available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/mycres

Armillaria luteobubalina mycelium develops air pores that conduct oxygen to rhizomorph clusters

Mamta PAREEK[a], *William G. ALLAWAY*[b], *Anne E. ASHFORD*[a,*]

[a]*School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, NSW 2052, Australia*
[b]*School of Biological Sciences, The University of Sydney, NSW 2006, Australia*

ARTICLE INFO

Article history:
Received 13 April 2005
Received in revised form
19 September 2005
Accepted 30 September 2005
Corresponding Editor:
Nicholas P. Money

Keywords:
Basidiomycota
Gas exchange
Hydrophobicity
Physiology
Sclerotia

ABSTRACT

Armillaria luteobubalina produces air pores in culture. They consist of two parts: a basal region of tissue elevated to form a mound covered with a rind continuous with that of the colony, but perforated; and an apical region of long parallel hyphae, cemented together by scattered patches of extracellular material. This forms a hydrophobic structure that is elevated above the general level of the mycelial crust and does not easily become waterlogged. Air pores develop near the inoculum plug shortly after inoculation, arising directly from the mycelium, and rhizomorphs are initiated from them. The air pore contains a complex system of gas space connecting the atmosphere with the central canal of each rhizomorph. The tissue beneath the melanised colony crust also contains gas space, especially near air pores. This is also connected with the gas space of each rhizomorph and of each air pore. Measurements with oxygen electrodes show that air pores and their associated rhizomorphs conduct oxygen. The average oxygen conductance of a group of air pores with associated rhizomorphs, within agar blocks, but with rhizomorph apices cut off, was about $700 \times 10^{-12}\ m^3\ s^{-1}$, equivalent to about $200 \times 10^{-12}\ m^3\ s^{-1}$ for each air-pore. We conclude that the air pores conduct oxygen into the gas space below the pigmented mycelium of the colony, where the rhizomorphs - which also conduct oxygen - originate. *A. luteobubalina* thus has a complex aerating system which allows efficient diffusion of oxygen into rhizomorphs, and this is likely to facilitate extension of inoculum into low-oxygen environments.

© 2005 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.

Introduction

Several observations indicate that rhizomorphs of *Armillaria* species need a good supply of oxygen for their initiation and growth. They range from observations that submerged mycelium must reach the substrate-air interface for rhizomorphs to be initiated and grow (Snider 1959; Smith & Griffin 1971; Pareek *et al.* 2001), and their abundance in the upper horizons of soil (e.g. Ono 1965, 1970; Redfern 1973; Morrison 1976) to data on the effects of restricting oxygen supply on rhizomorph growth in soil and culture (Münch 1909; Reitsma 1932; Jacques-Félix 1968; Smith & Griffin 1971; Rishbeth 1978; Worrall *et al.* 1986). Rhizomorphs grow faster than free hyphal tips (Rishbeth 1968) and they contain a large consolidated mass of cells (Motta 1969, 1971; Motta & Peabody 1982), so it can be assumed that they have a high oxygen requirement.

Many authors have alluded to gas spaces within rhizomorphs of *Armillaria* (e.g. Brefeld 1877; De Bary 1887), and some have postulated a role for rhizomorphs in aeration, and indicated that the central space of the rhizomorph is a gas-channel that might be involved in conducting oxygen (*vide* Garraway *et al.* 1991; Fox 2000). For example, the

* Corresponding author.
E-mail address: a.ashford@unsw.edu.au
0953-7562/$ – see front matter © 2005 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2005.09.006

experiments of Reitsma (1932) indicated that the oxygen for growing apices in *Armillaria mellea* is supplied through rhizomorphs. Smith & Griffin (1971) in *Armillaria luteobubalina* (then known as *Armillariella elegans*) showed that high partial pressures of oxygen at the origin of rhizomorphs promote their growth, but oxygen at the rhizomorph surface inhibits growth, as Jacques-Félix (1968) had thought for *A. mellea*. Smith & Griffin (1971) reported that when oxygen becomes limiting for apical growth, rhizomorphs flatten and become lobed, and growth becomes much slower; the distance at which flattening occurred depended on the partial pressure of oxygen applied. Investigations such as these are widely quoted in support of a role for rhizomorphs in aeration (e.g. Watkinson 1979) and for oxygen in their development (e.g. Rayner *et al.* 1985). However, the precise pathways by which oxygen from the atmosphere may reach the tips of the first cluster of rhizomorphs produced have never been elucidated.

In the seminal paper of Brefeld (1877), *A. mellea* rhizomorphs developed in culture were described as arising from clusters of sclerotia, presumably the same structures that Snider (1959) termed 'microsclerotia'. There are many reports of the presence of structures described as 'air pores' or 'breathing pores' on various parts of the mycelium, but particularly on rhizomorphs. Hartig (1874) described tufts of hyphae extending from rhizomorphs of *A. mellea*. Smith & Griffin (1971) showed tufts of hyphae on aborted side branches of rhizomorphs of *A. luteobubalina* growing into the air, and interpreted them to be 'breathing-pores'. The central canal of the aborted branch bearing the 'breathing-pores' was shown to be directly connected with the central canal of the main rhizomorph, and Smith & Griffin postulated that oxygen diffused through the intertwining hyphae about 0.5 cm behind the tip of the aborted branch into the central canal of the rhizomorph. Granlund *et al.* (1984) and Intini (1987) used the term 'air pores' for structures on the surface of the rhizomorphs of *A. mellea* and *A. obscura* respectively.

Pareek *et al.* (2001) noted fluffy structures developed when the mycelium reached the substrate-air interface in tubes of liquid medium inoculated with *A. luteobubalina*. These were necessary for the initiation or growth of rhizomorphs, but they did not look like sclerotia except in very early stages. Closer inspection of these fluffy structures at the substrate-air interface suggested a similarity between them and the air pores developed on rhizomorphs. In this paper we report the occurrence of 'air pores' consistently at inoculation sites, air-pore development and internal structure, and their connection with rhizomorphs. Finally, we examined their role in aeration quantitatively by estimating oxygen conductances with an oxygen electrode. We conclude that rhizomorphs develop beneath clusters of air pores, not clusters of microsclerotia, and that the air pores and their subtending rhizomorphs conduct oxygen.

Materials and methods

Fungal material

Armillaria luteobubalina Watling and Kile (isolate 930199KGS) from the culture collection of the University of Western Sydney was used throughout. This was originally isolated from fruit bodies collected from Gore Hill Park, (33.5° S, 151.15° E) North Sydney in June 1993. Stock cultures were maintained in Petri dishes on malt marmite agar (2 % malt extract (from a local health-food shop), 2 % agar (Amyl Media, Scoresby, VIC) and 0.1 % Marmite (Sanitarium Health Food, Wahroonga, NSW) w/v) at 23 °C, and sub-cultured every 4-8 weeks. To produce 'air pores' and rhizomorphs, 10 mm plugs of inoculum were cut from the edge of actively growing colonies, transferred to 2 % malt marmite agar in Petri dishes, and incubated in the dark at 23 °C.

Observations of air pores

Cultures were monitored daily for at least 10 d using a Leica dissecting microscope, and photomicrographs of air pore development were taken with a Zeiss Axiocam digital camera. For light microscopy of sections, air pores at various stages of development with associated mycelium were excised from the colony using a double-edged razor blade and rinsed in purified (reverse osmosis) water. They were then fixed *in situ* in a glass vial with 5 % (v/v) glutaraldehyde in 0.08 M piperazine-N-N′-*bis*-(2-ethanol sulphonic acid) (PIPES) buffer at pH 7.0 for 3 h, rinsed 3-4 times in 0.3 M PIPES buffer at pH 7.2 (Salema & Brandão 1973), dehydrated through a graded ethanol-water series and gradually infiltrated with medium grade LR White resin (London Resin, Woking) over 5 d, all at 4 °C. Samples were polymerised at 60 °C for 10-12 h in gelatin capsules. Semi-thin sections (1.5-2 μm) were cut on a Reichert Ultracut microtome using glass knives, collected on drops of water on slides and dried on a hot plate at 35 °C for 12 h. Sections were stained either with 0.05 % toluidine blue at pH 4.4 and observed with bright-field optics, or with 0.1 % Calcofluor White M2R and observed by fluorescence microscopy (O'Brien & McCully 1981). Photomicrographs were taken on Kodak 100 ISO film.

