Three-dimensional X-ray imaging and analysis of fungi on and in wood

Running head: X-ray imaging of fungi

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Abstract

As wood is prone to fungal degradation, fundamental research is necessary to increase our knowledge aiming at product improvement. Several imaging modalities are capable of visualizing fungi, but the X-ray equipment presented in this paper can envisage fungal mycelium in wood non-destructively in three dimensions with sub-micron resolution. Four types of wood subjected to the action of the white rot fungus *Coriolus versicolor* (Linnaeus) Quélet (CTB 863 A) were scanned using an X-ray based approach. Comparison of wood volumes before and after fungal exposure, segmented manually or semi-automatically, showed the presence of the fungal mass on and in the wood samples and therefore demonstrated the usefulness of computed X-ray tomography for mycological and wood research. Further improvements to the experimental set-up are necessary in order to resolve individual hyphae and enhance segmentation.

1. Introduction

As a sustainable material, wood has a considerable advantage compared to other building materials such as concrete and steel. Its susceptibility to attack and degradation by microorganisms is considered a disadvantage. Fungi cause the most serious kind of microbiological deterioration, leading to rapid structural failure (Green & Highley, 1997). Wood biodeterioration also affects the artistic and cultural value of historical buildings and monuments (Gutiérrez et al., 1995). Apart from its detrimental action, fungal degradation of lignocellulose is also probably the most important process for recycling carbon in nature (Eriksson et al., 1990).

Traditionally, wood is protected using biocidal treatment by impregnation or superficial application. Increasing environmental concerns during the last decade have initiated a shift to less toxic methods for the preservation of wood and an increased interest in protection by design. This could be facilitated if the process of degradation was better understood. Furthermore, the lack of accurate and rapid non-destructive methods to detect and quantify wood decay is one of the factors that hinders service life prediction of wooden constructions and commodities (Råberg et al., 2007).

In general, three types of wood rot can be distinguished: soft rot, brown rot and white rot. Especially the white rot basidiomycetes are known to be active wood degraders since they secrete a wide range of hydrolytic and oxidative enzymes (Nicole et al., 1993) and as such are able to degrade lignin efficiently (Mansur et al., 2003). Like most other fungi, they are able to persist in dynamic, heterogeneous environments because of the capacity to take locally immobilized internal resources and remobilize these into a form capable of being reused locally or directed to new internal sinks through their hyphal network (Falconer et al., 2005). These hyphae, the basic units of the mycelium, are the core of their successful invading

capabilities. Part of the research concerning wood damaging fungi involves localization by visualizing the entire network as well as their basic units, which is difficult due to their small dimensions. Apart from conventional light microscopy of stained wood sections, many other techniques enable researchers imaging fungi. Hickey et al. (2005) have given a review of live-cell imaging of filamentous fungi using vital fluorescent dyes and confocal microscopy. Dickson & Kolesik (1999) also used confocal microscopy for the visualisation of mycorrhizal fungal structures and quantification of their surface area and volume. Muller et al. (2001, 2002) employed magnetic resonance imaging for the detection of incipient fungal attack in wood. Other imaging modalities are cryo-FE-SEM and TEM immuno-techniques (Daniel et al., 2004). Especially low temperature SEM is a valuable tool (Refshauge et al., 2006) to examine samples with minimum disruption to structural integrity by rapidly freezing the specimen to temperatures below -130°C in which state the specimen is considered to be in a fully frozen hydrated condition (Beckett & Read 1986; Abu Ali et al., 1999). At last, X-ray analysis is a promising technique for mycological research. Illman and Dowd (1999) successfully used synchrotron-based X-ray microtomography to characterize wood degraded by decay fungi. Macchioni et al. (2007) explored X-ray microdensitometry for measuring fungal wood decay and Van den Bulcke et al. (2008) explored X-ray sub-micron tomography for examination of coated wood disfigured by blue stain fungi. Advantages are the non-destructiveness, penetrative power, acquisition speed and three-dimensional imaging of the substrate giving a large field of view on the internal structure at sub-micron resolution.

To assess the usefulness of X-ray tomography for wood decay research, four types of wood were inoculated with the white rot fungus *Coriolus versicolor* (Linnaeus) Quélet (CTB 863 A) and scanned with a state-of-the-art X-ray instrument. Samples were examined before and after exposure to the fungal culture and compared using manual segmentation. An attempt to use differential imaging is presented as well.

