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Short Note

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Imaging hyphal growth of *Physisporinus vitreus* in Norway spruce wood by means of confocal laser scanning microscopy (CLSM)

Abstract: Light microscopy and electron microscopy are the most common methods for analyzing wood-decay fungi. However, the 3D visualization and quantification of the filamentous structure of fungi in wood is difficult to realize by means of these traditional techniques. In the present work, confocal laser scanning microscopy (CLSM) was further developed for the quantitative imaging of the 3D microscopic hyphal growth of *Physisporinus vitreus*, a versatile fungus for engineering value-added wood products. To this purpose, the fungus was stained with a fluorescent dye Alexa Fluor. The 3D information obtained by CLSM has a high potential as a basis for the development of mathematical models for a more precise observation of the growth behavior of wood-decay fungi.

Keywords: confocal laser scanning microscopy, filamentous mycelium, fluorescence dye, hyphal growth indices, *Physisporinus vitreus*

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Introduction

Physisporinus vitreus is a selective white-rot fungus that degrades mainly bordered pit membranes without causing significant wood strength losses. A treatment with *P. vitreus* enhances the uptake of preservatives and other modification substances in refractory wood species ("bioincising") (Schwarze and Landmesser 2000; Lehringer et al. 2011a,b; Schwarze and Schubert 2011; Schwarze et al. 2006). A fungal growth model was developed for *P. vitreus* for an optimized engineering strategy (Fuhr et al. 2011). Mathematical modeling in this context is very helpful. To this purpose, qualitative and quantitative data concerning the 3D hyphal growth within the wood are required. Light microscopy and electron microscopy in combination with specific staining techniques are very useful (Schwarze 2007), but they provide limited information for the development of 3D images. Confocal laser scanning microscopy (CLSM) allows the imaging of microorganisms in a 3D environment and opens new avenues in biological science. For instance, it has been applied in various areas of wood research (Kim and Singh 1999; Speranza et al. 2009; Taguchi et al. 2010).

A seldom-applied opportunity is to visualize fungal hyphae by CLSM. As wood is transparent for CLSM up to 200 µm (Mannes et al. 2009), the visualization of individual hyphae is possible, but preferably the fungus should be stained by a fluorescent dye (Xiao et al. 1999, 2000). The purpose of the present work is the development of a CLSM-based quantitative analysis of the 3D filamentous growth of *P. vitreus* in Norway spruce wood by application of a fluorescent compound.

Materials and methods

Specimens were obtained from the heartwood of living 40- to 50-year-old Norway spruce trees (*Picea abies* L. Karst.). Their respective dimensions were 10×1–2×1–2 mm^3 (l×t×r). The side in focus of the specimen was left free and the other sides were sealed with a topcoat (Nuvovern ACR Emaillack, Walter Mäder AG, Killwangen, Switzerland) by brushing. After 24 h, this procedure was repeated to ensure a complete sealing. Before incubation with *P. vitreus*, wood specimens were sterilized at 121°C, 20 min, and 2 bar vapor pressure.

Physisporinus vitreus Empa strain 642 was precultivated in 9 cm Petri dishes on 2% malt-extract agar (MEA) for 1 week at 22°C and 70% relative humidity (RH). Wood specimens were placed on the

colonized Petri dishes sealed with parafilm and incubated at 22°C and 70% RH. After the incubation time (from 1 week up to 2 months), thin sections (10×3×0.3 mm^3) were processed from the specimens with a sledge microtome (Bauknecht, Germany). For labeling the hyphae, the sections were stained for 10 to 15 min with the fluorescence dye Alexa Fluor (2.5-5 µg ml^{-1} distilled H_2O_2) conjugated with wheat germ agglutinin (WGA) (Lubio Science, Switzerland). The analysis of the sections was carried out at the Light Microscopy Centre (ETH Hönggerberg, Zurich, Switzerland) with a CLSM (Leica SP2-AOBS). Excitation occurred simultaneously at a wavelength of 405 nm (UV-diode laser at 25%) for wood autofluorescence and 633 nm (HeNe laser at 75%) for Alexa Fluor fluorescence. The distinct fluorescence of wood at approximately 400 nm and Alexa Fluor at approximately 630 nm enables the separation of the signal into two channels. Thus, an individual TIFF image for each radius of interest (ROI) for wood and the fungus is recorded. The pixels of such a grayscale image represent the fluorescence intensity of a material (i.e., wood, fungus, or air).

