FULL PAPER

Effect of Cu^{2+}, Mn^{2+} and aromatic compounds on the production of laccase isoforms by *Coprinus comatus*

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Abstract Six different extracellular laccase isoforms were identified in submerged cultures of the commercially important edible mushroom, *Coprinus comatus*. Although laccase activity ($\sim$55 IU/L) was readily detectable in unsupplemented control cultures containing 1.6 μM Cu^{2+} after 22-day incubation, mean enzyme levels ($\sim$150–185 IU/L) were 2.7–3.4-fold higher in cultures supplemented with 0.5–3.0 mM Cu^{2+}. Laccase production was also stimulated by Mn supplementation over the range 0.05–0.8 mM Mn^{2+}, and the peak value of $\sim$225 IU/L recorded after 22 days in cultures containing 0.8 mM added Mn^{2+} was 4.5-fold higher compared with unsupplemented controls. Of 12 aromatic compounds tested for their effect on laccase isozyme production by *C. comatus*, highest laccase levels ($\sim$188 IU/L), equivalent to a 4.4-fold increase compared with unsupplemented controls ($\sim$43 IU/L), were recorded after 22 days in cultures supplemented with 3.0 mM caffeic acid. Other aromatic compounds tested all stimulated laccase production, with peak enzyme levels 1.3–3.3-fold higher compared with unsupplemented controls. Extracellular laccase levels in cultures supplemented with optimal concentrations of Mn^{2+} and caffeic acid together were 38% and 15% lower, respectively, compared with cultures containing the separate supplements. Lac1 was the most abundant laccase isoform produced under all the conditions tested, but marked differences were observed in the production patterns of Lac2–Lac6.

Introduction

The mushroom industry is expanding globally, with world production currently in excess of 5×10^6 tonnes fresh weight annually (Kues and Lui 2000) and expected to increase further due to growing market demand. Mushrooms are normally cultivated on an industrial scale using different lignocellulosic materials comprising a broad range of wastes emanating from the agricultural, food and forest industries. A range of enzymes are synthesized during mushroom growth on these substrates including laccases [benzenediol:oxygen oxidoreductases (EC 1.10.3.2)], copper-containing enzymes that catalyze the oxidation of a broad spectrum of phenolic compounds and non-phenolic substrates using molecular oxygen as the electron acceptor. Laccases are widely distributed among fungi, where they have been assigned roles in lignin degradation (Bourbonnais et al. 1995; Eggert et al. 1997), in rendering phenolic compounds less toxic via oxidative coupling and polymerisation (Bollag et al. 1988; Moldes and Sanroman 2006) and in mushroom fruit-body morphogenesis (Zhao and Kwan 1999; Chen et al. 2004). Laccases may be either constitutive or inducible, and many factors including culture parameters (Fu et al. 1997), heavy metals (Baldrian and Gabriel 2002; Chen et al. 2003) and aromatic compounds (Scheel et al. 2000) are reported to influence enzyme production in different fungal species.

Since all three of the aforementioned functions of laccases are of fundamental importance for substrate colonisation and mushroom sporophore development, laccase production has been studied extensively in several mushroom species

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(Perry et al. 1993; Soden and Dobson 2001). However, one notable exception is the basidiomycete, *Coprinus comatus* (O.F. Müll.) Pers., a delicious and highly nutritious edible fungus that is also recognized to be a source of various bioactive compounds exhibiting a range of medicinal properties (Fan et al. 2006; Hana et al. 2006). In this paper, we now report the production of multiple extracellular laccase isozymes by this commercially important mushroom and describe various parameters affecting isozyme induction during growth of the fungus in submerged culture.

Materials and methods

Organism and growth conditions

Coprinus comatus was obtained from the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, Shanghai, People's Republic of China and maintained on potato dextrose agar (PDA) at 4°C with periodic transfer.