2. Materials and Methods

Wood from the following tree species was used in this study: Scots pine (*Pinus silvestris* - sapwood), beech (*Fagus sylvatica*), movingui (*Disthemonanthus benthamianus*) and afzelia (*Afzelia bipindensis*), representing hardwood and softwood as well as temperate and tropical wood types. Pine sapwood and beech are often used in European standards related to Basidiomycota testing whereas movingui and afzelia are durable tropical species on the market. Samples were prepared by slicing a thin wood section of a larger block and subdividing it with a microtome into needle-shaped specimens. The tip of the wood sample, measuring approximately one mm^3, was scanned before exposure to fungi, using the X-ray equipment built at the Centre for X-ray Tomography at Ghent University (UGCT - http://www.ugct.ugent.be). This is a state-of-the-art scanner (Masschaele et al., 2007), highly flexible, with in-house developed software for scanner control, sample reconstruction, analysis and visualization. The X-ray source, a Feinfocus nano-focus tube, has a focal spot size < 1 μm. All samples were scanned at an average tube voltage of 50 kV, a current of 40 μA and an exposure time of 1500 ms per image. A rotation step size of 0.36° was used. Reconstruction took 20 min with Octopus, a server/client tomography reconstruction package for parallel and cone beam geometry (Vlassenbroeck et al., 2007). With the described set-up, sub-micron resolution can be reached, resulting in scans with voxels sizing approximately 0.7 x 0.7 x 0.7 μm. The small voxel size gives a clear view of anatomical features as described in Van den Bulcke et al. (2008). After scanning the non-decayed specimen, needle-shaped samples were exposed to *Coriolus versicolor*. The fungus was cultured in several Petri dishes on a nutrient medium (20 g agar, 40 g malt extract in 1 L water) at 23°C for one week, after which wood samples were put on the fungus. Scans were acquired twice, once after 1 week

and once after 6 weeks with similar tuning of the equipment. Obviously, as samples were scanned in a non-sterile environment, contamination of the samples and of the culture upon replacement could not be excluded due to the presence of airborne fungal spores. This was not a major issue as the objective of the experiment was mainly to assess the use of X-ray tomography for fungal visualization. Furthermore, as samples were exposed to non-sterile conditions only after one week of incubation, the cultured fungus already grew on and in the sample and was most likely the dominant fungal species. The radiation dose during a standard scan was monitored by measuring the optical density of a radiochromic fluid with a FWT-200 opti-chromic reader system and was found to be approximately 20 Gy.

Reconstructed images of the different samples before and after fungal attack were visualized with VGStudio MAX®, Matlab® and Drishti (Limaye, 2006). Fungal tissue was manually separated from noise and substrate. Green is used as an arbitrary assignment of colour to the specimen density where fungal hyphae are located in the reconstructed images. An attempt was made for differential imaging by subtracting two volumes using the Drop software (Glocker et al., 2007; Komodakis et al., 2007) for non-rigid body registration. Accurate registration required pre-processing including histogram equalization, affine volume rotation (Van den Bulcke et al., 2008), resolution correction and volume cropping as a first rough alignment.

3. Results

At first, the wood samples were scanned before exposure to white rot fungi (Fig. 1).

Figure 1.

The images so obtained clearly illustrate the possibilities of sub-micron CT-scanning and resemble classical SEM recordings (McMillin, 1977). As a resolution below one μm is reached, cell walls, pits and other small scale structures are discernable on the radial cross-sections that accompany the volumes in Fig. 1. The range of greyscale levels equals 2^{16}.

3.1 Fruiting bodies outside wood

Scanning of the wood samples involved removal from the Petri dish and exposure to airborne fungi resulting in the contamination of both the beech and the pine sample. Thelarge fungal fruiting bodies on beech, present a few weeks after the second scan, were used as a test case for scanning and visualization of fungi with ionizing radiation. The black globular structures at the outside were identified as an *Aspergillus* species and other unidentified moulds. Fig. 2a is an image of beech with *Coriolus versicolor* and *Aspergillus* growing on the wood while Fig. 2b is a pine sample covered with *Coriolus versicolor* and other unidentified mould fungi. Both images were taken with a standard camera in the visible wavelength domain. Fig. 2c and 2d represent their X-ray counterparts.

Figure 2.

The fruiting bodies are visualised easily, most likely due to their size and melanin content. A covering network of hyphae, either *Coriolus versicolor* (most probably) or a contaminant is also visible, but individual hyphae are very small and fuse easily with noise. Although the hyphal mass is discernable, individual hyphae are difficult to distinguish and exhibit a low contrast and unconnected links. Still, these small hyphae are of interest, particularly to what extent they are able to penetrate in the wood.