The grayscale image is transformed to a binary image (image of pixels with two possible values of 0=air or 1=material) by means of "thresholding." Each grayscale pixel is allocated to a specific threshold, which is low intensity (no material) or high intensity (pixel "occupied" by material). Based on such a binary image, quantitative analysis can be performed, that is, the mycelium density can be calculated by counting the number of "occupied" pixels in a defined area.

Objective magnification was 10-fold. Voxel size was 1.4648×1.4648×1.7910 µm with overall 1024×1024 pixels. 3D optical sections (tomograms) with x-y plane of approximately 2 mm^2 and thickness (z-plane) of approximately 150 µm were generated (Claxton et al. 2005). Stacks of optical sections were stitched by XuvTools (Emmenlauer et al. 2009) and further processed with Imaris V 7.0 (Bitplane, Scientific Software, Switzerland) for virtual reality imaging, which rendered hyphal structures (Xiao et al. 1999, 2000).

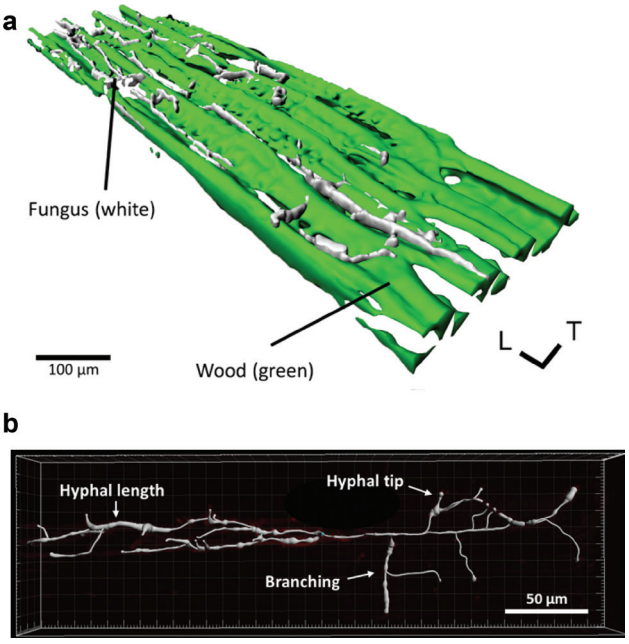

Figure 1 (a) CLSM image of a transverse section of Norway spruce heartwood (green) colonized by hyphae (white) of *P. vitreus*. Hyphae are stained with Alexa Fluor 633+WGA. Wood shows autofluorescence at approximately 405 nm and is transparent for CLSM up to a range of 200 µm. Wood and hyphae are visualized and rendered via isosurfaces. (b) Segmentation of the hyphal structure of *P. vitreus* in wood. The principle directions of wood are usually termed as longitudinal, radial, and tangential. L, longitudinal direction; T, tangential direction.

