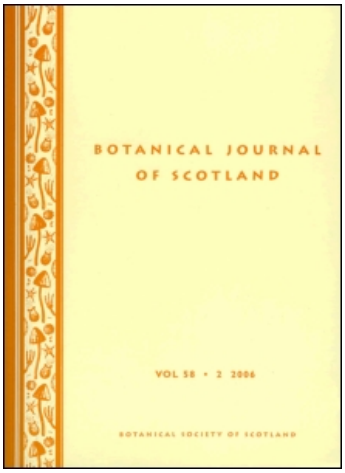

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In the midst of death we are in life: Further advances in the study of higher fungi

David Moore[a]; Siu Wai Chiu[b]; M. Halit Umar[c]; Carmen Sánchez[ad]
[a] School of Biological Sciences, The University of Manchester, Manchester, UK [b] Department of Biology, The Chinese University of Hong Kong, Hong Kong [c] Mushroom Experimental Station, Horst, The Netherlands [d] Laboratory of Biotechnology, Research Centre for Biological Sciences, Universidad Autonoma de Tlaxcala, Mexico

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In the Midst of Death we are in Life: Further Advances in the Study of Higher Fungi

DAVID MOORE,* SIU WAI CHIU,† M. HALIT UMAR‡ & CARMEN SÁNCHEZ*§

**School of Biological Sciences, The University of Manchester, Manchester M13 9PT, UK; †Department of Biology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, and ‡Mushroom Experimental Station, PO Box 6042, 5960 AA Horst, The Netherlands, §current address: Laboratory of Biotechnology, Research Centre for Biological Sciences, Universidad Autonoma de Tlaxcala, México*

Summary

Senescence and death of mushroom fruit bodies are the natural terminations of their development but not the end of their life because a new batch of fruit bodies can arise directly from the old. A fungal version of programmed cell death seems to be involved in sculpturing fruit body shapes. Fungal differentiation appears to be quite flexible and greatly influenced by the immediate microenvironment. Techniques are now emerging which will enable the earliest stages in fruit body initiation to be studied.

Introduction

In a northern Michigan hardwood forest, with little more than the breeze to disturb the leaves, there lives a monster. It is eating the forest. The locals say the monster is an individual '... that occupies a minimum of 15 hectares, weighs in excess of 10,000 kg and has remained genetically stable for more than 1,500 years ...' (Smith, Bruhn & Anderson, 1992). If true, this would place the monster amongst the largest, heaviest and oldest living things known on this planet. But this is no Scully-chasing alien. It truly belongs here. Its kin have seen the dinosaurs come and go. They have seen the mammals emerge and the higher primates rise to think to rule the Earth, and may well see those primates follow the terrible reptiles into extinction. The monster is a mushroom, a clone of the tree root pathogen *Armillaria bulbosa*.

In selfless pursuit of beasts like this Roy Watling has boldly gone where no mycologist has gone before. Though a redoubtable collector and field biologist, he has not neglected experimental and laboratory studies of mushrooms, nor critical thought about their nature. It is these aspects of his work we wish to celebrate. In 1978, Roy published a paper in this journal entitled 'From infancy to adolescence: advances in the study of higher fungi'. We wish to start our discussion at the other end of the life span, though, and ask, with reference to Kingdom Fungi, 'O death, where is thy sting?'

Mushrooms are not individual organisms, of course, they are merely the

fruiting appendages of an underlying mycelium. Yet in many ways a mushroom can be treated as an individual. The early development of a mushroom has many of the characteristics of an embryonic development (Moore, 1984a, 1998a & b) and like the larval and embryonic forms of animals, study of the different patterns and modes of that development can reveal taxonomic and phylogenetic relationships (Watling, 1985, 1996; Watling & Moore, 1994). Surveys of mushrooms, again treating them effectively as individuals, are used to reveal changing distributions for conservational and ecological analyses (Watling, 1996).

Programming life and death

Some bracket fungi are described as 'perennial' and, at the other end of the life-span spectrum, some small agarics are known to be short lived in the sense that the fruit body may last only a few hours. This again, of course, treats the fruit body as an individual rather than an appendage, but this is not unreasonable. With one exception, these casual comments are all that relates to life-span in the literature. Yet in the other two major Kingdoms it is self evident that death is an important aspect of their biology. Removal of old individuals makes way for the young and allows populations to evolve, and in recent years programmed cell death (PCD) has been recognized as a crucial contributor to morphogenesis in both animals and plants.