3.2 Time series scanning

Time recordings of the four wood species, before and after one and six weeks of exposure to the fungal culture, are examined in Fig. 3. Preprocessing of the original volumes included noise removal by histogram clipping, resampling and recoding of the grey scale values. Volumes are translated and rotated equally to facilitate comparison. All volumes consist of 2^{16} grey levels with a resolution ranging from 1.9 μm down to 0.6 μm.

Figure 3.

Apparently, the beech sample was damaged during preparation as can be seen from the rugged top section. Other samples are more or less parallelepiped shaped. The hyphae wind around the substrates, obviously more pronounced after 6 weeks. Especially the beech and movingui sample illustrate the presence of a mycelial coat at the outer boundaries. When taking a closer look, individual hyphae are discernible albeit difficult and the network is not fully connected. The pine images do not satisfy the aim of hyphal tracking and growth monitoring, as hyphae are difficult to see in the wood itself. For afzelia, hyphal masses are more pronounced due to the heterogeneous anatomy of the substrate, especially in the vessel region, probably because hyphae tend to coalesce in voids such as lumens and deteriorated rays and this is perhaps the reason why a signal for fungal density is given in these areas.

3.3 Segmentation of fungal hyphae

188 The X-ray density of the fungal hyphae is rather low, between air and wood, and especially
189 the isolated hyphae tend to fade into noise due to their small size and partial volume effects.
190 Segmentation based on the histogram is therefore rather difficult yet will give an idea of the
191 distribution of the fungal hyphae in the larger voids of the substrate. It is crucial to find the
192 range of grey level values that enclose the fungal hyphae. Fig. 4 illustrates the result of
193 manual classification of mycelium (green) for the four wood species.
194
195 **Figure 4.**
196
197 Green patches are present in all four samples, with explicit growth in large anatomical
198 structures such as vessels. When looking more closely to the pinewood sample, it is
199 remarkable that fungal penetration in depth in the wood is limited. When examining the
200 bordered pits, degradation of the tori is visible, similar to what is shown in Schwarze (2007).
201 Although fungal tissue is visualised with manual segmentation, the partial volume effect at
202 the edge of wood can result in falsely detected fungus on this position. It is also subjective,
203 incriminating quantitative and even precise qualitative analysis. To investigate the difference
204 between samples in an objective way, differential imaging would be appropriate.
205
206 **3.4 Differential imaging**
207
208 Application of Drop software permitted image subtraction to reveal areas with differing
209 density on a pre-processed subvolume of pine sapwood. These areas included fungal hyphae
210 primarily, also apparently included degraded wood zones, and potentially other features with
211 changed density. A 2D cross-section is shown with the original images (Fig. 5a and 5b) and
212 their difference (Fig. 5c).

Figure 5.

Although fungal tissue is visible as well (bottom left hand corner), a considerable amount of incorrect transformation artefacts are masking most of them. The edges of the images seem difficult to register. Especially the inner parts are deformed correctly as only a faint print of the wood structure remains.

4. Discussion

In the present study it is shown that fungi on and in wood can be imaged non-destructively using X-ray computed tomography with sub-micron resolution. It should be stressed that scanning at high resolution is non-trivial. Sub-micron scans for small samples require small spot sizes implying low fluxes and hitherto long scanning times. Deviations in the stability of the sample and of the X-ray spot by thermal effects of the X-ray tube should be overcome, just as inaccuracies in the positioning of the rotation motor and anomalies of the geometric accuracy of the detector. A motion compensation procedure is imperative (Vidal et al., 2005) in addition to filtering and normalisation. Results give evidence for the enormous potential for wood research in general and mycology in specific, though it should be remarked that although sub-micron resolution is obtained, resolution is lower even than conventional light microscopy. As illustrated by Trtik et al. (2007), scanning with a resolution below 1 μm results in a volume of the internal structure of wood (Fig. 1) down to details at cell wall level. These details are of importance when dealing with fungal growth in and decay of wood. It is common knowledge that fungi can penetrate very small openings. Bardage & Daniel (1997) tested the penetrative capacity of seven decay fungi. All of them were capable of penetrating

