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# Laboratory test to assess sensitivity of bio-based earth materials to fungal growth

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## Abstract

The effect of molds present in buildings on the health of the occupants is a major issue hence, when a building material is developed, its sensitivity to microbial growth should be assessed. However, few studies have investigated fungal growth on bio-based building materials with the resources available in a laboratory specializing in materials. The objective of this paper is thus to propose a simple and efficient experimental method useful for construction materials laboratories, adapted from methods proposed in the literature. For this purpose, fungal growth was investigated under different environmental conditions on earth-based material with or without the addition of straw or hemp shiv. Samples were inoculated with a strain of *Aspergillus brasiliensis* and were incubated for 12 weeks at 76, 84 or 93% RH, and 30 °C or 20 °C. Reproducible results showed that earth-based materials were more sensitive to fungi when they were enriched in plant aggregates. Fungal development was observed on earth material containing plant aggregates after 4 weeks of exposure at 93% RH and 30 °C, whereas it was observed after 8 weeks on raw earth material under the same conditions. Additionally, the possibility of quantifying fungal development with increased sensitivity by using image analysis is proposed. Due to the growth of fungal species other than *A. brasiliensis*, a natural inoculation approach is recommended. One of the conclusions is that liquid water is more favorable to mold growth than relative humidity alone. The addition of liquid water is thus recommended to accelerate the test.

Keywords: fungal growth, building material, unfired earth, plant aggregate, indoor environment, *Aspergillus brasiliensis*

Highlights:

- Mold grows only under the most severe conditions (30 °C and 93% RH).

- Fungal proliferation is activated by liquid water rather than water vapor.
- Straw favors fungal growth.

## **1. Introduction**

Development of construction materials is often planned to meet objectives and requirements concerning mechanical and/or thermal properties. However, the objective is not necessarily twofold, and other constraints can be defined, which may be as various as fire safety, visual aspect, durability and occupants' health. On this last point, mold risk has been the focus of increasing interest in recent years, for two main reasons. First, buildings are being designed to be increasingly airtight, which limits unwanted air infiltrations but also leads to increased levels of indoor relative humidity. Second, numerous research projects and recent constructions are considering bio-based materials, mainly for the low embodied energy and for the renewability of the raw material. These are claimed to be healthy and to increase the indoor comfort of the occupants [1] but it is commonly accepted that the use of plant matter would lead to an increased risk of mold growth. Furthermore, it is estimated that 20% to 40% of the housing in Northern Europe and North America is affected by indoor molds [2]. Microbial Volatile Organic Compounds (MVOC), responsible for the unpleasant odor, and spores and mycotoxins, which are responsible for various health issues [3,4], are by-products of this active fungal growth. The incidence of the spores on human health depends on the concentration, exposure time and host factors. An exposure to these fungal by-products can generate allergic conditions and impact asthmatic well-being particularly among immunocompromised persons [2]. The set of such health problems is part of the Sick Building Syndrome (SBS) or Building Related Illness (BRI). As people spend more than 80% of their time inside buildings [5], the impact is large.

For these reasons, more and more attention is being paid to mold growth on building materials [6-14], with applications of bio-based material becoming a topical issue. Some studies investigating such growth deal with wood-based or paper-based materials and also inorganic materials such as cement or gypsum plaster board [3,6,12]. Fungal growth has also been studied *in situ* on straw bales with lime-based render for building envelopes [15]. Hoang et al. [7] have shown that bio-based materials are more sensitive to fungal growth because of the nutrients they contain and their high hygric capacity. However, the methodologies followed in these studies were varied and mostly adapted from practices in biology laboratories [16,17]. Although such access to relevant devices has its importance, there are some significant differences in terms of purpose between the field of construction materials and that of biology. To develop new materials, it is important to elaborate a method to evaluate their sensitivity to fungal growth that would be easy to implement in a materials laboratory, without necessarily identifying the fungal species developed. Moreover, a screening method able to test various samples in a reproducible manner may help to select relevant materials, in the very early stages of the development of new materials in laboratories.

Some standard protocols exist to evaluate mold growth on materials, e.g., the standard ASTM D3273 [18] concerning the growth of mold on coated surfaces, in which the material is tested for only four weeks in one set of environmental conditions. However, no standard is specific to bio-based materials.

While different microbiological studies on construction materials have already been documented, this field of investigation is still emerging due to the diversity of environmental conditions and materials, and the multiplicity of questions addressed. Indoor microbial

growth on building materials was recently reviewed by Verdier et al. [19], who compared several methods for sampling and analyzing the proliferation of micro-organisms, and described the most common microbial communities and the building parameters. Mold growth is dependent on various environmental factors, which have to be taken into account when developing a testing protocol. Some of them have been identified as having a particularly strong influence:

1. Water availability. In steady state conditions, fungal growth begins at around 80% of relative humidity according to Nielsen [4]. A minimum relative humidity of 77% was reviewed by Krijgsheld et al. [20] for fungal species, but the optimal value was 97%.
2. Substrate (or medium, or material). The proliferation depends on the quantity of nutrients available and the porosity and roughness of the material [21].
3. Temperature. The optimal temperature for many fungal species is between 20 °C and 30 °C [22-24]. However, some microbial growth has been recorded between 0 and 50 °C depending on the species [25].
4. Time of exposure. The longer the material is exposed to humidity, the higher is the risk of microbial growth [4].

There is a clear need to move forward on this topic, one of the most obvious reasons being the absence of a suitable standardized protocol. This is one of the issues identified in the framework of the Bioterra project, a national collaborative project involving members from both materials and microbiology laboratories. This project is focused on earth-based materials including plant aggregates, as they are assumed to sometimes present mold growth and as limited research has been published on this topic up to now. Mold formation was observed on earth panels containing hemp shiv ten days after manufacturing [26]. It

was also observed after removal of the formwork of earth-straw walls [27], particularly inside the building, where ventilation was less effective.

One of the main objectives of this paper is to propose a simple and efficient method, adapted from methods already proposed in the literature, for assessing the sensitivity of materials to fungal growth, which will be useful for laboratories interested in construction materials. To achieve this objective, the second section of this paper provides a short literature review of existing tests and procedures. These considerations led us to design an in vitro protocol for the study of fungal growth, as described in the third section, dedicated to the presentation of the materials and procedures. The protocol was applied to samples made of raw earth as the mineral matrix, with the addition of two types of plant aggregates: barley straw or hemp shiv. In the fourth section, the results are presented regarding the rate of mold proliferation on the material, and the experimental procedures applied are discussed.

