

Cell population dynamics in *Coprinus cinereus*: narrow and inflated hyphae in the basidiome stipe

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The stipes of post-meiotic basidiomes of *Coprinus cinereus* always contained a significant numerical proportion (23–54%) of narrow (cross-sectional area < 20 μm^2) as well as inflated hyphae, although the former only contributed 1–4% to the overall cross-sectional area of the stipe. At the extreme bases and apices of elongating stipes, the central region was occupied exclusively by narrow hyphae, which were also concentrated on the outside of the stipe and fringing the central lumen. Elsewhere, narrow hyphae were interspersed between the inflated hyphae.

Nearest-neighbour analysis showed that inflated hyphae were evenly rather than randomly distributed regardless of the age of the basidiome (27–70 mm tall) and position within the stipe. The spatial distribution of narrow hyphae differed significantly from randomness only in the upper middle and upper apical regions of 27 mm and 70 mm tall basidiomes, respectively, where their distribution was more even. The proportion of narrow hyphae decreased with time, possibly due to some becoming inflated. The even distribution of inflated hyphae could be due to some sort of organizational control over their pattern of differentiation from narrow hyphae. During normal stipe extension the greatest cell expansion was seen in the inflated cells situated between the mid-cortex and the lumen, and not at the periphery of the stipe. Narrow hyphae tended to stain differentially and to have varied spatial arrangements, suggesting that although morphologically alike, they serve distinct functions.

Although *Coprinus* is one of the most studied fungal genera there is surprisingly little information concerning the structure of the stipe of its basidiomes, other than that they are composed of greatly inflated and elongated cells. Correspondingly, the most comprehensive description of stipe structure in *Coprinus cinereus* (Schaeff.: Fr.) S. F. Gray appears to be the following: 'The stipe includes a central column of dikaryotic hyphae and a cortex of giant multinucleate cells' (Lu, 1974). However, Gooday (1975) noted the presence of narrow hyphae showing apical labelling with *N*-acetylglucosamine (a precursor of chitin) rather than the uniformly distributed labelling shown by the other, inflated hyphae. Lu's (1974) observation referred to a pre-meiotic basidiome and implies that the cortex is made up exclusively of inflated cells. It is not: the cortex comprises both narrow hyphae and inflated cells. However, there is no clear account of the distribution of narrow cortical hyphae, and where they have been observed they tend to be dismissed as fragments of generative hyphae from the young primordium (A. F. M. Reijnders, personal communication). The aim of the present study was therefore to obtain a clearer picture of the quantitative significance, distribution and function of the narrow hyphae.

MATERIALS AND METHODS

Organism and culture methods

All experiments were conducted with the 'Meathop' dikaryon of *Coprinus cinereus* and cultured as described by Hammad *et al.* (1993).

Video recording

VHS video recordings were made of basidiome growth under low-intensity illumination. Measurements of stipe height (= basidiome overall height), stipe width, pileus height and pileus width were made directly on the video screen. In the first videos, where the whole of the growth of the basidiome was recorded in a single take, the screen image used for measurement was 0.82 times natural size. Later cultures were filmed so that screen images were 1.3 times natural size. Images were sampled routinely at 10 min intervals, and at shorter intervals when graphical analysis required intermediate data points.

Microscopic preparations

Stipe segments, ca 5 mm long, and other small pieces of basidiomes at various stages of development were fixed in formol–acetic acid–alcohol (FAA; 95 ml 50% ethanol + 5 ml

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10% formalin + 3 ml glacial acetic acid + 1 drop of detergent), infiltration being enhanced by 20 s exposure to reduced pressure. The tissue was dehydrated through an ethanol series, then placed in acetone for 1 h before being embedded in 'Historesin' (Reichert–Jung) following the manufacturer's instructions. The resin block was mounted on a perspex block using cyanoacrylate adhesive. Sections (5 µm) were cut on an LKB 2218 Historange microtome using glass knives. Both longitudinal and transverse sections of different tissues within the basidiome were cut. The sections were floated on to drops of distilled water on a slide and dried on a hot block. They were then stained. Twenty-four histological staining procedures were tested, but most use was found for Mayer's haemalum and the periodic acid–Schiff reagent.

Video micrography and image analysis

Light-microscope preparations were also observed using a video camera attached to the microscope. To provide illustrations, video frames were digitized with a Screen Machine real-time video digitizer card and software (Magnifeye, Studio 6, Walmer Studios, 235–239 Walmer Road, London W11 4EY) and transferred to film with a Polaroid Image Recorder. For numerical analyses video images were brought into an Opus PC computer using a Skye SI733 video digitizing card and Skye SI730 image analysis software (Skye Instruments Ltd, Unit 5, Ddole Industrial Estate, Llandrindod Wells, Powys LD1 6DF).

Preparation of material for SEM

For cryo-SEM, small pieces of basidiome tissue were excised, immediately frozen in nitrogen slush (−210 °C) and then transferred rapidly to the cryo pre-chamber of a Cambridge 200 SEM. Each specimen was initially observed uncoated; where ice crystals were present these were sublimed by raising the temperature to −80°. The specimen was then withdrawn from the pre-chamber and coated with gold before more extensive examination at −170°.