There is only one experimental study of the longevity of fungal fruit bodies. Umar & Van Griensven (1997a) grew the cultivated mushroom in artificial environments which protected the culture from pests and diseases and under these conditions they found that the life span of fruit bodies of *Agaricus bisporus* was 36 days. Ageing was first evident in fruit bodies about 18 days old, when localized nuclear and cytoplasmic lysis was seen. Remnants of lysed cells aggregated around and between the remaining hyphal cells. Eventually, most of the stem hyphae became empty cylinders, although other cells within the fruit body collapsed irregularly. Electron microscopy of specimens 36 days old and older showed most of the cells in the fruit body to be severely degenerated and malformed. Nevertheless, a number of basidia and subhymenial cells were alive and cytologically intact even on day 36.

Post-harvest physiology and morphology of mushrooms is of paramount importance for mushroom marketing and has been extensively studied, but post-harvest behaviour is usually described as senescence or as an ageing process. Umar & Van Griensven (1997a) emphasize, however, that the morphological changes which occur in naturally-senescent and post-harvest fruit bodies of *A. bisporus* are different. The harvested mushroom has suffered a traumatic injury and its post-harvest behaviour stems from that: in harvested *A. bisporus* fruit bodies (stored under various conditions) diffuse cell wall damage was observed first, this only later being accompanied by cytoplasmic degeneration. A major factor must be inability to replace water lost by evaporation. Exposed surfaces become desiccated and are damaged first. Thus, in what might be called 'post-harvest stress disorder', further damage is inflicted on the cell inwards, from the outside. In complete contrast, during the senescence which accompanies normal ageing the damage starts inside the cell and proceeds outwards. The nuclear and organelle genomes suffer first, then cytoplasmic integrity, and finally cell wall

Fig. 1. View of a fruiting culture of *Pleurotus ostreatus* which has a morchelloid fruit body on the left. At the top is a fruit body with more normal oyster mushroom morphology, but with a new generation of fruit body initials regenerating from its stem.

damage occurs as an aspect of the eventual necrosis suffered as the cell undergoes lysis.

Even in severely senescent fruit bodies Umar & Van Griensven (1997a) found healthy, living cells and these are presumably the source of origin of an unusual phenomenon known as renewed fruiting. Field-collected fruit body tissues of a mushroom usually generate abundant vegetative hyphae when inoculated onto nutrient agar plates. Such reversion from the fruiting stage into the vegetative stage is not an abrupt process, rather there appears to be some sort of 'memory' of the differentiated state. Initial hyphal outgrowth from gill lamellae usually mimics the densely packed branching and intertwined hyphal pattern of the gill tissues at first, being quite unlike the pattern of normal vegetative hyphae in culture. The 'memory' need be no more than the residual expression of differentiation-specific genes (such as the hydrophobins, (Wessels, 1994a & b, 1996)) before their products are diluted out by continued vegetative growth.

In the extreme, however, entirely new crops of fruit bodies may appear on the remains of the old (Fig. 1). This renewed fruiting (the formation of fruit bodies directly on fruiting tissue) is not uncommon, and it can occur at various locations (cap, stem and/or gills) in improperly stored excised fruit bodies. Ex-