## **2. Literature review of mold growth evaluation on building materials**

As underlined in the introduction, there is no consensus on the methodology that should be applied to study mold growth on building materials, although research has already been done on this topic. In this section, the main techniques found in the literature are presented. Some laboratory tests are based on standards intended for plastics (ISO 846 [28]) or insulation materials and their facings (ASTM C1338 [29]), for example. Recently, Johansson et al. [30] summarized these standards and proposed an innovative method intended for building materials. This constitutes the main basis for the present work.

### 2.1. Decontamination

Decontamination has to be performed just before starting the study of mold growth in order to remove the fungi already present in the material. A simple method is to expose the samples to high temperature for a given time. It is mentioned by Simons et al. [31] that, even if not all the bacteria were removed with a heat treatment at 100 °C, almost all molds were eliminated. Some authors have sterilized materials with gamma rays [7,12], which proved much more effective. However, this technique is costly and the device is rather unusual in material development laboratories. Some authors chose not to sterilize their samples to avoid unknown changes in the substrate [15,32].

### 2.2. Fungal selection and inoculation

Regarding inoculation, some authors suggest that natural inoculation would be more representative of real conditions [7,32], while artificial inoculation is preferred by others. The latter technique consists of inoculating the specimens with an inoculum preparation, which accelerates the test and improves repeatability [30]. Moreover, it is easier to quantify and compare the fungal growth when the initial state (spore quantity) is known.

Various species identified on indoor building materials have been listed by Verdier et al. [19]. The most frequent genera of species isolated are *Cladosporium*, *Penicillium*, *Aspergillus* and *Stachybotrys*. *Aspergillus niger* is used in many references [7,17] because it is often observed on building materials and has been involved in health issues [33,34]. This is a filamentous fungus, which has been observed all over the world in various environments (forests, dunes, indoors, etc.). It can be pathogenic for humans and its presence is not accepted in a hospital environment. The optimal water activity ( $a_w$ ) for its growth is around 0.95 [35]. According to different authors, the optimal temperature is around 30 °C. Krijgsheld et al. [20] observed

the greatest growth between 35 °C and 37 °C, it occurred between 27 °C and 37 °C according to Passamani et al. [35], and the proliferation was greater at 28 °C than at 20 °C in the study done by Lasram et al. [36]. *A. niger* may thus be considered as a representative species for a global evaluation in vitro.

Hoang et al. [7] inoculated a single strain (*A. niger*), but various fungal species have been used by others [6,9,12,17,37]. The inoculation was performed by means of a spray [6], micropipette [7] or dry cotton swab [12]. The latter was used in order not to modify the water activity.

### *2.3. Incubation*

Most of the time, samples were put in a single climatic chamber that regulated the hygrothermal conditions, or in a closed chamber at controlled temperature where saturated salt solutions were used to maintain constant relative humidity. The incubation time was found to differ significantly among the studies, ranging from 42 days [32] to 30 weeks [12]. However, the temperature and relative humidity conditions were similar. Often, the temperature was set close to 30 °C and the relative humidity was kept high (above 90% [732]). These are taken to be optimal conditions for fungal growth, leading to fast tests.

Some authors have investigated cyclic conditions to better represent indoor conditions in dwellings. In the study by Latif et al. [10], for example, samples were placed at 90% RH for 2 days, then at 55% for 4 days. The alternation of these two relative humidities lasted 16 days.

### *2.4. Observation techniques and result analysis*

The proliferation of fungi may be assessed by measuring the CO<sub>2</sub> production due to their aerobic respiration [15] or quantified by measuring the mass loss of the sample (consumption of nutrients by the fungi) [32]. However, the latter method presents problems

of material loss during handling, nutrient intake by the fungi, which will also decrease the mass, and hyphal growth, leading to an increase of the mass. In fact, the most widespread technique used to follow fungal growth consists of periodic observations with a microscope [6,12,32]. Johansson et al. [6] made their observations under a laminar flow to minimize contamination from the room but, again, in a building materials laboratory, such devices are not common.

The observations are often analyzed through a classification of different stages of proliferation. One example is presented by Johansson et al. [6], where the rating scale ranges from 0 to 4:

- 0 means no mold growth;
- 1 is for the start of growth, with one or few hyphae and no conidiophores;
- 2 means sparse growth but with some conidiophores;
- 3 means patchy or heavy growth with many well-developed conidiophores;
- 4 means growth over practically the entire surface.

This technique does not depend on the fungus or its inoculation, so it can be applied in many different cases. Moreover, only a microscope is required, which is a widely available and affordable device. A sample is considered to fail the test if stage 2 on the above scale is reached or exceeded as the sporulation phase is one of the most harmful to human health because spores and aerosolized hyphal fragments can be released and cause allergies, among other pathologies [38,39]. When several samples are considered, a choice can be made between two methods: considering either a median stage equal to two, or the first sample to fail. Note that a similar analysis method, named the “mold index”, was proposed by Viitanen [40], but 7 stages (from 0 to 6) were distinguished. Up to 2, the growth was

visible only with a microscope, whereas it was visible with the naked eye from 3 to 6 with a surface coverage ranging from 30% to 100%. The main drawback is that the classification is subjective. This was acknowledged by Johansson et al. [6] but it was also demonstrated that four different investigators obtained the same result, leading to the conclusion that the method was relevant. Other rating scales have been proposed, such as the one from the ASTM standard D3274 [41] (from 10 to 0), which considers the surface area covered by fungal growth. However, this analysis may be more relevant if spray is used for inoculation [42] rather than a pipette.

Despite these proposals, this methodology remains subjective and qualitative, which stresses the need for other techniques allowing mold growth to be quantified. One simple alternative is to take pictures of the samples then to carry out image analysis to quantify the area covered by fungal growth. Nielsen et al. [12] used stereo-microscopy, while Bekker et al. [42] developed a set-up called the “Fungal Observatory Climate controlled aUtomized Set-up” (FOCUS), which is based on the discoloration caused by the proliferation. Hoang et al. [7] used the software ImageJ for their image analysis.

### **3. Materials and procedures**

#### *3.1. Raw materials and sample preparation*

Raw earth material is increasingly studied for its low environmental impact and its ability to buffer indoor moisture. Depending on the intended use, plant aggregates can be incorporated into the earth matrix to lighten the material and improve some properties of the composite, such as lowering its thermal conductivity [43].