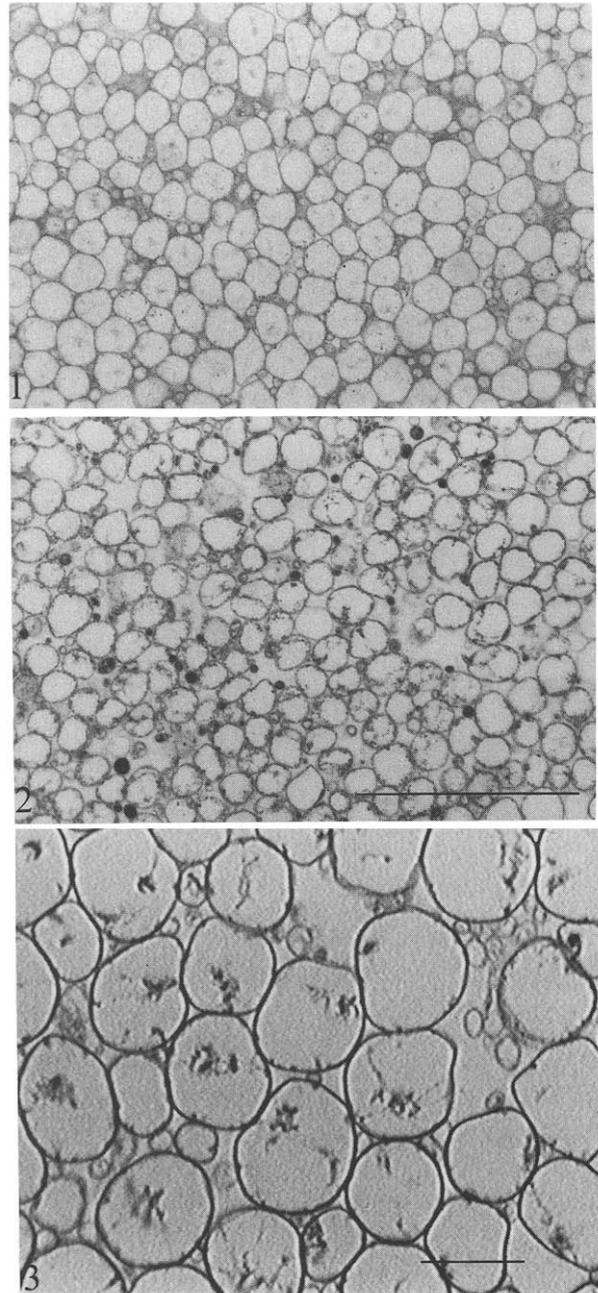
For conventional SEM the specimens were rapidly frozen in nitrogen slush before transferring to the chamber and freeze-drying at −60°. The dried specimens were mounted on silver DAG-coated stubs before sputter-coating with gold, and observed using a Cambridge 90 SEM. Alternatively, specimens were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) for 2 h and washed in 0.1 M sodium cacodylate buffer for 5 min before secondary fixation using 1% osmium tetroxide in the same buffer (1 h). The specimens were washed again for 5 min in the cacodylate buffer, dehydrated through an alcohol series and critical-point dried, then mounted and viewed as before.

RESULTS

Overview of stipe structure in transverse and longitudinal sections

Low-magnification images of transverse sections of stipes of any basidiome more than a few mm tall were dominated by

highly inflated cells (Figs 1, 2), but also had a scattering of very much narrower hyphal profiles, more evident at higher magnification (Fig. 3). The narrow hyphae were also clearly visible in longitudinal sections and SEM images (Figs 4–9) and selectively stained by Mayer's haemalum, the periodic acid–Schiff reagent (PAS), toluidine blue, Delafield's haema-



Figs 1–3. Light micrographs of transverse sections of the stipe of *Coprinus cinereus*. **Figs 1 and 2** are conventional light micrographs (from Kher, 1992). **Fig. 1** was stained with the periodic acid–Schiff reagent and **Fig. 2** with toluidine blue. Note that narrow hyphae are scattered among the inflated ones throughout the field of view. **Fig. 2** in particular shows that narrow hyphae stain heterogeneously (scale bar = 100 µm). **Fig. 3** is a light micrograph digitized from video images with Screen Machine. The section was stained with Mayer's haemalum. Narrow hyphae are present in the interstices between most inflated hyphae (scale bar, 5 µm).