Results and discussion

The CLSM images are clear and the filamentous mycelia of *P. vitreus* could be distinguished, segmented, and analyzed (Figure 1). The prerequisites for high-quality images with fully resolved structures at the submicron level are blur-free basic images with high signal-to-noise ratio (S/N ratio; which means high fluorescent signals compared to the noise from the surrounding background). Stitching (co-adding) of several optical sections increases the computing time strongly. Furthermore, it may lead to faults, which influence the analysis negatively. Nevertheless, the stitching of the optical sections with XuvTools (Emmenlauer et al. 2009) turned out to be useful to analyze wood sections not only at the microscopic but also at the mesoscopic scale (Figure 2). *P. vitreus* shows a dendritic filamentous growth pattern with a single hyphal mean diameter of approximately 2 µm. Generally, there is much higher fungal activity in earlywood and transition wood than in latewood with regard to the amount of mycelium (density allocation), growth front, and number of degraded pits membranes (Figure 3). With CLSM, the following hyphal growth indices could be analyzed and quantified: hyphae diameter, hyphal length, branching, tips, biomass of the mycelium, density allocation of the mycelium, and the hyphal growth unit (HGU). The HGU is the ratio between the total length of mycelium and the total number of tips (Plomley 1958). At this point, one should mention that the outcome of the analysis of the HGU might be influenced by the adjustments of the software (e.g., point spread function) and the chosen thresholds. Although the quantification might be only an approximation, these data are valuable information of hyphal growth in wood and enhance the knowledge about underlying processes of fungi-wood interactions. Results from the visualization can be directly incorporated into a mathematical growth model (Fuhr et al. 2011).

The advantages of confocal microscopy include the ability to control depth of field, elimination or reduction of background information from layers above and below the focal plane (which leads to image distortion), and particularly the capability to collect serial optical sections from thick specimens (Claxton et al., 2005). CLSM requires

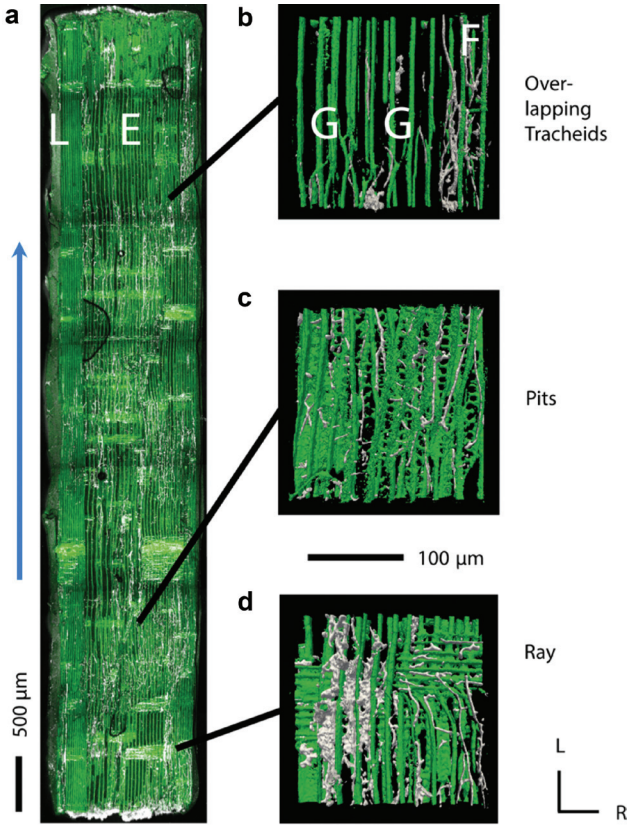

Figure 2 (a) Hyphal colonization of the white-rot fungus *P. vitreus* (white) into a longitudinal-tangential section of Norway spruce heartwood (green) visualized by CLSM (blue arrow indicates the direction from which wood samples were colonized). The terms E and L denote earlywood and latewood. (b)–(d) An overlapping region, hyphal activity at the pits, and a xylem ray, respectively. The terms F and G denote blocked and growing leading hyphae, respectively. L, longitudinal direction; R, radial direction.

staining of the microorganisms with fluorescent stains. In the present work, the labeling of the hyphae with Alexa Fluor 633+WGA enabled easy visualization of the fungal hyphae and good contrast of the filamentous mycelia to the wood autofluorescence.

