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Visualisation of ectomycorrhizal rhizomorph structure using laser scanning confocal microscopy

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A method for the observation of the three-dimensional structure of intact ectomycorrhizal rhizomorphs is described. The method is based on a combination of clearing the material with KOH followed by staining with congo red and subsequent imaging under a laser scanning confocal microscope (LSCM). The images obtained are of a much higher three-dimensional resolution than those obtained previously by use of conventional light microscopical techniques. The structure of highly differentiated and undifferentiated rhizomorphs is described. Applications of the method are briefly discussed.

INTRODUCTION

The extramatrical mycelium of many ectomycorrhizal fungi aggregates into linear, multi-hyphal organs. These rhizomorphs are an effective means of inoculum spread through soil. They also are organs of carbon, nutrient and water translocation (Cairney 1992). Their structure ranges from undifferentiated, with loosely interwoven hyphae of equal diameter, to highly differentiated, with centrally arranged thicker hyphae (Agerer 1995).

The organization of rhizomorphs has been studied on transverse as well as on longitudinal sections of embedded and non-embedded material (Cairney 1991). Neither approach, however, reveals all the characteristics such as septum dissolution or hyphal branching, ramification and anastomoses. These features are important to note since rhizomorph structure is one of the most valuable anatomical characters for the classification of ectomycorrhizas (Agerer 1995). Agerer (1995) therefore recommends the use of whole preparations of rhizomorphs viewed with interference or phase contrast.

Recently, the use of a laser scanning confocal microscope (LSCM) resulted in images of the 3D structure of arbuscular mycorrhizal structures of much higher resolution than possible with conventional light microscopical techniques (Dickson & Kolesik 1999).

The aim of the present study was to apply LSCM technology to the study of ectomycorrhizal rhizomorph structure. It became apparent during the course of our study, that a method yielding suitable material for imaging rhizomorphs under a LSCM had to be developed.

MATERIALS AND METHODS

Ectomycorrhizas of *Paxillus involutus* (isolate ATCC 200175) were synthesized on *Pinus contorta* and *Betula pendula* seedlings as previously described (Finlay *et al.* 1988, Brun *et al.* 1995). *Paxillus* was chosen because it produces rhizomorphs of the most highly evolved type. Rhizomorphs of that kind are 'highly differentiated, with centrally arranged thicker hyphae; septa are partially or completely dissolved' (Agerer 1995). *Paxillus* readily forms rhizomorphs (Massicotte *et al.* 1999) which have been the subject of a detailed ontogenetic study (Agerer 1988). An additional advantage of using *Paxillus* is its ability to form mycorrhizas with a large number of different hosts (Wallander & Söderström 2000). For comparison, mycorrhizas were also synthesized between a pink fungal isolate (Erland & Söderström 1990) and *Pinus contorta*. This isolate (isolate 88.015, maintained in the collection of the Department of Microbial Ecology) originates from a stand of *Pinus sylvestris* in south Sweden and has been identified as *Tomentellopsis submollis* by ITS rDNA sequence analysis (Robin Sen, pers. comm.).

Seedlings with well-developed mycorrhizal roots were transferred to perspex observation chambers that contained a layer of non-sterile, unfertilized peat (Finlay *et al.* 1988). The plants were kept in a growth