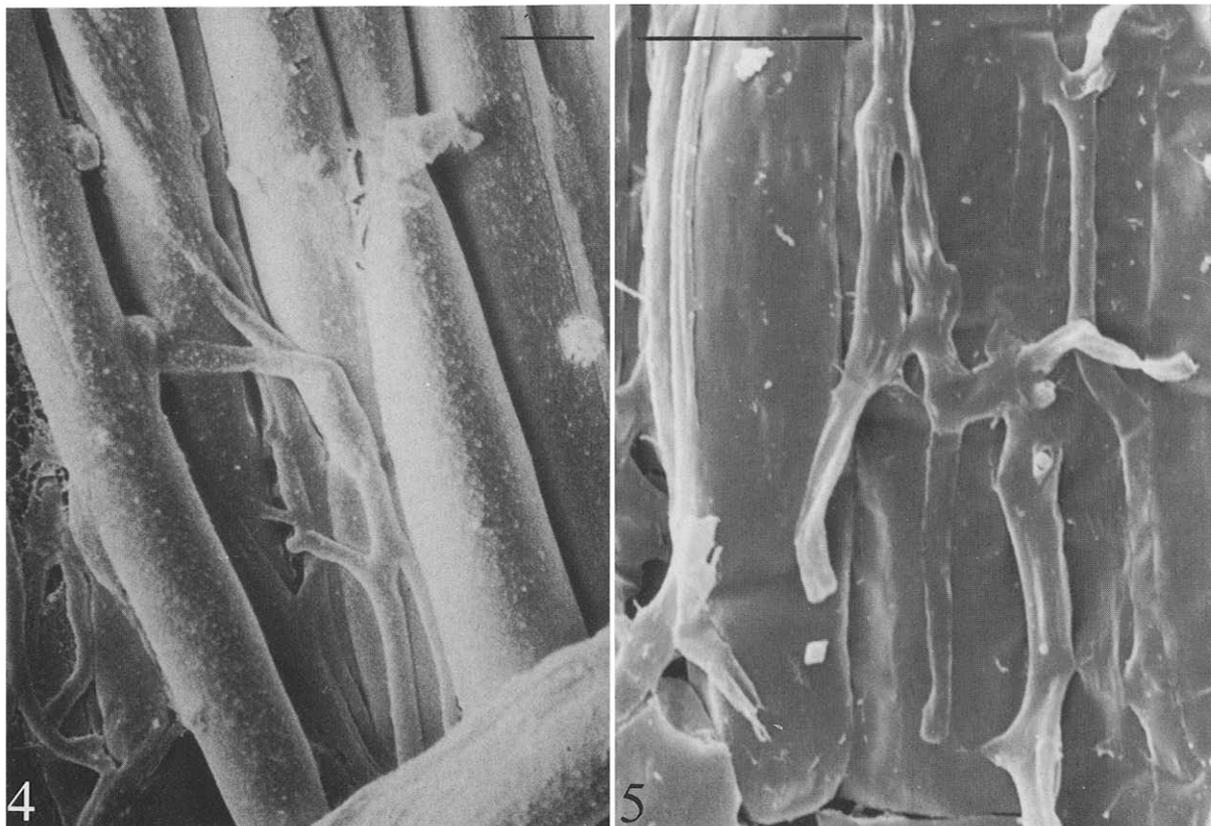


Fig. 4. A cryo-SEM image of a stipe fractured longitudinally (from Kher, 1992). Narrow hyphae are interwoven with their inflated counterparts (scale bar, 20 μm). **Fig. 5.** An SEM image of a longitudinally fractured stipe after chemical fixation and critical-point drying. Despite the collapse of the cells narrow hyphae can be seen clearly to be associated in networks amongst the inflated hyphae (scale bar, 20 μm).

toxylin, 0.5% (w/v) acid fuchsin in 0.5% (v/v) acetic acid, alcian blue/safranin, Luxol fast blue, and 1% aqueous acid fuchsin. Alcian blue and aniline blue were non-selective light general stains, and stains with no specificity and a high background were: light green, Mallory A, Mallory B, phosphotungstic acid/haematoxylin, Mann's methylene blue/eosin, lissamine rhodamine B, lissamine green, eosin, eosin/methylene blue and cotton blue/lactophenol. Neutral red, Verhoeff's iodine, Sudan black and celestine blue did not stain the preparations.

Although Mayer's haemalum and the periodic acid-Schiff reagent both stained narrow hyphae much more intensely than the inflated hyphae, these and all other staining reactions were differential, in that only some narrow hyphae were stained in any one transverse section and adjacent compartments in longitudinal sections could stain differently (Figs 6–9).

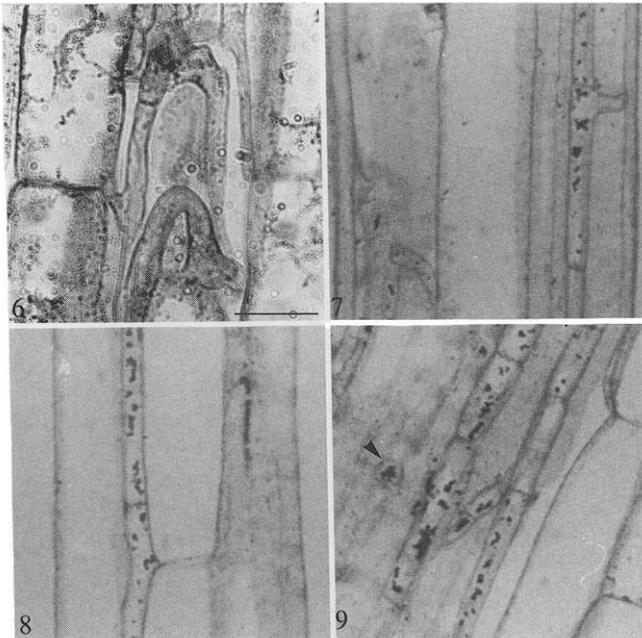
Cell-size spectrum in transverse sections of stipes

