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New approaches to investigating the function of mycelial networks

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Fungi play a key role in ecosystem nutrient cycles by scavenging, concentrating, translocating and redistributing nitrogen. To quantify and predict fungal nitrogen redistribution, and assess the importance of the integrity of fungal networks in soil for ecosystem function, we need better understanding of the structures and processes involved. Until recently nitrogen translocation has been experimentally intractable owing to the lack of a suitable radioisotope tracer for nitrogen, and the impossibility of observing nitrogen translocation in real time under realistic conditions. We have developed an imaging method for recording the magnitude and direction of amino acid flow through the whole mycelial network as it captures, assimilates and channels its carbon and nitrogen resources, while growing in realistically heterogeneous soil microcosms. Computer analysis and modeling, based on these digitized video records, can reveal patterns in transport that suggest experimentally testable hypotheses. Experimental approaches that we are developing include genomics and stable isotope NMR to investigate where in the system nitrogen compounds are being acquired and stored, and where they are mobilized for transport or broken down. The results are elucidating the interplay between environment, metabolism, and the development and function of transport networks as mycelium forages in soil. The highly adapted and selected foraging networks of fungi may illuminate fundamental principles applicable to other supply networks.

Keywords: Fungal development, fungal translocation, nitrogen cycling

Mycelial networks sustain forest ecosystems, which occupy a third of the Earth's land surface. Fungal mycelium dominates the microbial biomass of forest soils, commonly contributing 70-110g dry wt m⁻² (Markkola *et al.*, 1995). Fungi are the major agents of decomposition and play a key role in nutrient cycles as saprotrophs and mycorrhizal symbionts. Central to their importance is the extent that they influence control of the nitrogen and phosphorus supply (Boddy & Watkinson, 1995; Dighton, 1997). Microbial biomass can accumulate and retain mineral nutrients, immobilising as much as 20% and 18% respectively of the total soil nitrogen and phosphorus (Bäath & Söderstrom, 1979). Mycelia scavenge and sequester nutrients from soil, concentrate mineral nutrients from

plant litter that they decompose (Sen, 2000), relocate nutrients between different organic resources, and ultimately make nutrients available to plants via the 'wood-wide web' to maintain primary productivity (Read, 1997). It is believed that the spatial integrity of the networks may be critical for ecosystem function, by preventing nutrient loss by leaching (Miller & Lodge, 1997; Swift & Anderson, 1996).

There is evidence that fungi are capable of affecting the rate of release of sequestered carbon by collecting and transporting nitrogen/phosphorus to freshly colonized woody resources (Boddy & Watkinson, 1995). Lignocellulosic plant remains are carbon-rich but nitrogen-poor, with decomposition rates in the field correlating with N content (Dighton, 1997). Mycelial import of nitrogen accelerates the decomposition of pure cellulose in microcosms designed to mimic cellulosic plant litter being colonized by mycelium

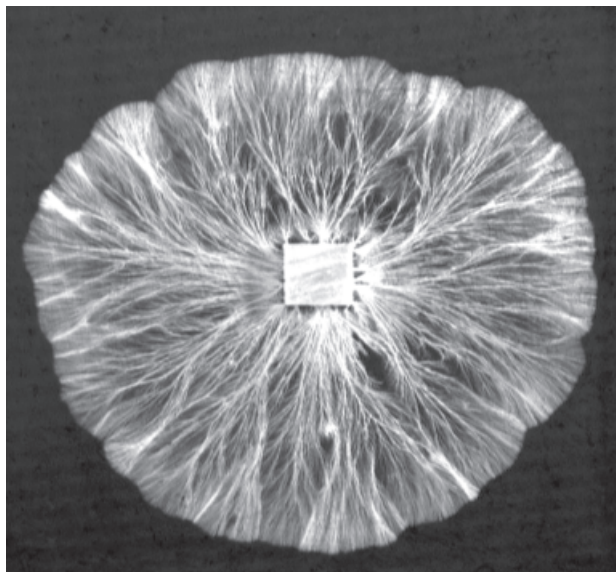


Fig 1 Digital image of a mycelial system of *Hypholoma fasciculare* growing from wood block of 2 cm side in 24 x 24 cm tray of compressed non-sterile soil. Mass fractal dimension > 1.9. Photo by George Tordoff.