The fungus was cultivated at 25°C in stationary 250-ml Erlenmeyer flasks containing 50 ml basal medium, pH 6.0, as described previously (Ding et al. 2006). Basal medium contained (per liter): 1.0 g KH_2PO_4, 0.4 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.013 g $CaCl_2 \cdot 2H_2O$, 0.1 g yeast extract, 0.5 g NH_4NO_3, 3.0 g asparagine and 2 ml Tween 80. After autoclaving and cooling to room temperature, 2.5 mg/l thiamine and 1 ml/l of a trace-elements solution consisting of (per liter): 4.8 g $FeC_6H_5O_7 \cdot 5H_2O$, 2.64 g $ZnSO_4 \cdot 4H_2O$, 2.0 g $MnCl_2 \cdot 4H_2O$, 0.4 g $CoCl_2 \cdot 6H_2O$ and 0.4 g $CuSO_4 \cdot 5H_2O$ was added. Four agar discs (1 cm diameter), cut from the growing edge of a 7-day PDA culture, were used to inoculate each flask.

Induction of laccase isozymes by aromatic compounds was determined by adding different test compounds (0.05–3.0 mM final concentration) to 10-day-old cultures grown on basal medium. Induction of laccase isozymes by Cu^{2+} or Mn^{2+} was determined by adding $CuSO_4$ or $MnSO_4$ (0.05–8.0 mM final concentration) to 10-day-old cultures grown on basal medium. All experiments were carried out in triplicate.

Sampling procedures

For time-course experiments, 0.3 ml sample was taken from each of triplicate flasks every 3 days and centrifuged, and the supernatant was used for enzyme activity measurements. In induction experiments, samples were harvested as above after 22 days from flasks supplemented with metal ions and aromatic compounds, respectively.

Enzyme assay

Laccase activity was determined using guaiacol as substrate in reaction mixtures containing 2.0 ml 50 mM succinic acid buffer (pH 5.0) with 1 mM guaiacol, 0.1 ml culture supernatant and 0.3 ml dH_2O. After incubation in a 37°C water bath for 30 min, samples were immersed in ice/water to terminate the reaction, and the amount of oxidized product ($\varepsilon = 12,100$ M^{-1} cm^{-1}) was determined by measuring absorbance at 465 nm. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 µmol guaiacol per min under the assay conditions.

Polyacrylamide gel electrophoresis and activity staining of gels

Native polyacrylamide gel electrophoresis (PAGE) (10% w/v gel) was performed on aliquots of 22-day culture liquids (24 µL) using the Mini-Protean II system (Bio-Rad). Protein bands exhibiting laccase activity stained green with ABTS (0.03% w/v) in 0.125 M succinic acid buffer, pH 5.0.

Chemicals

Vanillin, cinnamic acid, 3,4-dimethoxybenzaldehyde, 4-methoxybenzoic acid, 3,4,5-trimethoxycinnamic acid, 4-methoxycinnamic acid, 4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, 3-(4-methoxyphenyl) propionic acid, 3-(4-hydroxyphenyl) propionic acid, caffeic acid and guaiacol were purchased from Aldrich. All other chemicals were from Chinese sources and were of analytical-reagent (AR) grade.

Results

Time course of extracellular laccase production in unsupplemented cultures of *C. comatus*

Prior to studying the effects of various media supplements on extracellular laccase isozyme induction, total enzyme activity in the extracellular fluid of unsupplemented *C. comatus* cultures was measured over time courses in order to determine optimal sampling and harvesting times. Low levels of laccase were detectable after 3 days and then increased rapidly from 9 days to reach a maximum of 58.5 IU/L before termination of the experiment after a 24-day growth period (Fig. 1).

