

Location of hyphal differentiation in the agar pore field of the basidiome of *Phellinus contiguus*

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The spatial distribution of differentiated hyphal elements in the 'agar pore field' of the developing basidiome of *Phellinus contiguus* was studied by light and scanning electron microscopy. The first setae and basidia differentiated approximately 3 mm, and the first aerial skeletal hyphae approximately 4 mm behind the submerged mycelial margin. Thereafter formation of new setae and basidia was continuous. Rapid lateral growth of the aerial fascicles which delimited pores occurred 6–7 mm behind the margin. In the pre-dissemination zone there was a weak positive association between the distributions of basidia and setae.

Corner (1932*a*) defined the pore field in pileate polypore fungi as an annulus near the margin on the underside of the pileus in which localized development of different modes of hyphal growth resulted in initiation of pore dissepiments and delimitation of pore areas. Nuss (1980) extended this concept to a wider range of Aphyllophorales, using the term hymenophore field. By analogy an 'agar pore field' can be distinguished close behind the mycelial margin in light-grown agar cultures of the resupinate basidiome of *Phellinus contiguus* (Pers.:Fr.) Pat. (Butler, 1988). This differs from the pore field of pileate species in the absence of a distinct region of sterile basidiome tissue on which the pore dissepiments develop.

P. contiguus may be a useful model system in which to investigate developmental patterns in the pore field. It is first necessary to establish the location within the agar pore field of differentiation of hymenial and dissepiment hyphae. Corner (1953) described differentiation of the first hymenial elements in pore bases more or less simultaneously with initiation of the pore dissepiments in the pore field. In dimittic and trimitic species differentiation of hyphal systems in the pileal trama preceded hymenophore differentiation.

This paper describes investigations of the location of development of different hyphal types in the agar pore field of *P. contiguus*.

MATERIALS AND METHODS

The strain of *P. contiguus* and the culture system were as used in previous work (Butler & Wood, 1988; Butler, 1988). Cultures were grown in Petri dishes on 2% malt extract agar in a controlled environment room at 25 °C with light between 400 and 700 nm at 26 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 18 h d^{-1} and at 1.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 6 h d^{-1} . Dark cultures were grown in

black paper envelopes and light cultures in similar envelopes with transparent acetate windows.

Marginal samples of 4- to 6-wk-old colonies were taken between 5 and 2 h before the end of the period of high light intensity and mounted in aniline blue in lactic acid. Two or four samples were taken from each of between 3 and 6 replicate cultures. In the whole mount method 15 mm agar squares were covered with a few drops of 70% ethanol before draining and mounting. Thin vertical radial slices of colony margins were prepared similarly. In the Sellotape method approx 15 × 15 mm squares of Sellotape were pressed on the colony surface, with one edge parallel to the colony margin, before mounting.

At each sampling position the location of differentiated hyphae and the density of setae were recorded using an eyepiece graticule with 10 × 10 squares each of side 127.5 μm . Data were recorded for successive rows of 10 squares aligned parallel to the mycelial margin. The extent of dissepiment tissue was scored as the number of squares in which this tissue occupied at least half of the square area, as estimated by eye. Maximum seta density in the pre-dissepiment zone was the maximum number occurring in any one 10 × 10 squares per sample. Regression analysis on changes in seta density in the pre-dissepiment zone was carried out on the rolling totals for 10 × 10 squares, in steps of one row of squares and starting with the first 10 × 10 squares in which one or more setae was present in the most marginal row. For measurements of seta density in pore bases one eyepiece graticule square was placed over the centre of each pore base.

For analysis of the degree of association between the distributions of basidia and setae the presence or absence of each structure in each square was recorded for two adjacent rows of 10 squares aligned parallel to the mycelial margin and 1275 μm inside the position of the seta nearest the margin.

Statistical analysis was by a contingency χ^2 test (Greig-Smith, 1983).

Material for SEM was prepared as in Butler (1988) using glutaraldehyde fixation and critical point drying.

