a small incubation chamber, allowing for microscopic hyphal observation. The relative humidity in this chamber varied multiple times between a high (close to 100%) and low value (35%, 55%, or 75%). The hyphae reacted to a lowered relative humidity by an immediate growth stop and dehydration. When the relative humidity was increased again, the hyphae re-hydrated and three responses were found: regrowing after approximately 4 h, after a time equal to the germination time, or no regrowth at all. No substantial regrowth was found for fluctuations faster than 4 h. This time-scale was found for multiple decreases in relative humidity, and has been reported for the first time.

Key points

- Hyphae restart growth after a characteristic time of approximately 4 h.
- Relative humidity fluctuations of 3 h can suppress hyphal growth.
- Hyphae do not regrow after a severe desiccation and short periods of high humidity.

Keywords Dynamic relative humidity · Hyphal growth · Microscopy

Introduction

Esthetic and medical problems can be associated with indoor mold growth (Flannigan 2001; Green et al. 2011; Miller 1992; Samson et al. 2010). In Europe, people spend approximately 90% of their time in indoor environments (Schweizer et al. 2006). According to the World Health Organization's Large Analysis and Review of European Housing and Health Status, WHO LARES, project, the indoor environment of 25% of all 3373 investigated dwellings (eight European cities: Vilnius, Geneva, Forli, Ferreira, Budapest, Bratislava, Bonn, and Angers) suffer from mold growth in at least one room

(WHO 2009). Prediction and prevention strategies for mold growth are thus needed.

The basis of such strategies lies in detailed knowledge of the colonization process of indoor surfaces. Many controllable factors such as moisture, nutrients, temperature, oxygen, and pH influence this colonization (Burgain et al. 2013; Grant et al. 1989; Griffin 1996; Sautour et al. 2001; van Laarhoven et al. 2015; Xie et al. 1997). A profound understanding of the effect on colonization of each of these factors is vital.

One factor which is essential for germination of all fungal spores is water (Adan et al. 2011; Gottlieb 1950). Studies on the relation between water and fungal growth are often studies done at constant humidity conditions. From these, it is known that a lower water availability results in an increased germination time and a decreased growth rate (Ayerst 1969; Burgain et al. 2013; Gervais et al. 1988; Judet et al. 2008; Nanguy et al. 2010; Segers et al. 2016). Indoor conditions encountered by fungi are characterized by porous substrates, e.g., concrete, wood, and gypsum (Andersen et al. 2011), as well as varying water-related conditions, e.g., due to temperature fluctuations, or household activities such as cooking or

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bathing (Adan et al. 2011). Therefore, there is a need for information on the fungal response to dynamic relative humidity conditions on porous media. The relative humidity, RH, is a measure of the water vapor in the air.

Several studies have focused on the dependence of macroscopic growth on a non-steady relative humidity environment on porous materials (Adan 1994; Bekker et al. 2015; Johansson et al. 2013; Segers et al. 2016; Viitanen and Bjurman 1995).

Complementary to the macroscopic studies executed, information on fungal cell level, i.e., microscopic information, can help to create prevention strategies. Microscopic studies in this type of environment are limited to a single decrease in relative humidity (Luard 1982b; Segers et al. 2016; van Laarhoven et al. 2016). It was concluded that hyphae can regain growth at a higher growth rate after a desiccation. Microscopic experiments with the more realistic situation of multiple wetting-drying environments are thus needed to fully understand hyphal growth after multiple desiccations.

The aim of this paper is to examine how hyphae of *Penicillium rubens* react to multiple periods of lowered relative humidity, while growing on a porous substrate. In this study, we vary the number of RH-fluctuations, the RH of the dry period, and the duration of the dry periods. An experimental set-up is used which allows for real-time microscopic observation of an incubation chamber, in which the RH can be varied between two defined values at well-defined moments in time. It is hypothesized that the hyphae will restart growth, depending on the characteristics of the desiccation. Results are also expected to give an indication of RH values needed in indoor environments that ensure no germination, making this environment unsusceptible to mold growth.

Materials and method

Fungal strain, spore harvesting, substrate, and inoculation

P. rubens was chosen as model organism, as it is a dominant indoor fungus, often colonizing indoor surfaces (Adan et al. 2011; Nevalainen et al. 2015; Verdier et al. 2014). It has a minimal water activity, a_w , for growth of 0.82 at 25 °C (Zalar et al. 2007; Segers 2017). In equilibrium, we have $RH = a_w \times 100\%$. Stock conidial suspensions of *P. rubens* (CBS 401.92; CBS Fungal Biodiversity Centre, Utrecht, the Netherlands, WDCM 133) with a concentration of $7 \cdot 10^6$ spores ml^{-1} were spread on autoclaved Malt Extract Agar (MEA). These MEA plates have a water activity, a_w , of 0.995. These inoculated plates were incubated at 23 °C in a climate room, until sporulation occurred, which consistently happened after 5 to 7 days. Conidia, developed on the MEA plates, were harvested

with sterile cotton swabs (ClassiqSwabs, Copan Diagnostics, Murrieta, CA, USA).

The gypsum samples are inoculated with conidia from these plates using a cotton swab. Harvesting methods using solutions can have an influence on subsequent growth (Dantigny and Nanguy 2009; Nanguy et al. 2010; Nguyen Van Long et al. 2017; Nickerson et al. 1981). Therefore, dry harvesting is opted for.

The substrates for growth experiments were made by mixing gypsum ($CaSO_4 \cdot 1/2 H_2O$, Sigma Aldrich, Saint Louis, MO, USA) with an aqueous solution of Czapek Dox Broth (Oxoid, 8.76 g l^{-1} , ThermoFisher, Waltham, MA, USA) and trace metals $ZnSO_4 \cdot 7 H_2O$ ($2.5 \cdot 10^{-3} \text{ g l}^{-1}$, Sigma Aldrich, Saint Louis, MO, USA) and $CuSO_4 \cdot 5 H_2O$ ($1.25 \cdot 10^{-1} \text{ g l}^{-1}$, Sigma Aldrich, Saint Louis, MO, USA). The solution was autoclaved, mixed with gypsum at a mass ratio of 2 : 3, and cast into 3 mm thick casts. These gypsum samples were dried for 48 h at room temperature in a Bio Safety Cabinet (BSC) (CleanAir, Class II–EF/B, Utrecht, The Netherlands) to remove excess water. The surface of each sample was colored by pipetting 5 μl of Fe_3O_4 suspension (33.3 g l^{-1} water, Metzger Black, Chempur, Karlsruhe, Germany), to provide sufficient contrast for the microscopy. This method is that of van Laarhoven et al. (2016).

Set-up for growth experiments

Inoculated samples were placed in incubation chambers in our climate room, at 23 °C, as seen in Fig. 1. Growth is observed through a transparent lid.

Tap water, creating an RH of just below 100%, was used during all “wet” periods, and thus referred to as RH_{wet} . By using tap water, we ensure a consistent RH_{wet} . It should be noted that at around 100% the growth rate is very dependent on RH: a slightly lower RH results in a relatively big change in growth rate (Segers 2017). To add to that, the RH is highly temperature dependent, so a small temperature fluctuation induces a significant fluctuation in RH, e.g., one degree difference in temperature corresponds to six percent RH difference. This choice of RH_{wet} thus entails some risks, but was measured periodically and our a_w -meter (Labtouch- a_w Basic, Novasina AG, Lachen, Switzerland), with an accuracy of 0.005, consistently read an RH of 100%. The temperature in our climate chamber was constant up to 1 °C. The temperature in the inoculation chambers, however, varied less than 0.1 °C, due to cooling of all electrical equipment present in the climate chamber. This was checked by thermocouple measurements (NI USB-9213, National Instruments, Austin, TX, USA). Regular checks for condensation were done, and no condensation was found in any experiment, ensuring a constant RH of just below 100%.