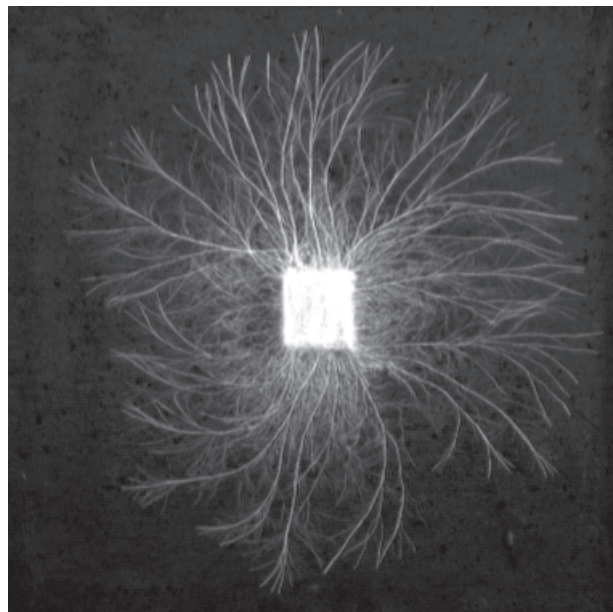


Fig 2 Mycelial system of *Phanerochaete velutina* in soil as in Fig 1. Mass fractal dimension approx. 1.6. Photo by George Tordoff.

growing into it from underlying N-rich soil (Watkinson *et al.*, 1981). Fungicide treatment of field soil reduces the nitrogen content of surface plant litter compared with untreated controls, and reduces decomposition rate (Frey *et al.*, 2000). However, quantifying fungal nitrogen translocation in mycelium, and predicting its contribution to decomposition in ecosystems, presents formidable methodological problems.

There is good understanding of the physiology underlying nitrogen acquisition and metabolism and its regulation from 'laboratory' species such as yeast, *Neurospora* and *Aspergillus* (Griffin, 1994). Fungi actively scavenge nitrogen and are equipped with a remarkable complement of constitutive and induced membrane transport systems in the plasmalemma and tonoplast (Klionsky *et al.*, 1990), enabling them to acquire and store both inorganic and organic nitrogen opportunistically wherever they encounter it in the course of their growth. It is accumulated first in expandable pools of free, soluble amino acids (Griffin, 1994). Close control on biochemical pathways of nitrogen metabolism is operated through transcription regulating genes, for example *NIT2* of *Neurospora* and *AREA* of *Aspergillus nidulans*, that regulate the expression of genes for enzymes of nitrogen assimilation and catabolism according to whether the mycelium is experiencing nitrogen starvation or glut (Griffin, 1994; Marzluf, 1996).

The main difference between growth of model fungi such as *Aspergillus* and *Neurospora* in routine laboratory culture, and fungi living in real ecosystems, is in the

structural complexity and duration of mycelial networks. In large basidiomycete colonies growing in natural habitats, both starvation and excess are likely to be encountered at the same time in different parts of the network. Basidiomycetes typically are able to translocate water and solutes long distances within the hyphae (Jennings, 1987), allowing exploratory outward growth from a food base over a nutritionally inert surface. Many cord-forming species develop persistent networks (Thompson, 1984) with specialised high-conductivity channels - mycelial cords - that carry nutrients between separate carbon-rich food resources. Wood decay basidiomycetes concentrate nitrogen acquired from wood and other sources, accumulating it opportunistically in excess of requirements in the form of free amino acids stored in cytoplasm and vacuoles (Venables & Watkinson, 1989). These amino acids are transported in cords, and become incorporated into protein and cell walls. Development in such fungi is closely linked to the C/N ratio and the intracellular nitrogen concentration within the mycelium (Watkinson, 1999).