Cross-sectional areas of hyphal profiles in 5 μm -thick sections were measured using an image analysis program (Skye Instruments Ltd). Transverse sections were cut from stipes of basidiomes of a range of developmental ages. The younger stipes (i.e. pre-rapid elongation phase) were divided up into three pieces (base, middle and apex) and sections cut from

each. Elongated stipes (up to 70 mm long) were divided into as many as six pieces. For each section of each piece of stipe, the area of every cell within two randomly chosen radial transects 12 μm wide was measured. Individual cells in a transect were measured in strict order, starting from the exterior of the stipe and ending at the lumen. Fig. 10 shows an example transect and Figs 11 and 12 the graphical plots derived from it.

For analysis of the size spectrum of the cells, the rank order in the transect was ignored and their cross-sectional areas were grouped together in frequency classes of 10 μm^2 , the number of cells falling within each frequency class being expressed as a percentage of the total number of cells in the transect (see Figs 12 and 13). In all transects the 0–10 and 10–20 μm^2 classes were the most numerous and the cell area of larger hyphae was widely dispersed. The frequency distribution, in 5 μm^2 classes, of the combined data for 3794 cells is shown in Fig. 14. On the basis of these frequency distributions two distinct populations of hyphae were identified and categorized as narrow hyphae, with cross-sectional area < 20 μm^2 , and inflated hyphae, with cross-sectional area \geq 20 μm^2 .

Narrow hyphae always constituted a significant numerical proportion (23–54%) of the cells in transverse sections of the stipe, but only contributed 1–4% of the overall cross-sectional area (Table 1).



Figs 6–9. Light micrographs of longitudinal sections of the stipe of *Coprinus cinereus*. **Figs 7–9** were digitized from video images with Screen Machine. **Fig. 6** was stained with Mayer’s haemalum and shows network formation by the narrow hyphae. **Figs 7–9** were stained with the periodic acid–Schiff reagent, and it can be seen that narrow hyphae show differential staining. The narrow hyphae stain intensely while the inflated hyphae remain unstained. **Fig. 7** shows that adjacent narrow hyphal compartments may also stain differentially. The intensely staining narrow hypha in this figure has formed a lateral branch. **Fig. 9** also shows branch formation by the narrow hyphae. The arrow indicates an intensely staining narrow hypha in cross-section which was running at right angles to the longitudinal axis of the stipe (scale bar, 5 μm).

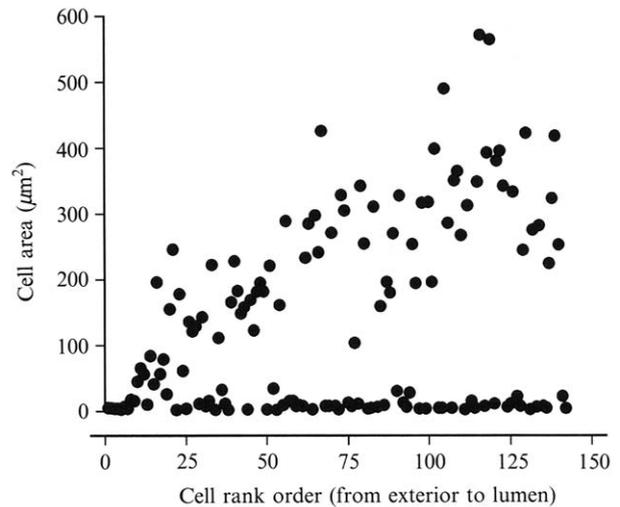


Fig. 11. Plot comparing cell cross-sectional area with position of the cell in the transect (= rank order) for the transect illustrated in Fig. 10.

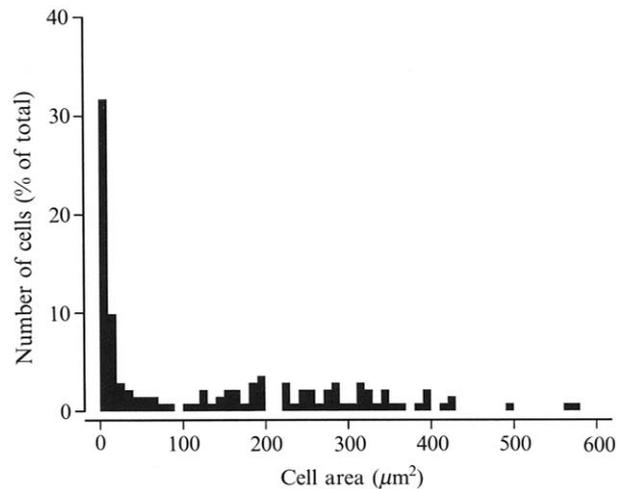


Fig. 12. Plot showing the cell size distribution of the transect illustrated in Fig. 10.