Oxygen conductance

Conductance of air pores and other tissues to oxygen from the air was measured with gas diffusion chambers fitted with oxygen electrodes, as described by Curran (1985) except that the chamber volume was reduced to about 10 ml to increase sensitivity. The electrode output was displayed on an iMac computer (Apple Computer, Cupertino, CA) through a PowerLab/4SP analog:digital converter using Chart 3.6.6 software (ADInstruments, Castle Hill, NSW). Chambers were routinely leak-tested, and electrodes were calibrated twice daily and whenever significant drift was detected.

Fungal tissue was mounted in 6 mm plastic tubes, to allow its insertion into the electrode chamber. Tubes of 1.5 ml capacity cut from 3 ml plastic pipettes were used to punch pieces of agar with attached mycelium out of the cultures. Each sample was then pushed slightly out of the tube and a thin slice of agar cut off with a double-edged razor blade, to expose cut ends of the rhizomorphs. A thin layer of Vaseline petroleum jelly was applied around the circumference of the sample to prevent any leakage and it was pushed back into the tube. Extreme care was necessary to avoid any breaks in the layer of mycelium or agar, or contamination of the

exposed surface with Vaseline. The outside of the plastic pipette tube with the sample in it was then lubricated and inserted through the rubber disc of the electrode chamber, as described by Curran (1985). The layer of agar and mycelium formed a barrier between the air and the inside of the oxygen electrode chamber, and entry of oxygen depended on conduction through the fungus and agar.

To identify the oxygen conducting capacity of different parts of the fungus, the experiments were divided into several sets. Oxygen conductances of groups of air pores with an inoculum plug, pigmented mycelium, aerial rhizomorphs and plain agar were measured. To clarify whether the inoculum plug was involved in conduction of oxygen, conductivities of groups of air pores grown on cultures without an inoculum plug, and pigmented mycelium and aerial rhizomorphs, were measured. To ascertain whether the oxygen conducted from the air pores travelled through the rhizomorph, conductance with uncut and cut ends of rhizomorphs was compared. Aerial rhizomorphs with one or both ends cut were also investigated. All sets of experiments were replicated 6 times, each replicate from a different plate. Conductances were calculated as in Curran (1985) and analysed after Sokal & Rohlf (1981) by Wilcoxon paired-sample test, or Kruskal-Wallis test followed when necessary by nonparametric multiple comparisons (*STP*).

Results

Development of air pores

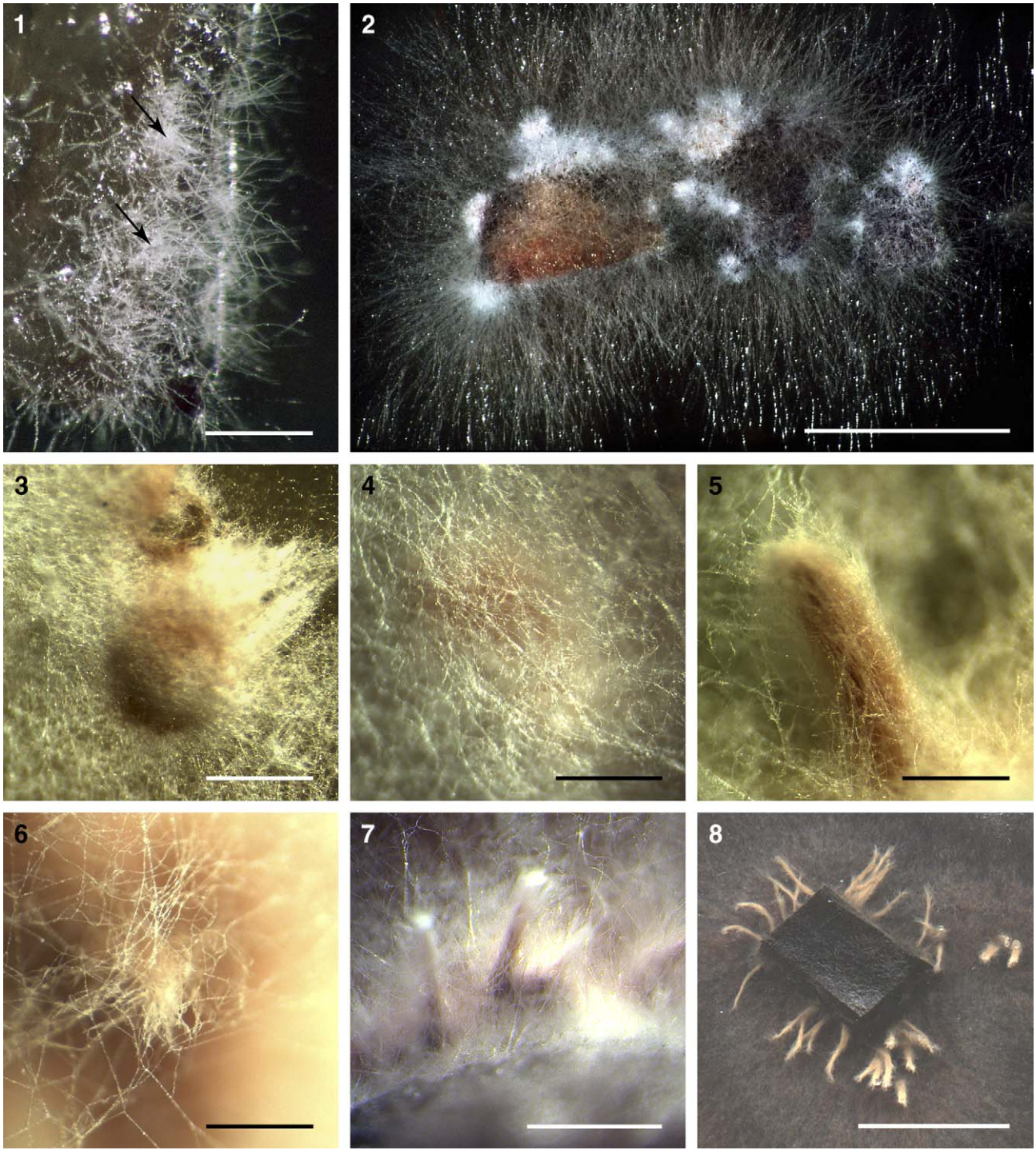
Three to four days after inoculation a radially spreading sparse white mycelium was observed around the inoculation plug. On this several balls of clustered white hyphae appeared (Fig 1). These hyphal clusters were the first distinctive stage of air pore development and under our conditions they first became obvious on day 4 or 5 after inoculation. They were about 0.5 mm diam and white. They occurred on the agar surface in close proximity to the inoculum plug, or on it, and the hyphae of the basal part were submerged in the agar. There was no sign of any rhizomorph tips at this stage. The developing air pores continued to grow, and measured 0.5-0.7 mm in diam on day 6. They were still white and appeared as undifferentiated aggregates of hyphae. At this time several rhizomorph tips were seen in the agar, located specifically beneath the developing air pore clusters. The next stage of air-pore development was defined by the onset of pigmentation. Under our conditions this usually occurred by day 7 after inoculation, but timing was variable due to variability in time of initiation and rate of development of air pores (compare Figs 2 and 3). At this second stage the developing air pore was larger (about 1 mm diam) and was lightly pigmented (Fig 3). This consolidated ball of pigmented hyphae was overlain by a fluffy mass of white hyphae from the colony (Fig 3). By day 8 after inoculation, beneath the cover of white mycelium, a structure of aggregated hyphae extended away from the agar surface. This created a lightly pigmented cylinder that was best seen after removing the mycelial covering (compare Figs 4 and 5). On day 9, with further growth, the tip of the air pore emerged through the mycelium (Fig 6). By day 10 the air pores had attained a height of 3-7 mm and were viewed as being fully extended (Fig 7). At this stage, the apical part of the air pore was fluffy and white in contrast to the lightly pigmented basal part (Fig 7). On day 10 the mycelium of the colony showed first signs of colour. Pigmentation spread from the inoculum plug and by day 28 most of the surface of the mycelium was darkly pigmented (Fig 8). The air pores were also pigmented but much more lightly than the colony as a whole, and the apical region of each air pore still retained its fluffy appearance (Fig 8). From 28 d onwards the air pores were regarded as being mature. They were hydrophobic and were oriented in various planes relative to the agar surface. At no stage of their development did air pores resemble rhizomorph tips and there were no indications of an organised meristem.

