Conventional histological stains selectively stain fruit body initials of basidiomycetes

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A method to identify the earliest stages (initials) of *Pleurotus pulmonarius* and *Coprinus cinereus* fruit body formation was developed. Light microscopy and Cryo-SEM were used to confirm that even the smallest structures to take up stain were initials of fruit bodies. An approach combining histological staining (flooding the Petri dish with 1% toluidine blue in 1% boric acid (w/v) for 15 min) and image analysis allowed the number of fruit bodies formed on Petri dishes to be quantified easily. Use of the vital stain Janus green (0·001% aqueous, w/v) allowed continued observation of living tissue so that the proportion of fruit bodies that matured (30%) could be established. The method was also effective on wheat straw cultures and could be used to monitor development of mature fruit bodies. It is a promising tool in the study of physiological processes involved in fruit body initiation.

The formation of fruit body initials is a crucial change in the behaviour of a mycelium. The shift from vegetative growth to multicellular development is not merely a profound scientific curiosity, but is the cornerstone of every cultivation programme. Fruit body initials in Agaricales and Aphyllophorales generally arise at points which express a heightened frequency of branching hyphae (Wessels, 1993). In *Coprinus cinereus*, fruit body initials have been described as tangled masses of hyphae (Niederpruem, 1978) originating as hyphal lattices (Matthews & Niederpruem, 1972), and the early steps in formation of fruit bodies are shared with the initiation of the asexual sclerotia (Waters, Moore & Butler, 1975; Moore, 1981). In *Flammulina velutipes* fruit body initials appear as the aggregation of widely spaced, interwoven hyphae (Williams, Beckett & Read, 1985). A mathematical model describing the initiation and growth of *Agaricus bisporus* crops has been developed (Chanter & Thornley, 1978) which assumes that initiation of fruit bodies only occurs when the substrate mycelium has reached a certain threshold hyphal density.

Because it is difficult to identify the youngest structures with certainty, most work on fruit body formation depends on the production of more recognizable immature primordia or mature fruit bodies. Yet it is a common observation that only a minority of initials which are formed subsequently progress to maturity. Evidently, initiation and maturation are different processes, but quantitative experimental analysis is demanding simply because it is difficult to identify the earliest stages in fruit body development (Niederpruem, 1978; Moore, 1998) and even more difficult to determine which of those initials will survive to maturity.

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During an investigation of the progress of fruit body development in *Pleurotus pulmonarius* we found that some standard histological stains enable differential staining of fruit body initials. In this report we validate the technique and indicate how it can be used for objective and quantitative studies of fruit body initiation.

MATERIALS AND METHODS

Organisms. *Pleurotus pulmonarius* (Fr.) Quél. PL27 from the culture collection at the Chinese University of Hong King (Shatin, Hong Kong) and *Coprinus cinereus* (Schaeff.: Fr.) Gray (Meathop strain) from MycoTech Ltd (Campus Ventures Centre Ltd, The University of Manchester M13 9PL, U.K.) were used. Stock cultures of both were grown on malt extract agar (MEA, see below) in the dark at 25 °C in Petri dishes for 7 and 10 d respectively and then stored at 4°. Cultures were transferred to fresh culture media periodically.

Culture media and culture conditions. Fruit bodies were formed either on 9 cm diam. Petri dishes containing Potato Extract Agar (PEA) or MEA, or in crystallizing dishes (9 cm diam., 5 cm high) containing wheat straw.

MEA was obtained from Oxoid (England) and was prepared according to the manufacturer's instructions. It contained (l^{-1}): 30 g malt extract, 5 g mycological peptone and 15 g agar. PEA was prepared from fresh potatoes. 1800 g potatoes were peeled and diced, suspended in muslin in 4500 ml of water and boiled for 10 min. The potatoes were discarded and the liquor placed in glass containers and autoclaved at 121° for 15 min. Agar to a final concentration of 15 g l^{-1} was added prior to autoclaving (Booth, 1971). Wheat straw (Higher Barn Farm, Birch, Lancashire) was prepared as a growth substrate by