New techniques are needed that can probe the full range of developmental, physiological and biochemical responses during the establishment and maintenance of complex networks under realistic conditions. Fungi have to grow to feed, and the adaptive development of the mycelium as it encounters and exploits its scattered woody food sources is described as 'foraging'. Modern imaging methods can now be combined with mathematical modelling to investigate development of

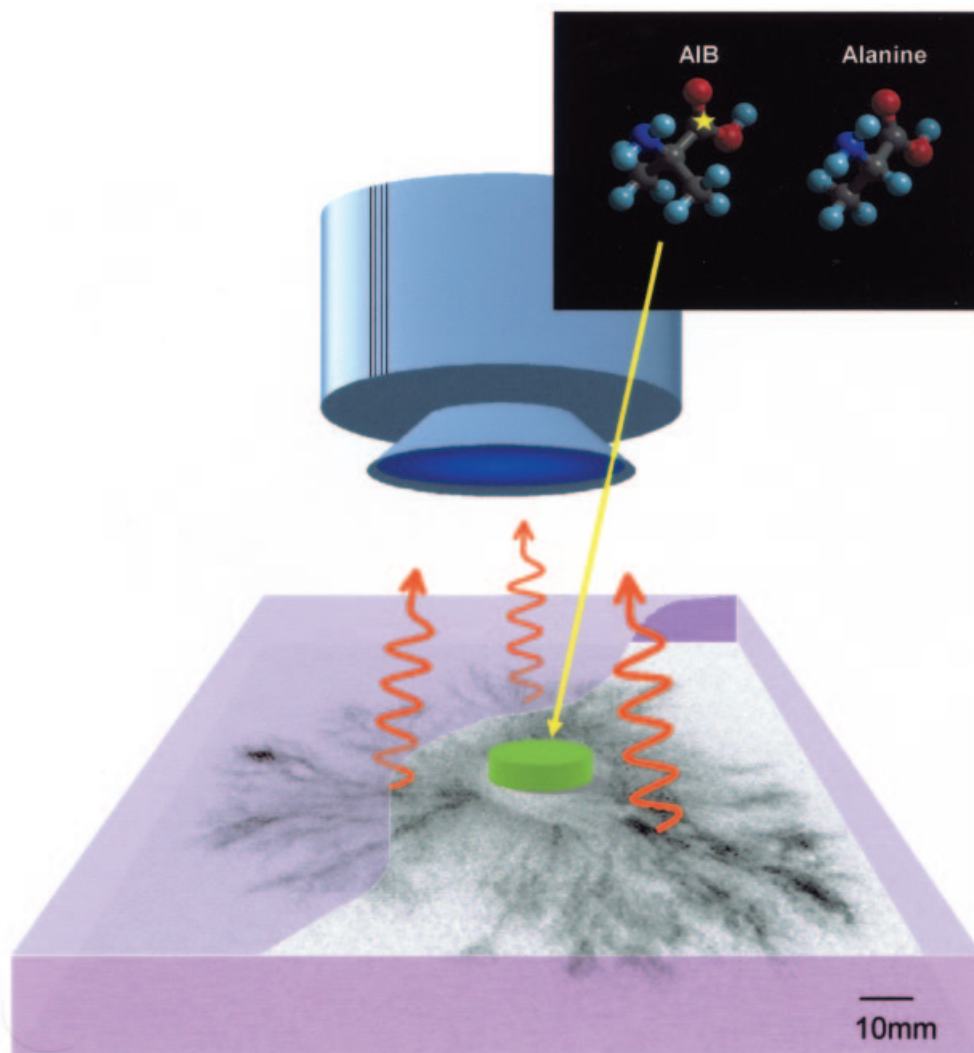


Fig 3 Photon counting camera system used to image transport of ¹⁴C-labelled (yellow star) non-metabolized amino acid analogue, AIB, through the mycelium of *Phanerochaete velutina* growing over an intensifying screen in a microcosm.