Following Forney and Brandl (1992), glycerol solutions were used to create the RH_{dry} values of the

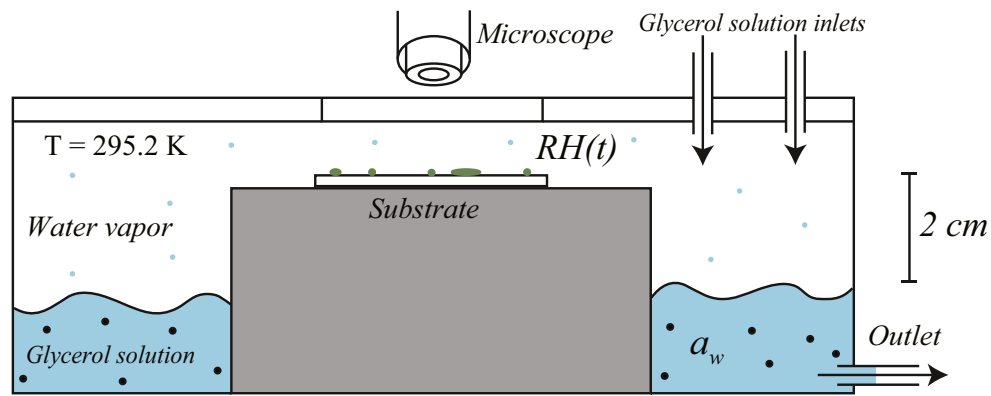


Fig. 1 Schematic representation of the set-up for growth experiments. An inoculated sample is stored in the incubation chamber above a glycerol solution that controls the chamber RH. In equilibrium, we have $RH = 100\% \cdot a_w$, and the solution a_w followed from Fomey and Brandl (1992).

The RH in the container is controlled by the glycerol solution. Two inlets and one outlet allow for switching between two RH values. Growth on the substrate is recorded with video microscopy through the transparent cover of the chamber

chambers during the experiments. To vary the relative humidity, the measurement chamber was emptied, flushed with the preceding water-glycerol mixture, and filled again to ensure that no residue was left behind. This was done using peristaltic pumps (BT100L, Lead Fluid). The resulting RH_{dry} was verified with a digital humidity sensor (SHT7X, Sensirion, Staefa, Switzerland). No residual solution was left between switching, and no splashing on the substrate was found.

During the dry periods, the RH was lowered to a lower value, indicated by RH_{dry} . The mild RH value of 75% is below the minimal value of 86% for which growth can be sustained in the same climate room and analogous incubation chambers, on the same substrate (van Laarhoven et al. 2015). Modest and severe desiccations to 55% and 35% are imposed as well. These are the typical median and minimal values, respectively, of RH of the indoor environment, as found in literature (Telejko and Koruba 2019). These three values are therefore chosen as RH_{dry} .

Analogous to van Laarhoven et al. (2015), growth was monitored by time-lapse recording of images. The time between two images was 1 h for all experiments considered. One hour is thus the experimental time resolution.

When switching between two liquids, i.e., switching between RH_{dry} and RH_{wet} , two elements of equilibration have to be taken into consideration: the air, and the gypsum substrate. Adan (1994) showed that the surface moisture condition is critical for fungal growth, and focus will thus be on the surface of the growth substrate. In the case of an increase in RH to just below 100%, the surface a_w always reaches 0.95 within about 3 h, independently of the initial RH difference (van Laarhoven 2016).

Appendix A gives details about the equilibration of the vapor in the air surrounding the substrate and fungus. It is concluded that after a change in glycerol solution in the inoculation chamber, vapor equilibration is reached in a matter of seconds.

Two types of microscopes are used. A 7013MZT4 (Dino-Lite, Almere, The Netherlands) with magnification of 470 \times , corresponding to a field of view (FOV) of 0.84 mm \times 0.63 mm, with pixels sized 0.6 μm \times 0.6 μm , and an Edge AM 4515T8 (Dino-Lite, Almere, The Netherlands) with magnification of 900 \times , corresponding to a FOV of 0.4 mm \times 0.3 mm, with pixels sizes 0.3 μm \times 0.3 μm . Both have numeric aperture 0.22 and an optical resolution of approximately 1.5 μm . The focus depth of the microscopes is equal to 0.1 mm for the former and 0.07 mm for the latter.

Experimental conditions and measured parameters

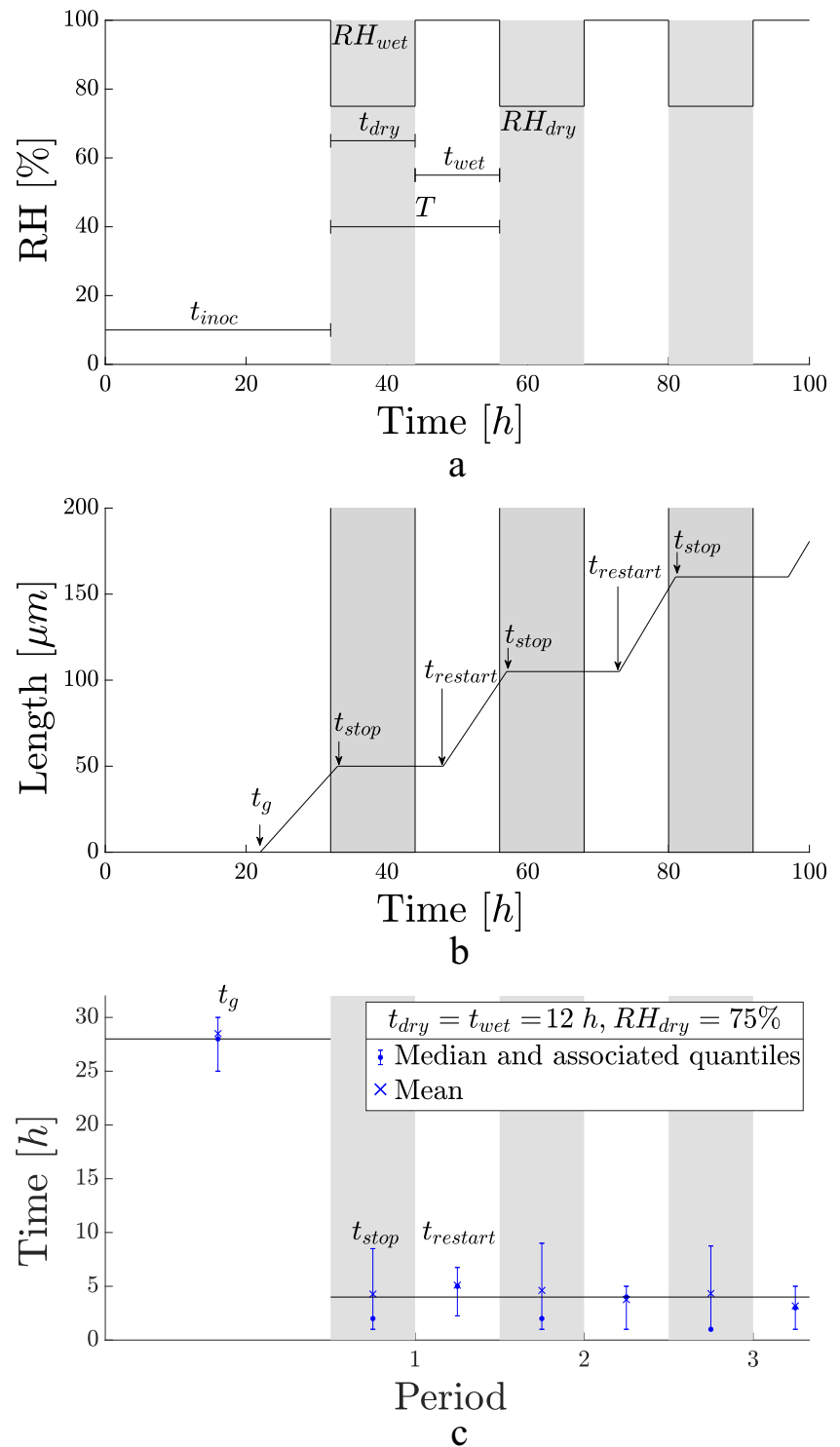
In this section, the conditions of the conducted experiments are discussed, as well as the measured parameters. All experiments began at RH_{wet} for a time period of 31 ± 1 h, indicated by t_{inoc} . This was done in order to ensure germination. Subsequently, a repetitive change in RH was applied, RH_{dry} for t_{dry} hours, and RH_{wet} for t_{wet} hours. Switching between RH_{dry} and RH_{wet} continued, with indicated duration t_{dry} and t_{wet} respectively, resulting in a period $T \equiv t_{dry} + t_{wet}$. The time of wetness (TOW) is defined as $TOW \equiv t_{wet}/T$, following the definition of Adan (1994). The parameters are depicted in Fig. 2a.