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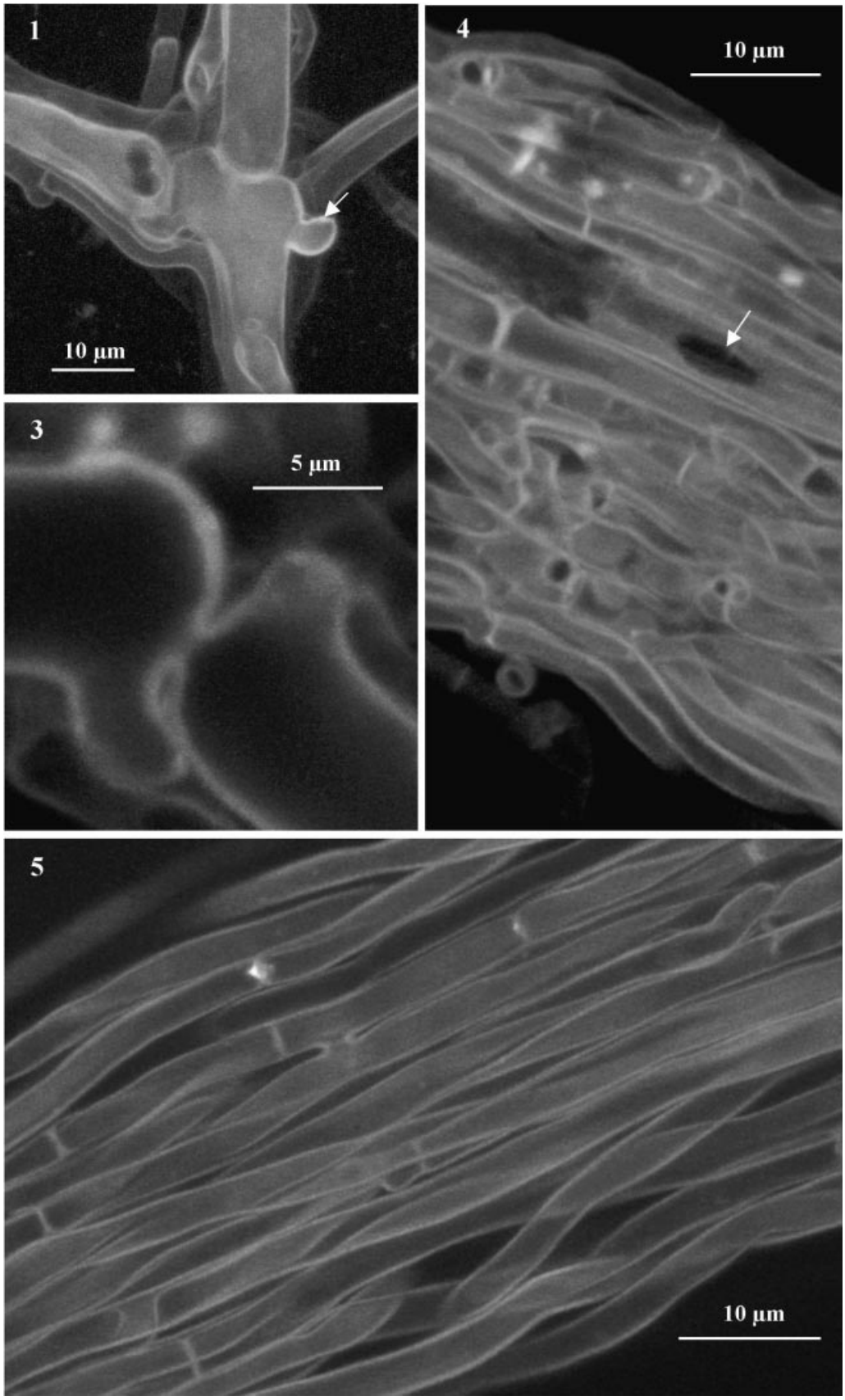

Fig. 1. For caption see opposite.

chamber under controlled light (300 μE; 18 h/6 h day/night), temperature (18 °C/16 ° day/night) and humidity (80 %) conditions. Extending from the mycorrhizas, the fungi colonized the peat surface. Rhizomorphs eventually developed behind the undifferentiated fan-like front of the spreading hyphae.

For microscopical examination, squares were cut of the peat substrate and the overlaying mycelium. These squares were transferred to a petri dish filled with water where the peat was separated from the mycelium. Most peat particles settled to the bottom of the dish following light swirling. The remaining were removed with dissecting needles. The hydrophobic mycelium stayed on the surface of the water and was transferred onto glass coverslips. It was thereafter subjected to various pretreatment, staining and destaining procedures (see Results and Discussion). Finally, the mycelium was mounted on slides. The LSCM used in this work was a Leica DM RBE equipped with an argon/krypton laser operated at excitation wavelengths of 480 ± 10 nm and 568 nm. Emission filter settings were adjusted according to the stain used. A × 63 water (PL APO; NA 1.20) and a × 40 oil (PL APO; NA 1.25) immersion objective were used for adequate resolution. Pinhole size, gain and photo multiplier tube signal amplification were adjusted manually. Most images were further magnified by electronic zoom. Each optical slice was collected as an average of 4 individual scans. Confocal images were recorded and stored as Leica files. After conversion to Biorad format, the final image layouts were adjusted in Confocal Assistant and Adobe Photoshop.