Cell wall damages were not clearly visible with the CLSM because of the weak autofluorescence of wood. Therefore, to combine the fungal growth with its impact on the cell wall structure, Fuhr et al. (2012) developed a computer-based method for the quantification of the impact of the wood-decay fungus *P. vitreus* by means of high-resolution X-ray computed tomographic microscopy. Based on these quantitative data, the use of *P. vitreus* can be optimized by linking the microscopic growth behavior with macroscopic wood properties.

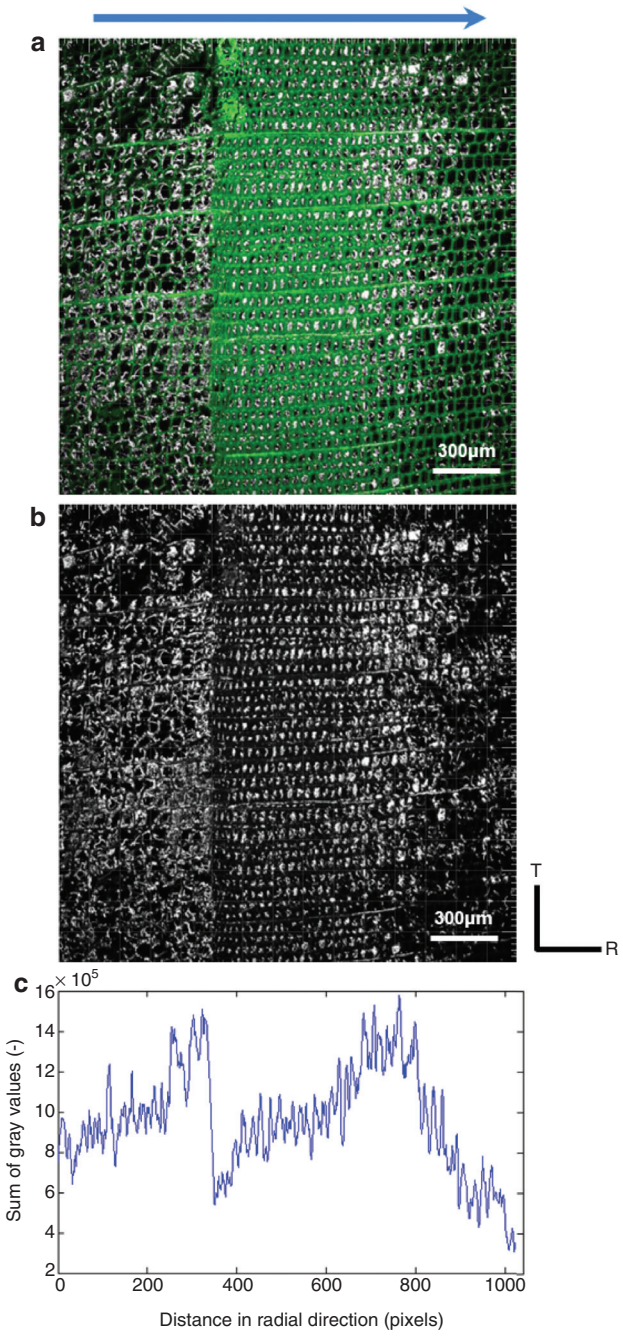

Figure 3 Density allocation of the mycelium of *P. vitreus* in Norway spruce heartwood.
The blue arrow indicates the direction from which wood samples were colonized. (a) Overlapping of the two CLSM channels, that is, mycelium (white; excitation at 405 nm) and wood (green; excitation at 633 nm). (b) Segmentation of the mycelium (white; excitation at 405 nm) using a threshold (i.e., a pixel is occupied by fungus if its gray values is above a specific threshold). (c) Quantification of the density allocation by adding up the gray values of each pixel in tangential and longitudinal direction. R, radial direction; T, tangential direction.

Conclusions

The application of the CLSM provides 3D images of the filamentous mycelium of *P. vitreus* in Norway spruce wood. Relevant hyphal growth indices can be quantified by this method. The method is also suited for live-cell imaging and time-lapse experiments in the course of wood colonization and degradation by fungi.

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