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# Association of Rhizomorph Formation with Laccase Activity in *Armillaria* spp.

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Ethanol and other substances that induce rhizomorphs in *Armillaria* spp. also induced laccase formation. In a range of isolates rhizomorph production and laccase activities were positively correlated. Laccase was first detectable just before the appearance of rhizomorph initials. Its activity reached a peak when rhizomorph growth rate was highest and fell to near zero when rhizomorph growth ceased. Laccase was not detected in non-induced cultures. Laccase reaction with chromogenic substrates was much higher in rhizomorphs than in mycelia. Laccase activity and rhizomorph production, but not mycelial growth, were decreased in the presence of enzyme inhibitors with activity against laccase. The results suggest a role for laccase in morphogenesis and/or growth of rhizomorphs.

## INTRODUCTION

Members of the genus *Armillaria* are among the most damaging root- and butt-rot pathogens of forest and orchard trees world-wide. Rhizomorphs, similar to roots in external appearance, are produced by these fungi on infected root systems; they grow through the soil, where they may contact roots of neighbouring trees. Their importance in the spread of the fungus through the soil has led to much interest in the physiology of rhizomorph induction and growth.

A submerged thallus produces mycelium, but no rhizomorphs, until an atmosphere–medium interface is reached (Snider, 1959). $O_2$ is required for the initial stage of morphogenesis of pseudo- or microsclerotia, which precede rhizomorph formation (Lopez-Real & Swift, 1977). Higher $O_2$ concentrations at the point of origin of rhizomorphs support greater growth (Smith & Griffin, 1971), and rhizomorph production in soil is less when the $O_2$ content of the atmosphere above the soil is reduced (Rishbeth, 1978).

It is commonly observed that rhizomorph tips that contact the atmosphere become brown and cease growth. Indeed, Smith & Griffin (1971) found that $O_2$ partial pressures above 0·04 atm at the rhizomorph surface are inhibitory, although growth requires high concentrations of $O_2$ at the rhizomorph origin or at connections which occasionally form between the rhizomorph canal and substrate–air interfaces. A brown pigment, formed by the action of laccase (EC 1.10.3.2), was thought to inhibit growth in the presence of high $O_2$ concentrations by blocking either nutrient assimilation or excretion of waste products (Smith & Griffin, 1971).

Although rhizomorphs may be produced abundantly on natural substrates and plant extracts, none are produced on a defined glucose–asparagine medium. Weinhold (1963) has shown that the addition of ethanol in low concentrations stimulates mycelial growth and induces abundant rhizomorph growth. Lipids (Moody & Weinhold, 1972) and some phenols such as tannic acid

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*Abbreviation*: DMOP, 2,6-dimethoxyphenol.

(Cheo, 1982) also induce rhizomorphs. Jacques-Felix (1968) found that a phenol oxidase was produced only in cultures producing rhizomorphs. Our initial observations suggested a close association between laccase activity and rhizomorph formation. The results reported here indicate that laccase may be responsible not only for eventual inhibition of rhizomorphs, as Smith & Griffin (1971) suggested, but also for their induction and/or growth.

## METHODS

*Organism. Armillaria mellea* (Vahl: Fr.) Kummer isolate PP, isolated from peach in France, was used for most experiments. A collection of isolates including *A. mellea*, *A. obscura* (Persoon) Herink in Hasek, *A. bulbosa* (Barla) Kile et Watling and *A. cepistipes* Velenovsky was used for a survey of laccase activity and rhizomorph production. A detailed description of these isolates is available on request.

*Culture.* A defined glucose–asparagine medium (Moody & Weinhold, 1972) and inoculum consisting of water agar cultures 2–6 weeks old were used after preliminary experiments to verify the optimum C:N ratio of the medium. To 1 litre were added 5 ml of a solution containing 2 mg $FeSO_4.7H_2O$ $ml^{-1}$, and 10 ml of a solution containing 0·8 mg $Mn(CH_3COO)_2.4H_2O$, 0·2 mg $Zn(NO_3)_2.4H_2O$, 5 mg $Ca(NO_3)_2.4H_2O$ and 0·3 mg $CuSO_4.5H_2O$ $ml^{-1}$. The pH was adjusted to 5. Substances tested for their effect on laccase and rhizomorph production were filter sterilized and added as stock solutions to the autoclaved medium unless stated otherwise. When used, ethanol was added directly to the autoclaved medium to give 0·1% (v/v) in liquid and 0·3% (v/v) in agar (to which it was added just before pouring).

