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Simple staining detects ultrastructural and biochemical differentiation of vegetative hyphae and fruit body initials in colonies of *Pleurotus pulmonarius*

C. Sánchez[1], M. Téllez-Téllez[1], G. Díaz-Godínez[1] and D. Moore[2]
[1]*Laboratory of Biotechnology, Research Centre for Biological Sciences, Universidad Autónoma de Tlaxcala, Tlaxcala, México, and* [2]*School of Biological Sciences, The University of Manchester, Manchester, UK*

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ABSTRACT

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Aims: To know the ultrastructural and biochemical differences of vegetative hyphae and fruit body initials in colonies of *Pleurotus pulmonarius*.

Methods and Results: Feulgen reagent was used to detects differentiation of hyphae. The intracellular laccases, proteases and β-1,3-glucanases activity, content of cytoplasmic protein, glycogen and glucans in the cell wall were evaluated in hyphae of fruit body initials and in vegetative hyphae. The thickness of hyphal walls of the vegetative hyphae was also evaluated. Substantial biochemical changes were observed in hyphae of different zones of the fruiting colony. Hyphae at the periphery had thinner walls than in the centre of the colony.

Conclusion, Significance and Impact of the Study: Staining correlated with the enzymatic activity, protein, glycogen and glucans, in mycelium and in fruit body initials. The implications are that hyphal maturity in *P. pulmonarius* involves storage of glucans, in part at least, in the form of a thickened hyphal wall.

Keywords: basidiomycetes, enzymatic activity, fruit body initials, hyphal differentiation, vegetative mycelium.

INTRODUCTION

Most of the information about development in higher fungi (basidiomycetes) has come from the study of less economically important species such as *Schizophyllum commune* and *Coprinus cinereus* (Wessels 1993; Moore 1998). However, in recent years, *Pleurotus* species, which are economically important, are increasingly being used to study mushroom development, as they are relatively easy to grow and fruit *in vitro*. Mushrooms have the ability to degrade ligninocellulosic substrates and can be produced on agricultural wastes such as wheat, barley or maize straw. In 1994, the value of the world mushroom crop was estimated to be worth US \$$9{\cdot}8 \times 10^{12}$, *Pleurotus* spp. being the third most cultivated with a production of $797{\cdot}4 \times 10^{3}$ tonnes (Kües and Liu 2000). The growth of all fungi occurs at the hyphal tip (Bartnicki-Garcia and Lippman 1972; Bartnicki-Garcia *et al.* 1989), except for expansion growth in the fruit bodies of agarics (Gooday 1972; Craig *et al.* 1977; Moore 1998). In a fungal colony, as the peripheral growth zone advances mycelium is left behind that apparently does not participate in growth (Wessels 1993). These are the 'mature' regions of the colony that can become competent to produce fruiting structures (Moore 1998). There is little knowledge about the characteristics of hyphae in different zones in a well-developed colony, but hyphal development could be important in understanding cellular function in the development of both the vegetative and reproductive phases in mushroom-producing fungi. In this research, the intracellular laccases, proteases and β-1,3-glucanases activity, content of cytoplasmic protein, glycogen and insoluble and soluble glucans in the cell wall, were evaluated in hyphae of fruit body initials and in hyphae of the periphery and central zone of a well-developed colony of *Pleurotus pulmonarius*.

Correspondence to: Carmen Sánchez, Laboratory of Biotechnology, Research Centre for Biological Sciences, Universidad Autónoma de Tlaxcala, Apartado postal 129, Tlaxcala, Tlax., CP 90000, México (Tel./fax: +52 2484815482; e-mail: sanher@cci.uatx.mx).

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The thickness of hyphal walls of the peripheral and central zones of *P. pulmonarius* colonies was also evaluated.

MATERIALS AND METHODS

Strain and culture conditions

Pleurotus pulmonarius PPL27 from the culture collection of the Chinese University of Hong Kong (Shatin, Hong Kong) was used. Stock cultures were grown on malt extract agar in the dark at 28°C in Petri dishes for 10 days and then stored at 4°C. Cultures were transferred to fresh culture media periodically. Cultures of *P. pulmonarius* grown on Petri dishes containing potato dextrose agar (Difco Laboratories, Detroit, MI, USA) and incubated for 7 days at 28°C were used as inoculum. In all experiments, fruiting colonies of *P. pulmonarius* grown on Petri dishes (containing potato dextrose agar) for 12 days at 28°C in a 16/8 h light/dark cycle were used.