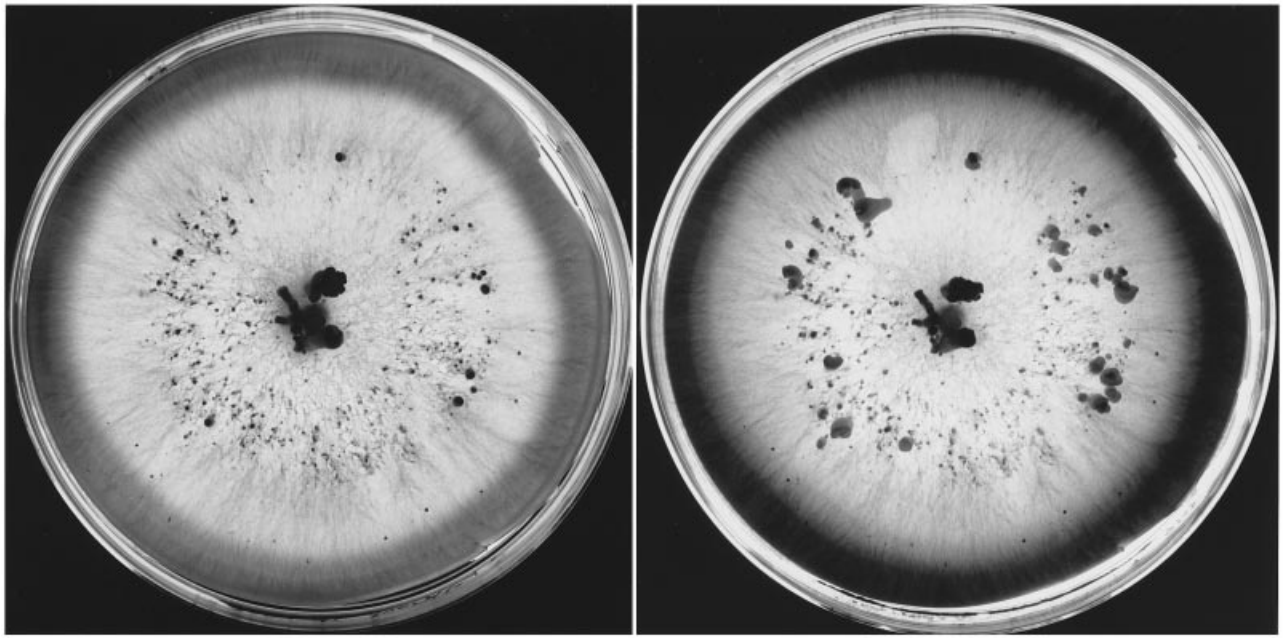


Fig. 1. A *P. pulmonarius* colony in a 9 cm Petri dish stained with 1% toluidine blue in 1% boric acid. The image at left shows the appearance immediately after the 12 d old colony was stained. Note the extreme contrast between the densely-stained fruiting structures of various sizes, the well-stained peripheral growth zone and the unstained body of the colony. The right hand image panel shows the same dish after incubation for a further 2 d. Growth of many of the pre-existing initials is evident although the fruit bodies which were close to maturity when first stained (at the centre of the colony) did not continue to grow.

soaking in tap water at 85° for 1 h, the excess of water was drained off. The crystallizing dishes contained 70 g of wet wheat straw and were autoclaved at 121° for 30 min.

Fruiting cultures of both fungi were incubated at 28° in a 16/8 h light/dark cycle. Illumination was by white fluorescent lights, with an average illuminance of 800 lx. Covered crystallizing dishes containing wheat straw already inoculated with *P. pulmonarius* were incubated at 25° for 15 d in the dark and then placed uncovered in a Perspex chamber at 28° in a 16/8 h light/dark cycle to induce fruiting. Humidity in the cultures was maintained by spraying the crystallizing dishes with water. Fruit bodies of *P. pulmonarius* and *C. cinereus* appeared on Petri dishes after approx. 7 and 10 d incubation respectively. *P. pulmonarius* fruit bodies were obtained after 21 d on wheat straw.

Staining. Cultures of *P. pulmonarius* grown on PEA, MEA or wheat straw and *C. cinereus* grown either on MEA or PEA were stained using 1% toluidine blue in 1% boric acid (w/v), Schiff reagent (Gurr, 1963) or 0·001% aqueous Janus green (w/v) (Sigma). The cultures were flooded and left to react with the stain for 15 min. at room temperature and then drained and rinsed with distilled water. The fruit body initials and the fruit bodies themselves were entirely stained, but not the surrounding mycelia. The technique was used to monitor the development of initials and fruit bodies to evaluate fruit body abortion.