238 micropores of 0.6 μm under certain experimental conditions but none of them could enter
239 diameters smaller than 0.1 μm. Therefore, anatomical structures such as pits, rays and axially
240 oriented elements (Rayner & Boddy 1988) are important pathways of least resistance for
241 fungal propagation in the wood, in addition to the bore holes created by hyphal tunnelling or
242 cell wall degradation (Schwarze, 2007). Apparently, the mycelium can be discerned rather
243 easily from the background noise when located outside the wood structure. A clear example is
244 the picture of the fruiting bodies of *Aspergillus* and the tubular network of *Coriolus versicolor*
245 (Fig. 2). The main difference between the two fungi is the pigmentation of the *Aspergillus*
246 species. The melanin pigments, negatively charged and hydrophobic, confer a survival
247 advantage to environmental microbes (Nosanchuk & Casadevall 2006) and to UV, solar and
248 gamma radiation (Nosanchuk & Casadevall 2003). Moreover, some pigmented species exhibit
249 a general attractive response to ionization (Zhdanova et al., 2004) or might even profit from
250 radiation by using it as an energy source (Dadachova et al., 2007). This advantage also results
251 in a better X-ray absorption and consequently a better visualization of melanin containing
252 fungi. Other research of Van den Bulcke et al. (2008) already demonstrated the possible use
253 of X-ray tomography for mycological research, more specifically by visualization of
254 *Aureobasidium pullulans* on and in coated wood. This blue stain fungus was observable in the
255 X-ray domain with a resolution down to 0.7 μm. The difference with current research is
256 twofold. First, the diameter of a blue stain fungus is on average larger (2-10 μm and even up
257 to 20 μm) than a white rot fungus (1.5-3.5 μm). Second, blue stain hyphae contain pigments.
258 Therefore, it is expected that scanning of the smaller non-pigmented white rot fungi will be
259 more difficult. For non-pigmented species the absence of strong X-ray absorbing component
260 makes X-ray CT scanning difficult. Furthermore, while outside the wood, there is only
261 interference from air, inside the substrate visualization and segmentation is more complicated,
262 especially when dealing with the non-pigmented fungal species. Artefacts during scanning

and reconstruction can obscure their presence and that of interesting features. Scattering, fluorescence, polychromatic X-rays and noise are disturbing the ideal acquisition (Vidal et al., 2005). The phase contrast phenomenon can be another interfering factor, although it can be an advantage as well. While current tomography is absorption based, only recently the application of 3D phase contrast X-ray imaging is explored (Bronnikov, 2002; Groso et al., 2006; Trtik et al., 2007; Boone et al., 2009). For quantitative tomography, for instance when mapping density distributions, it is an artefact. But for qualitative tomography, it could help in visualizing biological tissue demonstrating weak absorption contrast. Possibly, further algorithmic filtering of the projections and of the reconstructed slices could also enhance image quality.

Comparison of the samples scanned before, after 1 week and after 6 weeks of exposure to the white rot fungus (Fig. 3) showed the formation of mycelial mats around the sample, yet with hyphae that were not fully connected. Segmentation of fungal tissue from wood and noise could clarify hyphal presence, yet interference with the phase contrast phenomenon decreased image quality in the interior of the wood substrate. Edges of wood had a similar grey level as fungi, which resulted in misclassification of some of the outer cell walls as fungal tissue. Furthermore, as the technique is based on density differences, it may not be possible to distinguish between degradation of the wood, fungal extracellular, fungal mucilage, fungal hyphae or other products/changes which also might lead to misclassifications. When examining the pine sapwood sample, penetration of hyphae seems to stop deeper than 40 μm in longitudinal direction beneath the surface. A plausible explanation is a combination of several effects, which resulted in a low growth of fungi and even complete arrest of growth. At first, contrary to pigmented species where a low radiation dose can be beneficial, for non-pigmented ones ionizing radiation imposes stress. During scanning this could have had an adverse effect, resulting in inactivation or even sterilization, which is only partly true

(radiation damage is a statistical process) as the dose at exposure only amounted up to 20 Gy, which is certainly not enough to eradicate all fungal growth (Uber & Goddard, 1934; Saleh et al., 1988; Dadachova et al., 2007). It should be remarked that radiation dose measurements with radiochromic fluids are not mimicking exposure conditions for the fungal tissue exactly, but the measured dose is far beneath the lethal doses mentioned in literature. Secondly, in order to obtain a high quality scan, the object of interest should sustain a stable position, which was a difficult task for the samples that were taken from the Petri-dish. Therefore, these samples needed some time to stabilize, causing dehydration. Third, small samples with even smaller objects of interest require scanning at very high magnification to achieve the sub-micron resolution. This implies that the samples must be located close to the warm X-ray tube during a sufficient long scantime due to the low flux. As such, a combination of radiation damage and dehydration / high temperature might have caused a growth stop. Therefore, old immobilized mycelial tissue might have blocked the pathways for the new hyphae, which could barely reach the wood axially, but could easily grow on the surface of the substrate. This is true for wood species with small wood cells, but for movingui and afzelia this effect is counteracted partly as pathways of penetration such as large vessels (Fig. 4) are not easily obstructed.