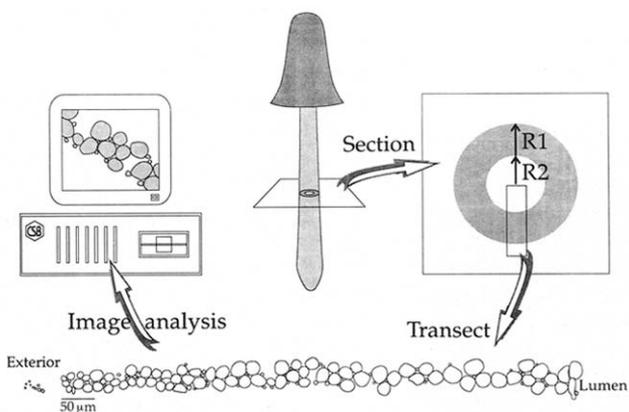


Fig. 10. Determination of cell population distributions. The diagram shows how glycolmethacrylate sections of the stipe were used for image analysis of cell cross-sectional areas. R1 and R2 refer to the radial extent of stipe tissue and stipe lumen, respectively, which are shown in Table 1. Transects were routinely 12 μm wide; a wider transect is shown here for illustrative convenience.

Spatial distribution of inflated and narrow hyphae

To analyse spatial distributions of cells in transverse sections of the stipes, cell area was plotted against cell rank order (where cell number one is deemed to be at the exterior of the

stipe and the last cell is at the lumen end of the radius, see Figs 10 and 11). The data set in Fig. 11 was representative of other transects and other basidiomes (Fig. 15). Narrow hyphae were particularly present coating the outside of the stipe and lining the lumen, but were also dispersed throughout the remaining tissue. Where no lumen was present, the central region was occupied exclusively by narrow hyphae, both in basidiomes < 6 mm tall in which the lumen had not yet developed (Fig. 16), and at the extreme apex of mature basidiomes above a well-developed lumen (Fig. 17).

These populations of hyphae may be randomly distributed, evenly distributed or clumped together. In a random distribution the presence of one individual does not affect the probability of another occurring near by; in an even distribution the probability is lowered, while in a clumped distribution it is raised. The nearest-neighbour method was used to determine the spatial distributions of the two populations of hyphae. Individual cells were chosen using random co-ordinates and the distance between the centre of

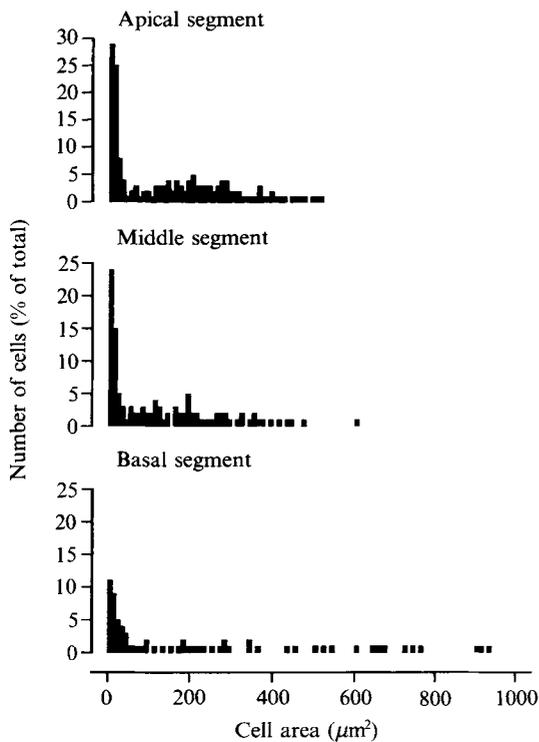


Fig. 13. Cell-size (cross-sectional area) frequency distributions of cells in transects of sections cut from the apical, middle and basal zones of a 45 mm long stipe. Note that throughout the fruit body narrow hyphae (up to 20 µm² in cross-sectional area, represented by the first two categories in the frequency histograms) were a major component of the hyphal population.

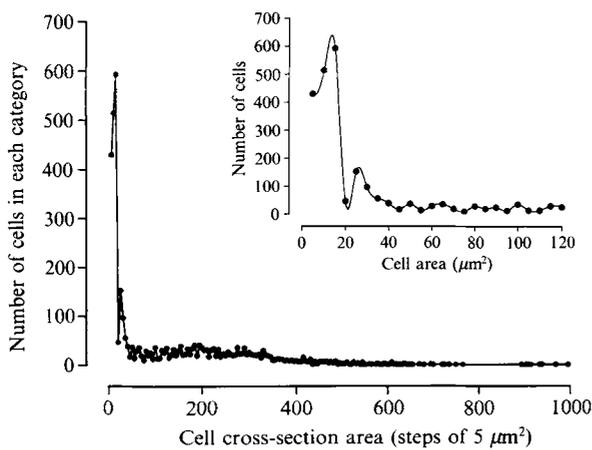


Fig. 14. Frequency distribution of cell cross-sectional areas in the cumulated data from all the transects analysed. A total of 3794 cells are represented here. Inset shows the clear demarcation between cell populations at the 20 µm² category.