Petri dishes (9 cm diam.) containing 25–30 ml medium were used for agar cultures. The effect of partial $O_2$ deprivation was observed in Petri dishes using three techniques. One week after inoculation, as rhizomorph initials just began to appear, the inoculum plugs were removed from one series of plates and the agar was inverted. The agar was then pressed against the plate around the edges to ensure a seal. In another series of plates, barely molten water agar was poured into the plates to cover the colonies. The inoculum plugs were left exposed above the agar surface in one half, and the plugs were cut off in the other half so that they were completely covered by the agar. The third technique consisted of providing an atmosphere with reduced $O_2$ concentration (3%, v/v) using an automatic gas incubator (Heraeus).

Our early experiments showed that best results with liquid cultures were obtained in 100 ml screw-cap medicine bottles containing 18 ml medium. Circular plugs 4 mm in diameter were cut from growing margins of the inoculum cultures and laid, mycelium down, either on the agar (solid cultures) or on sterile fibreglass cloth (1 × 1 cm) which was then floated on the liquid surface in the bottles. Bottles were stored flat with screw caps loosely fitted; all cultures were grown in the dark at 24 °C.

*Analyses.* After growth in liquid culture, thalli were removed from the bottles and rhizomorphs (including any mycelium growing from them) were separated from the central mycelium. These were weighed after drying at 75 °C for 24 h.

Phenol oxidase activity was detected in agar media by colour development after flooding with 10 mM-guaiacol or 0·05% (w/v) ethanolic syringaldazine. Syringaldazine is oxidized by laccase, but not by catechol oxidase, and produces an intense purple reaction product (Harkin *et al.*, 1974). Distribution of laccase in thalli from liquid culture was observed after treatment with a histological stain (Hermann *et al.*, 1983) based on the oxidation of 4-amino-2,6-dibromophenol and the coupling of the reaction product to 3,5-dimethylalanine.

For quantitative measurement of laccase in liquid media, cultures were first gently shaken, then a small volume (< 1 ml) of medium was removed and centrifuged for 5 min. The supernatant (referred to as culture filtrate) was used in one of two spectrophotometric assays. In one assay, 0·6 ml culture filtrate was added to 2·3 ml 2·9 mM-*p*-phenylenediamine in citrate/phosphate buffer (0·05 M-citric acid/0·1 M-disodium phosphate, pH 4). The increase in absorbance at 460 nm was monitored with a recorder over the absorbance range 0–1, where the reaction rate was constant. For routine assays, 0·4 mM-2,6-dimethoxyphenol (DMOP) in the same buffer was used as a substrate. Unless noted otherwise, 0·2 ml culture filtrate was added to 1·0 ml substrate. Increase in absorbance at 468 nm was monitored as above.

The contribution of peroxidase to the measured activity was determined by the addition of catalase (93 Sigma units $ml^{-1}$ final concentration) and ethanol (0·6%, v/v, final concentration) to the DMOP reaction mixture (0·3 ml culture filtrate in 3 ml final reaction mixture) (Mayer *et al.*, 1964). Water was added in place of catalase solution (0·375 ml) as a control.

The effect of phenylhydrazine, a catechol oxidase inhibitor (Lerner *et al.*, 1971), was tested by preincubating an enzyme mix containing 0·99 ml culture filtrate and 0·14 ml 8 mM-phenylhydrazine or water (control) for 30 min before adding 0·6 ml enzyme mix to 2·3 ml *p*-phenylenediamine substrate solution. Its effect could not be tested with DMOP as a substrate because it reduced absorbance by direct interaction with the oxidation product.

For extraction of the brown pigment from rhizomorphs, the fungus was first grown for three months on beech branch segments (3 cm diameter, 4 cm long). Soil was collected from a beech forest and placed in pots in the greenhouse; the segments were then buried in the pots for an additional three months. Rhizomorphs were

separated from the soil, washed in water, and dried at 70 °C. Rhizomorphs (20 g) were extracted in 100 ml 1 M-KOH for 2 h at 100 °C. The absorption spectrum of the extract was determined as described by Chet & Hüttermann (1977).