Staining of the colonies and differentiation of the zones of growth

Petri dishes containing fruiting colonies of *P. pulmonarius* were flooded with Feulgen reagent prepared as previously reported (Grimstone and Skaer 1972), and incubated for 20 min at room temperature. The stain was removed and the Petri dishes were rinsed with distilled water and dried at room temperature. The periphery of the colony (PC) and the fruit body (FB) initials (*ca* 0·5 mm high) formed at the centre of the colony were stained, whereas the central zone of the colony (CC) was not. The radius of the stained and unstained zones of the vegetative mycelium was measured in 30 Petri dishes by using an image analysis system (Image Pro-Plus, Media Cybernetics Inc., Silver Spring, MD, USA).

Determination of protein, glycogen and glucans

In unstained colonies, the content of intracellular protein and glycogen were determined in the biomass of the PC, CC and FB. The zone of the colony corresponding to the PC was separated from the CC with a scalpel and the FB were harvested with forceps. The intracellular enzymatic extract was obtained separately for mycelium scraped off the agar in the PC and of the CC, as well as for the FB. The mycelium of the PC, CC and the harvested FB were separately ground in 1 ml distilled water for each 0·1 mg fresh weight biomass, using a tissue grinder (Pyrex glass, Corning Inc., NY, USA). The resulting samples were centrifuged at 20 000 *g* for 10 min at 2°C and the supernatant collected as the intracellular enzymatic extract, and the sediment as the cell wall. Alternatively, the biomass, as dry weight (DW) was separately determined by drying at 60°C to constant weight (AOAC 1990). In the intracellular enzymatic extract, the total intracellular protein (mg g^{-1} DW) was measured by the Bradford (1976) method, using bovine serum albumin as standard. The glycogen content (mg g^{-1} DW) was considered as the difference between the total sugars minus the reducing sugars. Total and reducing sugars were determined using the Anthrone (Trevelyan and Harrison 1952) and DNS (Miller 1959) methods, respectively. The cell wall was rinsed first with water and then with a 76% (v/v) ethanol solution (three times with each), to remove membrane and cytoplasmic components. The glucans from the cell wall were separated into two fractions; soluble (S-glucans) and insoluble (R-glucans) in alkali (Wessels 1969), and reported as mg g^{-1} DW.

Enzymatic assay

Intracellular activity of laccases (EC 1.10.3.2), proteases, and β-1,3-glucanases (EC 3.2.1.39) was determined in the intracellular enzymatic extract obtained from the PC, CC and FB, as indicated above. Laccases activity was determined by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol as substrate (Ardon *et al.* 1998). Proteases activity was measured by changes in the absorbance at 280 nm, resulting from release of aromatic aminoacids, using casein as substrate (Kunitz 1947). One enzyme unit (U) of either laccase or protease was defined as the amount of enzyme, which gave an increase of 1·0 U of absorbance per minute in the reaction mixture. β-1,3-Glucanases activity was determined as previously reported (Sharma and Nakas 1987), using laminarine as substrate. One unit of β-1,3-glucanases activity was defined as the amount of enzyme that released 1 μmol of product per minute. The enzymatic activities were reported as U g^{-1} DW.

Measurements of hyphal wall

In unstained colonies, the wall of hyphae of the CC and PC was studied using a transmission electron microscope. Pieces of mycelium (*ca* 2 mm^2) were cut from both CC and PC. The samples were placed in small vials and fixed, dehydrated and embedded as previously described (Sánchez and Moore 1999) and the polymerized blocks were sectioned using an ultramicrotome LEICA OMU4 with glass knives. Sections (*ca* 50 nm thick) of approx. 0·7 mm^2 were placed on grids (G200HEX from Agar Scientific Ltd, Stansted, UK) and dried overnight at room temperature. Sections were stained sequentially for 20 min in 2% (w/v) uranyl acetate (Agar Scientific Ltd) in 70% (v/v) alcohol and for 5 min in 0·3% (w/v) lead citrate (Agar Scientific Ltd) in 0·1 M NaOH and washed in distilled water. Sections were observed using either a Philips 201 (Holland) or a Hitachi 600 (Tokyo) transmission electron microscope. Transmission electron micrographs were recorded on Kodak electron

microscope ESTAR 4489 film. The thickness of hyphal walls in the PC and CC was measured. The measurements were carried out in the middle part of hyphae using a digital micrometer. Data are the mean ± S.E.M. of 10 measurements taken in different hyphae from five different Petri dishes, which were randomly selected from a sample of 30 Petri dishes.