In the present study, quarry Fines from the Aggregate Washing processing (FWAS) were used as the earth material. These fines are waste generated by the washing of limestone aggregates produced for the concrete industry, among others. The sludge created is left in sedimentation basins until it is dry. It is then reduced to powder before its utilization. The fines used here were composed of calcite (60%), kaolinite (11%), illite (11%), quartz (10%), dolomite (6%) and goethite (3%). The FWAS had a pH of 7.8, which is an optimal value for the development of many microorganisms. The particles were extremely fine: 99% were smaller than 80  $\mu\text{m}$  and the average particle size (D50) determined using pipette analysis was 6.5  $\mu\text{m}$ . Before being used, they were stored in plastic bags at room temperature. Two plant aggregate types were studied in this work: barley straw, the part of the cereal stem rejected during the harvest, and hemp shiv, the lignin-rich part of the hemp stem.

Three formulations were studied: earth alone (FWAS), earth with 3% of barley straw (S3) and earth with 3% of hemp shiv (H3). The raw materials were mixed by hand before adding water to reach the optimum Proctor water content (corresponding to the highest density) and mixing mechanically until a homogeneous mixture was obtained. The specimens were manufactured by double static compression in cylindrical molds 5 cm in diameter and 5 cm high. Five specimens were made at once in the mold (Figure 1). They were separated by four PVC discs 5 cm in diameter and 1 mm thick. Specimens were first dried for 24h at 40°C, then the temperature was increased by 0.1 °C/min to 100 °C and kept at 100 °C until the weight became constant (weight variation less than 0.1% between two weighings 24 hours apart). This was done to accelerate the drying of the samples (in opposition to natural drying). Note that this temperature was set to be similar to the one used in brickyards before firing.



**Figure 1. S3 specimen manufacturing**

The sample surfaces were not perfectly flat. In order to facilitate the automated image acquisition on raw samples, the two faces of the specimens were polished to limit the surface roughness. The sides were also polished to allow the specimens to fit into the holder. The final thickness was around 0.8 cm, but the thickness was not expected to have a strong influence because mold growth mainly develops at the surface. Moreover, a faster proliferation was observed by Hoang et al. [7] on the edges of the specimens, i.e. where the fungus had not been inoculated. This might have been due to a higher roughness of the edges in comparison to the sides, which favored local water accumulation and mold growth. To solve this problem, Van den Bulcke et al. [8] proposed coating the sample edges with polyurethane. In the present study, the specimens were coated with a polyester resin (Synolite® 0288), except over a square area of 3x3 cm<sup>2</sup>, which was the surface area investigated.

## 3.2. Procedures

### 3.2.1. Initial state

The choice of drying the samples after the polishing and coating was made in order to impose the same initial state of the material. The specimens were placed in an oven at 100 °C for 24h. This value was the same as during the drying phase of the material and was not exceeded in order to avoid modifying the material and avoid combustion of the plant matter, which starts at 250 °C [44]. Sterilization of bio-based materials was thus complicated to achieve in a materials laboratory. However, this temperature was assumed to be high enough to eliminate some of the molds initially present, as mentioned above, and so to allow a better visualization of the inoculum added. Specimens were allowed to cool for 30 minutes after the decontamination before being inoculated.

### 3.2.2. Inoculation

The fungal species chosen for this study was *Aspergillus brasiliensis* (formerly *Aspergillus niger* [45]) as often used in the literature [7,17]. This was expected to make the phenomena occurring during the test easier to understand, and allow comparison with these earlier studies. Moreover, a previous study had shown that the main cultivable fungi growing on home-made raw earth specimens belonged to the *Aspergillus* and *Penicillium* genera [31]. Artificial inoculation with a pipette was preferred so that the spore quantity deposited on each specimen was known exactly. The fungal suspension of *Aspergillus brasiliensis* (ATCC 16404 / CBS 733.88) spores was prepared following the standards NF EN 1275 [46] and NF EN 12353 [47]. This inoculum was concentrated at  $3 \cdot 10^5$  conidia \* mL<sup>-1</sup> and 5 µl was pipetted onto each of 5 different spots of the surface, thus permitting rapid drying of each drop.

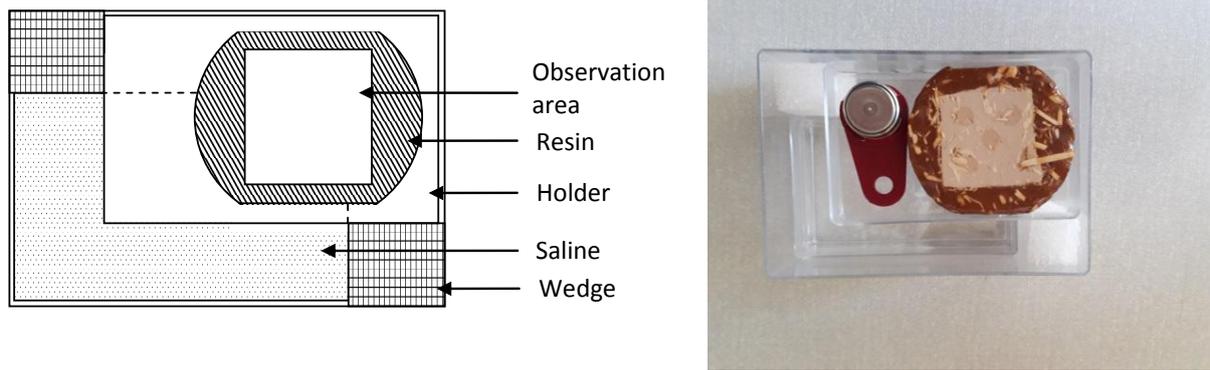
Therefore, each surface received 25  $\mu\text{L}$ , which represented about 7500 conidia, i.e. more than 800 conidia. $\text{cm}^{-2}$ . For each test, five samples were inoculated, referenced I1, I2, I3, I4 and I5, while two additional samples, serving as controls, were not.

### *3.2.3. Incubation*

In a preliminary study, it was shown that, when the samples were placed in the same climatic chamber, fungal growth could be detected on control samples (non-inoculated) as well as on the inoculated samples. To avoid this cross-contamination, the use of separate boxes was proposed by Thomson and Walker [15], according to the standard ASTM D6329 [48]. Several steady environmental conditions were investigated for 12 weeks, as recommended by Johansson et al. [30]. Saturated saline solution was placed inside the boxes to maintain a constant relative humidity level [49]. A similar protocol was followed here using different salts to obtain 3 different RH conditions:

- 93% of relative humidity with a solution of potassium nitrate ( $\text{KNO}_3$ );
- 84% with potassium chloride ( $\text{KCl}$ );
- 76% with sodium chloride ( $\text{NaCl}$ ).

The individual incubation set-up was composed of a plastic box in which saturated saline solution was placed. Above this, the specimen was placed on a holder supported by rigid foam wedges to ensure good stability (Figure 2). The box was sealed with Parafilm<sup>®</sup> in order to increase the air-tightness of the assembly. Prior to testing, all the plastic boxes were cleaned with alcohol to avoid any other source of contamination.