The average number of hyphal clusters per culture counted on day 5 after inoculation was 25 (Table 1) with a range from 13-39. It was difficult among these white hyphal clusters to pick out those that were destined to develop into air pores, and it was not until the air pores were all lightly pigmented on day 7 that this distinction could be made with confidence. The number of air pores (about 21 per dish at day 7) did not change over the next three days, although some of them failed to develop fully, and remained as small rounded pigmented aggregations surrounded by a dense white mycelium (Fig 9, values in parentheses in Table 1).

In all cases, the rhizomorphs were initiated specifically beneath clusters of growing air pores (Figs 10,11). Rhizomorph tips appeared after the initiation of the hyphal clusters but at an early stage of their development into air pores: rhizomorph tips first became apparent on day 6. The rhizomorphs rapidly grew down into the agar (Fig 12) until they reached the bottom of the dish where they bent and then grew horizontally outwards (Fig 13). From an early stage the central space of the rhizomorphs was filled with gas, as indicated by its shining appearance in Fig 12.

The internal structure of air pores and their gas space

The spatial relationship between air pores and rhizomorphs and their gas spaces is best shown in vertical sections through the site of emergence of individual air pores. However, since neither the air pores nor rhizomorphs are exactly perpendicular to the agar surface, they are sectioned in many planes. The earliest stage sectioned (day 6 after inoculation) shows two rhizomorph tips located beneath the edge of an obliquely sectioned developing air pore (Fig 14). The gas space in the medulla of these rhizomorphs is continuous with the atmosphere via gas spaces between the loosely arranged hyphae of the colony surface, and extends into the base of the air pore. Fully extended but not yet mature air pores are seen to consist of very loosely intertwined approximately parallel hyphae with intercellular spaces of various sizes (Fig 15). Serial sections indicate that the air pore at this stage is a roughly cylindrical loose mass of undifferentiated hyphae. Clusters of hyphal profiles appear to be bonded together in patches by an extracellular material that stains reddish purple with toluidine blue. Fig 15, a near median section, shows two distinct regions of more closely spaced hyphae on the flanks of a central space containing few hyphae located at the base of the air pore. The medulla in the base of a rhizomorph is situated immediately beneath the air pore and is in continuity



Figs 1-8 – Stages of air-pore development. Fig 1 - Loose clusters of non-pigmented hyphae (two examples indicated by arrows) at 4 d. Bar = 1 mm. Fig 2 - Day 7: numerous air-pore initials (dense clusters of hyphae). Bar = 10 mm. Fig 3 - Day 7: lightly pigmented air pores with associated white mycelium. Bar = 1 mm. Fig 4 - Day 8: denser white mycelium over a lightly pigmented developing air pore. Bar = 1 mm. Fig 5 - Extending cylindrical air pore after removal of the overlying white mycelium. Bar = 1 mm. Fig 6 - Day 9: loose mycelium at the tip of air pore emerging from the covering mycelium (viewed from above). Bar = 1 mm. Fig 7 - Day 10: fully extended air pores. Colony surface is pigmented. Bar = 5 mm. Fig 8 - A cluster of mature air pores 30 d after inoculation. Bar = 10 mm.

with a narrow gas space located beneath the surface of the colony. This region is shown better in Fig 16 where gas space continuity can be traced from the medulla of a submerged rhizomorph through the base of the air pore to the atmosphere. The pseudoparenchyma forming the cortex of the rhizomorph is easily distinguishable. The hyphae at the colony surface adjacent to the air pore are loosely arranged at this stage but pigmentation begins around the air pores and the surface hyphae

Table 1 – Initiation and growth of air pores and rhizomorphs in *Armillaria luteobubalina* cultures

Day after inoculation	Description of air pores	Number of air pores (mean ± S.E.M.)	Number of rhizomorph tips (mean ± S.E.M.)
1-4	mycelium	0	0
5	white hyphal aggregates	25.0 ± 4.0	3.5 ± 0.4
6	white hyphal aggregates	24.9 ± 3.0	4.8 ± 0.4
7	lightly pigmented	21.0 ± 3.0	9.4 ± 0.6
9	lightly pigmented, emerging	21.6 ± 2.4	26.2 ± 0.9
10	fully extended including (air pores	21.8 ± 2.2	n.d.
	that failed to develop fully)	(10.8 ± 2.0)	n.d.

Five culture plates were examined daily for 10 days after inoculation, and the numbers of air pores and rhizomorph tips were counted (n.d. = not determined). Greater numbers of air pores and variability on days 5 and 6 after inoculation indicate the difficulty in establishing whether an aggregation of hyphae would develop into an air pore.