In order to discern fungal tissue from its surroundings objectively, the subtraction of a volume before and after exposure should result, in ideal circumstances, in the segmentation of fungal tissue and decayed wood parts, but the feasibility of doing so is limited for several reasons. First, as volumes are scanned on different times, registration is obligatory. The size of the volumes makes such a mathematical operation rather impracticable. Although rigid-body registration (Rajapakse et al., 2008) might be an option when compressed volumes are used (downscaled resolution and grey levels), this is problematic when dealing with wood and fungus. The wood substrate and the fungus as well represent a non-rigid structure that

manifests swell and shrink behaviour. Non-rigid registration (Crum et al., 2004) with free form transformation is necessary. Apparently, results (Fig. 5) are highly dependent on the quality of the scans and therefore insufficient for quantitative processing. This was expected as samples were scanned before incubation, put back and scanned again after fungal degradation and thus it was nearly impossible to retain the same position for every scan. As such, the orientation of the two volumes was quite different which complicated the problem, especially when dealing with an anisotropic substrate as wood. Pre-alignment was necessary in the first place. Secondly, resolution of two scans was not exactly the same and fungal degradation of the samples altered the structure substantially, making deformation registration a difficult task. An ideal set-up would fix the exposed sample in a sample-holder in the X-ray scanner and several scans should be performed without removal. However, if not workable the sample will be moved away from the X-ray scanner, which makes marking of the first position for rough re-placement of subsequent scans obligatory. Further fine-alignment could then be done as described in Viot et al. (2007). In fact, to overcome problems of dehydration, radiation induced stress, registration errors, etc the sample should be fixed in a miniaturized climate chamber.

Eventually, as form and function of decay fungi are difficult to study due to their action on different spatial and temporal scales and the complexity of the substrate, mathematical modelling could be a powerful complementary tool to amass knowledge concerning their growth and decay on solid wood, wood-based materials and re-engineered materials. In silico growth and decay on X-ray scanned volumes offers the possibility to compare with lab-degraded specimen. Theories of attack by white rot and by extension of brown rot, can be put to the test once a realistic set of parameters and environmental conditions concert the growth of the intercommunicating tubular network. In fact, any substrate could be subjected to fungal attack.

5. Conclusions

The mycelium of filamentous fungi consists of a network of tubular hyphae. Some of them are able to degrade wood and can cause substantial losses in the building industry. However, these fungi also play an important role in nutrient recycling and are applied in several industrial processes. In order to investigate their colonization strategies, research makes use of various imaging tools, of which sub-micron X-ray computed tomography is presented in this paper. Seemingly, pigmented fungal species are rather easy to visualize when growing outside the wood. The small hyphae of the most important non-pigmented wood decay fungi are more difficult to visualize due to their weak absorption and small size. Within the wood substrate, the combined effect of noise, artefacts and weak absorption obscures their presence even more. Manual segmentation based on the grey level histogram gave a clear view on the hyphal mass, but misclassifications occurred because of reconstruction artefacts. Apparently, growth was arrested due to radiation, dehydration and heat stress, blocking smaller anatomical structures. Further analysis of the samples with differential imaging was very complex. Changes to the set-up such as sterile working scan conditions, pre-fixation of the samples and alignment before scanning will considerably improve scan quality and increase the chances of success of differential imaging. Although automatic segmentation and quantification of fungal colonization was not possible in this stage of research, the hyphal mass was pictured using X-rays even in spite of noise interference. Future research will focus on pretreatment of the samples and fine tuning of the experimental set-up, hopefully leading to a contrast enhancement, better resolution and visualization of separate hyphae within the mycelium. Preferably, low doses and phase-contrast imaging could also contribute to superior scans. Ultimately, by combining non-destructive scanning and modelling of fungal growth with

realistic morphological and physiological characteristics, a powerful tool can be developed for predicting the influence of substrate and environmental conditions on growth and vice-versa in complex substrata such as wood.