Table 1. Mean nearest distance (mm) and s.e.m. of various structures behind the aerial mycelial margin of light and dark-grown colonies of *P. contiguus*

Structure	Light	Dark
Whole agar samples (2 from each of 6 light- and 3 dark-grown dishes)		
Submerged hyphae	-1.073 ± 0.055*	-1.381 ± 0.077
Setae	2.242 ± 0.102	2.678 ± 0.447
Groups of basidia	3.273 ± 0.201	None
Dissepiment tissue (stage I)	5.493 ± 0.274	None
Dissepiment tissue (stage II)	6.031 ± 0.261†	None
Sellotape samples (4 from each of 4 dishes per treatment)		
Setae	1.857 ± 0.176	2.450 ± 0.201‡
Basidia	1.928 ± 0.141	None
Thick-walled hyphae	3.044 ± 0.356	3.092 ± 0.487‡

* Minus sign indicates structures in front of the aerial margin.
† Excluding 2 out of 12 samples in which dissepiment tissue did not reach stage II within 7 mm of the aerial margin.
‡ Excluding 2 (setae) and 8 (thick-walled hyphae) samples out of 16 in which these structures did not occur within 6 mm of the aerial margin.

Table 2. Mean density and s.e.m. of setae in the pre-dissepiment setal zone of light and dark-grown colonies of *P. contiguus* (samples as in Table 1)

	Light	Dark
Mean maximum seta density (no. mm ⁻²)	34.40 ± 6.19	2.56 ± 0.64
Number of samples (out of 12) showing significant increase in seta density across pre-dissepiment seta zone	12	Not tested
Mean rate of increase in seta density (no. mm ⁻² mm ⁻¹)	10.35 ± 2.89	—

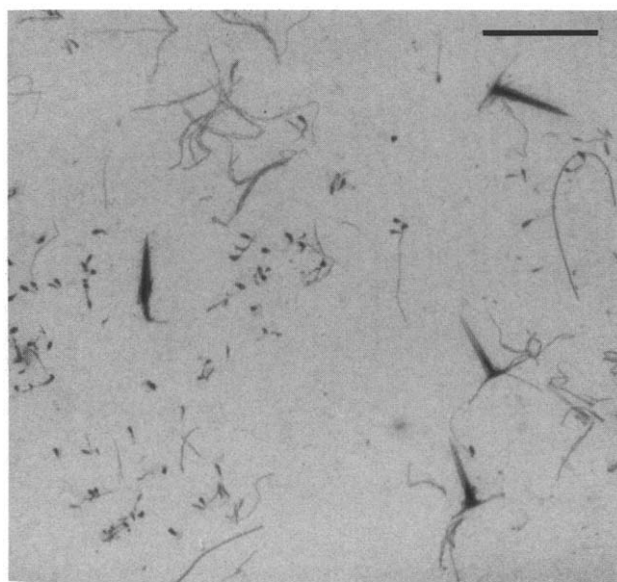


Fig. 1. Sellotape pull preparation from the pre-dissepiment zone of *P. contiguus* showing aerial hyphae, setae and basidia (scale bar, 100 µm).

Table 3. Seta density in the bases of developing pores at different positions in light-grown colonies of *P. contiguus*

Position	Seta density (no. mm ⁻²)
0 (pores first recognizable)	139.0 ± 12.50*
10 mm behind	342.0 ± 15.19

* Mean + s.e.m. from 20 pore bases from each of five colonies.

RESULTS

All the light grown colonies had an annulus of paler-coloured developing basidiome tissue between the brown area of defined pores and the extending colony margin. Dissepiment tissue and basidia were only formed in the light (Table 1). Although some setae occurred in the dark, their density was small in comparison with that in the light (Table 2).