## RESULTS

### *Co-induction and timing of laccase and rhizomorphs*

Ethanol in low concentrations induces rhizomorphs and stimulates mycelial growth on a defined glucose–asparagine medium (Weinhold, 1963). In agar media with ethanol, flooding with guaiacol or syringaldazine resulted in colour development within minutes. The guaiacol continued to darken whereas the syringaldazine began losing colour after 20–30 min. The substrates were oxidized in the agar around the protruding rhizomorphs as well as between them. The central mycelium did not appear to react. Cultures without ethanol showed a barely visible reaction after 0·5–1 h.

The identity of the phenoloxidase as laccase was suggested by its extracellular nature, a characteristic of laccase, and by the oxidation of syringaldazine, a substrate of laccase and peroxidase but not of catechol oxidase (Harkin *et al.*, 1974). Another laccase substrate, *p*-phenylenediamine, was oxidized in a spectrophotometric assay ($\Delta A$ min$^{-1}$ = 0·02); phenylhydrazine, a catechol oxidase inhibitor, did not inhibit this activity. The possibility that peroxidases were responsible for the phenoloxidase activity was tested by destruction of peroxides with catalase in the DMOP assay. No decrease in activity was observed.

In liquid media, substances which induced rhizomorphs, including ethanol, invariably induced laccase as well (Table 1).

In order to observe the sequence and progress of laccase and rhizomorph production, liquid cultures with and without ethanol were inoculated and sampled every 2 d for laccase and dry matter production. Normal S-shaped curves resulted for mycelium and rhizomorph growth in the presence of ethanol, while no rhizomorphs and less mycelium were produced without ethanol (Fig. 1). Laccase was first detected 2 d before appearance of the first rhizomorphs. Its activity was proportional to rhizomorph growth rate and decreased as rhizomorph growth ceased. No laccase was detected in the absence of ethanol.

### *Co-inhibition of laccase and rhizomorphs*

The effect of inhibitors of laccase activity was examined. Thioglycolic acid, 2-mercaptoethanol and $Na_2EDTA$ (autoclaved with medium) substantially inhibited rhizomorph formation at the concentrations tested (Table 2). In a separate experiment, the effects of $Na_2EDTA$ on rhizomorph production and laccase activity were reversed by the addition of $Cu^{2+}$, $Mn^{2+}$ and, to a lesser extent, $Zn^{2+}$ (all 1 mM) (data not shown). Mycelial growth was either unaffected or increased in the presence of these inhibitors. However, sodium azide, at a concentration that resulted in comparable inhibition of rhizomorph and laccase production, inhibited mycelial growth as well (Table 2).

Inversion of agar cultures and a complete overlay of water agar delayed or prevented rhizomorph formation (Fig. 2). Abundant rhizomorphs formed in undisturbed controls, in those in which the inoculum plug was removed, and in those receiving incomplete water agar overlay

Table 1. *Co-induction of laccase activity and rhizomorph growth in A. mellea*

Test substances were added on day 0, laccase (DMOP substrate) was measured on day 17, and cultures were harvested on day 20. Each value is the mean of five replicates, ± SE.

| Test substance | Dry wt (mg) | | Laccase activity ($\Delta A$ min$^{-1}$) |
|---|---|---|---|
| | Mycelium | Rhizomorphs | |
| None (control) | 9 ± 3 | 0 | 0·00 |
| Ethanol (0·1%) | 26 ± 4 | 85 ± 7 | 0·22 ± 0·04 |
| Lignosulphonic acid (0·2%) | 22 ± 3 | 99 ± 7 | 0·27 ± 0·04 |
| Yeast extract (0·2%) | 24 ± 6 | 107 ± 6 | 0·62 ± 0·14 |

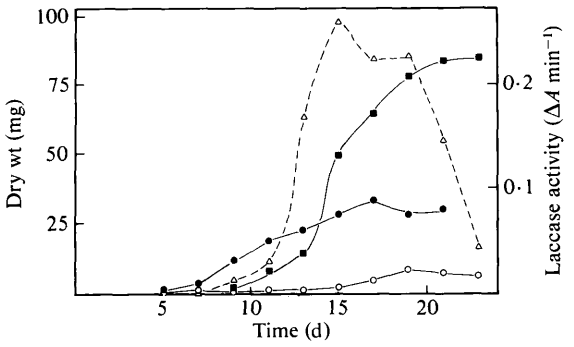


Fig. 1. Laccase activity (△), mycelium dry wt (○, ●) and rhizomorph dry wt (■) of *A. mellea* in liquid cultures with (●, ■, △) or without (○) ethanol. Each point is the mean of five replicates (with ethanol) or three replicates (without ethanol). The laccase assay mixture contained 0·2 ml culture filtrate and 0·8 ml substrate solution.