RESULTS

Differentiation of the zones of growth

After 20 min of incubation, Feulgen reagent densely stained the FB and the PC. However, the CC did not take up Feulgen reagent. The agar-solidified culture medium was lightly stained (Fig. 1). With longer incubation (2 h) hyphae in the CC and the culture medium also became densely stained. The radius (measured from the inoculum to the colony periphery) of the colonies of *P. pulmonarius* at the time of staining was of 30·2 ± 1·1 mm, from which 23·3 ± 1·0 mm corresponded to the CC and 6·8 ± 0·3 to the PC. The total area of the colony was 28·65 cm^2, of which 17·05 cm^2 (60%) corresponded to the CC and 11·6 cm^2 (40%) to the PC.

Determination of glycogen, protein and glucans

The content of intracellular protein and glycogen in hyphae of the PC was higher than that observed in hyphae of the CC (by approx. twofold) or the FB (1·6 to twofold higher; Table 1). However, the content of soluble and insoluble glucans in hyphae of the CC was approximately twice that observed in hyphae of the PC. The FB had approx. seven- and threefold higher content of soluble glucans than that observed in the PC and CC, respectively (Table 1). The content of insoluble glucans in the FB was approx. four- and twofold higher than that observed in the PC and the CC, respectively. The hyphae of the FB had lower moisture content than hyphae of the PC or CC (Table 1).

Table 1 Moisture content, intracellular protein and glycogen and cell wall glucan of hyphae of the PC, CC and FB of colonies of *P. pulmonarius* developed on potato dextrose agar for 12 days at 28°C

Mycelial zone	Moisture (%)	Cellular component (mg g^{-1} DW)			
		Cytoplasmic content		Cell wall glucans	
		Glycogen	Protein	Soluble	Insoluble
CC	92 ± 0·3[b]	237 ± 7[c]	11·7 ± 0·3[b]	7·7 ± 0·2[b]	6·4 ± 0·6[b]
PC	96 ± 0·8[a]	507 ± 18[a]	18·0 ± 0·3[a]	3·2 ± 0·2[c]	3·4 ± 0·3[c]
FB	80 ± 0·6[c]	316 ± 17[b]	10·0 ± 0·5[c]	21·5 ± 0·6[a]	13·6 ± 0·5[a]

Mean ± S.E. from three separate experiments. In the same column with the same letter are not significantly different ($P < 0·05$).

Enzymatic assays

The PC contained 30-fold higher laccase activity than the CC, whereas no laccase activity was observed in the FB. Protease activity was only observed in the CC and FB, with higher activity in the FB than in the CC. β-1,3-Glucanases activity in the PC was approximately twice than that observed in the CC (Table 2).

Ultrastructure and measurements of the hyphal wall

The wall of hyphae from the CC (0·151 ± 0·013 μm) was approximately twice the thickness of the wall of hyphae of the PC (0·073 ± 0·008 μm). Hyphae from the CC were less densely stained than hyphae from the PC. The hyphae from the PC also showed typical staining of glycogen in the cytoplasm. None of the hyphae contained large vacuoles, but hyphae from both regions of the colony contained mitochondria and nuclei (Fig. 2).

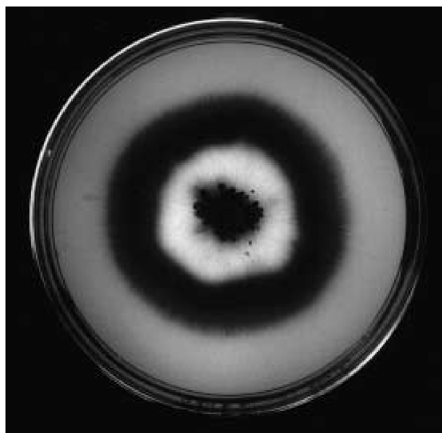

Fig. 1 Fruiting colony of *P. pulmonarius* developed for 12 days at 28°C. The fruit bodies are seen at the centre zone of the colony

Table 2 Intracellular laccases, proteases, and β-1,3-glucanases activity in the PC, CC and FB of a colony of *P. pulmonarius* developed on potato dextrose agar for 12 days at 28°C

Mycelial zone	Enzyme activity (U g^{-1} DW)		
	Laccases	Proteases	β-1,3-Glucanases
CC	1·6 ± 0·2[b]	1·7 ± 1·0[b]	2·5 ± 0·3[b]
PC	47·1 ± 1·7[a]	0·0 ± 0·0[c]	5·4 ± 0·6[a]
FB	0·0 ± 0·0[c]	11·2 ± 0·4[a]	0·9 ± 0·1[c]

Mean ± S.E. from three separate experiments. In the same column with the same letter are not significantly different ($P < 0·05$).