In the light dissepiment tissue first occupied the minimum area defined in this work, namely more than half the area of 1 out of 10 quadrat squares (stage I), 5.5 mm behind the aerial mycelial margin. Behind this there was usually a dramatic increase in amount of dissepiment tissue and stage II in which 5 out of 10 quadrat squares were at least half-filled with dissepiment tissue, occurred 6 mm behind the aerial margin (Table 1). At this stage the pore areas were more or less defined but the dissepiments were incomplete. Both setae and clusters of basidia occurred closer to the aerial margin with the first setae significantly nearer (Table 1). In whole mounts solitary immature basidia were difficult to distinguish from undifferentiated branch tips and Sellotape pulls provided a clearer view of the location of hyphal differentiation (Fig. 1). In these preparations although setae tended to occur nearer the aerial margin than basidia (Table 1) the difference was not statistically significant. Sellotape collected 60–70% of the setae and an unknown proportion of the basidia. Aerial hyphae were formed in both light and dark-grown cultures and it was not possible to distinguish the first dissepiment hyphae from other aerial hyphae. Thick-walled uninflated hyphae also occurred, mainly as skeletal hyphae, i.e. thick-walled unbranched apical compartments with thin-walled tips. These were present in teased out dissepiment tissue and were common on Sellotape pull preparations from the pore region, of extending dissepiments. They occurred less frequently in preparations from younger pore field regions, the first ones being detected significantly, and approximately 1 mm, behind the first setae and basidia (Table 1, $p < 0.01$). Within the agar thick-walled hyphae were absent from the mycelial margin and were rarely detected nearer to the margin than the first aerial aggregations which formed dissepiment tissue.

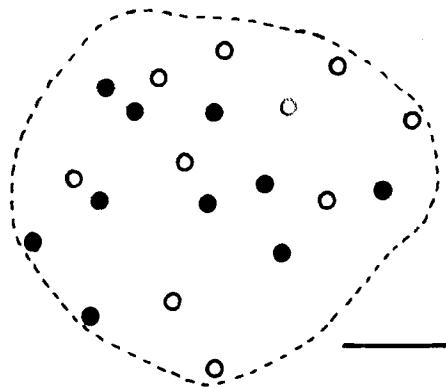
Thus there was a recognizable pre-dissepiment zone in which basidia, setae and skeletal hyphae were distinguishable, situated between the undifferentiated marginal zone and the zone of substantial dissepiments. This zone was approximately 3 mm wide and was highlighted in Sellotape pull preparations by the abundance of setae and basidia in comparison with both older and younger regions. In older regions few setae and basidia were picked up from the dissepiment edges.

New basidia and basidiospores were produced daily not only in the dissepiment zone but also in this pre-dissepiment zone. In all samples the density of setae increased significantly

Table 4. Changes over time in seta density in the bases of developing pores at one position in light-grown colonies of *P. contiguum*

Time	Seta density (no. mm ⁻²)
0 (pores first recognizable)	119.9 ± 8.87*
8 d later	228.4 ± 11.0

* Mean + s.e.m. from 20 pore bases from each of four colonies.

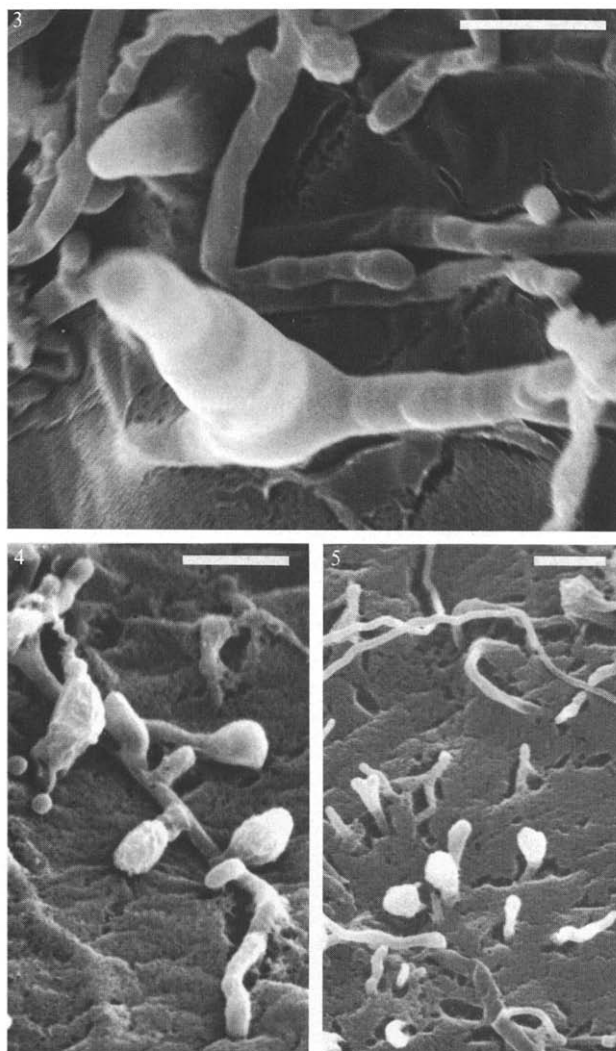
**Fig. 2.** Diagram of the location of setae in the base of a developing pore of *P. contiguum* at time 0 (open circles) and 6 d later (new setae as solid circles). Scale bar, 50 µm.**Table 5.** Association analysis of occurrence of setae and basidia in the pre-dissepiment zone of *P. contiguum*