Effect of copper on laccase isozyme induction

The effect of a range of Cu^{2+} concentrations (0.05–8.0 mM) on total extracellular laccase production in submerged cultures of *C. comatus* is shown in Fig. 2a. Laccase activity ($\sim$55 IU/L) was readily detectable in unsupplemented control cultures (1.6 µM Cu^{2+}) after 22-day
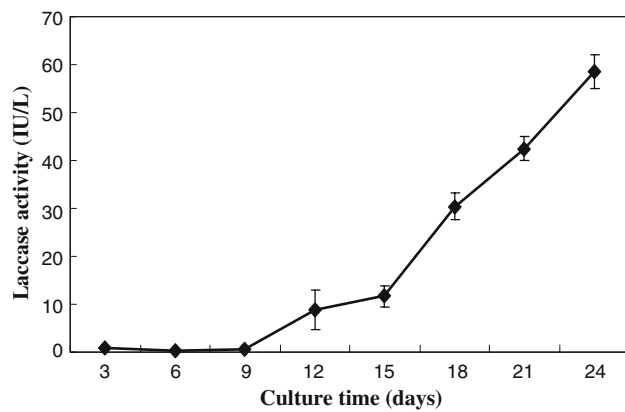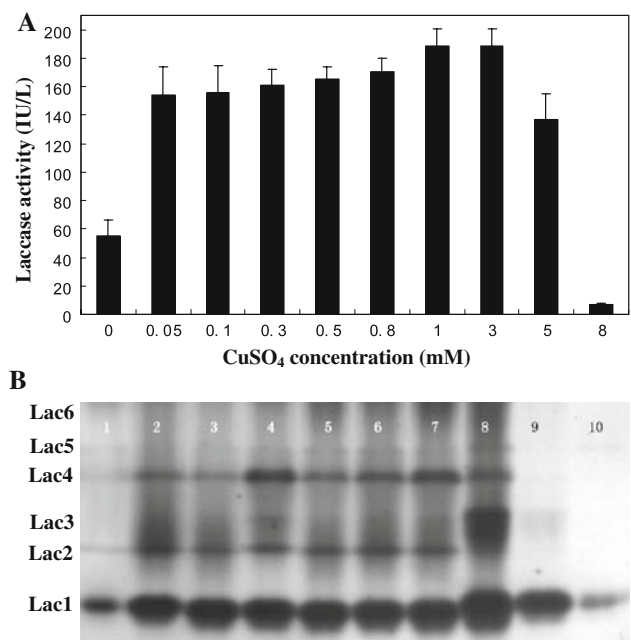
Fig. 1 Time course of laccase production by *Coprinus comatus* grown in basal medium. Values are the means of triplicate determinations; *error bars* represent standard deviation (SD)

Fig. 2 **a** Effect of CuSO$_4$ concentration on extracellular laccase production by *Coprinus comatus*. Values are the mean of triplicate determinations after 22-day incubation; error bars represent SD. **b** Zymogram of laccase isozymes in copper-supplemented cultures. *Lanes 1–10* samples (24 µL) from cultures supplemented with 0, 0.05, 0.1, 0.3, 0.5, 0.8, 1.0, 3.0, 5.0 and 8.0 mM CuSO$_4$, respectively

incubation, but mean enzyme levels ($\sim$150–185 IU/L) were 2.7–3.4-fold higher in cultures supplemented with 0.5–3.0 mM Cu^{2+}. Enzyme activity was marginally lower (135 IU/L) in cultures supplemented with 5 mM Cu^{2+}, although still 2.5-fold higher than controls, whereas only very low laccase activity ($\sim$8 IU/L, 15% of controls) was detectable in cultures supplemented with 8 mM Cu^{2+}.

Native PAGE analysis of culture fluids revealed at least six protein bands exhibiting laccase activity, all of which, with the exception of the band designated Lac3, were detectable in both unsupplemented and supplemented samples (Fig. 2b). The most prominent band, designated Lac1, was clearly evident in unsupplemented samples, and was even more intense in samples supplemented with 0.05–5.0 mM Cu^{2+}. Isozymes designated Lac2, Lac4 and Lac6 were less evident than Lac1 in unsupplemented cultures, stained more intensely in cultures containing 0.05–1.0 mM added Cu^{2+} and were absent from samples supplemented with higher Cu^{2+} concentrations. A band designated Lac3 was strongly induced in 3.0 mM Cu^{2+} supplemented culture, but was absent from unsupplemented culture and cultures supplemented with other Cu^{2+} concentrations. A band designated Lac5 exhibited similar intensity in both unsupplemented and supplemented (0.05–3.0 mM) samples.