Microscopic techniques. Light microscopy (LM) and Cryo-Scanning Electron Microscopy (CSEM) were used to confirm that the stained structures were truly initials of fruit bodies. Light microscopy was done on sections of pieces of mycelium (2 mm^2 approx.) cut from 7 d-old *P. pulmonarius* colonies grown on MEA which had been stained *in situ* with 1% toluidine blue. The samples were placed in small vials and fixed in 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer (TAAB Laboratory Equipment Ltd), pH 7·2–7·4 overnight, under vacuum (to help the fixative penetrate the specimen). The samples were washed in sodium cacodylate buffer for 5 min, postfixed in 1% osmium tetroxide in sodium cacodylate buffer for 2 h and then washed in sodium cacodylate buffer for 5 min. After being fixed, the samples were dehydrated in 50%, 70% and 90% laboratory grade alcohol for 20 min in each solution and four times in absolute alcohol for 1 h each time.

The specimens were infiltrated in 1:1 and 1:3 (v/v) absolute alcohol and Spurr's resin (TAAB Laboratory Equipment Ltd) for 1 d in each solution and then in 100% fresh resin three times for 1 d. The samples were gently agitated on a rotating turntable during the entire infiltration procedure. Once the specimens had been infiltrated with 100% resin they were labelled, embedded individually with resin in capsules, and then polymerized overnight at 60°. Blocks were sectioned using a Leica OMU4 ultramicrotome with glass knives. Sections (1·5 μm thick) were cut onto water and collected using a thin wire, and then placed on glass slides and dried on a hot plate for 10 min. The sections were routinely restained with 1% toludine blue in 1% boric acid (w/v), which stains most of the cytoplasm, the cell walls and nuclei of the cells. The slides were left in the stain on a hotplate for 15 min and then rinsed with distilled water and dried on a hot plate. The specimens were observed using a Leitz-dialux 20 EB photoautomatic microscope. Light micrographs were recorded using a Wild MPS 51S SPOT camera

attached to the microscope. Technical pan Kodak B & W film was used.

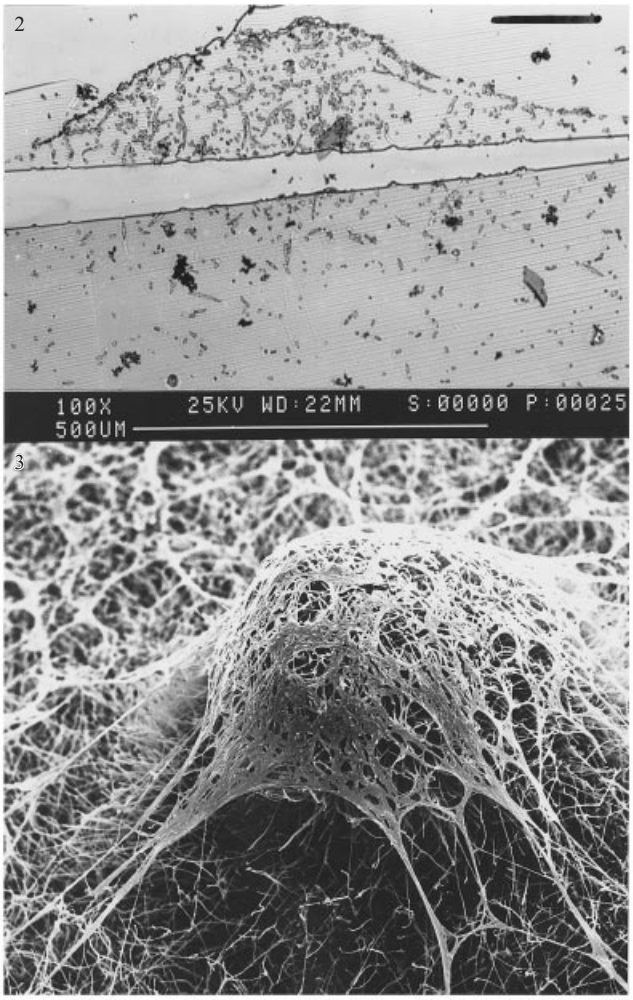
CSEM studies were carried out using pieces of approx. 7 mm² of 7 d old *P. pulmonarius* and 10 d old *C. cinereus* fruiting colonies grown on PEA either untreated or stained *in situ* with 0·05% toluidine blue. Tissues were frozen by immersion in liquid nitrogen slush (−210°). The samples were then transferred under vacuum to a cooled microscope stage. Ice was sublimed from the tissue (at −70°), and after cooling to −180° the specimen was coated with gold. The samples were examined in the hydrated frozen state using a Cambridge Instruments S200 Scanning Electron microscope fitted with an Oxford instrument CT1000 low temperature stage. CSE micrographs were taken using an attached 35 mm camera and Ilford B & W Delta film.