**Figure 2. Scheme (left) and picture (right) of the individual incubation set-up**

The materials were tested under two different temperatures (20 and 30°C), leading to a total of six different sets of hygrothermal conditions, as summarized in Table 1. Assuming that the least favorable conditions would lead to no mold growth, fewer samples were prepared and used for the tests at low temperature and low relative humidity (20 °C with 76% or 84% RH). For the tests at 30 °C, the incubation set-ups were placed in a climatic chamber. For the tests at 20 °C, they were placed in a room where the temperature was maintained at 20 °C. The conditions were monitored by hygrothermal sensors placed in the specimen holder, within the plastic box (Figure 2). As no surface condensation was detected in the boxes, it was concluded that this protocol avoided liquid water droplets falling onto the samples, which would have biased the experiment. For microscopic observation, the plastic box was opened and the sample was removed with its holder in order to avoid the sample being touched and so contaminated.

**Table 1. Conditions of incubation of the materials and quantification of the mixtures tested**

Incubation	Temperature	30°C			20°C		
	RH	75%	84%	93%	75%	84%	93%
	Time (weeks)	12			12		
Materials	FWAS (35 samples)	5 <sup>i</sup> , 2 <sup>n</sup>	5 <sup>i</sup> , 2 <sup>n</sup>	5 <sup>i</sup> , 2 <sup>n</sup>	2 <sup>i</sup>	5 <sup>i</sup>	5 <sup>i</sup> , 2 <sup>n</sup>

	S3 (35 samples)	5 <sup>i</sup> , 2 <sup>n</sup>	5 <sup>i</sup> , 2 <sup>n</sup>	5 <sup>i</sup> , 2 <sup>n</sup>	2 <sup>i</sup>	5 <sup>i</sup>	5 <sup>i</sup> , 2 <sup>n</sup>
	H3 (14 samples)	-	-	-	2 <sup>i</sup>	5 <sup>i</sup>	5 <sup>i</sup> , 2 <sup>n</sup>
<sup>i</sup> Inoculated, <sup>n</sup> Non-inoculated							

### 3.2.4. Microscopic observation

In the present study, observations were made with an optical microscope (magnification 10X) once a week to follow any microbial growth. The proliferation of mold growth was evaluated using the rating scale ranging from 0 (no mold growth) to 4 (growth over practically the entire surface) described in part 2.4.

To complete microscopy observations, automated image acquisition was also performed under a binocular microscope in order to assess the fungal growth by image analysis. The motorized stage allowed 165 snapshots of the sample to be taken over an area of 9 cm<sup>2</sup>. The global image was then recomposed by the computer software Ellix from Microvision Instruments. The objective was to determine the contaminated area of the surface by comparing the images at the end of the test with those from the beginning. However, the automated acquisition lasted for around 20 minutes for each sample, which made it hardly suitable for on-going monitoring of mold growth for all the samples. Consequently, this technique was applied only at the beginning and end of the whole period of incubation.

### 3.2.5. Additional analysis technique

Finally, this study was completed with a third analysis technique to identify the predominant fungal species, in order to check if *Aspergillus brasiliensis* is the main detected fungi. This test, referred to below as the "identification test", was carried out in a microbiology

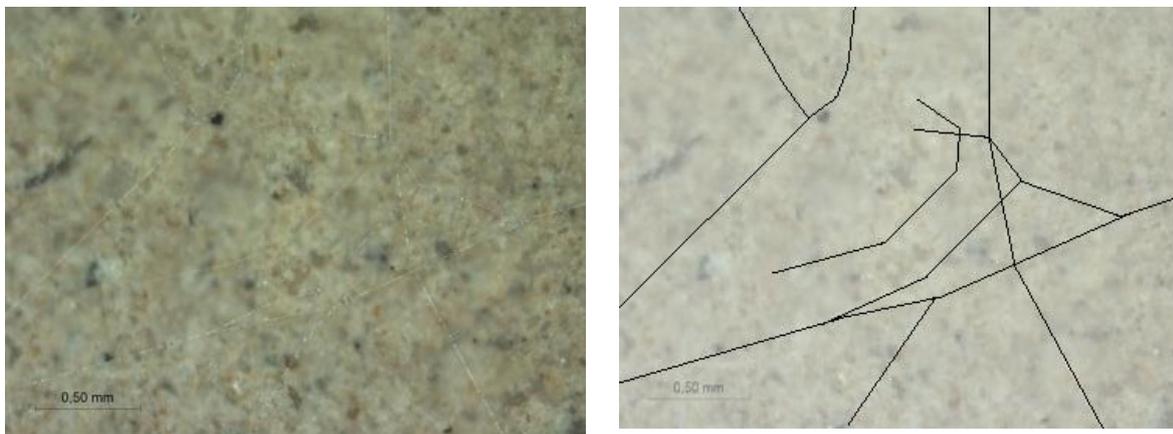
laboratory. This non-destructive (for the material) analysis was carried out on the five inoculated specimens of mixture S3 following a protocol established by Simons et al. [31]. First, the surface of a specimen was sampled by applying a 9 cm<sup>2</sup> adhesive dressing (Hydrofilm - Hartmann®) to it. The dressing was then removed, put into a tube with 10 mL of a recovery medium (sterile Phosphate Buffer Saline (PBS) + 1% of sterile detergent (Tween80)) and vortexed for 2 minutes. The suspension obtained was serially diluted and each dilution was deposited on a nutrient medium of Potato Dextrose Agar (PDA) with 0.05 mg.mL<sup>-1</sup> of Chloramphenicol (Cm) in triplicate technique. The specimens were finally incubated for 4 to 5 days at 22 °C before the fungi were enumerated. The Colony Forming Units (CFU) were counted and the fungal CFU concentration in the initial suspension was calculated. The fungal isolates were identified by observing some aspects of the mycelia such as their shape or color. The hyphae and conidiophores were also observed by optical microscopy (magnification 400X) with a safranin-stain.

This technique is complementary to microscopic observations but it should be noted that sampling by the adhesive method is not free from bias [31]. First, although the whole surface area of the specimen was sampled, the inside of the material was not. Second, there is no guarantee that all the molds were sampled by the adhesive film or that all adhered molds were released during the vortexing. Nevertheless, this technique focuses on spore production and may reveal whether various molds are present, and, if so, their respective proportions. Here, this technique was used on S3 samples only, as mold growth was not significant elsewhere.