Effect of manganese on laccase isozyme induction

The effect of a range of Mn^{2+} concentrations (0.05–8.0 mM) on total extracellular laccase production in submerged cultures of *C. comatus* is shown in Fig. 3a. Laccase activity ($\sim$50 IU/L) was again readily detectable in unsupplemented cultures ($\sim$10 µM Mn^{2+}). Enzyme production increased with increasing supplementation over the range 0.05–0.8 mM Mn^{2+}, and the peak value of $\sim$225 IU/L recorded after 22 days in cultures containing 0.8 mM added Mn^{2+} was 4.5-fold higher compared with

unsupplemented cultures. Gel electrophoresis revealed that Lac1 was again the dominant isozyme, and an increase in the intensity of this band corresponded to the higher enzyme levels recorded in culture supernatants (Fig. 3b). Mn^{2+} supplementation over the range 0.3–1.0 mM stimulated Lac2 and Lac3 activity, although two bands were very diffuse and unclear.

Effect of aromatic compounds on laccase isozyme induction

Twelve aromatic compounds were evaluated for their effect on laccase isoenzyme production by *C. comatus* grown on a basal medium containing trace amounts of Cu^{2+} and Mn^{2+} (1.6 and 10 µM, respectively). Highest laccase levels ($\sim$188 IU/L), equivalent to a 4.4-fold increase compared with unsupplemented controls ($\sim$43 IU/L), were recorded after 22 days in cultures supplemented with 3.0 mM caffeic acid (Fig. 4a). All other aromatic compounds tested stimulated laccase production, and peak fold-increases in enzyme levels (compared with controls) together with the corresponding concentration of aromatic compound were as follows: cinnamic acid, 1.75/0.05 mM; 3,5-dimethoxy-4-hydroxycinnamic acid, 1.94/0.5 mM; 4-hydroxycinnamic acid, 2.8/3.0 mM; guaiacol, 2.2/0.8 mM; 3-(4-hydroxyphenyl) propionic acid, 1.99/3.0 mM; 3-(4-methoxyphenyl)
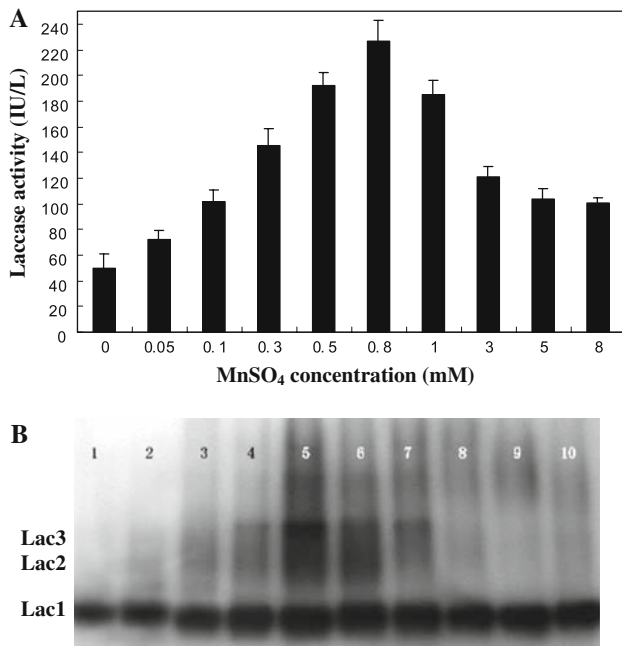
Fig. 3 a Effect of MnSO₄ concentration on extracellular laccase production by *Coprinus comatus*. Values are the mean of triplicate determinations after 22-day incubation; *error bars* represent SD. **b** Zymogram of laccase isozymes in manganese-supplemented cultures. *Lanes 1–10*: samples (24 μL) from cultures supplemented with 0, 0.05, 0.1, 0.3, 0.5, 0.8, 1.0, 3.0, 5.0 and 8.0 mM MnSO₄, respectively