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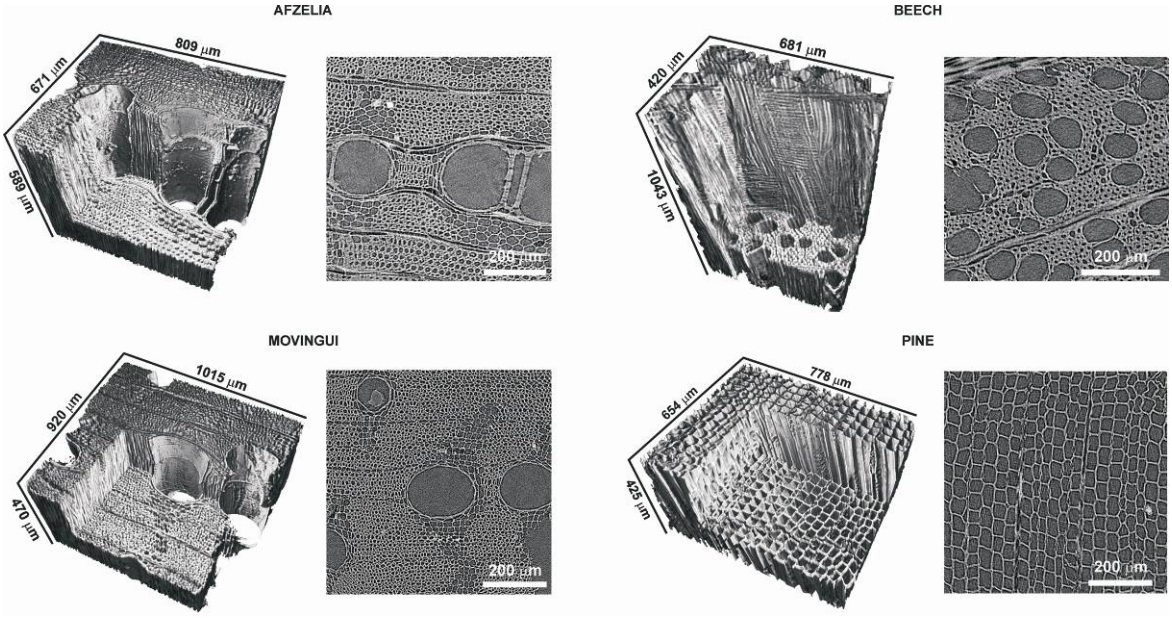

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522 **Figure 1.** Three-dimensional reconstructions of the tested wood samples before exposure to
523 the fungal culture of *Coriolus versicolor* and a cross-sectional view as an illustration of the
524 level of detail.

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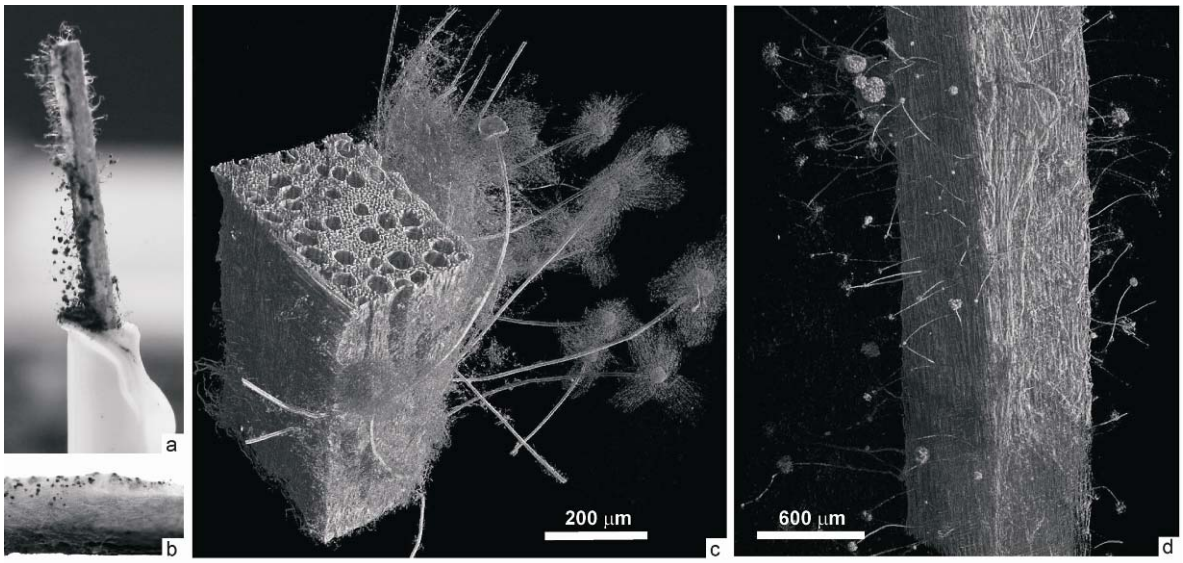