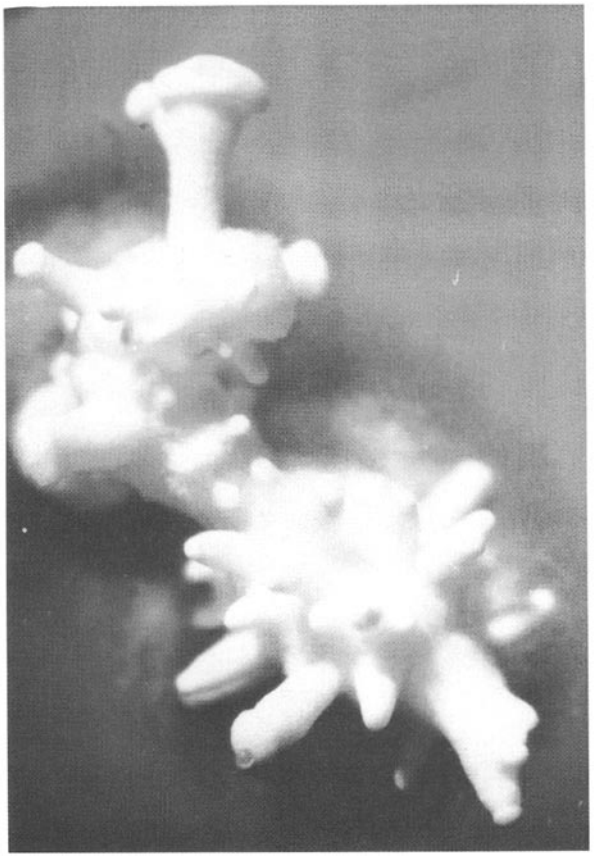

Fig. 2. Fruit body initials and primordia regenerating from gills of *Pleurotus pulmonarius* which have been explanted from their parent fruit body to an agar medium.

periments *in vitro* show that numerous primordia can arise on excised fruit body tissues and can mature into normal, though miniature, fruit bodies (Fig. 2). In comparison to vegetative cultures, the excised fruit body tissues form fruit bodies very rapidly. For example, in *Coprinus cinereus*, renewed fruiting occurred within four days, compared with cultures inoculated with vegetative dikaryon which, under the same conditions, formed fruit bodies in 10-14 days (Chiu & Moore, 1988; Brunt & Moore, 1989). Renewed fruiting may have an important role in survival, consuming and immediately recycling the resource in the dying fruit body tissue to disperse further crops of spores. For the fungi, in the midst of death, there is life!

For experimentalists it may be more important that renewed fruiting provides an excellent experimental system for study of fruit body morphogenesis (Bourne, Chiu & Moore, 1996). It has proved especially useful for bioassay of fruiting modulators such as heavy metal pollutants like cadmium (Chiu *et al.*, 1998).

Cell death in shaping a mushroom

Programmed cell death is the removal of tissue in a manner controlled in time and position. In animals it is called apoptosis and involves a programme of well regulated processes, including synthetic ones, which lead to internal cell degeneration and eventual removal of the dying cell by phagocytosis. It is important that apoptotic elimination of cells is intracellular in higher animals to avoid escape of antigens and the consequent danger of an immune response to components of the animal's own cells (autoimmunity). This is not a consideration in plants and fungi. The most obvious example of fungal PCD is the autolysis which occurs in the later stages of development of fruit bodies of many species of *Coprinus*, which Buller (1924, 1931) interpreted as a part of the developmental programme (autolysis removes gill tissue from the bottom of the cap to avoid interference with spore discharge from regions above). Autolysis involves production and organized release of a range of lytic enzymes (Iten, 1970; Iten & Matile, 1970). Consequently, autolytic destruction of these tissues is clearly a programmed cell death.

Umar & Van Griensven (1997b, 1998) have found that cell death is a common occurrence in various structures starting to differentiate, for example the formation of gill cavities in *A. bisporus*. The authors point out that specific timing and positioning imply that cell death is part of the differentiation process; a fungal kind of programmed cell death. Fungal PCD could play a role at many stages in development of many species (Umar & Van Griensven, 1998). Individual hyphal compartments can be sacrificed to trim hyphae to create particular tissue shaping. PCD is used, therefore, to sculpture the shape of the fruit body from the raw medium provided by the hyphal mass of the fruit body initial and primordium.

In several examples detailed by Umar & Van Griensven (1998) the programme leading to cell death involves the sacrificed cells over-producing mucilaginous materials which are released by cell lysis (Figs. 3 & 4). In the autolysing *Coprinus* gills the cell contents released on death contain heightened activities of lytic enzymes. Evidently, in fungal PCD the cell contents released when the sacrificed cells die could be specialized to particular functions too. This leads us to a discussion of the fungal strategy for differentiation.

True to form

The readiness of field-collected mushrooms to revert to vegetative growth when fragments are inoculated to culture medium has been remarked upon above as an aspect of developmental flexibility. However, the observation of this readiness to revert carries with it the implication that in the original fruit body the urge towards vegetative growth was suppressed in some way; perhaps by continual, but localized, reinforcement of the developmental information which drives differentiation. This raises the question: do fungal cells show the sort of developmental commitment or determination evident during animal and plant development?