Table 2. *Inhibition of rhizomorph production and laccase in A. mellea*

All cultures contained 0·1% ethanol. Putative inhibitors were added on day 0, laccase (DMOP substrate) was measured on day 16, and cultures were harvested on day 20. Each value is the mean of five replicates, ±SE.

| Inhibitor | Dry wt (mg) | | Laccase activity ($\Delta A$ min$^{-1}$) |
|---|---|---|---|
| | Mycelium | Rhizomorphs | |
| None (control) | 32 ± 4 | 74 ± 9 | 0·15 ± 0·04 |
| $Na_2EDTA$ (1 mM) | 44 ±11 | 14 ± 12 | 0·02 ± 0·01 |
| Thioglycollic acid (2 mM) | 51 ±8 | 12 ± 11 | 0·00 |
| Mercaptoethanol (1 mM) | 32 ± 3 | 14 ± 21 | 0·01 ± 0·02 |
| $NaN_3$ (6 μM) | 19 ± 9 | 10 ± 8 | 0·01 ± 0·02 |

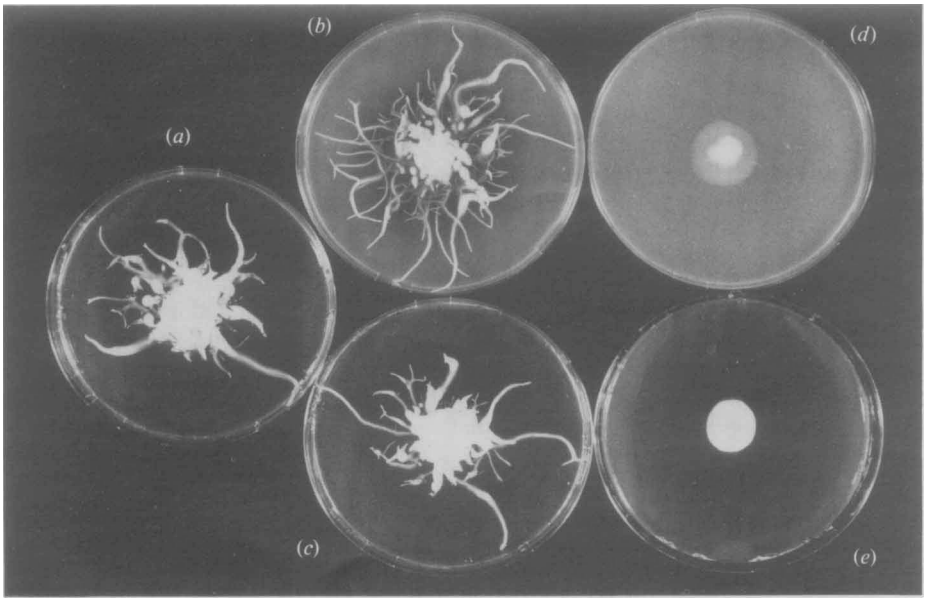


Fig. 2. Effect on *A. mellea* of treatments to reduce $O_2$ availability. All cultures contained 0·3% ethanol, and were photographed after 15 d growth. Treatments were done at day 7: (*a*) undisturbed control; (*b*) culture overlayed with water agar, inoculum plug still in contact with air; (*c*) inoculum plug cut; (*d*) inoculum plug cut and culture overlayed with water agar; (*e*) inoculum plug cut and culture inverted.