Figure 2. Images in the visible wavelength domain of beech (a) and pine (b) covered with *Coriolus versicolor*, *Aspergillus* and other unidentified moulds. Reconstructions of X-ray scans for beech (c) and pine (d) showing *Aspergillus* and a view on the internal structure of beech.

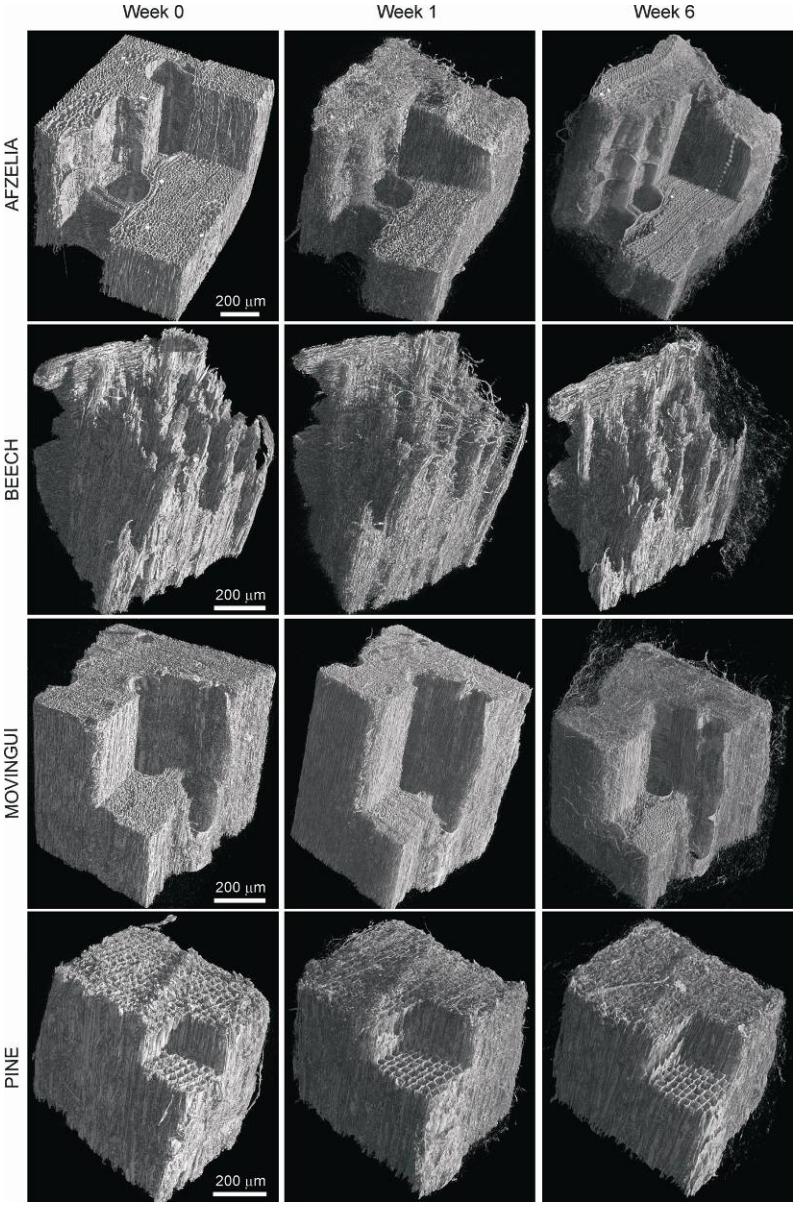

Figure 3. Time series scanning of the 4 wood samples under study: reconstruction before exposure to the fungi and after 1 and 6 weeks of incubation.