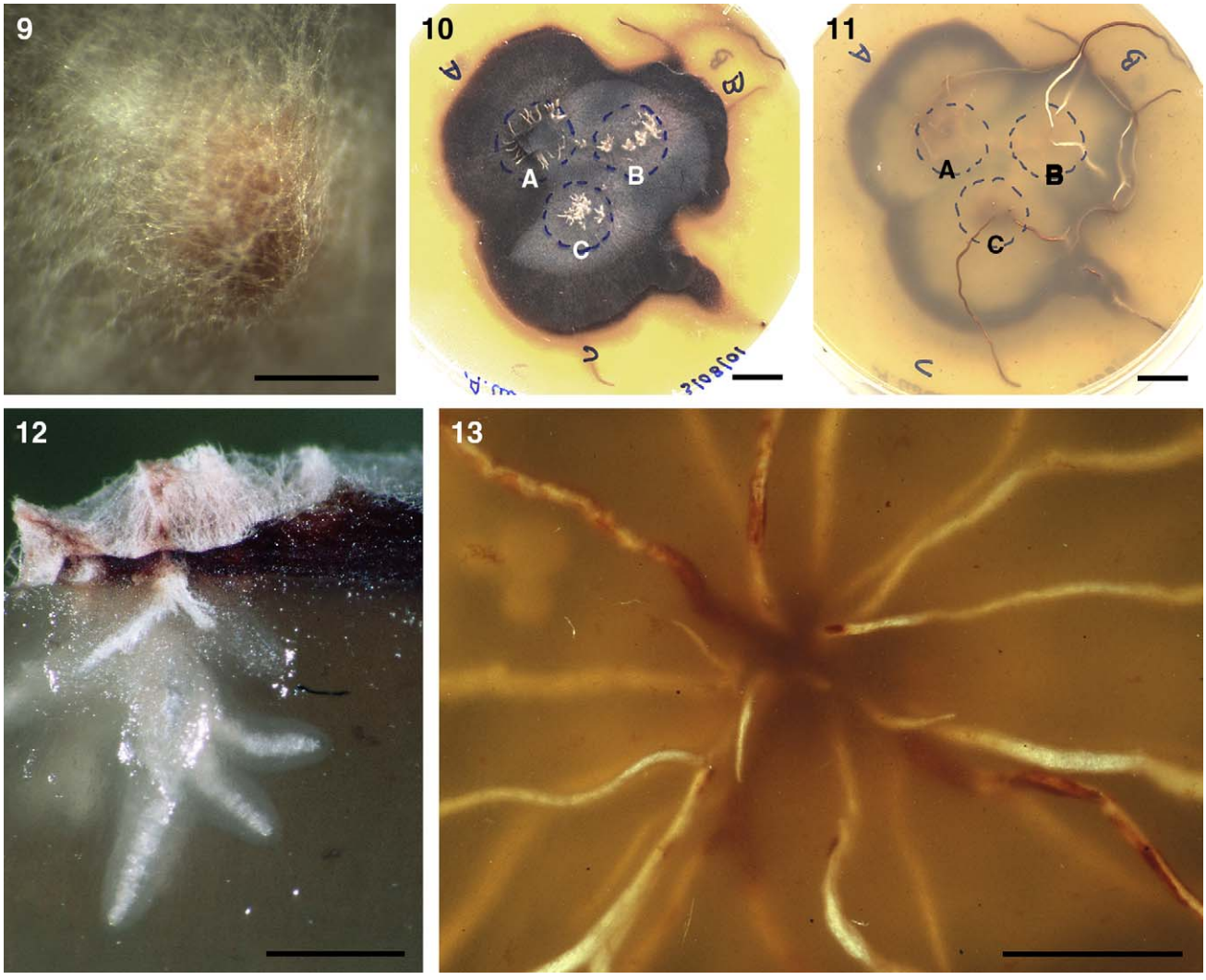
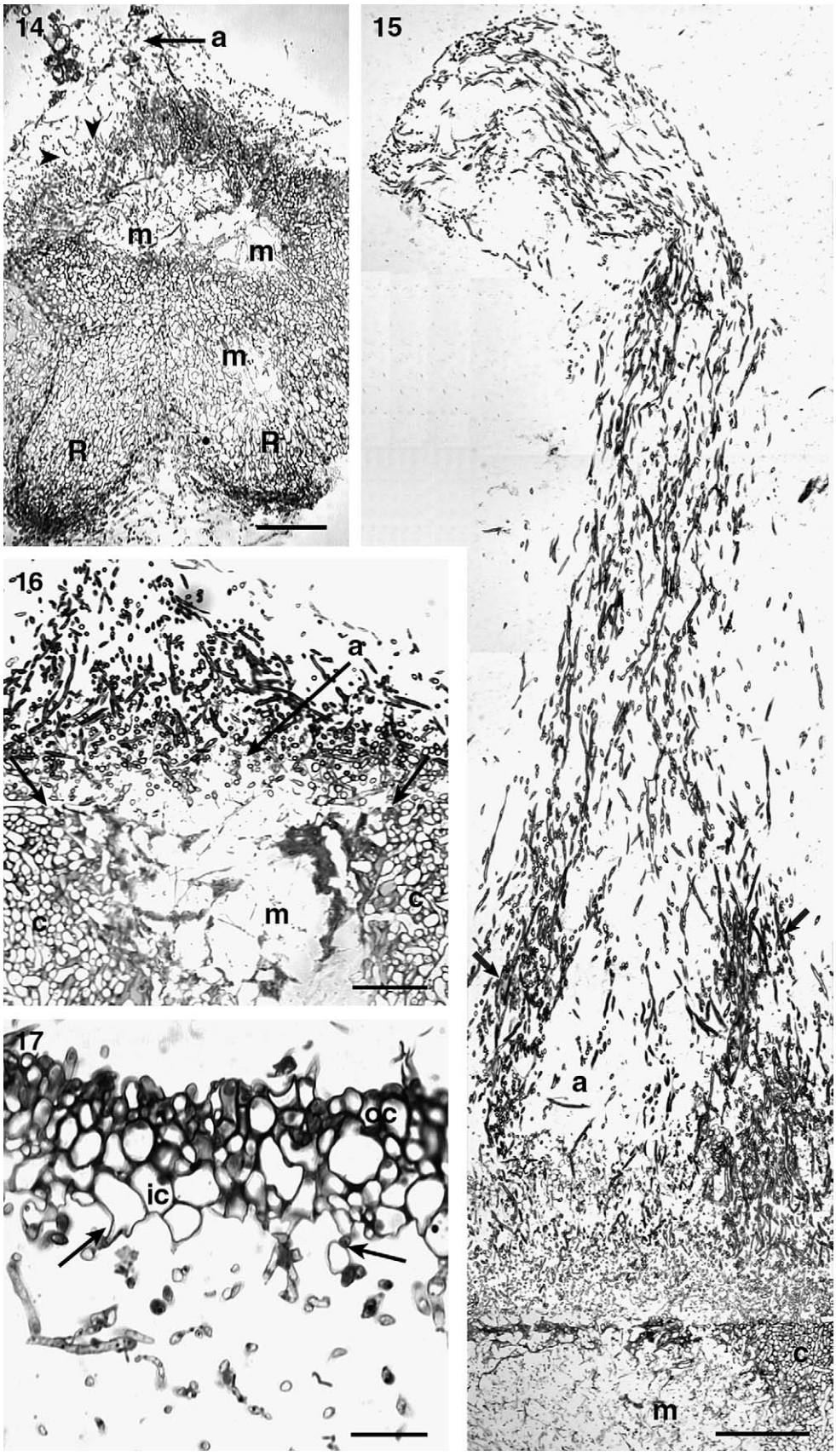


Fig 9 – Day 21: air pore that failed to develop fully: apparently arrested at an intermediate stage of development. Bar = 0.25 mm. Figs 10-11 - Relationship between emergent air pores and submerged rhizomorphs in Petri dish cultures. Fig 10 - Surface view of colony 30 d after inoculation, showing clusters of air pores in three distinct locations (circles A, B and C). Bar = 10 mm. Fig 11 - Underside of the same Petri dish on day 30, with the image digitally reversed to allow direct comparison, showing three sites of rhizomorph initiation beneath air pore clusters. Rhizomorphs emerging from position A are short, while those from B and C have reached the edge of the plate. Bar = 10 mm. Fig 12 - Vertical section through the agar in the centre of a group of developing air pores showing a cluster of rhizomorphs. Bar = 1 mm. Fig 13 - Dish of well-grown rhizomorphs viewed from the underside. Bar = 5 mm.


14
a
m
m
m
R
R
15
a
c
m
16
a
m
c
c
17
oc
ic

rapidly become consolidated into a pseudoparenchymatous crust with a developing rind at its surface (Fig 17). The outermost cortical cells that will form the rind are small in diameter (5-18 μm) and surrounded by extracellular lightly pigmented material, while inner cortical cells are larger (12-37 μm diam) and without pigmentation. A wide gas space containing scattered fine hyphae is present beneath this crust and separates it from the agar surface (Figs 17, 18).

The overall relationship between fully differentiated air pores, submerged rhizomorphs, and the radially spreading mycelium of the colony is shown in Fig 18. At higher resolution (Fig 19) the rind outside the air pores is seen to consist of tightly packed cells embedded in pigmented extracellular material, with no obvious gas spaces. Several fine hyphae branch from wider hyphae in the underlying cortex and traverse the rind. Some emerge into the atmosphere while others appear cut off or broken. Below the consolidated pseudoparenchyma layer of the crust there is a large gas space containing loosely arranged hyphae (Fig 18). It is widest beneath each air pore and tapers off with distance from air pore clusters. The medullary gas space in the base of rhizomorphs, which is also filled with loosely arranged hyphae, is continuous with this gas space underlying the colony crust (Figs 18, 22).

The relationship between the tissues of individual air pores and underlying rhizomorphs at maturity is shown most clearly in sections that are near the median longitudinal plane of the air pore (Fig 22). The medullary gas space of an obliquely sectioned rhizomorph lies immediately below the air pore. The rhizomorph is surrounded by a pseudoparenchymatous cortex except at its base. A mound of heterogeneous tissue has now differentiated at the base of the air pore (Fig 22). This consists of an outer pigmented rind and a narrow loosely arranged and discontinuous cortex surrounding a central region of fine sparsely arranged hyphae, with a large amount of extracellular space (Figs 20, 22). Groups of more or less parallel hyphae that appear to have their origin at the rhizomorph cortex (as inferred from serial sections) have grown across the gas space and entered the basal region of the air pore in a plane oblique to the colony surface (Figs 22-23). Many are cemented together in groups and anastomoses are frequent. This results in a complex arrangement of individual and aggregated hyphae bridging the gas space between the rhizomorph into the basal region of the air pore. Most of these appear to enter the air pore at its periphery, leaving a central region with a large amount of extracellular space containing loosely arranged fine hyphae, resembling the other gas spaces (Figs 20, 22,23). Above the rind of the differentiated basal region the air pore comprises a cylinder of loosely arranged more or less parallel hyphae with a large amount of extracellular space that forms a continuum with the atmosphere (Figs 21,22), as also shown at the earlier stage (Fig 15). Many of the hyphae are pigmented and/or have granular deposits on their walls, and patches where hyphae are cemented together by extracellular material are still apparent (Fig 21). The main differences between fully extended and fully mature air pores are the presence in fully mature air pores of many more pigmented hyphae throughout, and the differentiated basal region.