## 4. Results and analysis

### 4.1. Microscopic observation

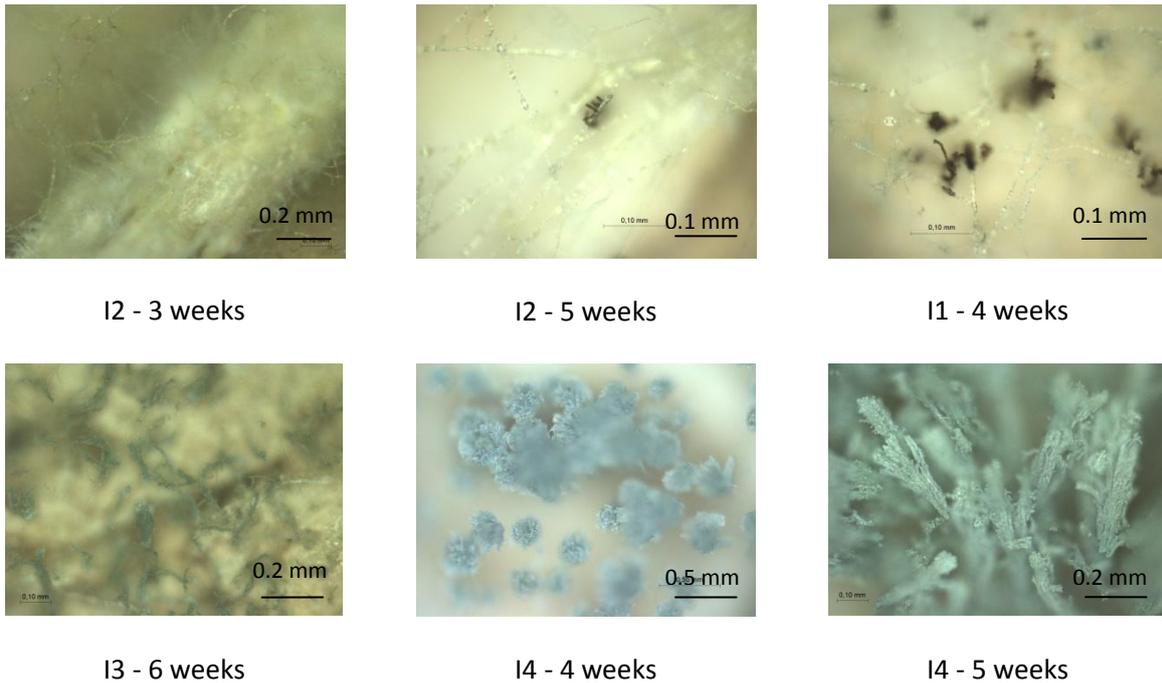
The proliferation started with a growth of tiny filaments a few micrometers in diameter (Figure 3), which extended over several millimeters in length above the material surface. The observation thus had to be made at high magnification and then the whole area had to be scanned to detect the hyphae. The magnification chosen, with a 10X objective, allowed a surface area of 0.93 mm<sup>2</sup> to be observed. At such high magnification, the specimen did not appear to be at all monochromatic, even for raw earth with no plant aggregate. Consequently, it was hard to detect the hyphae with this manual scanning method and they could even be confused with plant aggregates. In this case, the magnification 40X could be used to confirm the initial observation. This method was thus long and tedious, yet it seemed a good way to estimate the start of growth (rated 1 on the scale proposed [6]).



**Figure 3. Hyphae of a FWAS sample at 93% RH and 30°C after 12 weeks: microscopy photograph (left) and manual identification of hyphae routes (right)**

However, when the fungal growth was well established, it was easier to observe the molds. Marked fungal diversity was observed, with the varied appearances shown in Figure 4. On the first picture (I2 at 3 weeks), no conidia were observed, whereas black conidia could be

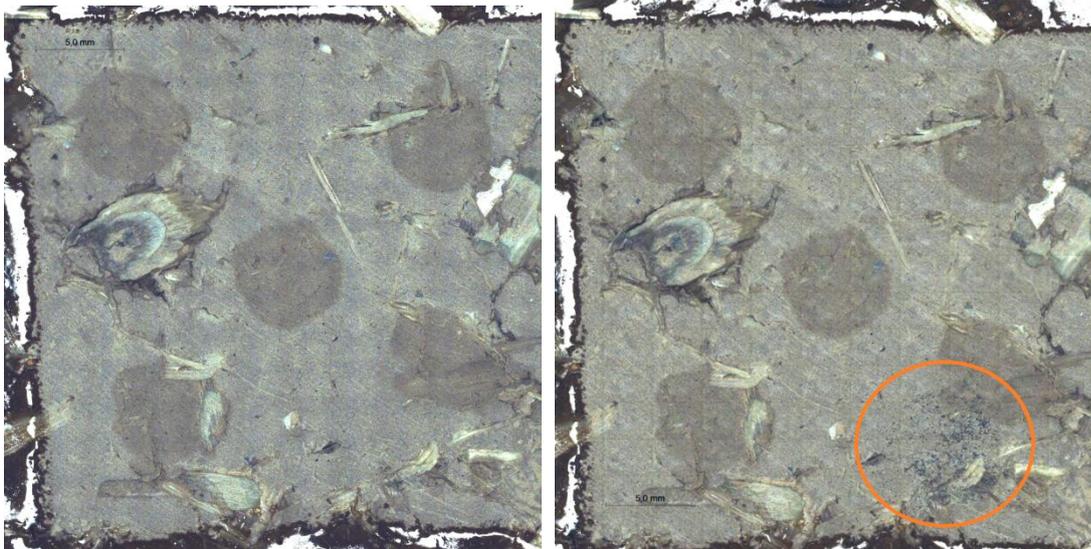
observed at 5 weeks (or on I1 at 4 weeks). Other molds observed on I3 or I4 presented other shapes, spherical or elongated, and colors: green or blue-green. These molds might not have been introduced by the inoculation only. The first occurrence of growth was not observed exclusively on inoculation sites; in the case of S3 specimens, the fungal proliferation often began on wisps of straw.



**Figure 4. Various fungal species observed with the microscope on S3 samples (30°C and 93% RH)**

#### *4.2. Image analysis*

Figure 5 presents a picture obtained at the beginning of the test (one week after inoculation) and another of the same sample 5 weeks later. Here, it is worth recalling that these images were obtained by juxtaposing the 165 snapshots taken by an objective of magnification 4X. The overall resolution was about 2300x2300 pixels.