this hypha and the centre of the nearest hypha of the same population (i.e. narrow or inflated) was measured (Clark & Evans, 1954). These observed values were compared with distances to the nearest-neighbour hyphae of the same population calculated on the assumption of a random distribution (Table 2). The inflated hyphae were very strongly evenly distributed, regardless of the age of the basidiome (from 27–70 mm tall) and regardless of position within the

Table 1. Comparison of the numbers of narrow hyphae and the area they contributed to the total area of cells in the transect for basidiomes of *Coprinus cinereus* at different stages of development and at different positions within each basidiome

Stipe length (mm)	Zone	Narrow hyphae (% of total)	% area contributed by narrow hyphae	Number of cells in the transect	R1* (tissue) (µm)	R2* (lumen) (µm)
6	Middle (full diameter)	47.2	4.2	496	1664	546
27	Apical	48.2	3.4	164		
	Apical	50.3	3.2	145	832	416
	Upper mid-region	45.1	3.4	173		
	Upper mid-region	46.6	3.0	189	966	312
	Lower mid-region	41.3	3.3	104		
	Lower mid-region	31.3	2.3	80	989	884
	Basal	45.2	3.9	146		
	Basal	40.4	4.4	146	839	780
27	Mean values	43.6	3.4			
45	Apical	38.8	2.7	165		
	Apical	32.6	2.2	132	1173	832
	Middle	34.5	1.7	87		
	Middle	40.2	3.9	117	973	1768
	Basal	27.8	1.4	54		
	Basal	37.1	1.5	62	875	ND
45	Mean values	35.2	2.2			
70	Apex (full diameter)	53.8	4.0	318	691	748
	Apical	23.8	1.1	105		
	Apical	28.7	1.6	108	856	1300
	Upper mid-region	25.0	1.4	104		
	Upper mid-region	23.3	1.4	103	821	1716
	Lower mid-region	30.8	1.7	117		
	Lower mid-region	29.9	1.6	107	834	1872
	Upper basal	38.7	2.5	119		
	Upper basal	37.8	2.0	111	948	1664
	Lower basal	43.6	2.6	117		
	Lower basal	50.0	2.8	152	1153	1092
70	Mean values	33.2	1.9			

The 6 mm basidiome was at a pre-karyogamy stage. Sporulation was occurring in the 27 mm long basidiome and had been completed in the 45 and 70 mm long basidiomes. Entries in the table show total cell numbers and narrow hypha proportions for radial transects except for the 6 mm and 70 mm apex transects, which were full diameters.

* R1 and R2 refer to the actual length of the radii spanning stipe tissue and lumen respectively (see diagram in Fig. 10). ND, not determined (specimen was damaged).

stipe. On the other hand, the spatial distribution of narrow hyphae differed significantly from randomness only in the upper middle region of the 27 mm tall basidiome and the upper apical region of the 70 mm tall basidiome, where there were slight tendencies towards even distributions.

Changes in the pattern of hyphal distribution

Generally, narrow hyphae were interspersed with inflated hyphae across the full radius of all stipes irrespective of

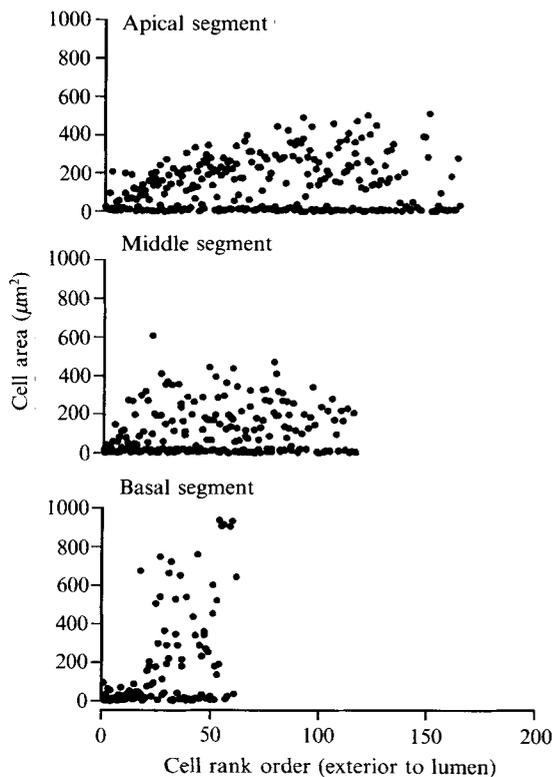


Fig. 15. Rank-order plots for transects of a 45 mm fruit body (same data as Fig. 13). Note that narrow hyphae were present throughout the fruit body.