Table 1 shows the combination of RH_{dry} , t_{dry} , RH_{wet} , and t_{wet} of all the experiments conducted. The results are divided by the three values chosen as RH_{dry} .

In each experiment, germination, restart, and stop times, t_g , $t_{restart}$, t_{stop} , [h], resp., and linear hyphal growth rate, μ [$\mu\text{m}/\text{h}$], were monitored. These measured parameters are schematically represented in Fig. 2b. They are all determined from the hyphal length traced as a function of time.

The germination time, t_g , is the time when no growth is visible for the last time. The time-parameter characterizing hyphal growth is the delay before growth restarts after switching from RH_{dry} to RH_{wet} . This is indicated by the

Fig. 2 (a) Schematic representation of the dynamic RH during growth experiments. An inoculated sample is stored in the incubation chamber at high RH, i.e., RH_{wet} , for a duration of $t_{inoc} = 31 \pm 1$ h. After this germination period, the RH is lowered to RH_{dry} for t_{dry} hours. Subsequently, the RH is increased again to RH_{wet} for t_{wet} hours, and the cycle is repeated, resulting in a period $T = t_{dry} + t_{wet}$. The gray areas show how dry periods will be indicated afterwards. (b) Generic hyphal growth curve, with germination, stop, and restart times, t_g , t_{stop} , and $t_{restart}$ resp., and linear growth rate μ . The gray areas indicate the periods of RH_{dry} . (c) Germination time t_g , equal to 28 ± 2 h, stopping times t_{stop} (gray indicates RH_{dry}), and starting times $t_{restart}$ (white indicates RH_{wet}) from the traced hyphae of Fig. 3: the first wet period is 32 h, and followed by periods of $t_{dry}=12$ h (gray) and $t_{wet}=12$ h (white). Note that t_g is indicated in the first period, while the other times are t_{stop} and $t_{restart}$



restart time, $t_{restart}$. The time when hyphal growth stops during a period of RH_{dry} is obtained: the stopping time, t_{stop} .

When the RH is high, i.e., RH_{wet} , the hyphae grow and growth is measured and a linear fit is made for the growth curve, resulting in a linear growth rate. The linear growth rate was found to be 10.8 ± 4 $\mu m/h$.

The values of t_g and $t_{restart}$ will be represented throughout this paper as in Fig. 2c. The dots represent the experimental median, the crosses represent the means, and the error bars are the associated first and third quartiles, as indicated in the figure legend.

The restarting times are shown in the white regions of RH_{wet} . The horizontal black line at 4 h serves as a guide for the eye.

Table 1 Parameters of experiments conducted, and associated growth rates

Experiment	RH _{dry} (%)	t _{wet} (h)	t _{dry} (h)	μ±σ [μm/h]
Mild desiccation	75	14	10	9.5±4
	75	8	8	6.9±3
	75	12	12	10.8±4
	75	8	16	11.5±5
Modest desiccation	55	16	12	12.9±7
	55	12	12	11.0±4
	55	12	16	18.6±8
Severe desiccation	35	24	32	15.5±6
	35	12	12	8.2±6
	35	12	16	8.8±5
	35	8	16	14.8±6
Variable fluctuation	75	3	3	No substantial growth
	75	12	12	13.2±5
	75	3	3	No substantial growth

The stopping times t_{stop} are represented in the gray zones of RH_{dry}. For all experiments considered, and for every period within an experiment, this stopping time was within the given experimental time resolution of 1 h, and will therefore not be shown.

Table 1 shows all the experiments conducted, with the associated growth rates.

Statistical analysis

Each experiment was repeated at least five times. Approximately five to fifteen hyphae were traceable in one experiment. Post-processing of the captured movies with a custom MATLAB (The Mathworks, Natick, Massachusetts, United States) script generated hyphal length, l , as a function of time, t , indicated by $l(t)$. Any small change in camera position during the experiment, was also compensated with a custom MATLAB script. Growing hyphae could be followed until they grew out of the FOV, until the FOV was covered densely with hyphae thereby obscuring their tips, or until the experiment terminated. Following Dantigny et al. (2007), a normal distribution for these parameters was assumed. All results discussed consist of at least 25 hyphae, unless stated otherwise.

For the purpose of comparing the t_{restart} with t_g , the function `ttest2`, MATLAB, with a significance level of 0.01 was used.

Results

Hyphal behavior

To show how hyphae of *P. rubens* behave in the experimental set-up considered, a case study is presented. The experiment

considered is characterized by: $t_{\text{inoc}} = 32$ h, $t_{\text{dry}} = 6$ h, $t_{\text{wet}} = 8$ h, and $\text{RH}_{\text{dry}} = 75\%$. Figure 3 shows snapshots of the fourth and fifth period of the experiment. [Supplementary material](#) contains a video of the growth of hyphae on a gypsum substrate during this experiment. In a period of RH_{wet} , Fig. 3a-c, a hypha grows. Figure 3d-f shows the hyphal response when the RH is changed to RH_{dry} : the hypha stops growing within an hour. After this sudden stop, the hypha dehydrates and changes its position slightly due to shrinking.

When the RH is increased again, the hypha regains its former position, i.e., it rehydrates after which growth is restarted. Both rehydration and regrowth are seen in Fig. 3g-i. Regrowth starts after approximately 4 h.

This can be summarized as follows: after a decrease in RH, growth stops within 1 h. After this, dehydration takes place. Rehydration sets in once the RH is increased, and after approximately 4 h, growth restarts.

The growth rates found for each period, for all experiments, are found in Table 1.