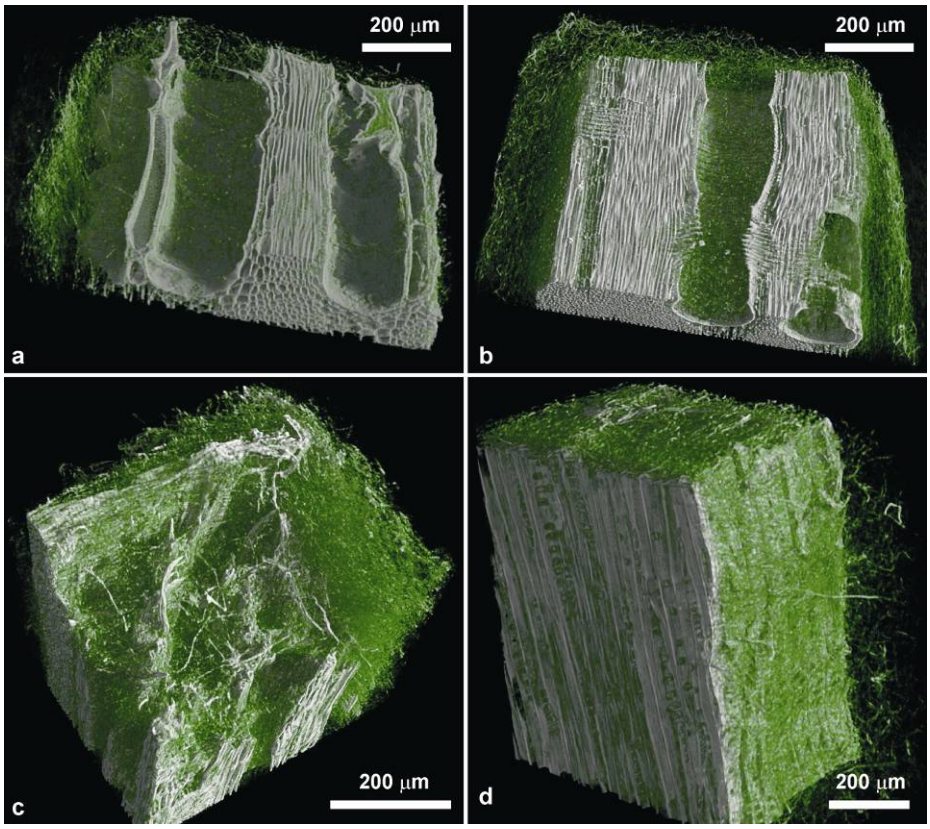

Figure 4. Segmentation of hyphae (green) on the afzelia (a), movingui (b), beech (c) and pine (d) volumes.

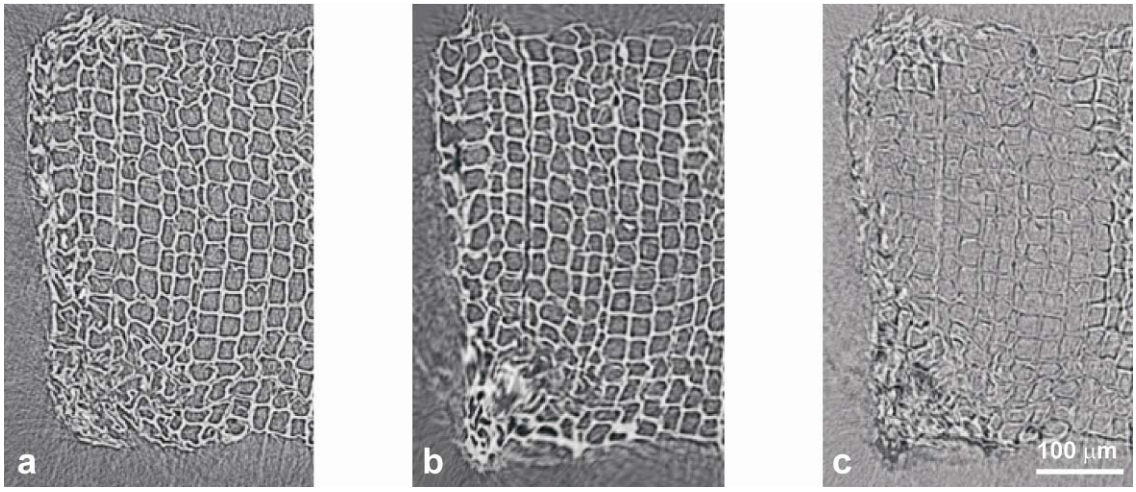

Figure 5. Reconstructed slice before exposure (a), image of the same slice after 6 weeks of exposure to the fungi and non-rigid transformation (b) and difference (c) between (a) and (b).