Fungal cells are certainly morphogenetically flexible. As an example of inherent flexibility consider the basidia of *A. bisporus*. Allen, Moore & Elliott (1992) examined the dynamic population structure of nuclear division in *A.*

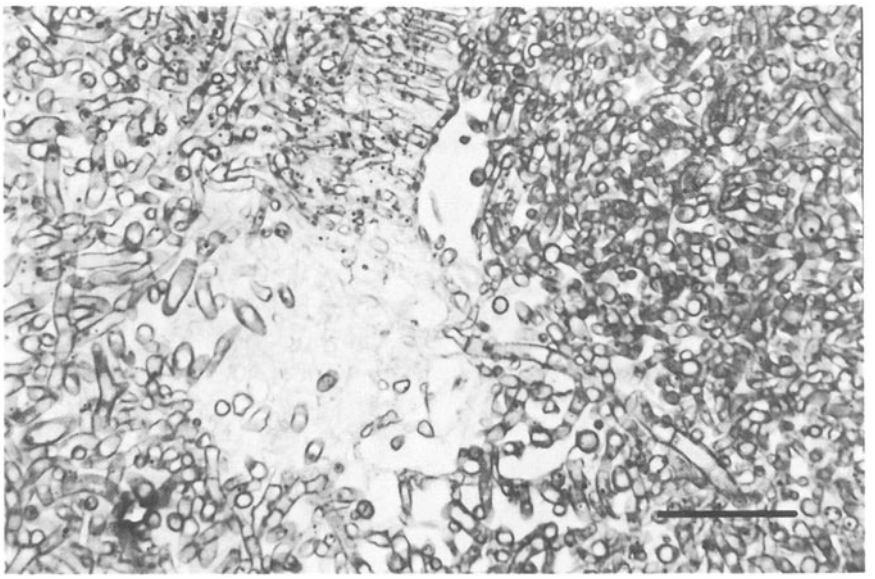

Fig. 3. Programmed cell death in *Agaricus bisporus*. Light micrograph of a section of a fruit body primordium in the region where the gill space is being formed. Diffusely-stained walls and cell contents identify a localized group of cells which are undergoing a transformation towards matrix accumulation which leads to their death by wall lysis. Scale bar = 50 μm.

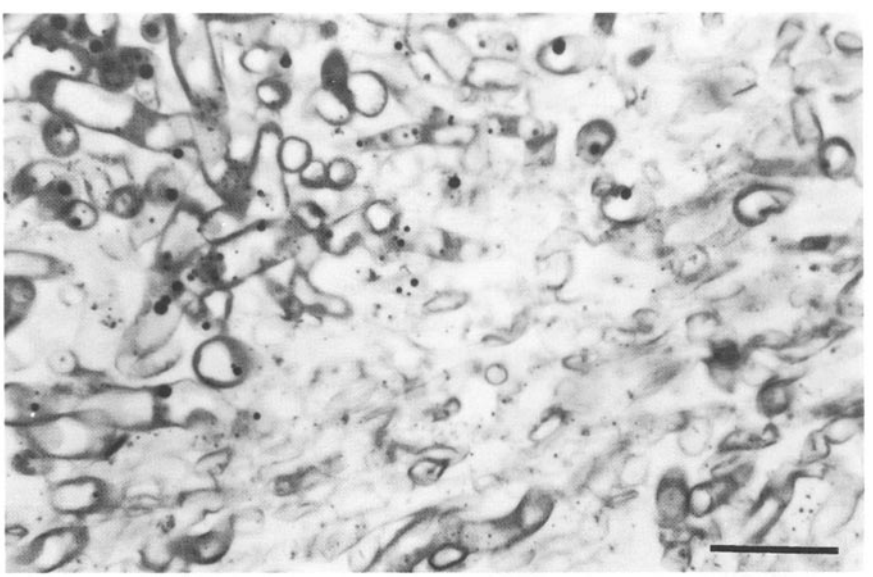

Fig. 4. Programmed cell death in *Agaricus bisporus*. Magnified view of a region of a fruit body primordium which is undergoing lysis following accumulation of extracellular matrix. Scale bar = 20 μm.

bisporus and found that basidia at prophase I predominate throughout the life of the fruit body. As prophase I is the longest stage of the nuclear cycle, such basidia will form the natural spacers of the maturing hymenium. That is, pre-sporulating basidia act as natural spacers between the sporulating basidia. The hymenium of *A. bisporus* lacks specifically differentiated sterile cells so this structural function of young basidia is especially important. The same is true for *Volvariella bombycina*, in which differential maturation of successive generations of basidia (emerging from different levels of the subhymenium) enables basidia to act as natural spacers (Chiu & Moore, 1990a). This is a 'make-do' strategy, which is quite different from that employed in the hymenium of *Coprinus*, for example, which has three major types of cells, the paraphysis, the cystidium and the basidium. Spacing of basidia in this hymenium is by the insertion and swelling of paraphyses which are highly differentiated to form the structural foundation of the whole gill (Rosin & Moore, 1985).