Periods of mild desiccation, $\text{RH}_{\text{dry}} = 75\%$

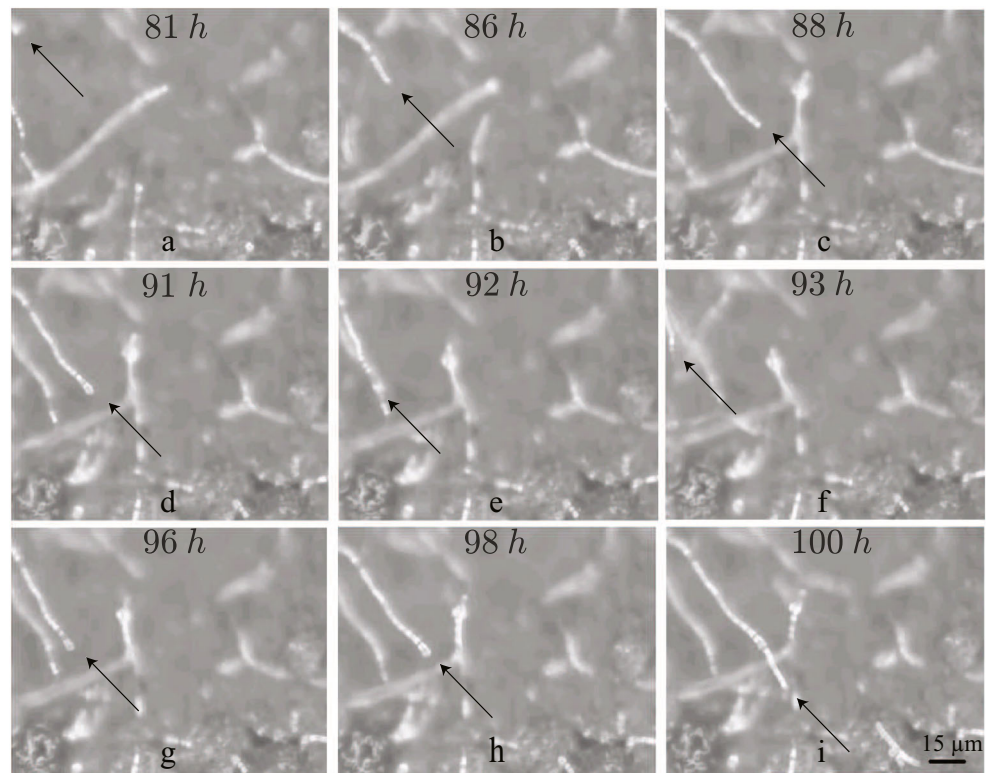
Here, we discuss in more detail the impact of a repetitive change of RH, alternating between 100 and 75%. The changes in RH can be considered instantaneous, as mentioned in the “[Materials and methods](#)” section.

Four experiments are represented wherein t_{dry} has values of 14 h, 8 h, 12 h, and 8 h, with associated t_{wet} equal to 10 h, 8 h, 12 h, and 16 h, respectively.

In all the results shown in Fig. 4a, the number of hyphae was at least 25.

Four RH-cycles were carried out in the experiment with $t_{\text{dry}} = 14$ h and $t_{\text{wet}} = 10$ h. The experiment was terminated after this. For the other experiments, more cycles were carried

Fig. 3 Images taken from the fourth and fifth cycle of the experiment with $t_{\text{inoc}}=32$ h, $t_{\text{dry}}=6$ h, $t_{\text{wet}}=8$ h, and $\text{RH}_{\text{dry}}=75\%$. The first row, (a–c), taken at $t=81$ h, 86 h, and 88 h respectively, is characterized by RH_{wet} . For the middle row, (d–g), are taken at $t=91$ h, 92 h, and 93 h, in an environment of RH_{dry} . The last row, (g–i), are again taken in an environment with RH_{wet} , taken at $t=96$ h, 98 h, and 100 h



out, until the field of view was so full of hyphae that their tips could not be traced anymore. Most switches between low and high RH could be monitored for the experiment with $t_{\text{dry}}=t_{\text{wet}}=8$ h. This is because it took more cycles before the FOV was fully overgrown by hyphae.

For all four experiments, t_g falls between 20 and 28 h, and they are all similar. This was expected as the experimental conditions are identical. The average t_g is 24.5 h and indicated by the horizontal line.

During the subsequent periods of RH_{wet} , the average start-times are similar, independent of t_{wet} and t_{dry} , with the

exception of the first wet period after a desiccation of 8 h, where no hyphae regrew. This is indicated by the arrow in Fig. 4a. In the first wet period after desiccation, no growing hyphae were registered. The horizontal line at 4 h is again a guide for the eye. In all experiments, very few hyphae started regrowing before this time.

The experiments with $t_{\text{dry}}=t_{\text{wet}}=8$ h and $t_{\text{dry}}=t_{\text{wet}}=12$ h have the same TOW, but a different period: $T=16$ h and $T=24$ h respectively.

The experiments with $t_{\text{wet}}=14, 12,$ and 8 h, and associated $t_{\text{dry}}=10, 12,$ and 16 h all have the same period $T=24$ h, but

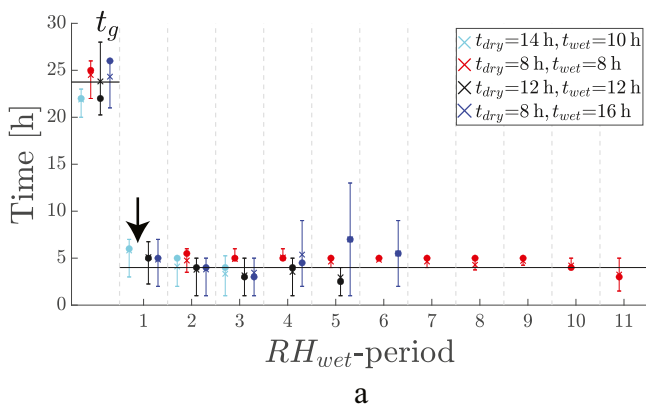
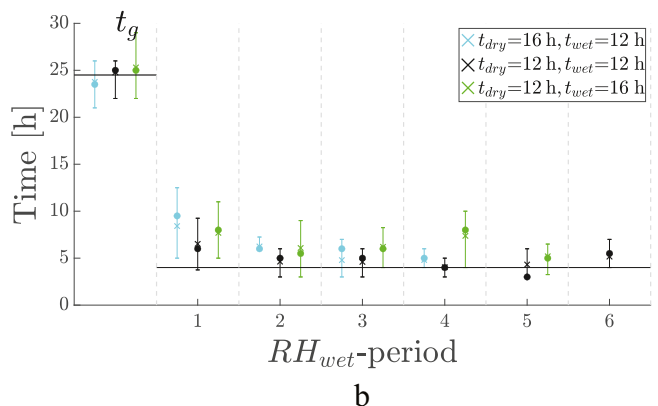


Fig. 4 t_g and t_{restart} during experiments with $\text{RH}_{\text{dry}}=75\%$ (a) and 55% (b). The horizontal axis indicates the RH_{wet} period, e.g., the first increase to RH_{wet} after RH_{dry} is indicated by “1,” etc. The values for t_{dry} and t_{wet} are shown in the legend. Dots indicate the median, crosses represent the



mean, and error bars indicate the associated first and third quartiles. Note that t_g is indicated in the first period, while the other times are t_{restart} . All data-points consist out of at least 25 hyphae. The horizontal line at 4 h is again a guide for the eye, below which few hyphae started regrowing

different TOW, namely, 0.42, 0.5, and 0.67 respectively. Although the TOW varied for these experiments, they show a similar t_{restart} at about 4 h.