Fig. 4 a Effect of caffeic acid concentration on extracellular laccase production by *Coprinus comatus*. Values are the mean of triplicate determinations after 22-day incubation; error bars represent SD. **b** Zymogram of laccase isozymes in caffeic acid-supplemented cultures. *Lanes 1–10* samples (24 μL) from cultures supplemented with 0, 0.05, 0.1, 0.3, 0.5, 0.8, 1.0 and 3.0 mM caffeic acid, respectively

propionic acid, 1.7/0.1 mM; 4-methoxybenzoic acid, 1.47/0.3 mM 3,4,5-trimethoxycinnamic acid, 1.5/0.3 mM; 4-methoxycinnamic acid, 1.95/0.1 mM; 3,4-dimethoxy-benzaldehyde, 3.3/3.0 mM; vanillin, 1.3/0.8 mM.
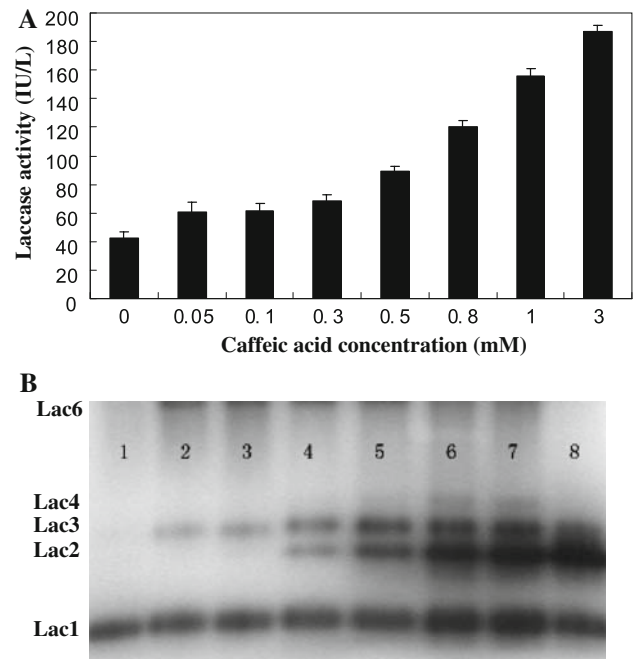
A very strong Lac1, two strong Lac2 and Lac3 and two fainter Lac4 and Lac6 bands were evident in gels prepared from cultures supplemented with caffeic acid (Fig. 4b). The concentration of caffeic acid added to the culture medium had a pronounced stimulatory effect on the levels of Lac2 and Lac3, and the latter was only evident in cultures supplemented with caffeic acid at or above 0.3 mM.

Laccase production in caffeic-acid-supplemented cultures was also influenced by the time of addition of the aromatic compound, with highest enzyme levels recorded in cultures supplemented immediately prior to, and 5 days following, inoculation (Fig. 5).
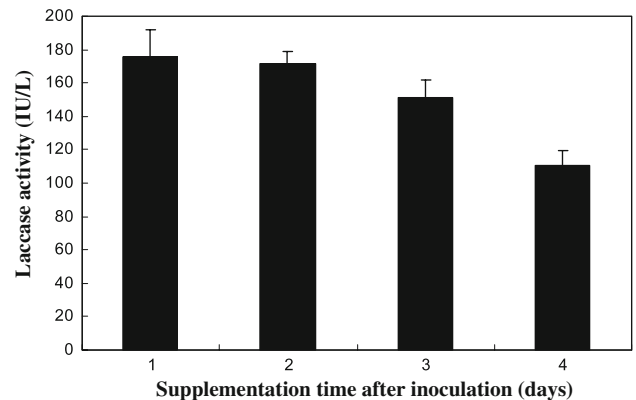
Extracellular laccase levels in cultures supplemented with optimal concentrations of Mn^{2+} and caffeic acid together were 38% and 15% lower, respectively, compared with cultures containing the separate supplements (Fig. 6).