Hymenia of both *Coprinus cinereus* and *Volvariella bombycina* feature cystidia as giant cells which extend into, and often span, the gill cavity. It has been speculated that cystidia function as buttresses to separate neighbouring gills. This may apply at very young stages but is unlikely in developing and mature fruit bodies. Mature cystidia in *V. bombycina* bear fluid droplets (Chiu & Moore, 1990a). This indicates a role in regulating the atmosphere of the gill space, either by actively excreting fluid for evaporation or by secreting hygroscopic substances to act as a focus for water condensation. In *C. cinereus*, the large cystidia act as tension ties to keep the two neighbouring hymenia together as the fruit body develops; growth and expansion of the cap produce mechanical forces which pull the gills into shape. Cystidia communicate and equalize these forces around the cap and consequently maintain the pattern of gills during development (Moore, 1996).

Both *C. cinereus* and *V. bombycina* have facial (on the gill surface) and marginal (on the gill edge) cystidia. Both types of cystidium in *V. bombycina* are established when the hymenium is first laid down and become multinucleate through repeated mitoses (Chiu & Moore, 1993). Facial cystidia in *C. cinereus* are also established as components of the very first population of dikaryotic hyphal tips which form hymenial tissue (Rosin & Moore, 1985; Horner & Moore, 1987) and were mostly binucleate as a result. Marginal cystidia in *C. cinereus* were the apical cells of branches from the multinucleate gill trama, which became swollen to repair the injury caused when primary gills pulled away from the stem (Moore, 1987); marginal cystidia retained the multinucleate character of their parental hyphae (Chiu & Moore, 1993). Thus, cells with very similar morphologies can have very different origins.

Fungal cells behave as though they have considerable latitude in expression; decisions between developmental pathways seem to be able to cope with a degree of uncertainty. Facial cystidia of *C. cinereus* are generally binucleate, reflecting their origin and the fact that they are sterile cells, yet occasional examples can be found of cystidia in which karyogamy has occurred (Chiu & Moore, 1993). This suggests that entry to the cystidial pathway of differentiation does not totally preclude expression of at least the start of the nuclear differentiation pathway characteristic of a different cell type. Another example is the observation of

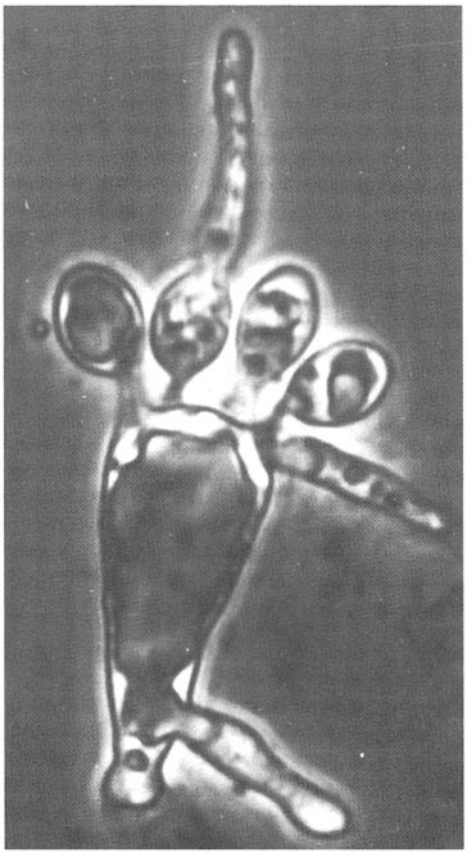

Fig. 5. A basidium of *Coprinus cinereus* showing the sort of reversion to vegetative hyphal growth which occurs when gills are removed from their parental fruit body and exposed to an *in vitro* medium containing ammonium ions.

cystidia bearing hyphal outgrowths looking like sterigmata in a spontaneous fruit body variant of *Psilocybe merdaria* (Watling, 1971).