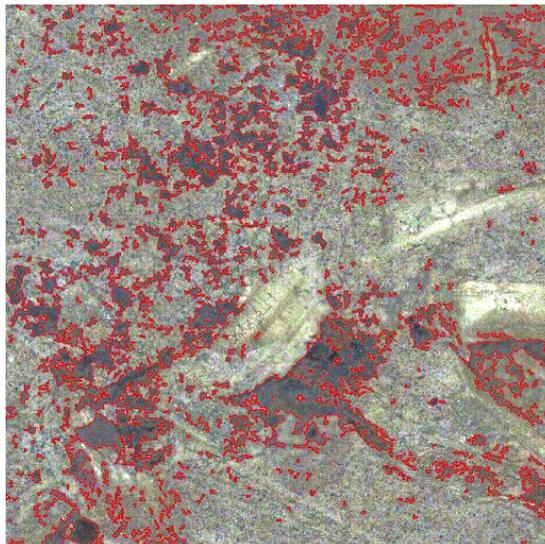
**Figure 5. Pictures obtained by the automated image acquisition device with an S3 sample (I3) after one week (left) and six weeks (right) of incubation at 30°C and 93%RH.**

Basically, image processing relies on visual changes (either in color or brightness). By modifying the basic image settings (brightness, contrast and gamma correction), mold growth could be identified at six weeks (orange circled zone in Figure 5). This is in line with other studies, e.g. [50], where a good correlation between the image analysis and the visual observation was obtained thanks to the discoloration caused by the fungi. However, this was not systematically the case, and some growth could be identified by microscopic observation while it remained undetected by image analysis. This agrees with the conclusions presented by Van den Bulcke [51], who stated that some molds were blue-green but most were colorless or had a similar color to the background. Apart from the color of the mold, it can be seen that the straw inclusions and the inoculation sites stand out from the background, which makes the detection of mold growth more difficult.

Image analysis was performed punctually when mold growth was detected with the naked eye. The methodology can be roughly broken down as follows:

- Segmentation: the objective was to reduce the number of grey levels needed to distinguish mold growth from the background. Here, the number and size of these segments was determined by using a Particle Swarm Optimization (PSO) algorithm [52]. Consequently, the number of grey levels was reduced from 256 to only 3;
- Binarizing: this step distinguished two zones, so that areas with mold growth could be made clear;
- Filtering: very small areas may have resulted from the methodology rather than from mold growth. Consequently, they were removed from the final image;
- Ratio calculation: the pixels corresponding to mold growth were summed so that the percentage of mold growth coverage could be computed.

This technique was successfully applied to a 7x7 mm<sup>2</sup> area extracted from Figure 5. The result of mold growth detection is presented in Figure 6, the resolution of which is about 512x512 pixels. The relative area covered with mold growth was computed as 22% in this case. This exemplifies the potential of image analysis applied to mold growth.



**Figure 6. Identification of mold growth (red contoured areas) by image analysis**

However, this technique could not be applied to the whole surface of the sample, because of the poor quality of the reconstructed image. As illustrated in Figure 7, the final image was an assembly of several snapshots, the boundaries of which were visible in some cases. Furthermore, the growth developed above the sample, i.e. in the third dimension. This caused small variations in the focal position, which is also visible on the final image, making it unsuitable for image analysis.



**Figure 7. Highlight of the poor quality of the reconstructed image**

Hence, the conclusions on the application of image analysis to the study of mold growth are mixed. On the one hand, this technique has been proved to be a good candidate for quantifying mold growth coverage. On the other hand, the present methodology is not robust enough to be applied systematically. As improving image analysis falls beyond the scope of this work, the study of mold growth will rely on microscopic observation alone in the remainder of the paper.

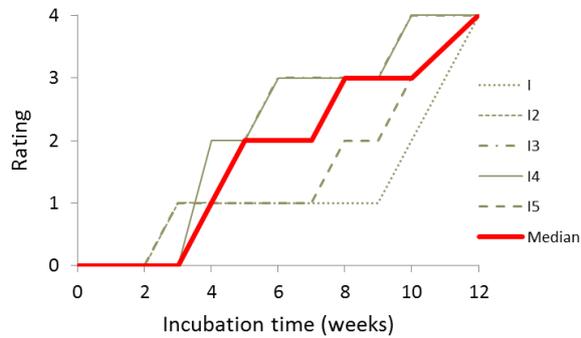
### *4.3. Rating of the mold growth*

After 12 weeks of monitoring, no growth was reported at any time during the test for the lower values of relative humidity (76% and 84% RH), for all temperatures and materials. At 20 °C, no growth was observed on FWAS or H3 specimens. For the S3 specimens, mold growth was detected for only two samples at 93% RH. One of them was classified 1 from the second week to the end of the test, showing only one hypha. On the other one, fungal growth appeared 12 weeks after inoculation, directly at stage 2. Mold growth occurred on all samples under one hygrothermal condition only: 30 °C and 93% RH. In addition, mold growth was found on inoculated samples only. Finally, the results differed according to the material. Overall, this increases confidence in the methodology.

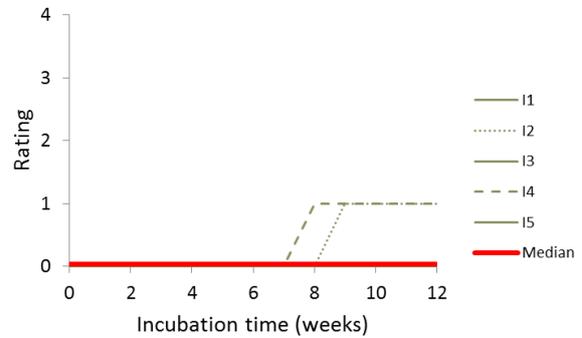
The fungal growth was described as a function of time and was analyzed using two criteria:

1. The rating attributed each week (to each sample and the median rating) for S3 specimens (Figure 8) and FWAS specimens (Figure 9) ;
2. The Kaplan-Meier survivor curves proposed by Singer and Willet [53] and presented by Johansson et al. [6]. A threshold is defined and corresponds to a sample obtaining a rating of 2 here. The survival rate is defined as the proportion of samples that passed the test at a given time (i.e. with a rating lower than 2). If a sample has a rate higher than or equal to 2, it is considered to be dead. This second criterion is somewhat more severe as the evaluation is binary and the scatter on the results has a more limited impact. Consequently, the times to observe results obtained with this criterion should be systematically shorter than the times obtained with the median value criterion. So, this criterion is useful for a quick comparison among different tests.

Only the five inoculated specimens of each formulation were used to plot Figure 8, Figure 9 and Figure 10.