RESULTS AND DISCUSSION

Test of stains

Choice of potentially useful fluorochromes was mainly based on information from the literature but was also dependent on the available equipment. Calcofluor was therefore not included in this work because it requires excitation with UV light. Additionally, it has been reported not to stain *Paxillus* (Brundrett, Melville & Peterson 1994). Its feature of selectively staining β-glucans makes it, however, an obvious candidate as a possibly suitable stain.

Mycelium of *Paxillus* showed no autofluorescence which prevented the use of unstained material for imaging with the LSCM. It still needs to be examined whether mycelium of other ectomycorrhizal fungi, especially of latex-producing species in the *Russulaceae* is sufficiently autofluorescent (Comandini, Pacioni & Rinaldi 1998). Possible induction of autofluorescence by fixation in glutaraldehyde as previously shown for plant material (Prior, Oparka & Roberts 1999) was not tested. In an attempt to stain the fungal cell walls selectively we tested wheat germ agglutinin conjugated with fluorescein isothiocyanate (WGA + FITC; 100 μg ml^{-1} 10 mM P-buffer, 0.15 M NaCl, pH 6.8). But staining was very patchy and it was confined to single hyphae and very thin rhizomorphs. Improved staining, but restricted to thin rhizomorphs, was obtained for lucifer yellow carbohydrazide (LYCH; 20 μg ml^{-1} diH_2O). As previously recorded (Cole, Hyde & Ashford 1997), this dye stained fungal cell walls and dolipore septa (Fig. 1). Trypan blue, which has previously been used with good results to stain mycelium of ectomycorrhizal fungi (0.05 % freshly made in lactoglycerol; Schelkle *et al.* 1996) did not yield satisfactory staining. Similarly, none of the xanthene dyes previously used for staining of embedded mycorrhizal material (1 % in diH_2O; Melville *et al.* 1998) resulted in satisfactory staining. One apparant problem was the hydrophobicity of *Paxillus* mycelium. This problem, which is due to the production of hydrophobins (Tagu, Kottke & Martin 1998), could not be circumvented by including the nonionic detergent tween 80 at 1.5 % (v/v) (Unestam & Sun 1995) or a number of other both anionic (8.5 mM lauryl sulfate; 5 mM sodium deoxycholate) and cationic (1 mM hexadecyltrimethylammonium bromide) surfactants in the staining solutions. The by far best result was obtained by staining with the fluorescent Schiff's reagent (Brundrett *et al.* 1994) which yielded distinctly stained mycelium. But LSCM images collected below the surface of rhizomorphs deteriorated rapidly and progressively with the depth of imaging. This was not due to a disability of acriflavine to penetrate into the rhizomorphs as cross sections revealed. We therefore assumed that the light brown pigmentation of *Paxillus* opposed satisfactory LSCM imaging by self-shadowing of lower-lying optical planes.

Specimens viewed under a LSCM should ideally be opaque and of optical homogeneity. Clearing of material may therefore be necessary to facilitate observation of internal structures. This can be achieved

Fig. 1. Node of young *Paxillus involutus* rhizomorph stained with 20 μg LYCH ml^{-1} diH_2O. Dolipore indicated by arrow. Projection of 21 horizontal optical slices spaced 1 μm along the vertical axis. Filter settings: excitation 480/10 nm, emission LP 515 nm. Objective × 63 water immersion. Electronic zoom 2.76. **Fig. 3.** A dissolving septum in a vessel-like hypha of a *Paxillus involutus* rhizomorph. Projection of three optical slices with a 0.3 μm interval along the vertical axis. Filter settings: excitation 480/10 nm, emission LP 590 nm. Objective × 63 water immersion. Electronic zoom 5.36. **Fig. 4.** Disintegration of the cell wall of a central vessel-like hypha in a thick, senescent *Paxillus involutus* rhizomorph. Arrow points at hole in cell wall. Projection of five horizontal slices spaced 0.5 μm along the vertical axis collected at 6 to 8 μm below the surface of the rhizomorph. Filter settings: excitation 480/10 nm, emission LP 590 nm. Objective × 40 oil immersion. Electronic zoom 3.59. **Fig. 5.** Rhizomorph of the pink isolate showing hyphae of equal diameter and a lateral anastomosis. Projection of two horizontal optical slices spaced 1 μm along the vertical axis at a depth of 10 μm below the surface of the rhizomorph. Filter settings: excitation 480/10 nm, emission LP 590 nm. Objective × 63 water immersion. Electronic zoom 2.22.