As discussed earlier, fruit body fragments excised and transplanted to a water or nutrient agar revert to vegetative hyphal growth. When this is done with fragments of immature gill, vegetative hyphae emerge from most cells of the hymenium and subhymenium, but basidia are an exception. Basidia in early stages of meiosis were arrested in development and failed even to revert; basidia at later stages were able to complete meiosis and sporulation. Consequently, it was concluded that basidia are the only cells which are committed to their pathway of differentiation (Chiu & Moore, 1988).

Interestingly, when ammonium solution was injected into the cap or included in agar medium, the meiotic pathway was affected, leaving white patches on the cap or leading to vegetative outgrowth from basidia (Fig. 5) (Chiu & Moore, 1990b). Thus, meiosis and sporulation are sensitive to inhibition by ammonium ions. But *C. cinereus* grows naturally in compost where ammonia is abundant and volatile and so ammonium assimilating enzymes are produced in the cap which act as an efficient ammonium-scavenging system (Moore, 1984b; Moore, Horner & Liu, 1987); clearly, the microenvironment surrounding the

hypha has enormous impact on its differentiation. This example also shows that intermediary metabolism is a major contributor to cell differentiation and fungal morphogenesis.

Beyond morphology

Evidently, study of fungal morphogenesis involves more than morphology and we can continue the theme of metabolic differentiation with brief discussion of the roles of urea and mannitol in mushroom development. Fruit body morphogenesis is usually associated with expansion or cell inflation. In *Coprinus cinereus*, urea is found to increase by a factor of 2.5 on a dry weight basis but the urea concentration on a fresh weight basis was essentially unchanged during cap development (Ewaze, Moore & Stewart, 1978; Moore, 1984b). Thus, urea behaves as an osmoticant for driving water into the cells for expansion of the cap in *C. cinereus*. *A. bisporus* and *Lentinula edodes*, however, synthesize mannitol (a sugar alcohol) for an analogous osmotic function (Rast, 1965; Hammond & Nichols, 1976; Tan & Moore, 1994). Cap expansion is due to hyphal inflation in *A. bisporus* but hyphal proliferation in *L. edodes*. Interestingly, *C. cinereus* (which expands by inflation) and *Schizophyllum commune* (which expands by proliferation) also show many metabolic similarities despite the fundamental difference between their strategies of expansion. Thus totally different tactics can be used to achieve the same strategic end. Presumably, during evolution the 'choice' between different metabolic mechanisms which enable a morphogenetic process to be put into effect, has been made independently of 'choices' among different processes which might contribute to that morphogenesis.

Casual observation suggests that the value of comparative physiology and biochemistry is lost on most mycologists. It also suggests that numerical methods are avoided like the plague. Most mycologists will be aware of, and understand the value of, the description of tissue construction in mushrooms and toadstools called hyphal analysis that was introduced by Corner (1932a, b, 1966; Redhead, 1987). Hyphal analysis is entirely descriptive, and its taxonomic importance is immense (Pegler, 1996). Yet the functional and morphogenetic purposes of the hyphal differentiation which hyphal analysis describes have only been considered in one study. Further, in the 60+ years that hyphal analysis has been in vogue, the only *quantitative* study is that done by Hammad *et al.* (1993a) and Hammad, Watling & Moore (1993b), who showed that enumerating cell types at different stages of development (in the fruit bodies of *C. cinereus*) is a powerful way of revealing how fruit body structure emerges during morphogenesis as a result of changes in hyphal type and distribution.

Measuring and counting cells (= hyphal compartments) in different regions of fruit bodies at different stages of development reveals specific patterns of cell differentiation (particularly inflation) which mechanically generate the final form of the fruit body. The patterns revealed must be organized by signalling molecules, so these studies establish the very foundation for detailed analysis of the control of fungal morphogenesis. By revealing that positional information is regulated in time and space, investigations of this sort raise crucial questions about the nature of the signalling molecule(s) involved, their transduction pathways and the responses they elicit.

We are only at the brink of this research, but it is going forward. Attempts to determine the nature of the signals used in fungi have been initiated (Novak Frazer, 1996) and attempts are under way to exploit further the numerical approach and computer-aided image analysis. The aim of the latter is to establish mathematical models describing mushroom morphogenesis. Too much reliance is currently placed on drawings of hyphal distributions in fruit body tissues. Such drawings are often a delight to behold from an artistic point of view, but suffer from subjective interpretation and lack of a quantitative dimension. At the moment we are trying to extend our image analysis of *C. cinereus* into three dimensions; using confocal microscopy to make digital records of optical sections of pieces of tissue, then visualization software to create virtual reconstructions through which we can wander to make cell counts and measurements. The counts and measurements are intended to define parameters for mathematical modelling of morphogenesis in three dimensions, rather than the one and two dimensional models which have already been achieved (Stočkus & Moore, 1996; Meškauskas, Moore & Novak Frazer, 1998). Overall, we are making two approaches, one coming from abstract mathematics towards a computer model, the other coming from real life towards a computer model.