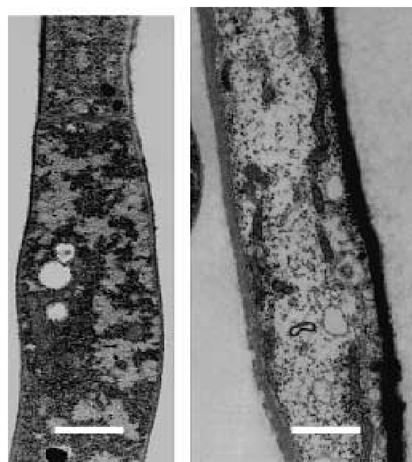

Fig. 2 Ultrastructure of the PC (left) and CC (right) of a fruiting colony of *P. pulmonarius* developed for 12 days at 28°C. Scale bar: 2 μm

DISCUSSION

We have shown here that hyphae at the periphery and the fruit bodies formed in the centre of a colony of *Pleurotus pulmonarius* rapidly took up Feulgen reagent, a stain which is typically used for its reactions with DNA and glycoproteins (Grimstone and Skaer 1972). In contrast, hyphae from the colony centre did not take up the stain rapidly.

Differential staining suggests physiological differentiation between the young and mature hyphae, and between mature hyphae and those undergoing differentiation within the fruit bodies. We have previously demonstrated (Sánchez and Moore 1999) that the earliest stages (initials) of *P. pulmonarius* and *C. cinereus* fruit body formation can be detected by staining with either toluidine blue (which kills the tissue) or Janus green (a vital stain). Such techniques provide valuable tools to monitor development of fruit bodies; however, the basis and cause of the differential staining is completely obscure in these cases.

The present results show that the differential staining of the vegetative colony with Feulgen reagent is correlated with particular biochemical features of the hyphae. Rapidly staining hyphae had only about half the content of both soluble and insoluble glucans observed in mature hyphae of the central zone. On the other hand, rapidly staining peripheral hyphae had the higher glycogen content. It was also observed that the cell walls of hyphae in the nonstaining zone of the colony were considerably thicker than those of hyphae in the staining zone. Basic fuchsin (pararosanilin), the main dye in Feulgen reagent, has been shown to react strongly and immediately with hydrophilic hyphae of *Agaricus bisporus*, but react only weakly and slower with hydrophobic hyphae (Umar and Van Griensven 1997). If this also applies to *Pleurotus*, it could be concluded that older, less active vegetative hyphae which did not readily take up Feulgen reagent, are more hydrophobic than actively growing hyphae at the colony periphery.

The implications are that hyphal maturity in *P. pulmonarius* involves storage of glucans, possibly, in part at least, in the form of a thickened hyphal wall; coupled with a change to the external biophysical properties of the wall. Glycogen, on the other hand, is evidently not employed for long-term storage, but may be the immediate transportable product of rapid assimilatory metabolism in peripheral hyphae. A similar conclusion was drawn about glycogen metabolism during fruit body development in *C. cinereus* (Ji and Moore 1993), where, although massive quantities of glycogen are metabolized as the fruit body matures, the metabolism is made up of repetitive synthesis/degradation cycles that continue through to fruit body maturation.

Hyphal maturation is also correlated with lesser activities of laccase and β-1,3-glucanases, while protease activity increases and the protein content is reduced accordingly. On the other hand, hyphae at the periphery of the colony, assumed to be young and actively extending into fresh substrate, have the highest activity of laccase and β-1,3-glucanases.

These changes during hyphal maturation appear to prepare the mycelia for the formation of fruit body initials. In these, protease activity is further increased, protein content reduced, glucan content increases above that of mature hyphae and laccases and β-1,3-glucanases become less active. Glycogen increases above the level found in the hyphae from which the fruit bodies develop, perhaps being utilized/transported from older regions of the colony. Clearly, as in *C. cinereus*, glycogen plays an important role in the carbohydrate economy of the fruit body, although glucans are much more significant in *Pleurotus* than they are in *Coprinus*.

The role of these changes in hyphal differentiation and their potential exploitation for improved fruit body production remains to be determined, but the ability to detect them with simple staining procedures provides us with a tool to develop a better understanding of production processes.

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