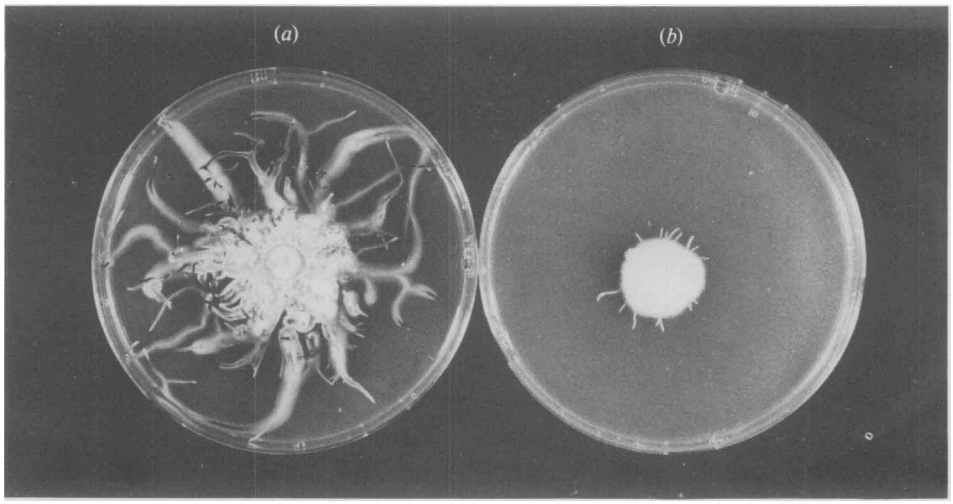


Fig. 3. Effect of reduced $O_2$ concentration on *A. mellea*. Photographed after 18 d growth. (*a*) 21% (v/v) $O_2$; (*b*) 3% (v/v) $O_2$.

(with intact inoculum plug still in contact with air). Though mycelial growth continued, rhizomorph formation was not observed in cultures where inoculum plug removal was combined with agar overlay, and was delayed 10 d in inverted cultures.

Similarly, incubation of cultures with 3% (v/v) $O_2$ also delayed rhizomorph formation (Fig. 3). Control cultures produced rhizomorphs on day 7, while rhizomorphs appeared on day 15 at reduced $O_2$ concentrations, when mycelial growth had already progressed well beyond the stage at which rhizomorphs normally appear. Additional cultures containing 0·2% (w/v) gallic acid showed minimal browning with reduced $O_2$ compared with those at 21% (v/v) $O_2$.

### *Laccase and melanin-like pigment in the rhizomorphs*

In an attempt to locate the enzyme more exactly, thalli from liquid cultures with actively growing rhizomorphs were rinsed and treated with a laccase stain (Hermann *et al.*, 1983). The colour development proved too diffuse to detect microscopically, but reaction in the rhizomorphs was obvious macroscopically. Little or no reaction occurred 0–5 mm behind growing rhizomorph tips or in the central mycelium.

The pigment extracted from rhizomorphs was brownish-black. Neutralization of the solution resulted in precipitation. The pigment was decolorized by 1% $H_2O_2$. Its absorption spectrum showed no peak in the range 200–650 nm. Plotting the logarithm of the absorbance *vs* wavelength gave a line with a slope of −0·0042. These features are characteristic of melanin-like substances (Chet & Hüttermann, 1977).

### *Survey of isolates for laccase and rhizomorphs*

A survey of 32 isolates representing four species of *Armillaria* showed a positive correlation ($r = 0{\cdot}85$) between laccase activity and rhizomorph production in ethanol-supplemented liquid cultures. A log–log plot resulted in a somewhat more linear trend (Fig. 4), but the data transformation did not significantly affect the correlation coefficient.

Although the species all followed the same trend of increasing rhizomorph weight with increasing laccase activity, they were not distributed equally across the ranges of those variables. Thus *A. mellea* isolates tended to have relatively high laccase activity and rhizomorph production, *A. obscura* isolates had low laccase activity and generally low rhizomorph production, while *A. bulbosa* was more variable in these respects.

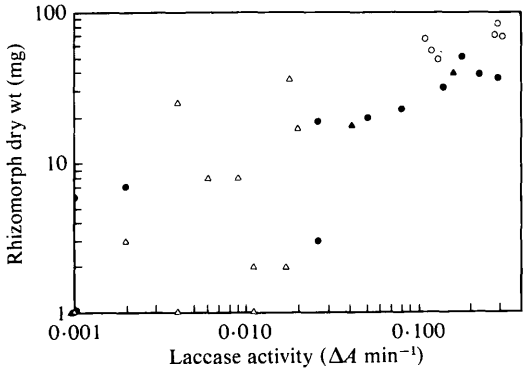


Fig. 4. Laccase activity and rhizomorph production of 32 isolates representing four species of *Armillaria* (○, *A. mellea*; △, *A. obscura*; ●, *A. bulbosa*; ▲, *A. cepistipes*). Laccase was measured on day 16 and cultures harvested on day 20. Correlation coefficient ($r$) using log-transformed data = 0·84.