No. cultures	No. quadrats/culture	Quadrat size (µm ²)	Mean frequency of quadrats			χ ²	Probability	
			With basidia	With setae				
				Basidial	Non-basidial			
10	200–400	4096	0.43	0.14	0.07	Range	< 0.01 (4) [†]	
							7.76–	< 0.05 (1)
							0.18	n.s. (5)
1	480	2430	0.53	0.086	0.036	2.62	n.s.	
	240	4861	0.69	0.15	0.054	3.63	n.s.	
	120	9722	0.83	0.28	0.048	4.02	< 0.05	

[†] Number of cultures in parentheses.

from the younger to the older side of the pre-dissepiment zone and the mean rate of increase was 10.35 mm⁻² mm⁻¹ (Table 2, $p < 0.001$ for 10 samples, 0.001–0.01 and 0.01–0.05 for one sample each). In older parts of the basidiome there was a further significant increase in seta density in the bases of developing pores. This increase was evident not only in comparisons between different positions in the extending basidiome (Table 3, $p < 0.001$) but also at the same location at successive times (Table 4, $p < 0.001$). This finding was confirmed by time lapse observation of the same living pores, in which new setae were interpolated between existing setae (Fig. 2).

In the pre-dissepiment zone basidia and setae could be seen to arise from segments of the sparse surface and submerged hyphae (Figs 3–5). In this region some of the basidia matured to form spores (Fig. 4). At this stage the two types of structure usually arose from separate portions of hyphae. Basidia tended

**Fig. 3.** SEM views of setae and basidia in the pre-dissepiment zone of *P. contiguum* (scale bars, 10 µm). **Fig. 3.** Foreshortened view of setae showing origin from a surface hyphal compartment (lower seta) and the tip region of a seta which arose from a hypha submerged in the agar. **Fig. 4.** Basidia arising from a surface hypha. **Fig. 5.** Basidia emerging through agar surface from a submerged hyphal origin.

to occur in irregular patches within the pre-dissepiment zone but this pattern did not match the pattern of pores and dissepiments in older regions. An association analysis of the spatial distribution of setae and basidia was carried out using a quadrat size which in mature pore areas yielded 26% entirely pore, 36% entirely dissepiment and 38% mixed pore and dissepiment quadrats. At a sampling position 1275 µm inside the position of the most marginal seta, basidia occurred in 43% of quadrats, many more than the 10% of quadrats with setae (Table 5). A χ^2 test revealed significant heterogeneity between dishes in the distribution pattern of basidia and setae. Within dishes there was evidence of a weak positive association between occurrence of setae and basidia, with a significant association in 5 out of 10 dishes (Table 5). However setae were only twice as frequent in basidial (14%) than in non-basidial (7%) quadrats. Where the effect of quadrat size was tested, the χ^2 value increased with increasing quadrat size and was only significant at the largest size (Table 5).

DISCUSSION

Since the mycelial margin and basidiome tissue were extending at similar rates (0.93 mm d^{-1} , Butler, 1988), the observed spatial sequence can be taken to represent the time sequence of development. Samples were taken when the daily crop of basidiospores were maturing, giving maximum recognition of basidia.