The growth rates of all experiments are given in Table 1. They are all in the same order of magnitude: varying between 6.9 and 11.5 $\mu\text{m/h}$.

Periods of modest desiccation, $\text{RH}_{\text{dry}} = 55\%$

The results of a set of experiments with multiple decreases to $\text{RH}_{\text{dry}} = 55\%$ can be found in Fig. 4b. The t_{dry} has values of 16 h, 12 h, and 12 h, with associated t_{wet} equal to 12 h, 12 h, and 16 h, respectively.

In all the results shown in Fig. 4b, the number of hyphae was at least 25.

The experiment with $t_{\text{wet}}=16$ h and $t_{\text{dry}}=12$ h in Fig. 4b only shows four periods because the experiment was terminated after 150 h. The two other experiments, with more cycles, were stopped when the FOV was too cluttered with hyphae.

The shown t_{restart} values for all experiments with $\text{RH}_{\text{dry}} = 55\%$ are all similar and approximately 4 h. The horizontal line

at 4 h is again a guide for the eye only: very few hyphae restart growing before this time-scale.

The growth rates of all experiments with RH decreases to 55% are given in Table 1. They are in the same order of magnitude: varying between 11.0 and 18.6 $\mu\text{m/h}$.

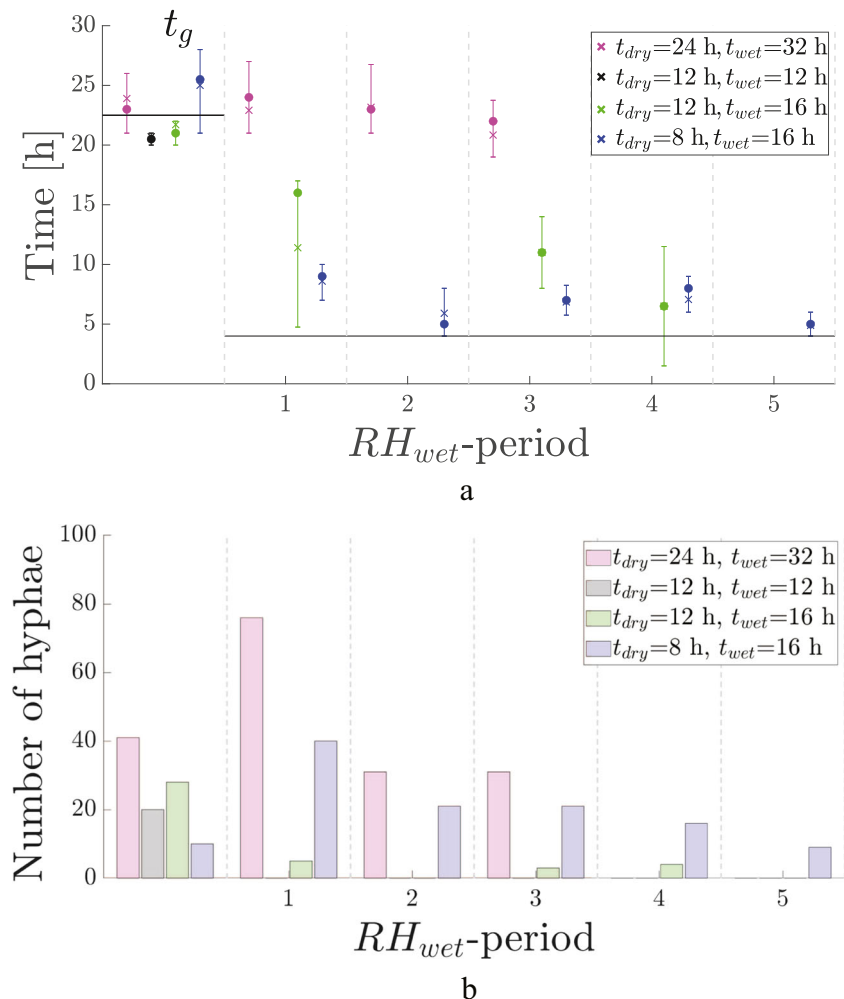
Periods of severe desiccation, $\text{RH}_{\text{dry}} = 35\%$

The growth behavior after multiple periods of $\text{RH}_{\text{dry}} = 35\%$ was investigated. The results are shown in Fig. 5a.

For a desiccation to 35%, three restart times are found: restart after a time-scale t_{restart} of about 4 to 10 h, regrowth with a longer time-scale of about 20–25 h, similar to t_g , or no regrowth.

Firstly, the situation without regrowth is discussed: in the experiment with $t_{\text{dry}} = t_{\text{wet}} = 12$ h, no other hyphae grew outside of this first period of inoculation. For decreases to 75 and 55%, multiple periods of growth were found for these values of t_{dry} and t_{wet} : no regrowth was seen within the wet periods of $t_{\text{wet}} = 12$ h, for a dry period of $t_{\text{dry}} = 12$ h. Although the experiment with $t_{\text{dry}} = 12$ h and $t_{\text{wet}} = 16$ h has data in multiple

Fig. 5 (a) t_g and t_{restart} during experiments with $\text{RH}_{\text{dry}} = 35\%$. The horizontal axis indicates the RH_{wet} period, e.g., the first increase to RH_{wet} after RH_{dry} is indicated by “1,” etc. The values for t_{dry} and t_{wet} are shown in the legend. Dots indicate the median, crosses represent the mean, and error bars indicate the associated first and third quartiles. Note that t_g is indicated in the first period, while the other times are t_{restart} . All data-points consist out of at least 25 hyphae. The horizontal line at 4 h is a guide for the eye, below which few hyphae started regrowing. (b) The corresponding number of traced hyphae per experiment and per period of the results shown in (a)



periods, the amount of hyphae there is considerably lower compared to the previous experiments. Only three-five hyphae were traced, as seen in Fig. 5b. This can be considered as very limited regrowth.

For t_{dry} is 8 h, with a t_{wet} of 16 h, i.e., a short period of dehydration and a long period of rehydration and regrowth, significant growth is observed. This is shown in Fig. 5b. The time-scale of regrowth, t_{restart} is between 4 and 10 h, and the horizontal line at 4 h is again shown as a guide for the eye only: few hyphae restart growth below this time-scale.

The experiment with $t_{\text{dry}} = 24$ h and $t_{\text{wet}} = 32$ h shows significant growth. The t_{restart} for this experiment, however, is significantly larger: approximately 20–25 h, compared to the 4 to 10 h found for before. It is equal to the t_g found throughout.

The growth rates of all experiments with decreases to 35% are given in Table 1, they fall between 8.2 and 15.5 $\mu\text{m}/\text{h}$.

The number of hyphae traced in experiments with RH_{dry} equal to 75% and 55% involved at least 25. In this case, RH_{dry} is equal to 35% and number of hyphae traced is much less, as is shown in Fig. 5b.

Changing RH fluctuations

Very little regrowth is seen for values of t_{restart} below 4 h. This is seen, by using the guide for the eye, in Fig. 3, 4, and 5. It is therefore investigated if growth is seen when $t_{\text{wet}} = t_{\text{dry}} < 4$ h.