## DISCUSSION

The results presented here demonstrate a close relationship between laccase activity and rhizomorph growth in *Armillaria* spp. Laccase was detected only when rhizomorphs were induced, and was localized primarily in the rhizomorphs rather than in the mycelium. The appearance of laccase shortly preceded that of rhizomorphs, and activity increased and declined with rhizomorph growth rate. The ability of isolates to produce rhizomorphs under our cultural conditions was correlated with their laccase activity. Because specific inhibitors of laccase are not known, enzyme inhibition cannot be used to determine unequivocally whether or not laccase is necessary for rhizomorph morphogenesis or growth. Nevertheless, the inhibitors decreased laccase activity and rhizomorph production while mycelial growth was uninhibited, suggesting that laccase may have a role in rhizomorph production.

Incubation of cultures at a reduced $O_2$ concentration inhibited rhizomorph growth while allowing continued mycelial growth. Such treatment reduced laccase activity (as indicated by less browning of gallic acid under the same conditions). Laccases of wood decay fungi, including *Armillaria* spp., are known to be more sensitive to $O_2$ deprivation than is mycelial growth (Smith & Griffin, 1971; Worrall & Parmeter, 1983). Inverting the agar and overlaying cultures with water agar had a similar effect which was probably not due to $CO_2$ accumulation as $CO_2$ has been shown to have very little or a slight stimulatory effect on *Armillaria* spp. (Smith & Griffin, 1971). The special $O_2$ requirement for rhizomorph formation observed by us and by other workers (Lopez-Real & Swift, 1977; Smith & Giffin, 1971; Snider, 1959) could thus be explained by a morphogenetic role for laccase.

It has been suggested that laccase may have various functions in fruiting body formation in certain basidiomycetes. Actively growing margins of fruiting bodies of wood-decay fungi often have high laccase activities (Harkin *et al.*, 1974), and several studies have described the association of laccase with fruit-body development in basidiomycetes (Leatham & Stahmann, 1981; Leslie & Leonard, 1979; Ross, 1982; Wood, 1980). There is also evidence suggesting a morphogenetic role for phenoloxidases in production of sclerotia by *Sclerotinia sclerotiorum* (Willets, 1978).

Electron micrographs of rhizomorphs show an electron-dense pigment in the intercellular spaces of the rinds of brown rhizomorphs which was shown to be a melanin-like polyphenol (Smith & Griffin, 1971). Phenols are produced by *A. mellea* in a defined medium, and lower amounts of soluble phenolics were found in the presence of ethanol (Vance & Garraway, 1973). It seems likely that oxidative polymerization by laccase, induced by ethanol, is responsible for this decrease in soluble phenols. The traditional Japanese method of lacquer production using laccase, and recent research on production of industrially useful wood adhesives using laccase and lignin wastes (Haars & Hüttermann, 1984) show the powerful binding properties of certain

phenolic materials polymerized by laccase. Thus, binding of hyphae by the polymerization of phenols in the intercellular spaces may be one function of laccase. The hypothesis that phenoloxidase-catalysed formation of extracellular pigments is coupled to oxidative polymerization of cell wall components, strengthening cell–cell adhesion, has been discussed recently with reference to morphogenesis in higher fungi (Leatham & Stahmann, 1981).

Since this intercellular polymerization and, as we have shown, laccase activity, occur approximately 5 mm behind the actively growing rhizomorph tip, it is unlikely that it has a strictly morphogenetic function. Such melanin-like pigments are associated with ripening and may protect structures from microbial lysis in the soil (Bloomfield & Alexander, 1967). Thus, laccase may be associated with ripening and, as Smith & Griffin (1971) suggested, eventual cessation of growth in conditions of excess aeration. The results presented here suggest that laccase may also have another, morphogenetic or developmental, function. Quite early stages of morphogenesis may be influenced by laccase in systems that have been more closely studied, such as *Coprinus congregatus*, where laccase appears to mediate the induction of fruiting by light (Ross, 1982).

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