The mushroom structure has been measured in terms of fruit body height, cap diameter and diameter of the stem. Ingold (1946) and Bond (1952) used published illustrations of a wide range of agarics to extract graphical relationships between these features, arriving at the conclusion that smaller fruit bodies have proportionately longer and more slender stems. Watling (1975) established a different graphical representation for the Bolbitiaceae, using measurements from fresh specimens. He pointed out faults with the earlier work which depended on published collections of illustrations which were selective in their inclusion of species and in their descriptive boundaries of species. Only facile generalizations can be expected if measurements of different species are combined; dimensional measurements have very limited value unless they are accompanied by measurements of the structural characteristics of the tissues and their components; and the developmental observations are essential to determine how dimensions change during morphogenesis. Morphological measurements have practical value, too. They have been used to define the 'normal' mushroom for the *A. bisporus* crop (Flegg, 1996) and image analysis of shape, form and colour of *A. bisporus* can be related statistically to crop development (Van Loon *et al.*, 1995). Such approaches contribute to devising control methods for machine automation of crop picking.

Back to the future

Aggregation of hyphae to form tissues begins from the vegetative hyphae of a mycelium; the aggregation of hyphae establishing a fruit body initial that will develop into a primordium and then in a mature fruit body. In *Coprinus lagopus* (=*cinereus*) fruit body initials were described as tangled masses of hyphae (Niederpruem, 1978) and in *Flammulina velutipes* as aggregates of widely spaced, interwoven hyphae (Williams, Beckett & Read, 1985). In one strain of *F. velutipes*, under the same culture conditions, different numbers of fruit bodies were recorded at the different stages of development. The number of fruit bodies

that matured was also variable, with some fruit bodies aborting regardless of their stage of development (Williams *et al.*, 1985). To study this variation in fruiting it is necessary to quantify the number of fruit bodies formed on the substrate in an objective manner. However, a limiting factor in the study of fruiting is that it is usually difficult to identify the earliest stages in fruit body development (the fruit body initials). Recent studies of *P. pulmonarius* showed that fruit body initiation was characterized by aggregation of densely staining hyphae (Sánchez, 1998) and this provides a simple histological method which distinguishes fruit body initials from the rest of the mycelium even at the very earliest stages. The stained cultures can be examined with an image analysis system to quantify *P. pulmonarius* fruit body initials and their growth potential as they age. Early results show that approximately 70% of the first formed initials aborted. The 36% which matured presumably recycled the nutrients from those which aborted.

Studies with the transmission electron microscope show that walls of vegetative hyphae were 2.5 times thicker than those of hyphae in the base of the fruit body initial and vegetative mycelium of *P. pulmonarius* (Sánchez, 1998). Similar characteristics have been identified in hyphae and walls of *A. bisporus* primordia (Heckman *et al.*, 1989). Histological staining has been used for many years, of course (*e.g.* Disbrey & Watling, 1967) though its promise has not been fully realized. Recent applications, for example the demonstration that the periodic acid-Schiff reaction was slow and weakly with hydrophobic hyphae of *A. bisporus* (Umar & Van Griensven, 1997c) show how conventional techniques can be combined with current knowledge to improve understanding. Sánchez (1998) developed a technique to stain fruit body initials of *P. pulmonarius* specifically with Janus Green and other dyes. Highly localized staining of fruit bodies and the peripheral growth zone of the mycelium (*i.e.* hyphal tips at the margin of the colony) but not the mature vegetative mycelium at the location of the developing fruit bodies occurs and no doubt indicates some (presently unknown) biochemical differentiation between these hyphae. Through the mist we start to see the light!

Most mushrooms and toadstools decompose dead substrates, recycling the nutrients to create new fungal life. In the context of differentiation and development, life arises from the midst of death. For the scientist, old facts are the nutrients which nurture new ideas and new interpretations but new facts must be won by experiment. Roy Watling has led the way in using experiment to expand on observational mycology, enabling new concepts and interpretations to remodel the old; extracting live ideas from dead and dying notions. Long may it be so.

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