An experiment was conducted with decreases to $\text{RH}_{\text{dry}} = 75\%$, with varying t_{dry} , and $t_{\text{dry}} = t_{\text{wet}}$. First, four periods with $t_{\text{wet}} = t_{\text{dry}} = 3$ h were conducted, followed by two periods of $t_{\text{wet}} = t_{\text{dry}} = 12$ h, and again four periods of $t_{\text{wet}} = t_{\text{dry}} = 3$ h. The value for $t_{\text{wet}} = 3$ h is below the observed typical short time-scale of restart of 4 h. The restart times are shown in Fig. 6a.

In the first period after the inoculation, eighteen hyphae started growing within 3 h. In the fourth and eighth period, no regrowth is seen at all. In period 2, 3, 7, 9, and 10 very few hyphae regrew. Thus after period 1, no significant growth is seen in the periods with $t_{\text{wet}} = t_{\text{dry}} = 3$ h. This can be seen in Fig. 6b.

In the two periods with $t_{\text{wet}} = t_{\text{dry}} = 12$ h, regrowth is seen with a t_{restart} similar to all the t_{restart} values found before. The amount of traceable hyphae is again substantial, and the growth rate, found in Table 1, is around 13 $\mu\text{m}/\text{h}$, similar to values discussed in the previous sections.

After the two 12 h periods, four periods with $t_{\text{wet}} = t_{\text{dry}} = 3$ h followed. Again no significant growth is found.

Discussion

Three different hyphal restart methods after a RH-decrease are found in the experiments conducted: regrowth after approximately 4 h, 25 h, or no regrowth at all. The short time-scale of

hyphal regrowth is seen throughout all the sections in the “Results” section. This time-scale, approximately 4 h, is consistently found, for multiple values of RH_{dry} , t_{wet} , t_{dry} , T , and TOW . When the relative humidity is decreased, the hyphae lose water and turgor. When the relative humidity is increased again, the hyphae rehydrate. After this, regrowth is initiated. After a long and severe desiccation, i.e., big t_{dry} and low RH_{dry} , regrowth is found after a t_{restart} equal to the germination time, or no regrowth is found within the investigated t_{wet} .

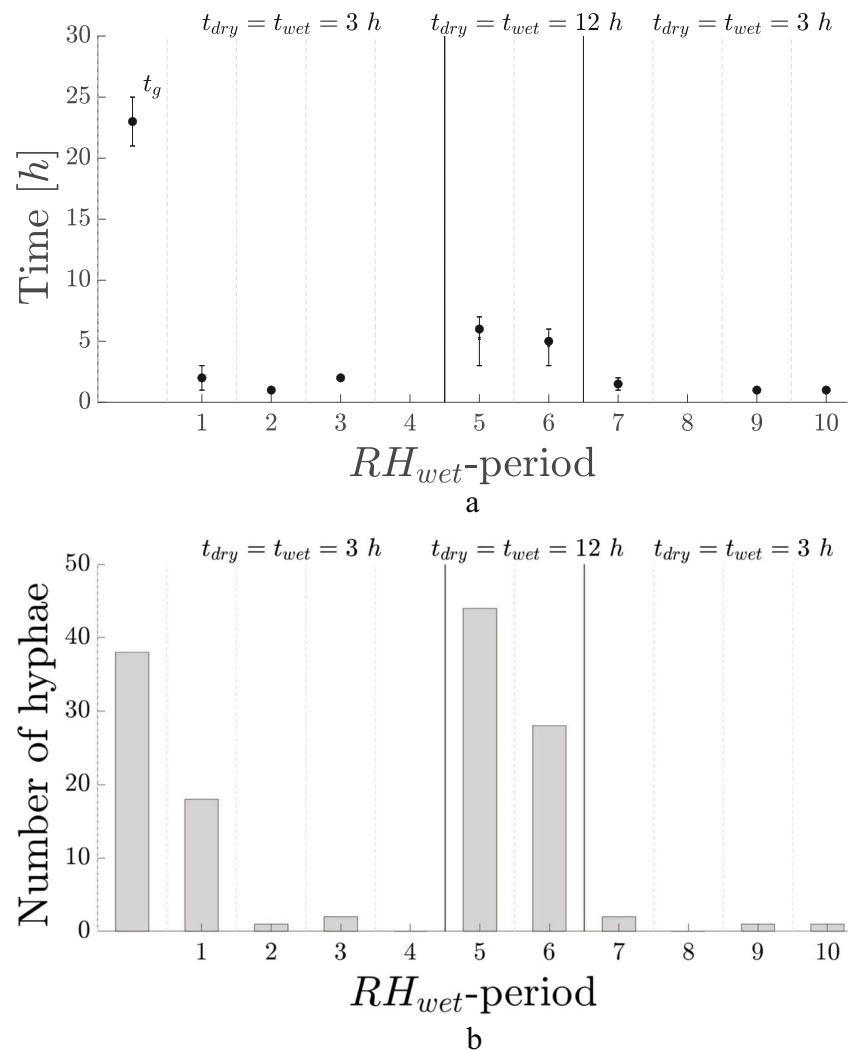
Next to the restart time, the growth rates of all experiments were monitored. The growth rates were represented in Table 1, and are all in the same order of magnitude and similar to literature (van Laarhoven et al. 2016). A single decrease in RH of 1 h to 50%, 60%, 80%, 80%, and 90% resulted in increased growth rates, with a 2 $\mu\text{m}/\text{h}$ increase from 8 $\mu\text{m}/\text{h}$. The porous substrate of the growth experiments was the same. The onset of the decrease in RH, however, was different to the onset of the first RH_{dry} in our work: 96 h by (van Laarhoven et al. 2016), vs. onset after 32 h in this work. Results presented here all have a first decrease in RH after 32 h. It was found by Bekker (2014) that the timing of the drought period had no influence on its effect, on a macroscopic scale: irrespective of the developmental stage of *P. rubens* prior to a RH decrease, the time after desiccation to sporulation remained the same.

Regrowth after a time-scale of approximately 25 h is only seen in the experiment with $\text{RH}_{\text{dry}} = 35\%$, $t_{\text{dry}} = 24$ h, and $t_{\text{wet}} = 32$ h. This time-scale is significantly larger than the characteristic regrowth time-scale of 4 h and equals the germination time t_g found in all experiments. It is hypothesized that growth in this experiment is a consequence of new germination: a considerable decrease in RH, to $\text{RH}_{\text{dry}} = 35\%$, continued for an extended time, $t_{\text{dry}} = 24$ h, prevents regrowth of hyphae. When this is followed by a lengthy period, $t_{\text{wet}} = 32$ h, of RH_{wet} , ungerminated spores can germinate. This would then imply that there is a typical time period and dry RH value, after which hyphae cannot regrow. Hyphal autolysis might occur (Perez-Leblic et al. 1982), but more research is needed to confirm this.

Literature can be found on the response of fungi to changing humidity conditions, e.g., (Adan 1994; Blomberg and Adler 1992; Park 1982; Segers et al. 2016; Viitanen and Bjurman 1995; Viitanen and Ojanen 2007), but comparison between these studies is difficult due to temperature and substrate variation etc. (Dedesko and Siegel 2015; Vereecken and Roels 2012). The work of van Laarhoven et al. (2016) and Bekker (2014), however, can be compared with our results. The substrates used are the same, as well as the type of fungus.