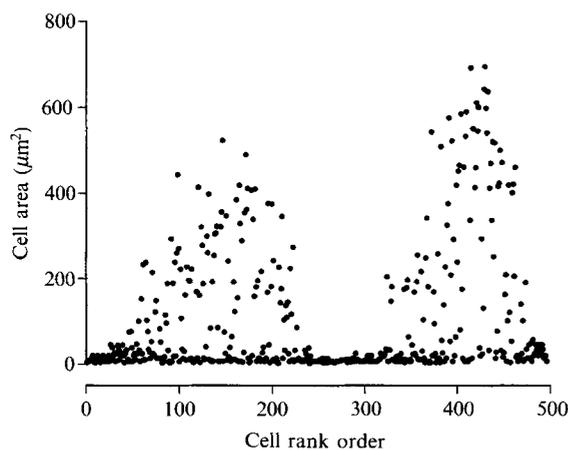


Fig. 16. Rank-order plot of a transect across the full diameter of the middle region of a 6 mm tall stipe. Narrow hyphae occupy the central column of tissue in the stipe.

position along the length of the stipe and irrespective of the developmental age of the stipe (compare Fig. 16, from a 6 mm tall basidiome, Fig. 15, from a 45 mm tall basidiome, and Figs 11 and 17, which come from a 70 mm tall basidiome). However, a comparison of the data from all transects between basidiomes of different size revealed a progressive change in the distribution of inflated hyphae (Fig. 18). In 6 and 27 mm tall basidiomes the inflated hyphae increased in cross-sectional area up to halfway across the cortex but then size declined towards the lumen (Figs 16, 18). In the 45 mm tall basidiome

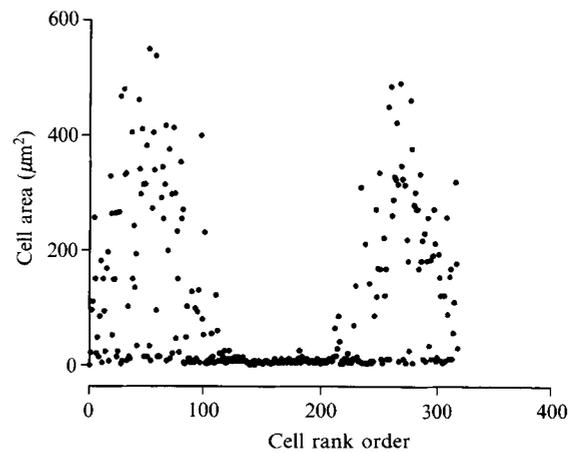


Fig. 17. Rank-order plot of a transect across the full diameter of the extreme apex of a 70 mm tall stipe. The section passed above the lumen, and narrow hyphae occupy the central region of the stipe.

Table 2. Nearest-neighbour analysis for hyphal distributions in transects of stipe sections of *Coprinus cinereus*.

Stipe zone	Observed mean distance (µm)	Expected mean distance (µm)	R*	Standard error	Standard variate (c)
(a) Narrow hyphae					
27 mm					
Upper middle 45 mm	9.7	7.7	1.2	0.57	3.3
Middle 70 mm	11.2	9.9	1.1	0.73	1.8
Base 70 mm	11.2	12.4	0.9	0.92	-1.3
Lower middle 70 mm	15.7	15.7	1.0	1.16	0.1
Upper apical 70 mm	17.0	12.8	1.3	0.95	4.5
(b) Inflated hyphae					
27 mm					
Upper middle 45 mm	18.9	6.2	3.0	0.46	27.5
Middle 70 mm	17.3	5.6	3.1	0.41	28.2
Base 70 mm	20.2	5.8	3.5	0.45	34.0
Lower middle 70 mm	21.7	6.1	3.6	0.45	35.0
Upper apical 70 mm	21.9	5.9	3.7	0.43	37.0

* R is the ratio of the observed mean distance to the expected mean distance and serves as the measure of departure from randomness. In a random distribution the observed mean distance and the expected mean distance will be identical, so that the ratio between them (R) is equal to 1. In a clumped distribution the ratio will be less than 1 and for an evenly spaced population the ratio will be greater than 1. The significance of the observed differences is measured by c, which is the standard variate of the normal curve. A positive value of c indicates that the observed value is more than the expected value and the distribution is therefore tending towards evenness. Similarly, a negative value indicates a tendency towards clumping. A value of c more than 1.96 (5% level) or less than -1.96 indicates a significantly non-random distribution.

the cross-sectional area of inflated hyphae increased gradually from the exterior to the lumen, and this pattern was even more pronounced in the 70 mm tall basidiome (Fig. 18).