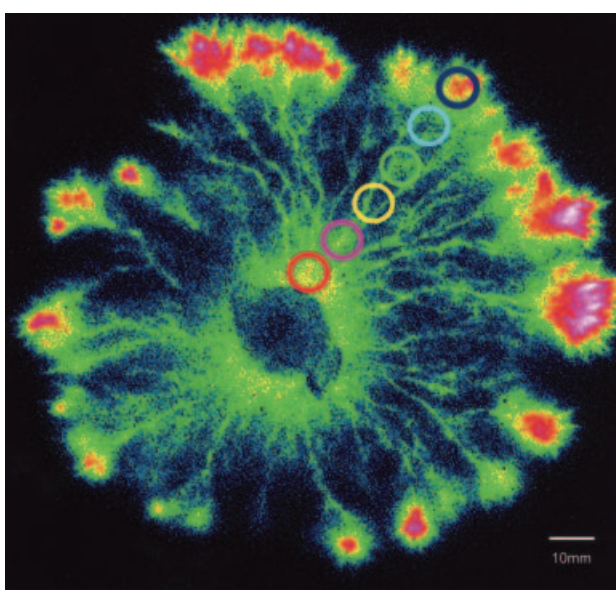


Fig 4 Distribution of ¹⁴C-labelled AIB through the mycelium of *P. velutina*.

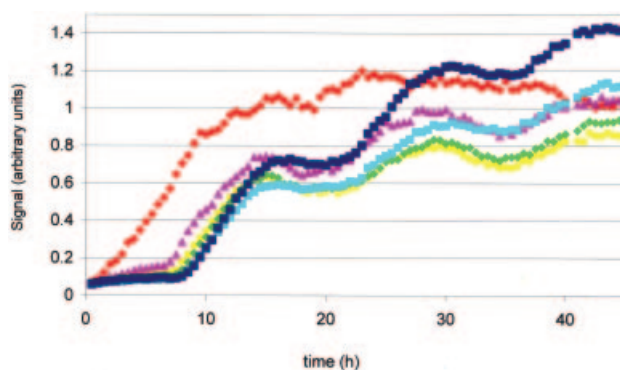


Fig 5 Regular pulses in transport recorded at the sites indicated in Fig 4.

foraging mycelium, and explore the path of nutrients into hyphae and throughout the network. Based on imaging techniques, theoretical models can generate precise hypotheses for experimentation. With current methods in molecular genetics (e.g. Breeze *et al.*, 2004),

it is now possible experimentally to compare the expression levels of genes with known metabolic functions, offering a new way of tracking changes in the metabolic state of the organism. Further, mathematical description of development in mycelium is advancing our understanding of the evolutionary adaptations in fungal development for foraging in the patchy, unpredictable nutrient supply of real ecosystems.

Mycelial cords, the main transport pathways in mycelial networks of those woodland fungi not confined to a single resource unit (e.g. a stump or twig), develop in a way that suggests each cord-forming species has evolved a different foraging strategy. Long range foragers such as *Phanerochaete velutina* grow fast for long distances, with cords developing early in growth to form long supply lines from the large wood food bases this organism prefers. Advance only halts when the mycelium meets, and pauses to exploit, a substantial new wood resource (Boddy, 1999). By contrast, *Hypholoma fasciculare*, which lives on dead leaves as well as substantial pieces of wood, forages over a short range, spreading mainly as fanned-out mycelium composed of separate hyphae, with shorter cords. The use of fractal dimension (Boddy *et al.*, 1998) to describe the organisation of the mycelial network, and its responses to various baits as it grows in suitable microcosms, can highlight developmental shifts – some so subtle as to evade simple observation – between diffuse assimilative and corded distributive growth. The fractal dimension provides a means to convey these patterns in quantitative form and to measure changes (Figs 1 and 2). Essentially, it measures space-filling, and allows description of structures that do not take Euclidean whole number dimensions of 1 (straight lines), 2 (flat surfaces) or 3 (volumes), but rather have fractional dimensions between these.