Bekker (2014) identified six different development stages for *P. rubens*. These stages are as follows: ungerminated conidia, onset and minimal germination, germ tubes and branching hyphae, aerial hyphae, initial conidiophore formation, and regular conidiophore formation. They are presented

Fig. 6 (a) Germination, and restart times of the experiment with $RH_{dry} = 75\%$, and t_{wet} and t_{dry} as indicated. The horizontal axis indicates the RH_{wet} period, e.g., the first increase to RH_{wet} after RH_{wet} is indicated by “1,” etc. The dots indicate the median, crosses represent the mean, and error bars indicate the associated first and third quartiles. (b) The corresponding number of traced hyphae per experiment and per period of the results shown in (a)



in Fig. 7. Bekker looked at the macroscopic scale by the discoloration by the formed conidia: two decreases in RH were given at different stages of development, resulting in a different discoloration. The results of Bekker can be summarized as follows: the timing of the decrease in RH, i.e., the stage of the development, affects its impact, and a 48 h-period of lowered RH, prior to sporulation, fully resets the development of *P. rubens*, while other structures can resume growth after a shorter desiccation. We hypothesize that a decrease of 24 h to 35% results only in new germination and not in regrowth of existing hyphae. Although our parameters are different, i.e., $t_{dry}=24$ h (vs. 48 h, Bekker (2014)) and $RH_{dry}=35\%$ (vs. 75%, Bekker (2014)), the concept is the same: a certain depth and length of desiccation stops regrowth of hyphae, and only new germination is possible. The work by Bekker (2014) indicates that other elements than spores regrow after a desiccation, which is confirmed by our results.

One of the few publications where hyphal level response was investigated in a dynamic environment is that of van Laarhoven et al. (2016). They investigated the mycelium at

a scale indicated by the blue square in Fig. 7. As shown in the previous section, the method of regrowth depends on the depth, duration, and moment of application of the RH decrease. This is represented by the two options of regrowth in Fig. 8a. Regrowth of hyphae was found by van Laarhoven et al. (2016), but with a higher growth rate after the period of desiccation, compared to before, a clear difference from the results in this work. The decrease in RH imposed by van Laarhoven et al. (2016), with $t_{dry} = 24$ h, was initiated after 96 h, which corresponds to a decrease in the fifth growth phase, where sporulation starts. It has been shown that the spore formation conditions determine their water adsorption behavior (van Laarhoven et al. 2017) and physiological state (Nguyen Van Long et al. 2017). Further, it has recently been found that sporulation in stressed conditions affects growth (Ruijten et al. 2020). We therefore hypothesize that these faster growing hyphae observed by van Laarhoven et al. (2016) are the result of a “second generation spores,” as is depicted in Fig. 8b. Second generation spores are then non-inoculated spores, but grown in the experimental set-up. It was

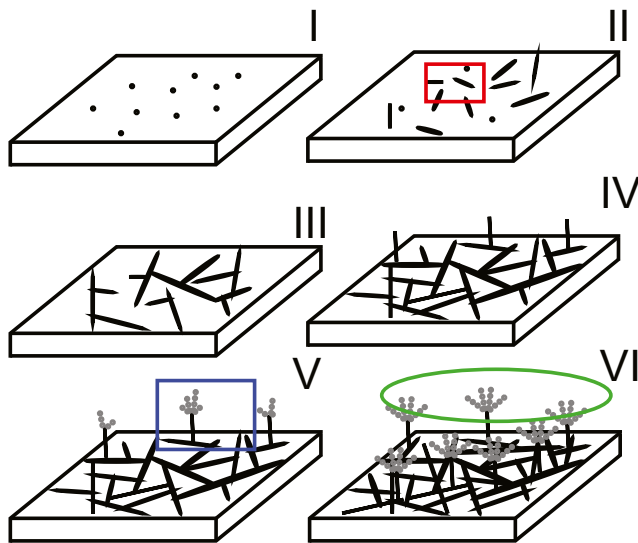
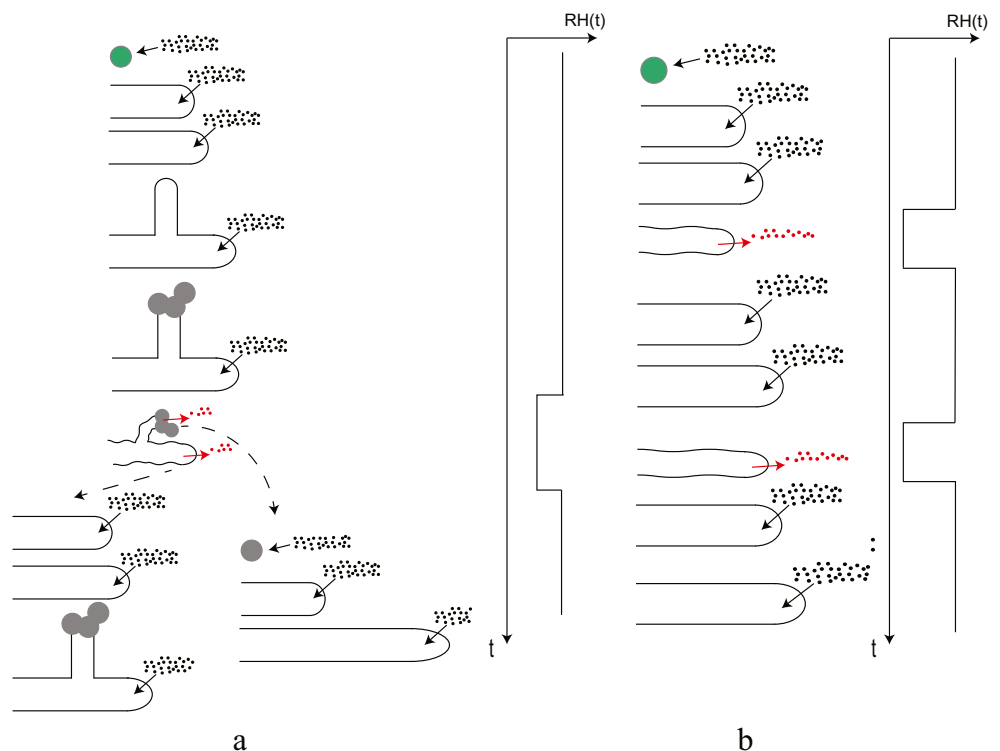


Fig. 7 Overview of different development stages of *P. rubens* on gypsum, from (Bekker 2014): (I) ungerminated conidia (12 h), (II) onset and minimal germination (18 h), (III) germ tubes and branching hyphae (24 h), (IV) aerial hyphae (48 h), (V) initial conidiophore formation (72 h), and (VI) regular conidiophore formation (96 h). The highlighted regions are the scales of interest of the work by Bekker (2014) (green), (van Laarhoven et al. 2016) (blue), and the work represented here (red)

concluded by van Laarhoven et al. (2016) that “growing tips exposed to a desiccation of any considered duration or RH become unviable for further growth afterwards.” This conclusion is thus proven incorrect.