This study confirms the development of pore dissepiments in *P. contiguus* from discrete aerial fascicles, i.e. clavarioid bundles of Reijnders (1991). There are two morphological clues to the first differentiation amongst aerial hyphae of the dissepiment-forming hyphae, namely convergent growth and differentiation of aerial skeletal hyphae. The onset of convergent growth is difficult to define but the first skeletal hyphae occurred significantly behind, and more than one day later, than the first hymenial elements. The earliest morphological differentiation was of the hymenial elements, setae and basidia arising directly from compartments of approximately 3-d-old divergently-growing mycelium (Gregory, 1984). At this stage both basidia and setae were sparse and there was no clear subhymenium. However, mature basidia with basidiospores were present. In the pore bases this discontinuous hymenium (Donk, 1964) gradually became more continuous as more basidia, and also setae, differentiated in parallel with dissepiment growth.

The developmental stage at which the first basidia mature has not been recorded in many of the Aphyllophorales. In a pileate member of the Hymenochaetaceae described by Corner (1932*b*) as *Fomes levigatus* Corner the pore and dissepiment areas could be distinguished before the first basidia with basidiospores. Similar earlier definition of pore areas has been described more recently for a pileate member of the Ganodermataceae, *Ganoderma lucidum* (Leysser ex Fr.) P. Karsten (Mims & Seabury, 1989). However Raudaskoski & Vauras (1982) illustrate a basidium with sterigmata in a developing basidiome 15 h after light induction in a culture of *Schizophyllum commune* Fr. Basidiospore production was subsequently delayed by a layer of slime until growth of the gills.

The formation of basidia directly from divergently growing vegetative mycelium has been reported in a variety of homobasidiomycetes mainly in cultures (Donk, 1964; McLaughlin *et al.*, 1978; Yamanaka & Sagara, 1990). However, in all these examples basidium formation is separated from other developmental processes of the basidiome. In cultures of *P. contiguus* the whole morphogenetic sequence is switched on, with continuous development to the complete polyporous basidiome. The Hymenochaetaceae include a wide range of basidiome morphology (Donk, 1964, 1971*a*) and such a switch could be important in their evolution (Donk, 1971*b*, Nuss, 1986).

The failure to recognize visually potential pore and potential dissepiment areas in the distribution of basidia and setae in the pre-dissepiment zone could be due either to the sparsity of these structures making recognition of pattern difficult or to the absence of a pore pattern at this stage. The setae would be poor indicators of such a spatial pattern since their mean maximum density in the pre-dissepiment zone (34 mm^{-2}) in

comparison with the mean pore density in such cultures ($5.3\text{--}9.9 \text{ mm}^{-2}$, Butler & Wood, 1988) is such that there would be only 3–6 setae per potential pore area. Moreover tramal setae are reported in this species (Ryvarden, 1978). However, basidia are much more abundant and their visual distribution supports the latter hypothesis, of absence of circumscribed pore areas. This is in agreement with previous observations that during lateral extension the aerial fascicles sometimes overgrow areas with basidia (Butler, 1988).

The rate of development of new setae was at a similar order of magnitude in both the pre-dissepiment and pore zones (10 and $20 \text{ setae mm}^{-2} \text{ mm}^{-1}$ respectively). This continuous development of setae as well as basidia is at the opposite pole of the range of hymenial development amongst basidiomycetes from the synchronized development in *Coprinus cinereus* (Schaeff. ex Fr.) S. F. Gray (Rosin & Moore, 1985; Horner & Moore, 1987), and is characteristic of many Aphyllophorales (Donk, 1964). Any model seeking to explain setal distribution in terms of spatial pattern of activators and inhibitors would need to include temporal changes in these activities (Meinhardt, 1984). This continuous development also means that care must be taken in sampling for the study of spatial pattern. In comparing the distribution of basidia and setae an attempt was made to take samples from a specific region in the pre-dissepiment zone. The looseness of the association between the distributions of these first-formed setae and basidia, taken together with the observation of their origin from non-adjacent compartments of the divergent mycelium, suggests independent initiation within larger, perhaps pre-hymenial patches. The clusters of basidia which arise sympodially from subsetal hyphae in the hymenochaetacean species *Polystictus microcycclus* Corner (Corner, 1953) do not occur at this earliest stage of pore field development.

There is thus evidence that three kinds of hyphae, setae, basidia and dissepiment hyphae, arise at separate locations in the agar pore field from divergently growing hyphae and later become organized into the characteristic polyporous form.

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