Discussion

In many fungal species, laccases occur as groups of isozymes encoded by gene families (Mansur et al. 1997,

Fig. 5 Effect of caffeic acid supplementation time on extracellular laccase production by *Coprinus comatus*. Caffeic acid (1.0 mM) was added to cultures immediately prior to, and 4, 10 and 15 days following, inoculation. Values are the mean of triplicate determinations after 22-day incubation; *error bars* represent SD

1998; Yaver and Golightly 1996). The largest laccase gene family identified so far, in the genome of *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys & Moncalvo, contains 17 non-allelic laccase genes and one gene footprint (Kilaru et al. 2006). The rationale for multiple laccase production is still unclear, although biochemical evidence suggests that the laccase isozymes synthesized by many fungi have different properties (Galhaup et al. 2002;
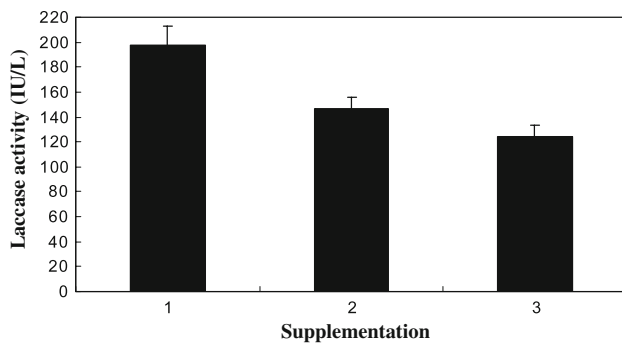
Fig. 6 Effect of Mn^{2+}, caffeic acid and combined Mn^{2+}/caffeic acid supplementation on extracellular laccase production by *Coprinus comatus*. *Lane 1* 0.8 mM $MnSO_4$, *Lane 2* 3.0 mM caffeic acid, *Lane 3* 0.8 mM $MnSO_4$ + 3.0 mM caffeic acid. Values are the mean of triplicate determinations after 22-day incubation; *error bars* represent SD

Palmieri et al. 2003; Susla et al. 2007). Therefore, isozymes with different functional roles may have evolved to allow fungi to succeed in a range of environments.

In this paper, we report for the first time the production of multiple laccase isoforms by the commercially important mushroom, *C. comatus*. In common with other fungi (Gnanamania et al. 2006; Ohga et al. 1999), laccase isozyme patterns varied considerably in response to changes in culture conditions during fungal growth in submerged culture. Supplementation of *C. comatus* cultures with copper markedly increased total enzyme activity and induced synthesis of an additional isoform. Lac3 was strongly induced by addition of 3 mM Cu^{2+}, while other concentrations of Cu^{2+} (0.05–1 mM) induced both Lac1 and Lac2. Copper is a strong inducer of laccase in many fungal species, including *Trametes versicolor* (L.) Lloyd (Collins and Dobson 1997), *Marasmius quercophilus* Pouzar (Klonowsk et al. 2001), *Pleurotus ostreatus* (Jacq.) P. Kumm. (Hana et al. 2006; Palmieri et al. 2000), *Volvariella volvacea* (Bull.) Singer (Chen et al. 2003) and *Neurospora crassa* Shear & B.O. Dodge (Huber and Lerch 1987). In *P. ostreatus*, copper not only regulated laccase gene expression but also positively affected the activity and stability of the enzyme (Baldrian and Gabriel 2002). The effect of copper on enzyme stability may be related to the inhibitory effect of the metal on the activity of an extracellular protease produced by *P. ostreatus* (PoS1) that is reported to degrade laccase (Palmieri et al. 2001). The optimal copper concentration (3.0 mM) for laccase production by *C. comatus* is significantly higher than the 2–600 µM Cu^{2+} typically added to culture media in studies on fungal laccases (Collins and Dobson 1997; Ohga et al. 1999; Chen et al. 2003; Susla et al. 2007). Copper concentrations in excess of 10 µM are normally toxic to microbial cells (Labbé and Thiele 1999). However, copper is normally incorporated into culture medium prior to

inoculation, and the stimulating effect of copper at the higher concentrations used here may be due in part to our procedure of supplementing cultures after 10-day incubation, when fungal biomass production was close to maximum.