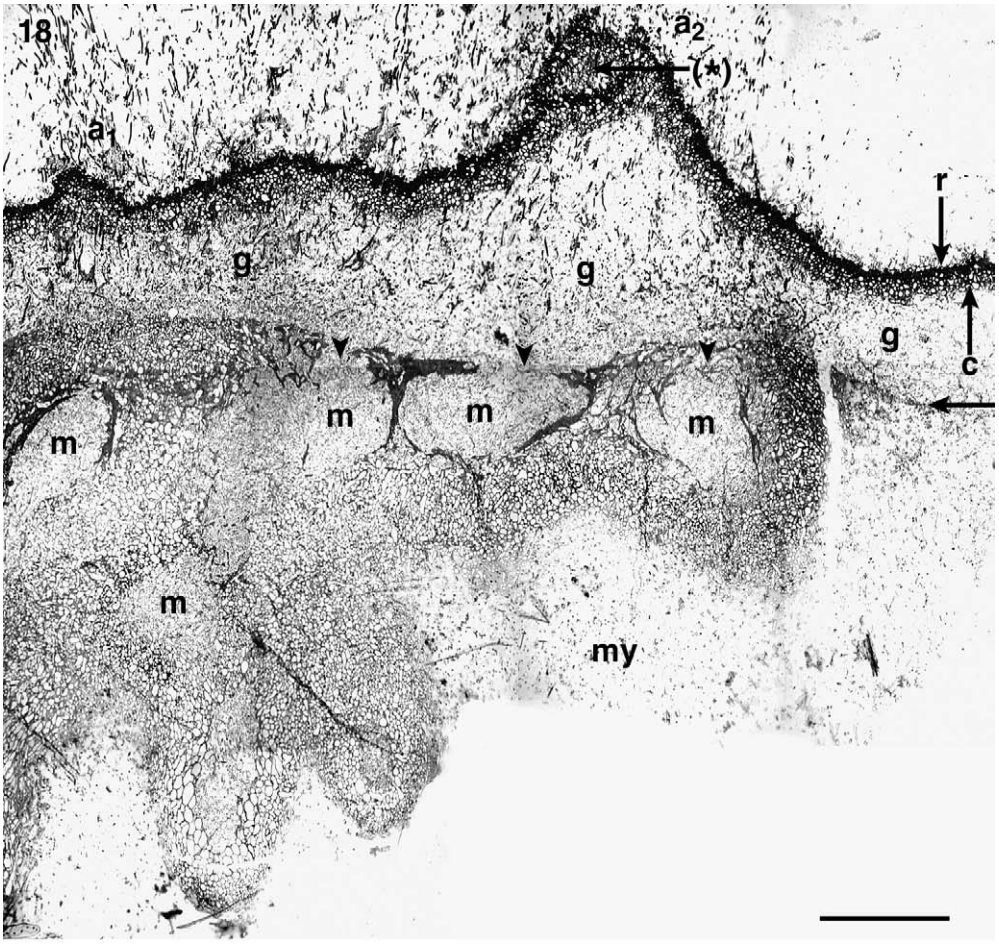
Continuity of gas space between the rhizomorph medulla and the atmosphere via the air pore depends crucially on the nature and amount of extracellular space in the rind specifically in the basal region of the air pore (Fig 22). Figure 24 shows that the tissue of the rind and cortex in the basal mound of the air pore is not consolidated, in contrast to that over the colony crust. There are many areas where there is a clear continuity between the gas space within the basal mound and the atmosphere via gaps in the rind (Fig 24). In many of these areas the cortex is poorly developed. Many of the hyphae that extend into the air pore from the rhizomorph cortex have grown through this unconsolidated rind and contribute to the hyphal mass of the cylinder of loosely arranged hyphae comprising the main body of the air pore. Many of these emergent hyphae have pigmented walls.

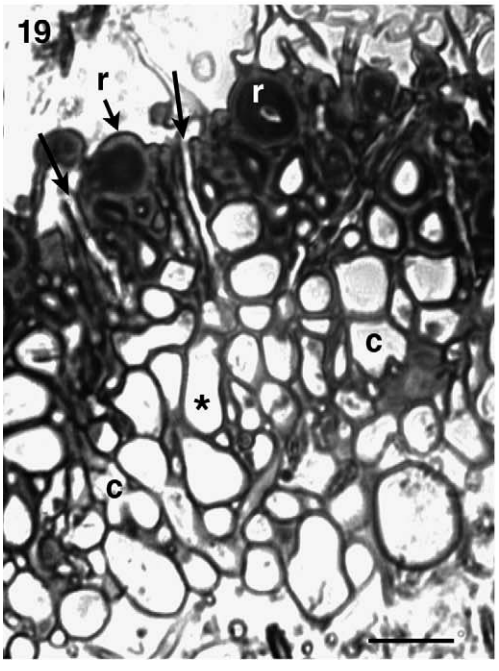
A schematic summary from sectioning data of the structural evidence (Fig 25) illustrates the spatial relationships of the various regions of the air pore at maturity, its relationships with the rhizomorphs, melanised crust and surrounding mycelium, and the distribution of gas spaces in these various regions and their interconnections.

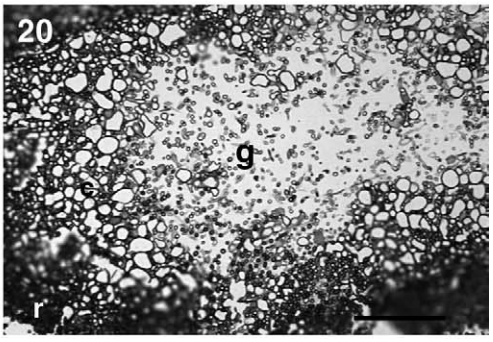
Oxygen conductance

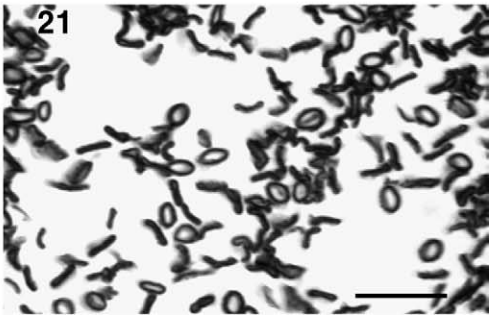
There was essentially no conductance to oxygen (average $4 \times 10^{-12}\ m^3\ s^{-1}$) unless the rhizomorphs originating from the region of the base of the air pore were sliced, exposing their cut ends inside the electrode chamber (Table 2a): this was done in all subsequent measurements. Conductances to oxygen of plain agar blocks without mycelium, and agar blocks covered with pigmented mycelium, were very low

Figs 14-17 – Vertical sections through air pores and adjacent areas at various stages of development. Fig 14 - Edge of a developing air pore fixed 6 d after inoculation, sectioned obliquely, with two very young rhizomorphs (R) in longitudinal section beneath. Gas spaces in rhizomorph medulla (m), among the hyphae of the colony surface (arrowheads), and in the base of the air pore (a). Note the darkly stained apical meristem of each rhizomorph. Stained with toluidine blue pH 4.4. Bar = 100 μm. Fig 15 - Median longitudinal section through a fully extended (but not yet mature) air pore. The basal region of the air pore is not yet fully differentiated. It consists of a central gas space (a) with few hyphae running in diverse directions, surrounded by a zone with more abundant hyphae around the periphery (arrows). There is as yet no differentiated rind within the air pore base. Bar = 100 μm. Fig 16 - Vertical section through the agar showing continuity between the gas space in the medulla (m) of a developing rhizomorph and gas space in the base of the air pore (a) which has loosely arranged hyphae and an extracellular space continuous with the atmosphere. The rhizomorph medullary gas space contains fine hyphae and is surrounded by the pseudoparenchymatous rhizomorph cortex (c). Unlabelled arrows indicate the agar surface. Bar = 50 μm. Fig 17 - Developing rind at the colony surface near a fully extended air pore. Pigmented outer cortex (oc) grades into non-pigmented pseudoparenchymatous inner cortex (ic). Gas space beneath has loosely arranged hyphae, in continuity with inner cortical hyphae (arrows). Bar = 30 μm.


18
a2
(*)
a1
r
g
g
g
c
m
m
m
m
m
my


19
r
r
c
*
c


20
g
r


21

(Table 2b, set 2), presumably representing the rate of oxygen leakage around the agar in the plastic tubes. Groups of air pores showed very much greater conductance to oxygen (Table 2b, set 2). Using cultures inoculated from a loop rather than an inoculum plug, conductance of groups of air pores again was significantly higher than pigmented mycelium or aerial rhizomorphs (Table 2b, set 3). Across all observations the conductance to oxygen per air pore was $198 \pm 27 \times 10^{-12}\ m^3\ s^{-1}$ (mean ± S.E. of the mean, $n = 25$, Table 2d). Aerial rhizomorph conductances were very variable, and were significantly greater than those of agar and pigmented mycelium in set 2, but not in set 3 (Table 2 b). The increase in conductance from cutting off the tops of aerial rhizomorphs was not statistically significant (Table 2 c).