Cords perform a remarkable range of transport functions, carrying carbon sources including sugars and polyols, amino acids, phosphate and water (Cairney, 1992; Boddy, 1999; Jennings, 1987; Watkinson, 1984). Substances can travel towards the growing margin and back to base at the same time, although not necessarily by the same transport system. However, we know little about the cellular and sub-cellular anatomy of the pathway, the mechanism of transport and its driving forces, and the information pathways through mycelium implicit in the coordinated system-wide responses that it displays in response to localised nutritional stimuli. One approach to measuring transport of solutes is to follow the movement of radio-labelled compounds through the mycelium. Often the final radiolabel distribution is

visualized using autoradiography techniques or analysed by destructive harvesting of the tissues followed by scintillation counting. To try to provide a more dynamic view of how the transport system develops and alters, we are exploring the use of a new technique, termed Photon Counting Scintillation Imaging (PCSI), to image amino acid translocation continuously in foraging mycelium (Tlalka *et al.*, 2002; Fig 3). Amino acid is chosen because nitrogen translocation by mycelium is a pivotal ecosystem process, and amino acid is presumed to be the chemical form in which accumulated nitrogen is translocated through hyphae, since it is soluble, mobile in mycelium, and is the form in which nitrogen acquired by the mycelium is stored until being converted into protein and used for growth. The existence of an amino acid, 2-aminoisobutyric acid (AIB), which is actively accumulated in the fungus, but neither lost as CO₂ by metabolism nor incorporated into protein (Watkinson, 1984), allows amino acid translocation to be tracked through the mycelial system using a ¹⁴C-label as marker – nitrogen itself has no convenient radioisotopes, so cannot be tracked directly.

In the PCSI technique, the fungus is grown in close proximity to a scintillation screen and the movement of the radiolabelled compound tracked from the photons of light that result from collision of the radioactive emissions from the compound with the screen. Using an extremely sensitive photon-counting camera it is possible to collect time-lapse images at intervals of 30-60 min for periods in excess of 4 weeks. In simple microcosms with mycelium growing out over a screen from a central inoculum, transport occurred towards the margin of the colony, concentrated in cords if these were present (Fig 4). The signal from different regions of the mycelium can be quantified from these images (Fig 5) and revealed that there was a strong pulsatile component associated with rapid transport, particularly through the corded system. Even faster rates of transport and more pronounced oscillations were observed in larger colonies grown across natural substrates such as sand and soil.

Many biological systems show pronounced oscillations with a period of around 24 hours and are termed circadian (*circa, diem*). These are often associated with anticipatory behaviour to adjust the metabolism and physiology of the organism to predictable changes in the environment such as daybreak. Such circadian rhythms are driven by a central 'clock' and are not affected by changes in temperature. In contrast, the oscillations observed here are significantly frequent than 24 h and alter markedly with temperature, the frequency doubling about every

10°C. This suggests that they arise directly from a metabolic process rather than reflect the underlying rhythm of the 'clock'. The frequency of the pulses was established using Fourier techniques. Fourier analysis is a family of mathematical techniques, all based on decomposing signals into sinusoids, each with a particular frequency, magnitude and phase. For a fairly regular oscillation, the dominant Fourier frequency provides a very convenient and compact description of the signal that captures most of its interesting features. On more detailed examination, the shape of the pulses was highly asymmetric, with an apparent switch between a gradual rise and a sudden drop in the intensity of the amino acid signal during each cycle. Furthermore, exactly the opposite profile was observed from the assimilatory hyphae growing on the inoculum compared to those that were foraging (Tlalka *et al.*, 2003; Fig 6). This suggested one plausible sequence of events to explain these data that is consistent with evidence for amino acid transport systems in the literature. Signal would initially increase with uptake of amino acid into cytoplasm and sequestration into vacuoles. Once these have reached a threshold level, a proportion of the amino acid would be released into a pathway for mass flow to the growing hyphae at the colony margin. A computer simulation model was constructed incorporating partial differential equations describing the concentration dependence of these processes, which generated a comparable pulse pattern to that observed when values based on physiological data were used for amino acid transport rates across plasmalemma and tonoplast (Tlalka *et al.*, 2003). Whether such a model is sufficient to explain all the experimental data remains to be established, however, at this stage it is consistent with the vacuole as a key compartment in amino-acid dynamics in mycelial fungi.