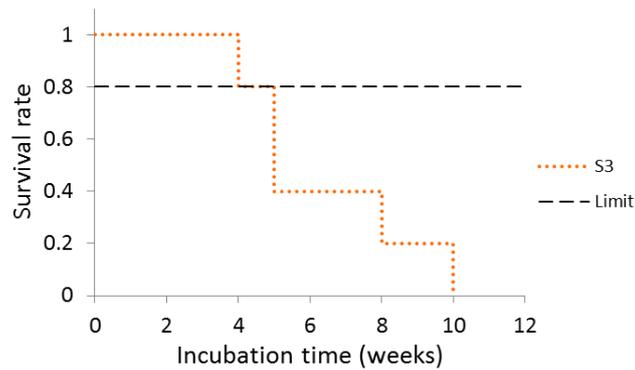
**Figure 8. Rating for inoculated samples of earth with 3% of straw (S3) - 93% RH, 30 °C**



**Figure 9. Rating for inoculated samples of raw earth (FWAS) - 93% RH, 30 °C**

The first observation of hyphae (rated 1) took place at 3 weeks after inoculation for an S3 sample at 93% RH and 30 °C, while its median rating reached 2 at 5 weeks of incubation (Figure 8). The graph shows a large variation of rating among the five specimens. For example, after 9 weeks, one sample was rated 1, one other was rated 2 and the other three were rated 3. Even with the same, strict protocol, the results were scattered.

Concerning FWAS specimens, the first observation of hyphae was made at 8 weeks after inoculation in the same conditions (Figure 9). Median rating for FWAS specimens was 0 until 12 weeks. As can be seen on Figure 9, only 2 samples out of 5 showed a start of growth within 12 weeks and it reached only the rating of 1.



**Figure 10. Survival rates of specimens made of earth with 3% of straw (S3) according to incubation time - 93% RH, 30 °C**

The second method is illustrated by Figure 10 and presents the survival rates of S3 specimens according to incubation time. The survival rate was set to 0.8, which, in our case, means that the formulation was assumed to fail the test as soon as a spot of mold growth reaching rating 2 was detected on one of the samples. For S3 specimens, the threshold was reached after 4 weeks, whereas it took 5 weeks with the first method (median value in Figure 8). Note that all S3 specimens failed the test at 10 weeks.

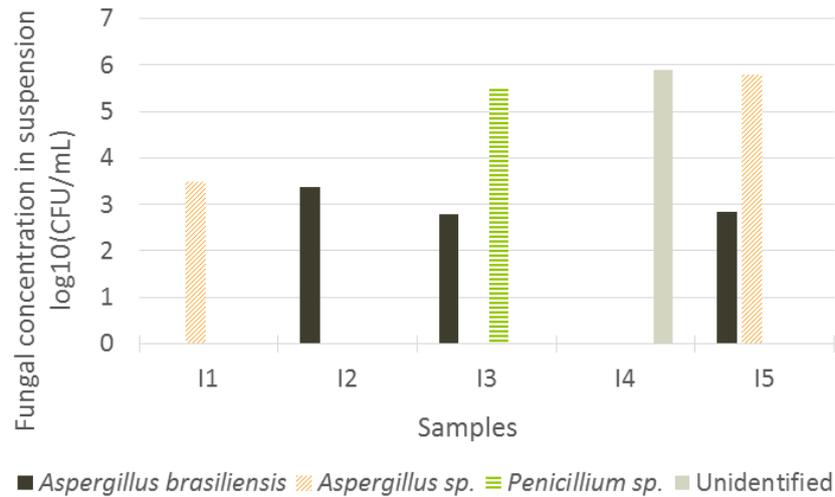
The longer the incubation time was, the more resistant to microbial growth the material was considered to be. As expected, straw seemed to be more favorable to fungal growth than earth or hemp shiv. Straw constitutes a carbon source that is useful for proliferation. This result was already observed by Hoang et al. [7] with the case of sunflower panels and plywood boards, which were very favorable to microbial growth. The growth may also have been facilitated by the inclusion of plant particles, which made the material more heterogeneous. It has thus been reported that rough surfaces or surfaces with cracks may concentrate moisture and nutrients more easily and thus provide favorable conditions for fungal attachment and growth [21,54]. The fact that fungal growth was less pronounced on hemp shiv samples than on straw samples can be explained by their chemical composition.

Harper and Lynch [55] have shown that lignin is resistant to microbial attacks. According to a previous paper [56], a lignin content of 17.2% was determined for hemp shiv, while it was only 5.5 for barley straw. This high resistance of hemp to fungal growth was already observed by Sedlbauer et al. [57], who showed that hemp had no risk of developing any mold at all below 95% of RH, whatever the temperature, while mold proliferation on straw was likely to occur from 70% RH.

Irregular observations were continued during an additional time for the samples at 30 °C. The main result was that the two non-inoculated samples of S3 and one of FWAS also presented fungal growth at 93% of RH. The two non-inoculated S3 samples reached a rating of 4 between 14 and 17 weeks and the non-inoculated FWAS sample reached a rating of 4 between 17 and 21 weeks. Moreover, the rating of the two inoculated samples of FWAS presenting fungal growth was increased to 2 between 15 and 16 weeks.

#### *4.4. Identification of the growing species*

After 12 weeks of incubation, all five S3 samples showed fungal growth with conidiophores, reaching the rating of 4. Thus the number of Colony-Forming Units per mL of suspension was counted (expressed in  $\log_{10}(\text{CFU}/\text{mL})$ ) and the growing species were identified on those samples (Figure 11).



**Figure 11. Colony-forming units sampled per mL of the suspension of inoculated S3 samples**

This diversity of molds was already noted with the microscopic observation (Figure 4). Strikingly, *Aspergillus brasiliensis*, which was the inoculated strain, was not the predominant species, except for one of the 5 samples (I2), and was not even found in two samples (I1 and I4). However, it was found in three specimens, albeit in an amount well below the other fungal species grown. *Aspergillus sp.* was identified in two specimens (I1 and I5), undoubtedly of the same genus but species other than *A. brasiliensis*. *Penicillium sp.* was identified as the major species on one sample (I3), whereas the major isolate of the last sample (I4) could not be identified because it was only a hyphal growth and no conidiophore production, but belonged to a genus that was not *Aspergillus* or *Penicillium*.

The diversity of the fungal species grown indicates initial and/or external contaminations on the material. These contaminations seem to grow more efficiently on earthen materials than *Aspergillus brasiliensis*. However, no growth was observed on control samples (non-inoculated) during the first 12 weeks, whereas inoculated samples had several mycelia on

their surface. A parameter other than contamination seems to facilitate unwanted mold growth.