Fig. 8 (a) Schematic representation of the experiments conducted by van Laarhoven et al. (2016), where a single decrease in RH was given after 96 h, when aerial hyphae and the first spores were already developed. The black dots indicate the water vapor, defining the RH. The red dots represent water expelled by the hyphae during dehydration. The new spores differ from the original spores present, and result in hyphae with a different growth rate. (b) Schematic representation of the regrowth response of a hypha. Multiple decreases in RH are applied to spores formed under one specific condition. The experimental parameters are RH_{dry} , t_{dry} , and t_{wet} , as discussed. At the onset of a decrease in RH, the hypha loses water, shrinks, and stops growing within the hour. When the RH is increased again, regrowth starts



The result that hyphae can restart growth with a short time-scale of approximately 4 h will now be discussed. In our study, the dry periods are initiated when the culture is in the growth phase II from Fig. 7. Regrowth after this short time-scale follows the following behavior, shown in Fig. 3: hyphae germinate, grow, dehydrate and shrink down, stay motionless, rehydrate, and regrow with a characteristic short time-scale. This is represented in Fig. 8b. This time-scale can be attributed to the time needed for a hypha to refill itself and restart growth. The fact that this time-scale was observed in most experiments might be explained by a hyphal “dormant” state, reached below a certain threshold of RH. When the RH increases again, the time to go from this dormant state to a state of regrowth is approximately 4 h. Fungal spore dormancy is well-known (Griffin 1996), and more research needs to be done to explore this possible hyphal dormancy. Regrowth is a combination of both active and passive events with their respective time-scales (Steinberg 2007). An example of the former is molecular motors supplying cargo to the hyphal tip. Osmosis is an example of a passive process. The latter is influenced by the amount of internal osmolites in the hyphae, which is species-dependent (Luard 1982a). Experiments with other species should be conducted to test if the observed time-scale depends on this internal osmolyte composition. It has recently been found that microbial growth in carpet dust can be sustained in the indoor environment even with short 6-h bursts of elevated relative humidity in a room (Haines et al. 2020). This time of high RH is longer than the minimum needed to sustain fungal growth found in this work.

The growth rates from Table 1 can be explained by assuming that the typical growth rate of the hyphae is determined by the conditions during sporogenesis. Spores formed at low water activity results in more osmolites (Luard 1982a; van Laarhoven et al. 2017), which leads to the ability of growth in more stressing environments (Ruijten et al. 2020). The growth rate in indoor environments differ from the incubation chamber: dust can be present, paper surrounds gypsum, etc. The results presented here should thus not be compared to work where other factors might influence the growth rate. It is not known if these other factors also influence the time-scale of 4 h.

It is noted that the growth rate depends on the moisture content, which was not discussed in this work. It has been found that on clean surfaces, only the RH is enough for early hyphal growth (Ruijten et al. 2020). From the appendix, it followed that the surface RH reached fast equilibrium, triggering hyphal regrowth.

In conclusion, this study investigates the effect of relative humidity, RH, fluctuations on the hyphal growth of *P. rubens* on a porous substrate. The hyphal response to a decrease in RH at a microscopic level was observed. It was found to be a growth-stop within an hour: the hypha loses water and shrinks, before becoming static. When the RH is increased again, the result of all fourteen represented experiments can be categorized in three different responses, depending on RH_{dry} , t_{dry} , and t_{wet} : regrowth after approximately 4 h, 25 h, or no regrowth at all.

Characteristic hyphal regrowth with a typical short time-scale, of about 4 h, has been observed for the first time. This regrowth with this short time-scale is found consistently in different experiments, and therefore considered characteristic for the fungal response to RH fluctuations. No regrowth below this characteristic time-scale is found.

It is hypothesized that the regrowth after approximately 24 h is a consequence of germination of ungerminated spores, and not hyphal regrowth. It is, however, stressed that this study does not prove that germination is the only source of growth after a long severe desiccation.

The final response is no regrowth, which is due to a long and severe desiccation, followed by a period of high RH, which lasts shorter than the germination time.

Hyphal regrowth at a microscopic level, after multiple decreases in RH, has been investigated for the first time, and refuted earlier findings. The importance of experiments at the microscopic scale mimicking a realistic environment thus lies in its use to unveil all hyphal response types to desiccation. The practical application of the results found here is linked to indoor RH recommendations: the indoor RH should be such that the period of growth-susceptible-RH does not last more than approximately 3 h. Other factors determining this general growth-susceptible-RH should be looked into. A pragmatic use of the results of this work also lies in fungal

modeling: when considering the time-scales at which fungi react to an increased RH, any model should incorporate this time-scale of regrowth, which is the main finding reported.

Besides multiple fluctuations between two set RH values, other combinations of RH decrease, their duration, and that of a subsequent high RH period, could be included in future experiments. It has been found that the indoor fungus *Cladosporium halotolerans* survives humidity dynamics markedly better than *Aspergillus niger* and even *P. rubens* (Segers et al. 2016). Therefore, similar experiments with other species can give new information. Besides other transient RH conditions and species, novel compounds or materials that might inhibit growth of molds on indoor surfaces under simulated real conditions should be investigated.

Appendix: air equilibration in the incubation chamber

For air equilibration, a diffusion constant of $D = 0.28 \text{ cm}^2\text{s}^{-1}$ for water vapor in air is found in the literature (Cussler 2009). A RH step from 99 to 35%, at 23 °C, corresponds to concentrations of $c_i = 20.3 \text{ gm}^{-3}$ and $c_f = 7.2 \text{ gm}^{-3}$ respectively. The general solution to the diffusion equation in this set-up, with $d = 2 \text{ cm}$ as indicated in Fig. 1, is given by:

$$c(x, t) = c_f + \frac{4}{\pi} (c_i - c_f) \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} \cos\left(\frac{(2n+1)\pi x}{2d}\right) e^{-\frac{(2n+1)^2 \pi^2 D t}{4d^2}} \quad (1)$$

Equation 1 has been evaluated, with 1000 terms, and the concentrations are shown in Fig. 9. In a matter of seconds, equilibration to the new RH has occurred. This fast air equilibration was also confirmed by van Laarhoven (2016).

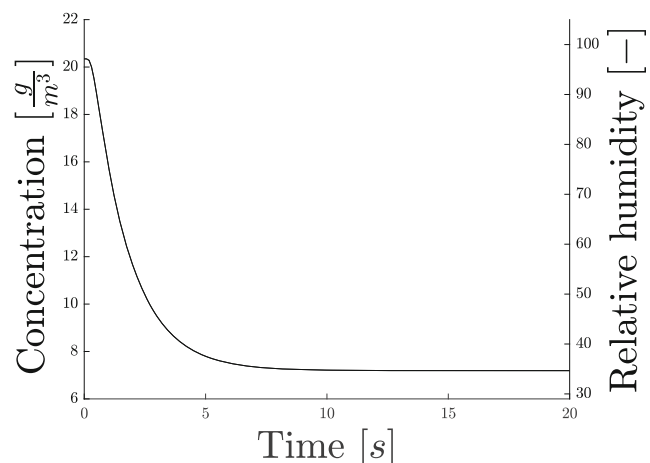


Fig. 9 Evaluation of the first 1000 terms of Equation 1, for a switch from $RH = 95$ to 35% . Within a matter of seconds, the air in the experimental set-up of Fig. 1 is equilibrated after a change in RH

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00253-021-11343-6>.

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Code availability Not applicable

Author contribution PR conducted and co-created the experimental set-up and wrote a first draft of the paper.

HPH and OCGA co-created the experimental set-up and revised multiple drafts of the paper.

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Data availability Raw data were generated at Eindhoven University of Technology. Derived data supporting the findings of this study are available from the corresponding author H.P.H. on request.

Declarations

Ethics approval and consent to participate Authors did not submit to more than one journal for simultaneous consideration. The submitted work is original and not published elsewhere. No material was re-used. Authors adhered to discipline-specific rules for acquiring, selecting, and processing data. No data, text, or theories by others are presented as if they were the author's own. Proper acknowledgements to other works are given, quotation marks (to indicate words taken from another source) are used for verbatim copying of material, and permissions secured for material that is copyrighted.

Consent for publication Not applicable

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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