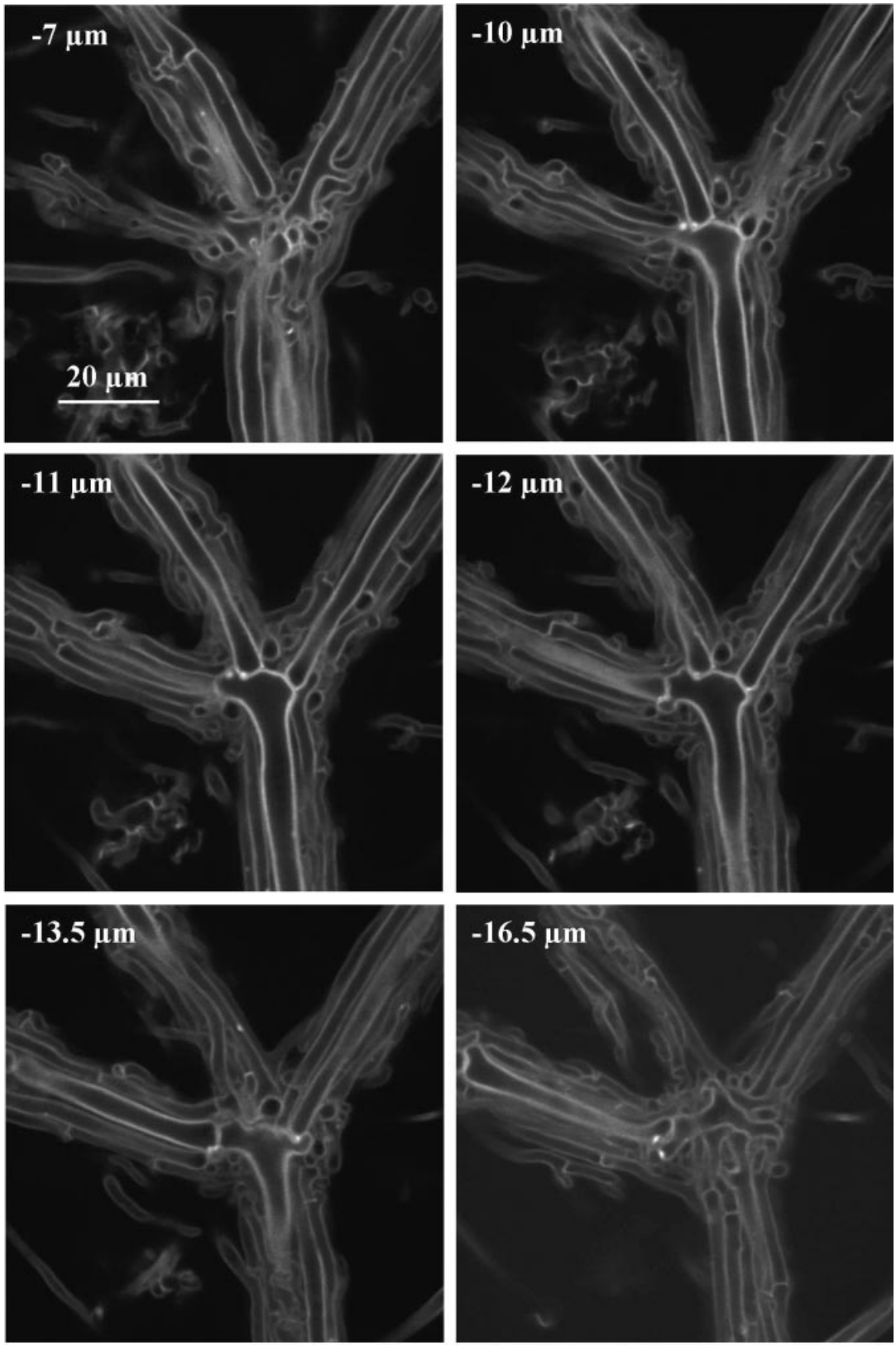

Fig. 2. Single optical slices collected at various depths below the surface of a medium-thick *Paxillus involutus* rhizomorph. Filter settings: excitation 480/10 nm, emission LP 590 nm. Objective × 40 oil immersion. Electronic zoom 2.88.