Discussion

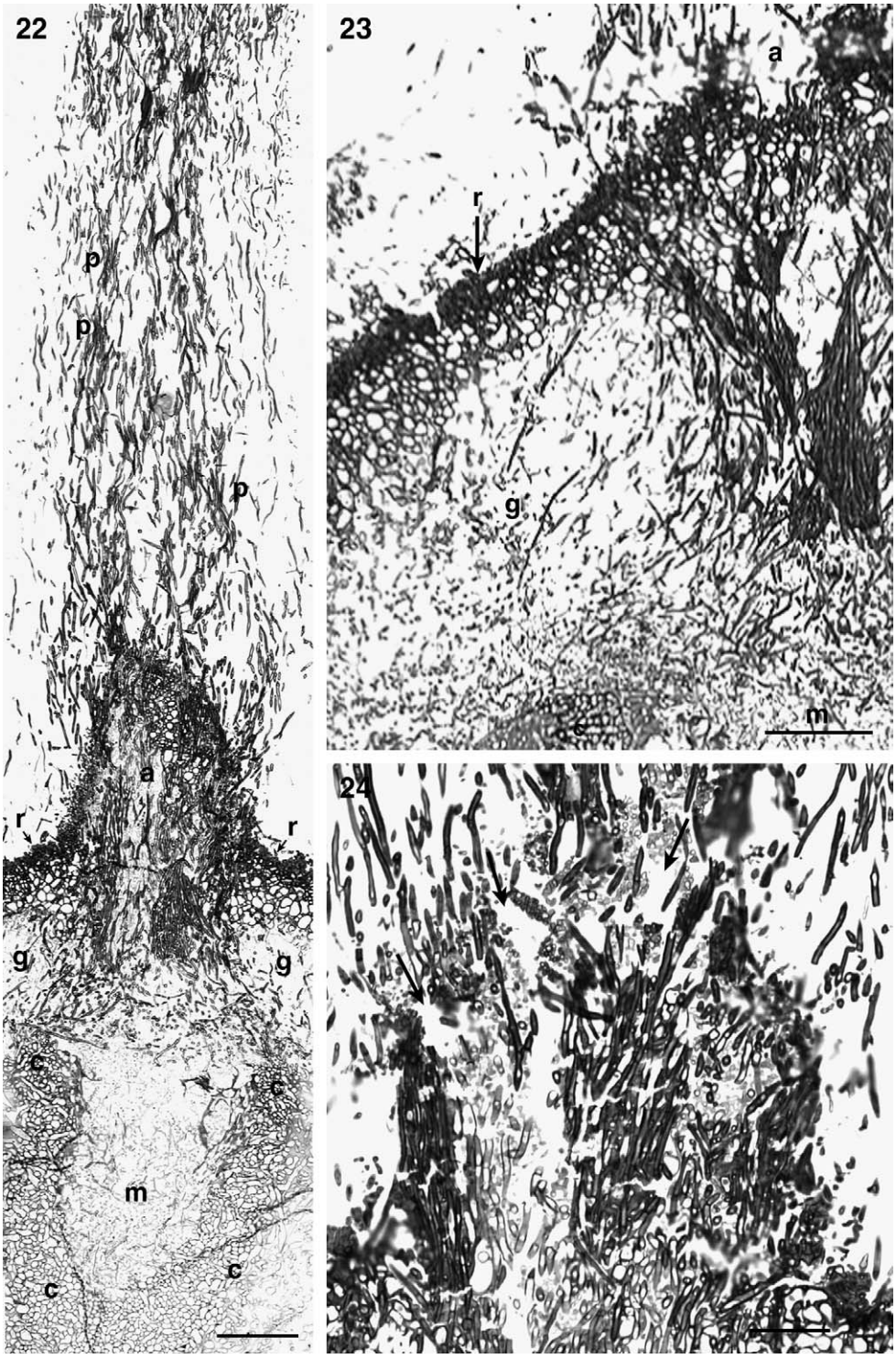
This paper reports the development of air pores at the substrate-air interface in pure cultures of *Armillaria luteobubalina* and confirms their role in conducting oxygen to rhizomorphs. These air pores are not borne on aborted side branches of rhizomorphs as were the 'breathing pores' of Smith & Griffin (1971), but are produced on recently inoculated mycelium and initiated before rhizomorphs. Nevertheless, there is a very clear spatial relationship between air pores and rhizomorphs. Of the names previously used, 'breathing pores' (Smith & Griffin 1971) and 'air pores' (Granlund *et al.* 1984; Intini 1987), we chose 'air pores' because gas exchange is presumably by diffusion rather than mechanical ventilation.

Air pores are distinct from sclerotia or microsclerotia, despite initially being very similar. Many sclerotia and microsclerotia arise, like air pores, as more or less spherical loose hyphal aggregates, and later become pseudoparenchymatous and brown-pigmented in the outer part (Willetts 1972, 1978; Willetts & Bullock 1992). However, air pores continue their development to become elongate structures with a totally different internal structure from microsclerotia. Initiation of air pores early in mycelial development, before rhizomorphs, explains why rhizomorphs are not initiated in culture unless there is an interface between the mycelium and air: air pores must develop first. It explains Rishbeth's (1968) observations that from their inception rhizomorphs grow much faster than hyphal tips. The timing of air pore development and their position suggest that they play a strategic role in gas exchange and aeration of *Armillaria* rhizomorphs, and perhaps associated mycelium as well.

There is no apparent barrier to oxygen diffusion among the hyphae of the air pore region above the rind, and oxygen would be expected to diffuse from the air throughout the intercellular spaces without impediment. The spaces in the rind at the basal region of the air pore would be expected to allow oxygen to diffuse readily into the mound of tissue beneath, which has large intercellular spaces. This gas space is continuous with that underlying the melanised crust of the colony, and is in direct continuum with the central canal of all the rhizomorphs via the gas space in the loose mycelium on the agar surface beneath the air pore. Its oxygen is replenished via the air pore, supplying the mycelium under the melanised crust and providing an extensive reservoir of oxygen for the rhizomorphs to draw upon. As oxygen diffuses through this gas space system as a whole it will be utilised by hyphae. The gas space will be depleted of oxygen, and gradients will be set up. Regions with the most hyphae relative to the volume of gas space they draw on will be the most depleted, as also will be those furthest from the atmosphere, and the depletion gradients will automatically direct the diffusion of oxygen to where it is needed. All this depends on the extracellular space being filled with gas and not liquid. This is difficult to assess from sections but it is obvious in fresh material. The silvery appearance of the rhizomorph canals in the agar indicates that they are filled with gas, and the melanised crust can be peeled away from the agar surface to reveal that the hyphae beneath are free and not consolidated by extracellular mucilage. There is gas space in rhizomorphs of *A. luteobubalina* (Smith & Griffin 1971; Pareek *et al.* 2001), and in *A. mellea* even at maturity when the medulla contains much hyphal tissue (Cairney *et al.* 1988).

The ultimate test of whether the system works is whether it conducts oxygen. Some aerial rhizomorphs, and all samples of air pores (attached to rhizomorphs that were cut below, near their apex) did so, indicating that air pores and rhizomorphs both conduct oxygen, and are connected. The difference between samples with and without cut rhizomorphs, however, does not allow us to conclude that the tip, or rind and cortex along the flanks of rhizomorphs, are impermeable to oxygen, since we know the agar in controls is not permeable. For similar reasons, we cannot assess from these data the oxygen permeability of the melanised rind. The closely packed hyphae of

Figs 18-21 – Sections showing mature (fully differentiated) air pores and surrounding regions of the colony. Fig 18 - Vertical section through the colony showing the base of two obliquely sectioned mature air-pores (a_1 and a_2) with a cluster of rhizomorphs (sectioned in various planes) beneath, surrounded by loosely arranged mycelium (my) growing submerged in the agar. The rind (r) and cortex (c) at the surface of the colony are fully differentiated. Between them and the agar surface (unlabelled arrow) is a gas space (g) containing fine loosely arranged hyphae, connected (arrowheads) to medullary gas space (m) of submerged rhizomorphs. Rind in base of air pores is continuous with rind of colony crust. Base of a_2 is sectioned approximately transversely so that the rind forms a ring of darkly pigmented tissue surrounding more loosely arranged central tissue (arrow with asterisk). Bar = 250 μm. Fig 19 - Vertical section through colony crust near mature air pore. Rind cells (r) have thick wall and small lumen; walls and surrounding matrix are a deep brown with no obvious gas spaces. Rind grades into underlying pseudoparenchymatous cortex (c): cells vary in size and wall thickness and are surrounded by a matrix that grades from brown to non-pigmented in the innermost region. Fine hyphae arising from the cortex traverse the rind (arrows). Bar = 20 μm. Fig 20 - TS of air pore basal region: central gas space (g) containing loosely arranged hyphae, surrounded by a more consolidated cortex and rind. Bar = 50 μm. Fig 21 - TS of air pore column above the basal region. Bar = 20 μm.