This growth could be explained by the addition of water through inoculation. The droplets might not have dried fast enough for the water effect to be negligible. Liquid water might help to activate or accelerate the fungal growth of species not removed by decontamination or deposited afterwards [30]. To validate this assumption, another test was carried out by making five drop deposits of 5  $\mu$ l of distilled water, as performed by Hoang et al. [7], instead of the *A. brasiliensis* inoculum. Fungal growth was observable on the two samples over the same time frame as the samples inoculated with *A. brasiliensis*. Hyphae were observed from the second week for one sample whereas hyphae and green spores were seen after 6 weeks of incubation for the other. Thus, more than the addition of fungal strains, the addition of liquid water through inoculation seems to initiate the fungal growth on/inside the material when combined with convenient RH and temperature.

The samples with water drop deposits clearly showed that fungal growth occurred without any inoculum. Thus, inoculation seems only to speed up the proliferation, through the addition of water. This finding that liquid water supports fungal growth better than relative humidity alone has been made elsewhere [16,58,59]. Even though the results obtained here were not those expected, the identification on growth medium stands as complementary to direct observation for the development of a methodology.

## **5. Discussion**

The assessment of mold growth on building materials is a major issue and the main objective of this research work was to propose a methodology that could be carried out in a materials

laboratory to evaluate a potential fungal proliferation. This objective was reached through some methodological and analysis choices summarized below, although the whole biological process of the experiment was not fully understood and controlled. Nevertheless, this study also led to valuable results and promising perspectives can be foreseen. As the point of view is twofold, the discussion is broken down into two parts.

From the biological point of view, the identification of unwanted mold fungi questions the efficiency and the relevance of the inoculation, and to a lesser extent, of decontamination. *Aspergillus brasiliensis* and *niger* are regularly found in indoor air and on indoor surfaces, but seem to be more associated with some other indoor sources like food rather than growing on construction materials [60,61]. Mold species associated with water damage, such as *Aspergillus versicolor* or *Penicillium chrysogenum*, could be better candidates for testing fungal proliferation on building materials [33,61,62]. However, fungal growth is initiated by the addition of water and starts mainly from a natural inoculum. Using no artificial inoculation could thus be an alternative which would allow to simplify the process and is a major advantage for materials laboratories in terms of resource requirements and cost savings. Also, scanning the whole surface with a microscope was not a successful way to quantify the microbial growth, even though the procedure was automated. However, these first results obtained by imagery are encouraging. The problem of the contrast between the mold and the material is well known. The use of fluorescence techniques or genetically modified strains could lead to significant improvements. Another alternative would be to use a reflectance spectroscopy approach (Fourier-transformed mid-infrared and near-infrared) to visualize fungal growth. Calderon et al. [63] indeed found promising results in this way concerning the identification of infrared markers on root fungi, based on the properties of chitin. Finally, it was found that the use of the identification technique, although not free

from bias, complemented to microscopic observation by highlighting the biological mechanisms occurring during the test. It was thus a relevant technique for developing a methodology allowing the fungal growth of a building material to be assessed. However, this should not be kept in the standardized method for assessing building materials.

From the point of view of material development, the assessment of the emergence of growth, i.e. the definition of rating increases from 0 to 1, was found to be cumbersome. However, as most of the mold fragments are released into the air from stage 2 (presence of spores), this is the most interesting stage for the choice of a material. Time could thus be saved by looking for molds only at stage 2, where the presence of spores facilitates the detection. Although this analysis is quite limited considering the complexity of mold growth, it nevertheless highlights differences between the formulations, as illustrated in Figure 10. All the samples containing straw failed the test after 10 weeks, while none of the FWAS samples failed. This result is significant and useful in the process of developing new construction materials. Besides, the tests presented here were time consuming and required a significant number of samples compared to current practice in material development. Therefore, it appears difficult to test fungal resistance when developing new materials if a detailed study, like the one presented here, is necessary. In the aim of providing a faster test, a simplified method can be proposed, based on the present findings. It is acknowledged that additional tests based on a wider range of material would strengthen this proposal but it still represent an appreciable step toward a wider assessment of the resistance of construction material to fungal growth.

The proposed method for assessing fungal growth on building materials is the following:

1. Sample preparation:

- a. The assay should be performed on samples prepared according to actual manufacturing conditions. Five samples plus two control samples seems to be a minimum for a mold growth study.
- b. Polishing the surfaces and coating the edges and sides of the samples is recommended to limit undesirable proliferation and make observation easier. This is because it is foreseen that high roughness would facilitates mold growth independently of the nature of the material and its preparation.

2. Decontamination and inoculation:

- a. As the decontamination of bio-based materials cannot be achieved in a laboratory of materials, the second heating process at 100 °C is not useful and natural inoculation should be considered.
- b. Depositing water drops was found to be effective and should be considered as an alternative to the use of an inoculum. An amount of distilled water of  $2.7 \mu\text{l}\cdot\text{cm}^{-2}$  deposited only once before inoculation is recommended.

3. Incubation:

- a. Each specimen should be tested in an individual box containing a saturated salt solution to maintain constant relative humidity. This would avoid cross-contamination.
- b. A single test performed under highly favorable hygrothermal conditions (e.g. 30 °C; 93% RH) should highlight how the resistance to mold growth varies between different materials.

4. Analysis technique:

- a. Weekly microscopic observations were found to be sufficient.

- b. These observations could be limited to the search for fungal growth only, which corresponds to stage 2. This would lead to simplified yet effective analysis, as exemplified by the survival rate.

## 6. Conclusion and outlook

In this paper, a methodology for studying mold growth has been proposed and applied to earthen bio-based materials. Various climatic conditions were tested on earth specimens or earth-with-straw specimens. The influence of the straw on the microbial resistance was demonstrated: earth with straw is more sensitive to fungal growth than earth alone. Fungal growth took place after 4 weeks of exposure at 93% of relative humidity and 30 °C. For earth with no plant aggregates, the mycelium was observed for the first time after 8 weeks of incubation under the same conditions. Finally, no proliferation at all was observed at 20 °C within the 12 weeks of this test, whereas it had already been observed in other studies. As the bio-resource used in a composite was found to influence the fungal growth, it would be interesting to evaluate the fungal resistance of a composite material containing rice husk, generally recognized as rot-proof [64], or cork particles, which are hydrophobic. Preliminary tests in our laboratory have given promising results. Rice husk composites seem to have a better resistance to molds than barley straw composites.

The methodology has also been discussed: some improvements have been suggested, such as a natural inoculation or an addition of liquid water, and image analysis appears to be encouraging even though it still needs to be studied for quantifying mold growth more precisely. Moreover, calibrating laboratory tests with *in situ* tests would be relevant to determine the “real” risks and kinetics of mold growth for a specific material. Finally, it is recognized that more tests would be necessary to determine the isopleth curves. These

could possibly be implemented within transient hygrothermal models, in order to compare the risk of mold growth for several materials under dynamic conditions representative of realistic indoor situations.

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