A role for the vacuole in solute transport has already been suggested for a number of mycorrhizal fungi by Ashford and colleagues (Ashford *et al.*, 2001) after they observed a highly dynamic tubular network using fluorescence microscopy. A similar dynamic network of tubular vacuoles interconnecting systems of larger and constantly changing vacuoles was found in *Phanerochaete velutina* using confocal scanning laser microscopy (CSLM; Fig 7). Simultaneous use of dyes preferentially accumulated in other subcellular compartments, such as mitochondria (Fig 8), is helping us to understand whether this dynamic behaviour is specific to vacuoles or a general feature of an extremely active cytoplasm. An important technical challenge is to apply similar microscopy techniques to study the internal structure of hyphae which have ceased to grow, but are in the process of differentiating to form

the very rapid, high conductivity pathway through mycelial cords revealed in PCSI.

Whilst PCSI and CSLM are proving useful tools to visualize dynamic processes, they can provide relatively little information on the biochemical processes that are normally associated with N-uptake and metabolism and how these processes may alter with changing nutrient supply and demand. We intend to apply stable-isotope NMR to profile the underlying metabolic pathways, and to test the hypothesis (Bago *et al.*, 2001) that the urea cycle is involved in vacuolar nitrogen transport. These studies will be linked with characterization of the molecular control systems for nitrogen distribution during mycelial foraging, using methods for fungal gene expression of nitrogen metabolism and mobilisation developed at Warwick-HRI (Eastwood *et al.*, 2001). Thus our experiments in 'spatial metabolomics' are designed to map the transcript level of relevant genes on to the patterns of mycelial differentiation and amino acid transport imaged during foraging development of mycelium in baited microcosms. The metabolic differentiation of assimilative and exploratory mycelium predicted by models based on imaging, will be tested by comparing levels of expression of genes coding for enzymes involved in nitrogen acquisition, metabolism and mobilisation.

While we expect that this multi-disciplinary approach will facilitate a much more comprehensive description of nitrogen cycling in ecologically important basidiomycetes, it also represents an opportunity to dissect how a self-organising, complex biological network develops. At a more abstract level, such a network represents a generic class of complex systems with loose parallels across a broad range of physical, biological and socio-economic fields. Complex systems in this context can be characterized as consisting of interacting populations (or networks) of autonomous and adaptive agents (or components) which allocate global resources with reasonable efficiency on the basis of incomplete and noisy information without the need for a central control mechanism. Examples of such systems permeate the physical, biological and social sciences, and include traffic flow, internet routing, the response of the immune system, financial markets and manufacturing supply chains. It is possible that the study of nutrient fluxes in such superbly well-adapted mycelial networks can help us understand how efficient resource allocation and robustness can be achieved in man-made networks, despite non-linear dynamics and the fact that the transmitted information is limited and noisy.