Figs 22-24 – Longitudinal sections through fully differentiated air pores fixed at 30d after inoculation. Fig 22 - Median longitudinal section: central region (a) of basal mound; rind of the colony (r). Occasional cemented patches (p) and variously sized intercellular spaces among more or less parallel loosely arranged hyphae above. Rhizomorph medullary gas space (m). Bar = 100 μm. Fig 23 - Detail from an adjacent section showing hyphal connections traversing the gas space from the rhizomorph cortex into the base of the air pore (a). Rind (r) at colony surface continues into base of air pore. Gas space with loose hyphae beneath rind and cortex: g. Medulla of base of rhizomorph: m. Bar = 100 μm. Fig 24 - LS showing detail of the basal region from an adjacent section. Large gaps in rind (arrows) and discontinuity of cortex allow connection from atmosphere to internal gas space. Bar = 50 μm.

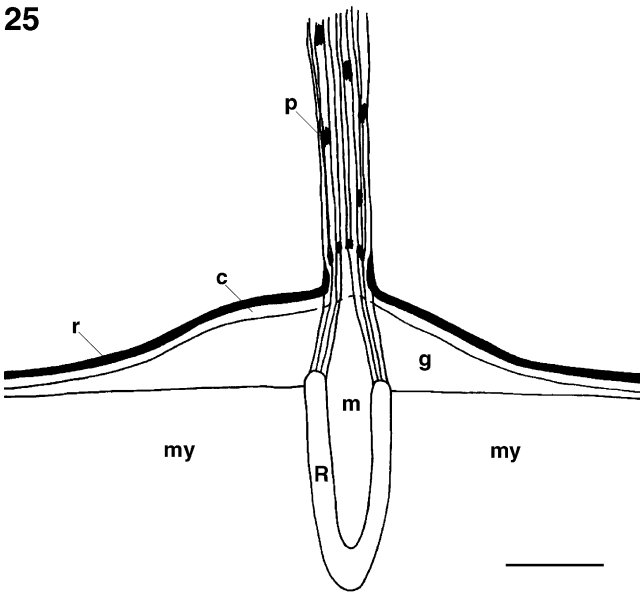


Fig 25 – Diagram showing the spatial relationship between air pore, rhizomorph (R) and mycelial crust, comprising a rind (r) and cortex (c). Gas space in the rhizomorph medulla (m). Parallel air pore hyphae cemented in small patches (p) by extracellular material. Gas space (g) surrounds the hyphae growing on the agar surface under the mycelial crust. Mycelium also grows submerged in the agar (my). Bar = 0.5 mm.

the rind pseudoparenchyma where the cells are thick walled, cemented and melanised suggests low permeability, but the layer is pierced by emergent hyphae and these may provide gas passages, especially if they are damaged.

There is evidence that air pores are common and widespread. Not only are they found in *A. luteobubalina* in culture, but a reassessment of the literature indicates that they occur in cultures of other *Armillaria* species, on or as branches of rhizomorphs, and as extensions of pseudosclerotial plates. For example, structures identical in position and external morphology are visible in illustrations of Kenyan rhizomorph-producing isolates in agar culture (Otieno *et al.* 2003). Illustrations in Garrett (1953) and Mwenje & Ride (1996) show what appear to be air pores. Granlund *et al.* (1984) tentatively identified an air pore on a rhizomorph which previously grew in air. They thought these were side branches that had stopped growing, and compared them with Smith & Griffin's (1971) 'breathing pores', but provided no further information on their structure or development. Intini (1987) showed air pores on rhizomorphs, and commented that their principal function appears to be improvement of gas exchange.

Secondary air pores form on mycelium on the colony surface in our agar cultures, and new rhizomorph clusters arise from them. We have also found air pores directly produced on rhizomorphs of *A. luteobubalina* in agar and soil grown cultures in Petri dishes (M. P., unpubl.). The *A. mellea* rhizomorphs many metres long found floating in water by Goffart (1903-4) and Findlay (1951) also point to a need for some kind of aeration structure at intervals along their length. The little tufts of hyphae associated with rhizomorph branching described by Hartig (1874) may also have been air pores; however, we never saw any air pores develop into rhizomorph branches or fruiting bodies. Air pores in our situations always retained their internal structure even in cultures several months old.

The capacity of rhizomorphs to conduct oxygen was strongly suspected, and the basic structure of rhizomorph-derived breathing pores was previously reported to be simple

Table 2 – Oxygen conductances in cultures of *Armillaria luteobubalina*

(a) Effects of cutting rhizomorphs beneath the agar

Set no.	*n*	Air pores (with uncut rhizomorphs below): conductance	Number of air pores	Conductance per air-pore	Air pores (with cut rhizomorphs below): conductance	Conductance per air pore
1	6	$6^{a} \pm 3$	3 ± 0.3	2 ± 1	$532^{b} \pm 132$	182 ± 55

(b) Conductances of parts of cultures

Set no.	*n*	Plain agar	Agar with pigmented mycelium	Air pores	Number of air pores	Conductance per air pore	Aerial rhizomorph
2	6	$18^{a} \pm 3$	$21^{a} \pm 7$	$987^{b} \pm 86$	4 ± 0.7	291 ± 83	$187^{c} \pm 131$
3	6	n.d.	$7^{a} \pm 12$	$619^{b} \pm 81$	5 ± 0.7	132 ± 27	$34^{a} \pm 26$

(c) Effects of cutting on aerial rhizomorph conductance

Set no.	*n*	Aerial rhizomorph, agar and rhizomorph cut below	Aerial rhizomorph, cut top	Aerial rhizomorph, cut top and below
4	6	$29^{a} \pm 30$	$21^{a} \pm 7$	$212^{a} \pm 94$

(d) Summary of oxygen conductances over all observations

Agar conductance	Pigmented mycelium conductance	Aerial rhizomorph conductance	Air pores conductance	Number of air pores	Conductance per air pore
18 ± 3 (6)	12 ± 6 (18)	56 ± 28 (31)	679 ± 68 (25)	4 ± 0.3 (25)	198 ± 27 (25)

A difference in superscript letter indicates a statistically significant difference ($p < 0.05$) within a row, for the measured conductances only. All conductances are in 10^{-12} m^3 s^{-1}; mean ± standard error of the mean are shown (n.d. = not determined).

tufts of hyphae bursting through the rind of rhizomorphs at the substrate air interface. The mature structure that we have found here is more complex, but nevertheless contains hyphae that have grown through a rind layer. Those hyphae which cross the sub-air-pore space into the basal tissue mound and thence through the spaces in the rind to form the apical cylinder of hyphae appear to have arisen from consolidated pseudoparenchyma around the site of origin of the developing rhizomorphs. The mature structure satisfies the requirement for longevity and survival and, since the crust and the air pore are both hydrophobic, should prevent waterlogging of the air pores even when it is sufficiently wet to cause pooling on the crust surface.

It appears that we have here an elaborate, sophisticated aeration system that extends and incorporates all the mycelial thallus. It meets the following requirements for a good aeration system: abundant gas space relative to tissue volume; good connectivity with all parts that need aeration (including longitudinal channels in conducting organs); and protected entry points that have longevity and survive adverse conditions, that resist waterlogging and yet also survive desiccation, and that have good support so that they do not collapse. The possession of a more elaborate gas space system than was formerly thought perhaps explains the success of some *Armillaria* spp. as pathogens in waterlogged situations. It may also be an important factor in pathogenesis in allowing a mycelium to grow on a wet root surface and to send hyphae or rhizomorphs as inoculum into live roots or cut stumps, in places where conditions may be hypoxic. It would seem that a preoccupation with terminology and a shift in focus to transport of nutrients and water rather than gas have distracted attention from the importance of gas transport in *Armillaria* rhizomorphs, and from their fundamental structural and functional differences from cords of wood-rotting and ectomycorrhizal fungi such as *Phanerochaete velutina* and *Suillus bovinus*. The morphology and anatomy of *Armillaria* rhizomorphs reflect the fact that they are strongly suited for gas transport, a point well understood by early workers, and this is what differentiates them from other structures. Their role in gas transport is supported by an apparent developmental plasticity and ubiquity of air pores, and an aeration system in associated mycelium, that were formerly unappreciated.