Manganese was also a highly effective inducer of laccase in *C. comatus*. Supplementation of *C. comatus* cultures with manganese markedly increased total enzyme activity and induced synthesis of additional isoforms (Lac2 and Lac3), although the resultant isoenzyme patterns were different compared with copper-supplemented cultures. Manganese increases both laccase activity and expression in *Pleurotus sajor-caju* (Fr.) Singer, and laccase production increased ~1.5-fold in fungal cultures supplementation with 300 µM $MnSO_4$ (Soden and Dobson 2001). Increased laccase messenger RNA (mRNA) levels have also been reported in manganese-supplemented cultures of the white-rot fungi *Clitocybula dusenii* (Bres.) Singer and *Naematoloma frowardii* (Speg.) E. Horak (Scheel et al. 2000). Relatively little is known about Mn^{2+}-dependent regulation of gene expression in fungi, although a putative Mn^{2+}-responsive element required for manganese regulation of manganese peroxidase isozyme 1 gene expression was identified in *Phanerochaete chrysosporium* Burds. (Ma et al. 2004).

Stimulation of laccase production in fungi by aromatic compounds has been widely reported, and all 12 aromatic compounds tested in this study had a positive effect on laccase biosynthesis in *C. comatus*. However, while Lac1 was the dominant isozyme present in all other aromatic-supplemented cultures (data not shown), the pattern of Lac2–Lac6 isozyme production depended very much on the nature of the supplementation. Thus, Lac1 was usually the only isozyme detected in cultures supplemented with 3,5-dimethoxy-4-hydroxycinnamic acid, 4-hydroxycinnamic acid, guaiacol and cinnamic acid, whereas Lac1 and a very weak Lac2 band were evident in 3,4-dimethoxy-benzaldehyde- and vanillin-supplemented cultures (data not shown). In addition to the dominant Lac1 band, two very weak bands corresponding to Lac4 and Lac5 were detected in cultures containing either 3-(4-hydroxyphenyl) propionic acid or 3-(4-methoxyphenyl) propionic acid, and three very weak Lac3, Lac4 and Lac5 bands were seen in cultures supplemented with 4-methoxy benzoic acid (data not shown).

Lac2 and Lac3 were strongly induced by caffeic acid, whereas both these isoforms were undetectable in cultures supplemented with the structurally related 4-hydroxycinnamic acid which lacks the 3-hydroxyl group on the aromatic ring. As far as we are aware, caffeic acid has not previously been reported to be an inducer of laccase. Interestingly, combined supplementation of *C. comatus* cultures with optimum concentrations of caffeic acid (0.8 mM) and Mn^{2+} (3.0 mM) led to a decrease in total

extracellular laccase production compared with cultures supplemented with caffeic acid alone. There is a possibility that, in the presence of high Mn^{2+} (0.8 mM), simultaneously addition of caffeic acid may interfere with Mn^{2+}, lowering the pool of soluble manganese ions and caffeic acid and therefore decreasing the induction of laccase.

Aromatic inducers with different substituted groups also stimulated *Trametes* sp. AH28-2 to synthesize different laccase isoforms, with *o*-toluidine inducing LacA and 3,5-dihydroxytoluene mainly inducing LacB (Xiao et al. 2006). Wide variations with respect to the induction of laccase gene transcription by aromatic compounds have also been documented (Yaver and Golightly 1996; Mansur et al. 1998). Terrón et al. (2004) reported that guaiacol and *p*-coumaric acid selectively induced expression of *lcc1* and *lcc2*, and ferulic acid induced *lcc3* expression, in *Trametes* sp. I-62. Transcription of one laccase gene, *lcc1*, from *Trametes villosa* was induced about 17-fold by the addition of 2,5-xylidine, but a second gene (*lcc2*) was constitutive under the conditions tested (Yaver et al. 1996). It has been suggested that laccase may play a role in protection against oxidative stress caused by oxygen radicals originating from the aromatic compounds (Collins and Dobson 1997; Eggert et al. 1996), although the manifold induction patterns indicate that only certain isoforms serve in this protective capacity.

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