Quantification of fruit bodies using image analysis system. Fruiting structures of *P. pulmonarius*, stained with toluidine blue as described above, formed on 20 Petri dishes containing PEA after 12 d of growth, were quantified, using a Quantimet Q570 image analysis system and a video camera (Panasonic model WV-CD20) which sent the culture image to the computer monitor. An image analysis program written by G. C. Paul (Birmingham University), to quantify conidial germination was used. To quantify abortion, cultures of *P. pulmonarius* grown for 10 d on PEA were stained using 0·001% Janus green. The fruit bodies formed on 18 Petri dishes were counted manually using a mechanical counter, the cultures were incubated for another two days and counted again (without restaining). Fruit bodies which had continued to grow were distinguishable by the absence of colour in areas of new growth.

RESULTS AND DISCUSSION

***Identification of 'initials' and fruit bodies of* P. pulmonarius *and* C. cinereus.** *P. pulmonarius* and *C. cinereus* cultures grown on Petri dishes were stained with Schiff reagent, toluidine blue or Janus green. When the cultures were stained using either Janus green or toluidine blue the peripheral growth zone of the colony and the fruiting structures, even those so small as to be on the verge of visibility to the unaided eye, were stained, but the surrounding vegetative mycelium was unstained (Fig. 1). Further incubation of stained cultures showed that stained structures generally continued to grow, although some of them aborted, especially after toluidine blue staining (Fig. 1). *P. pulmonarius* cultures stained slowly with Schiff reagent; after 5 min. of reaction with the stain the top of the fruit bodies and the transition zone between mature hyphae and young hyphae of the mycelium were stained and after 2 h the fruit bodies and the margin of the colony were completely stained. In *P. pulmonarius* cultures grown in crystallising dishes on wheat straw and stained using toluidine blue in 1% boric acid, the fruit bodies were stained but most of the wheat straw was not.

LM and CSEM observations confirmed that the smallest structures stained *in situ* were fruit body initials. Fig. 2 shows a light micrograph of a fruit body initial 115 µm tall that was identified by this *in situ* staining. Although the section shows various artefacts caused by the *in situ* staining prior to embedding, the structure shows clearly the typical hyphal arrangement and shape of a fruit body initial.



Figs 2–3. Fruit body initials of *P. pulmonarius* formed on MEA after 12 d growth and detected by staining the colony with 1% toluidine blue. **Fig. 2.** LM section which shows the object identified has a structure consistent with its being a fruit body initial (which has separated from the supporting mycelium during preparation). Scale bar = 100 µm. **Fig. 3.** Cryo-SEM image of a similar fruit body initial.

Stained and unstained *P. pulmonarius* and *C. cinereus* cultures examined using CSEM showed that fruit bodies of both fungi were clearly identifiable by shape and size, but much earlier stages of fruit body development were detected in cultures stained *in situ* with toluidine blue, as some contrast differences due to the effects of the stain were observable in the SEM (Fig. 3).

Clearly, the earliest stages of formation of fruit body initials as well as the fruit bodies themselves, could be discerned by staining the whole colony on which they were formed, using any of the stains described here. In all cases the fruiting structures were stained but not the vegetative mycelium. When Schiff's reagent was used the reaction progressed with time and could thus give more information about the nature of the hyphae in the different zones of growth. Fuchsin, which is the main dye in Schiff's reagent, stains mucopolysaccharides (Bullock & Willetts, 1996),

Table 1. Number of fruit body initials formed by *P. pulmonarius* on 9 cm Petri dishes of potato extract agar after 12 d growth

Dish	Initials formed*	Maturing (%)†
1	106	31·1
2	233	41·9
3	85	13·4
4	174	14·4
5	221	20·0
6	181	24·2
7	113	52·1
8	69	29·1
9	232	25·7
10	99	60·6
11	165	16·5
12	144	15·3
13	98	21·3
14	134	30·2
15	121	24·7
16	187	66·0
17	197	20·3
18	204	48·8
19	144	Not done
20	157	Not done
Mean ± S.E.M.	153 ± 49	30·9 ± 15·8

* Counted using Quantimet Q570 image analysis system after toludine blue staining.