Acknowledgements

We thank Danielle Davies, Mark Dickson and Basil Panayotakos for technical assistance, Louise Cole and Mark Curran for generous advice, and the Australian Research Council for research grants.

REFERENCES

Brefeld O, 1877. *Botanische Untersuchungen über Schimmelpilze. III. Heft. Basidiomyceten I*. Verlag von Arthur Felix, Leipzig.

Cairney JWG, Jennings DH, Veltkamp CJ, 1988. Structural differentiation in maturing rhizomorphs of *Armillaria mellea (Tricholomatales)*. *Nova Hedwigia* **46**: 1–25.

Curran M, 1985. Gas movements in the roots of *Avicennia marina* (Forsk.) Vierh. *Australian Journal of Plant Physiology* **12**: 97–108.

De Bary A, 1887. *Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria*. Clarendon Press, Oxford, [English translation by H.E.F. Garnsey, revised by I.B. Balfour].

Findlay WPK, 1951. The development of *Armillaria mellea* rhizomorphs in a water tunnel. *Transactions of the British Mycological Society* **34**: 146–147.

Fox RTV, 2000. Biology and life cycle. In: Fox RTV (ed.), *Armillaria Root Rot: Biology and Control of Honey Fungus*. Intercept Ltd, Andover, pp. 3–44.

Garraway MO, Hüttermann A, Wargo PM, 1991. Ontogeny and physiology. In: Shaw III CG, Kile GA (eds), *Armillaria Root Disease*. USDA, Washington DC, pp. 21–47 [Agriculture Handbook No. 691].

Garrett SD, 1953. Rhizomorph behaviour in *Armillaria mellea* (Vahl) Quél. I. Factors controlling rhizomorph initiation by *A. mellea* in pure culture. *Annals of Botany* **17**: 63–79.

Goffart J, 1903-4. Contribution à l'étude du rhizomorphe de l'*Armillaria mellea* Vahl. *Mémoires couronnés et Mémoires des Savants étrangers publiés par l'Académie Royale des Sciences, des Lettres et des Beaux-Arts de Belgique* **62**: 3–29.

Granlund HI, Jennings DH, Veltkamp K, 1984. Scanning electron microscope studies of rhizomorphs of *Armillaria mellea*. *Nova Hedwigia* **39**: 85–100.

Hartig R, 1874. *Agaricus (Armillaria) melleus* L. In: Hartig R (ed.), *Wichtige Krankheiten der Waldbäume. Beiträge zur Mycologie und Phytopathologie für Botaniker und Forstmänner*. Verlag von Julius Springer, Berlin [English translation by W. Merrill, D.H. Lambert & W. Liese (1974) *Important diseases of forest trees. Contributions to mycology and phytopathology for botanists and foresters*. Phytopathological Classics No. 12. American Phytopathological Society Press, St Paul, MN, pp. 12–41.].

Intini MG, 1987. Le rizomorfe di *Armillaria obscura* al microscopio elettronico a scansione. *Micologia Italiana* **2**: 40–48.

Jacques-Félix M, 1968. Recherches morphologiques, anatomiques, morphogénétiques et physiologiques sur des rhizomorphes de champignons supérieurs et sur le déterminisme de leur formation. Deuxième partie. Recherches sur la morphogenèse des rhizomorphes et télépodes en culture pure. *Bulletin trimestriel de la Société mycologique de France* **84**: 167–307.

Morrison DJ, 1976. Vertical distribution of *Armillaria mellea* rhizomorphs in soil. *Transactions of the British Mycological Society* **66**: 393–399.

Motta JJ, 1969. Cytology and morphogenesis in the rhizomorph of *Armillaria mellea*. *American Journal of Botany* **56**: 610–619.

Motta JJ, 1971. Histochemistry of the rhizomorph meristem of *Armillaria mellea*. *American Journal of Botany* **58**: 80–87.

Motta JJ, Peabody DC, 1982. Rhizomorph cytology and morphogenesis in *Armillaria tabescens*. *Mycologia* **74**: 671–674.

Münch E, 1909. Untersuchungen über Immunität und Krankheitsempfänglichkeit der Holzpflanzen. *Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft* **7**: 54–75; 87–114; 129–160.

Mwenje E, Ride JP, 1996. Morphological and biochemical characterization of *Armillaria* isolates from Zimbabwe. *Plant Pathology* **45**: 1036–1051.

O'Brien TP, McCully ME, 1981. *The Study of Plant Structure Principles and Selected Methods*. Termarcarphi, Melbourne.

Ono K, 1965. *Armillaria* root rot in plantations in Hokkaido. Effects of topography and soil conditions on its occurrence. *Bulletin of the Government Forest Experiment Station* **179**: 1–62 [In Japanese].

Ono K, 1970. Effect of soil conditions on the occurrence of *Armillaria* root rot of Japanese Larch. *Bulletin of the Government Forest Experiment Station* **229**: 123–219 [In Japanese].

Otieno W, Sierra AP, Termorshuizen A, 2003. Characterization of *Armillaria* isolates from tea (*Camellia sinensis*) in Kenya. *Mycologia* **95**: 160–175.

Pareek M, Cole L, Ashford AE, 2001. Variations in structure of aerial and submerged rhizomorphs of *Armillaria luteobubalina* indicate that they may be organs of absorption. *Mycological Research* **105**: 1377–1387.

Rayner ADM, Powell KA, Thompson W, Jennings DH, 1985. Morphogenesis of vegetative organs. In: Moore D, Casselton LA, Wood DA, Frankland JC (eds), *Developmental Biology of Higher Fungi*. Cambridge University Press, Cambridge, pp. 249–279.

Redfern DB, 1973. Growth and behaviour of *Armillaria mellea* rhizomorphs in soil. *Transactions of the British Mycological Society* **61**: 569–581.

Reitsma J, 1932. Studien über *Armillaria mellea* (Vahl) Quél. *Phytopathologisches Zeitschrift* **4**: 461–522.

Rishbeth J, 1968. The growth rate of *Armillaria mellea*. *Transactions of the British Mycological Society* **51**: 575–586.

Rishbeth J, 1978. Effects of soil temperature and atmosphere on growth of *Armillaria* rhizomorphs. *Transactions of the British Mycological Society* **70**: 213–220.

Salema B, Brandão I, 1973. The use of PIPES buffer in the fixation of plant cells for electron microscopy. *Journal of Microscopic Cytology* **5**: 79–96.

Smith AM, Griffin DM, 1971. Oxygen and the ecology of *Armillariella elegans* Heim. *Australian Journal of Biological Science* **24**: 231–262.

Snider PJ, 1959. Stages of development in rhizomorphic thalli of *Armillaria mellea*. *Mycologia* **51**: 693–707.

Sokal RR, Rohlf FJ, 1981. *Biometry*, second ed. W.H. Freeman, New York.

Watkinson SC, 1979. Growth of rhizomorphs, mycelial strands, coremia and sclerotia. In: Burnett JH, Trinci APJ (eds), *Fungal Walls and Hyphal Growth*. Cambridge University Press, Cambridge, pp. 93–113.

Willetts HJ, 1972. The morphogenesis and possible evolutionary origins of fungal sclerotia. *Biological Reviews* **47**: 515–536.

Willetts HJ, 1978. Sclerotium formation. In: Smith JE, Berry DR (eds), *The Filamentous Fungi. Developmental Mycology*, vol. 3. Edward Arnold, London 197–213.

Willetts HJ, Bullock S, 1992. Developmental biology of sclerotia. *Mycological Research* **96**: 801–816.

Worrall JJ, Chet I, Hüttermann A, 1986. Association of rhizomorph formation with laccase activity in *Armillaria* spp. *Journal of General Microbiology* **132**: 2527–2533.