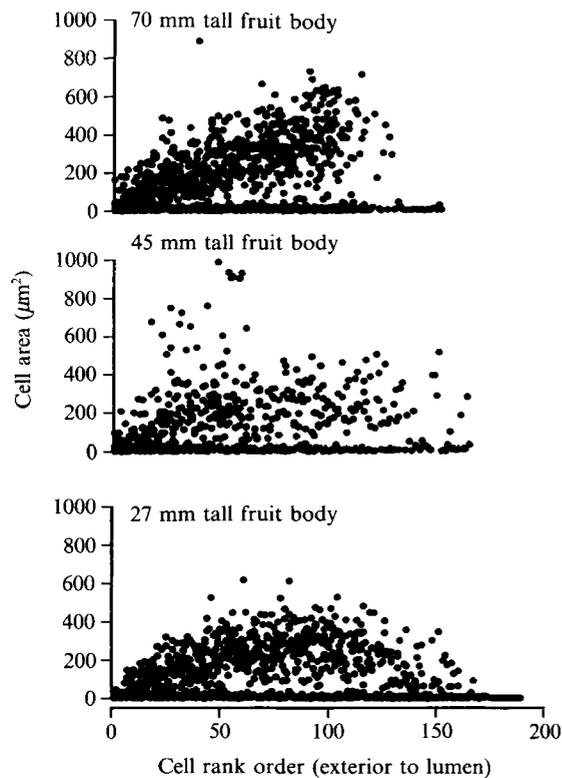


Fig. 18. Cumulated rank-order plots of 27, 45 and 70 mm stipes. Note how the cell-size distribution across the radius of the stipe changes with increasing stipe length.

DISCUSSION

Corner (1932) was largely responsible for introducing hyphal analysis as a procedure to encompass descriptive studies of hyphal systems in basidiomes, a system which is now used in routine classification. Over the years a number of different types of hyphae and a range of tissue types have been described. Corner coined the terms monomitic, dimitic and trimitic to describe tissues consisting of one, two or three kinds of hyphae, and hyphae in these different categories have been referred to as generative (because they ultimately give rise to the basidia and directly or indirectly to all other structures), skeletal (with thick walls and narrow lumen, but lacking branching and septation) or binding hyphae (which have limited growth and irregular, often repeated branching).

Corner (1966) introduced the terms sarcodimitic and sarcotrimitic to describe basidiomes where there are two or three types of hyphae, of which one is inflated and thickened. Redhead (1987) recognized a group of closely related agarics with such structures, but this included neither the Coprinaceae nor the Russulaceae. However, Fayod (1889) had already described the heteromerous trama in Russulaceae, composed of swollen cells (sphaerocysts) and filamentous hyphae (and see Reijnders, 1976, and Watling & Nicoll, 1980). Fayod was also aware of the presence of narrow hyphae amongst the more easily seen cells of the basidiome tissues he examined. These were called 'fundamental hyphae', but have played little part in agaric studies as they have not been used in identification in the way that the mitic system has in polypores.

To our knowledge no attempt has previously been made to undertake quantitative hyphal analysis, even though the relative sizes of differentiated hyphal populations, their distributions and the way the populations change during development are crucial to full appreciation of cell and tissue functions.

All the available evidence indicates that narrow hyphae in stipes of *Coprinus cinereus* have diverse functions. They tend to be particularly concentrated at the exterior of the stipe – where they may serve as an insulating layer, and as a lining to the lumen – where they may excrete material into the cavity or represent the remnants of the initially central core of dikaryotic hyphae. Cox & Niederpruem (1975) referred to a brown gel in the lumen which disappeared as the stipe extended. This gel might be produced by the narrow hyphae or consist of their degradation products if the lumen is produced by degradation of the initial central core. Our data show that expansion of the stipe is mainly due to increase in cross-sectional area of inflated hyphae in the region between the mid-cortex and the lumen. The geometrical consequences of this would be firstly that the central core would be torn apart, leaving its constituent cells as a remnant around the inner wall of the lumen so created; and secondly that the tissues in the outer zones would be stretched and reorganized.

Narrow hyphae stained densely with Mayer's haemalum, toluidine blue and aniline blue/safranin and revealed especially strong, particulate staining with the periodic acid–Schiff reagent for polysaccharide. However, not all narrow hyphal profiles in a transverse section and not all hyphal compartments belonging to any one narrow hypha in longitudinal sections stained equally. The reason for this differential staining is not known, but it might reflect differential function among the narrow hyphal population or, since narrow hyphae may be important in translocation of nutrients through the stipe, it may simply reflect inhomogeneities in the vertical distribution of cytoplasmic materials in course of translocation. The narrow hyphae seem to form networks independent of the inflated hyphae; they were seen to be branched and to be fused laterally with other narrow hyphae, but we saw no evidence that inflated hyphae were either branched or associated in networks (Figs 4–6).

What makes some hyphae become inflated and multinucleate while others remain morphologically similar to the vegetative mycelial hyphae is not known, although the even (i.e. non-random) distribution of the former implies some form of organizational control. This differentiation occurs at an extremely early stage, as both narrow and inflated hyphae can be seen in primordia 3 mm tall. However, during stipe elongation the numerical proportion of narrow hyphae decreased (Table 1), implying that at least some (approx. 25%) of them are recruited to the inflated category as the basidiome develops, and this seems to be a fourth way of expanding the basidiome. Already well documented are the heteromerous trama of Russulales, with inflating rosettes of sphaerocytes, sarcodimitism in some of the tougher pleurotoid/mycenoid agarics, and the dimitism of the aphylophoralean type in *Pleurotus* and *Lentinus*. It will be of interest to establish whether the coprinoid type of basidiome expansion which we

describe here is applicable to other organisms which develop rapidly on ephemeral substrates.

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