by, for example, leaving specimens in 96% ethanol which is standard procedure for preparation of material for electron microscopy. This may explain the previously observed usefulness of xanthene dyes for staining of fixed and embedded material (Melville *et al.* 1998). Viewing of stained, fresh material also yielded blurry images of quite bad resolution in that study. To achieve both opacity and a reduced hydrophobicity of the material we decided to use the strong base KOH. Potassium hydroxide is known to dissolve hydrophobic

material as well as to remove tannins and other pigments. It is routinely used in the clearing and staining of roots for visualisation of arbuscular mycorrhizal colonization (Brundrett *et al.* 1994). Clearing of *Paxillus* mycelium with near boiling 10% KOH for up to 1 h removed all the pigments and rendered the mycelium hydrophilic. However, it also made it extremely fragile for further processing. Following a rinse in water, the cleared mycelium was stained with either congo red (2% in 30% ethanol; using the supernatant of the otherwise colloidal suspension) or, following an acidification step with 1% HCl, with acid fuchsin (0.01% in lactoglycerol). Staining time was one minute with congo red and more than 12 h with acid fuchsin. After washing off excessive staining solution with water the specimens were mounted in non-fluorescent mounting media. Both of these stains, especially congo red, yielded very clear images with stable fluorescence (Fig. 2). Images with good resolution could be collected all through even the thickest rhizomorphs. Staining mycelium with congo red after clearing in KOH was therefore implemented as standard procedure. Congo red (CI 22120; also cotton red B or C) is a diazo dye that is used as a general counterstain but also as a stain for cellulose. It is an indicator stain, changing from red (basic) to blue (acidic) in the pH range 5.0 to 3.0. We found it to be much more fluorescent in the basic range and used it therefore directly on KOH-cleared and rinsed mycelium without an intermediate acidification step. As mounting media we recommend either water or glycerol. Mounting in immersion oil resulted in rapid fading of the fluorescence under excitation light.

Rhizomorph structure

Most of the features previously described as characteristic for rhizomorphs of *Paxillus* (Agerer 1988, Hahn & Agerer 1999) were observed in the present study. Rhizomorphs are structured into nodes, at which they branch, and internodes. Inflated hyphae are found at the centre of the rhizomorphs surrounded by much thinner hyphae. Outer hyphae grow towards each other and form anastomoses which can result in reversely oriented clamps. The number of thick, central hyphae varies with the general thickness of the rhizomorphs. A maximum of four vessel-like hyphae was observed in this study. In contrast to earlier reports, septum dissolution was very rarely observed. In fact we only found one dissolving septum (Fig. 3). The ringlike residual part of a septum left along the lateral hyphal wall was similarly only observed once in a slightly thicker hypha that lay next to the central vessel-like hypha. Initial clamps of vessel-like hyphae within the internodes have become inconspicuous as a consequence of inflation. Only a slight bulge remains at the original position of the clamp next to the septum. Visible clamps have however been retained in the nodes. They are inflated as the rest of thick hyphae resulting in a joint-like shape (Fig. 2). During senescence of the rhizomorph holes start to appear in the walls of the vessel-hyphae (Fig. 4). This process eventually results in large open spaces within the rhizomorphs that can even lead out of the rhizomorphs. Intrahyphal hyphae (as described by Franz & Acker 1995) were not observed within rhizomorphs and only infrequently in slightly inflated single hyphae.

Rhizomorphs of the pink isolate are of a much simpler structure. Hyphae of equal diameter are loosely interwoven with frequent lateral anastomoses (Fig. 5). Infrequent branching of hyphae and the absence of any clamps are additional distinguishing characters. Hyphae emanate from and merge into the rhizomorphs all along their lengths which gives them a rough surface. In relation to the classification by Agerer (1995), these rhizomorphs belong to the least differentiated type.

Transport in rhizomorphs is bi-directional but the exact routes of translocation are still unknown (Cairney 1992). Rate of nutrient translocation within rhizomorphs has been shown to differ between fungi (Timonen *et al.* 1996). The high degree of 3D resolution gained by the presented method allows quantification of parameters of the degree of differentiation of rhizomorphs formed by different fungi. The possible dependence of efficiency of translocation on structural differentiation could then be tested. For the calculation of fluxes through rhizomorphs the actual routes of translocation still need to be confirmed. Use of radioisotopes in combination with suitable fluorochromes followed by imaging under the LSCM may solve that question.

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