† Counted manually using a mechanical counter after staining with the vital stain Janus green.

glycoproteins, glycogen and chitins in fungal tissues (Gurr, 1963; Grimstone & Skaer, 1972). In studies on hyphal regeneration in *A. bisporus*, periodic acid Schiff reagent gave an immediate and strong reaction with the hydrophilic hyphae of tissues but a delayed and weak reaction with the hydrophobic hyphae (Umar & Van Griensven, 1997). Differences between hyphal surfaces could account for the differential staining of the colony observed with all the stains used here. Interaction of the stain with the hyphal surfaces could also explain the persistence of a contrast difference to the SEM (Fig. 3). Presumably, the *in situ* staining alters the wall surface in a way which affects reflection of electrons.

***Quantification of fruit bodies formed and aborted in* P. pulmonarius.** Toluidine blue provided excellent contrast, well suited to machine quantification of factors affecting fruit body formation. The toluidine blue treatment could, however be toxic to growth of the fruit bodies and this stain is better suited to experimental protocols in which the stained cultures can be discarded. On the other hand, Janus green is a vital stain and ideally suited to procedures depending on continued growth of the fruit bodies. Initial trials of the technique to quantify fruit body formation and further development (Table 1) showed it to be effective for both image analysis and manual counting approaches. Colonies of *P. pulmonarius* grown on PEA formed large numbers of fruit body initials, but only 30% matured. Such a circumstance has been remarked upon frequently. The method reported here provides an objective approach for study of fruit body initiation and progress to maturation in a quantitative manner.

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REFERENCES

Booth, C. (1971). *The Genus Fusarium*. Commonwealth Mycological Institute: Kew.

Bullock, S. & Willetts, H. J. (1996). Ultrastructural and histochemical studies on mycelial germination of sclerotia of *Sclerotinia minor*. *Mycological Research* **100**, 561–570.

Chanter, D. O & Thornley, J. H. M. (1978). Mycelial growth and the initiation and growth of sporophores in the mushroom crop: A mathematical model. *Journal of General Microbiology* **106**, 55–65.

Grimstone, A. V. & Skaer, R. J. (1972). *A Guide Book to Microscopical Methods*. Cambridge University Press: Cambridge, U.K.

Gurr, G. T. (1963). *Biological Staining Methods*. George T. Gurr Ltd: London.

Matthews, T. R. & Niederpruem, D. J. (1972). Differentiation in *Coprinus lagopus*. I. Control of fruiting and cytology of initial events. *Archives of Microbiology* **87**, 257–268.

Moore, D. (1981). Developmental genetics of *Coprinus cinereus*: genetic evidence that carpophores and sclerotia share a common pathway of initiation. *Current Genetics* **3**, 145–150.

Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York.

Niederpruem, D. J. (1978). Morphogenetic processes in *Schizophyllum* and *Coprinus*. In *Genetics and Morphogenesis in the Basidiomycetes* (ed. M. N. Schwalb & P. G. Miles), pp. 105–134. Academic Press: New York.

Umar, M. H. & Van Griensven, L. J. L. D. (1997). Hyphal regeneration and histogenesis in *Agaricus bisporus*. *Mycological Research* **101**, 1025–1032.

Waters, H., Moore, D. & Butler, R. D. (1975). Morphogenesis of aerial sclerotia of *Coprinus lagopus*. *New Phytologist* **74**, 207–213.

Wessels, J. G. H. (1993). Fruiting in higher fungi. *Advances in Microbial Physiology* **34**, 147–202.

Williams, M. A., Beckett, A. & Read, N. D. (1985). Ultrastructural aspects of fruit body differentiation in *Flammulina velutipes*. In *Developmental Biology of Higher Fungi* (ed. D. Moore, L. A. Casselton, D. A. Wood & J. C. Frankland), pp. 429–450. Cambridge University Press: Cambridge, U.K.

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