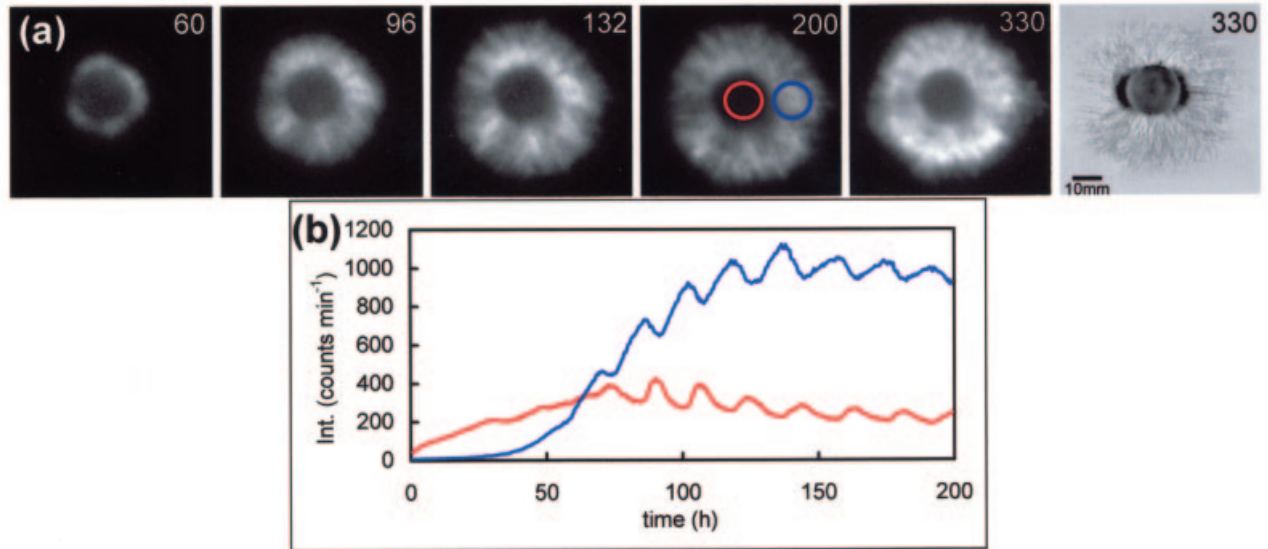


Fig 6 a Photon counting scintillation images of mycelium of *P. velutina* growing over glass from a central inoculum, with bright-field image shown at 330 h. **Fig 6 b** Pulse profiles from inoculum and outgrowing mycelium, at sites indicated on image at 200 h in Fig 6 a.

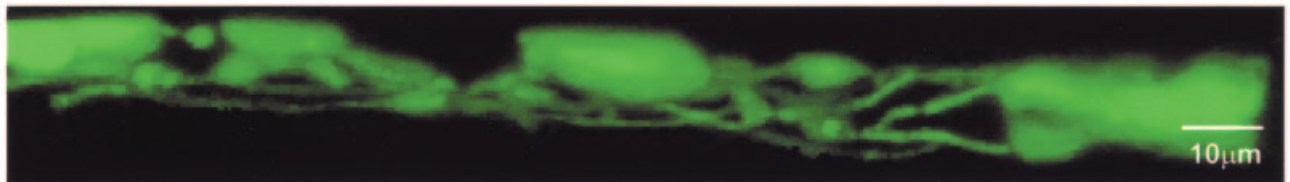


Fig 7 Dynamic vacuolar network – a possible nutrient transport channel – stained with Oregon Green

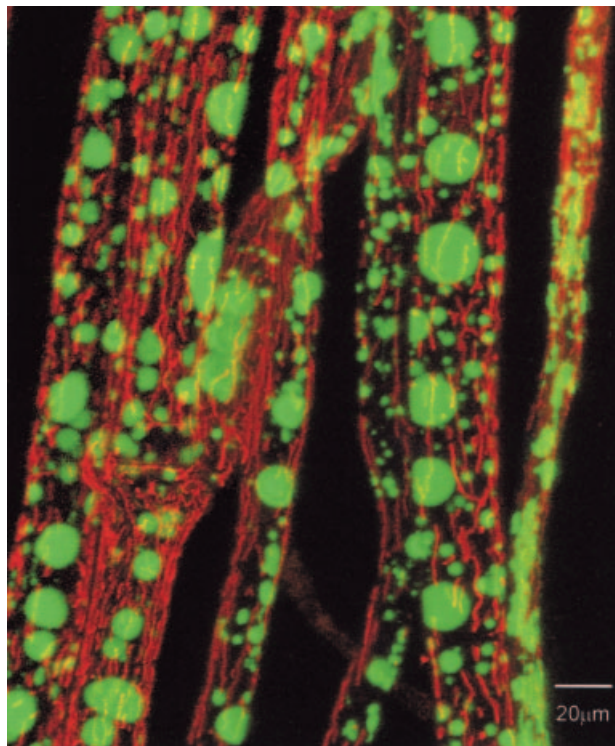


Fig 8 3-D confocal image of *P. velutina* vacuoles (green) and mitochondria (red) labelled with fluorescent dyes Oregon Green and Rhodamine B.

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