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Original

Phenol-Oxidizing Enzyme Expression in *Lentinula edodes* by the Addition of Sawdust Extract, Aromatic Compounds, or Copper in Liquid Culture Media

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This study examined how the addition of a sawdust extract from *Castanopsis cuspidata*, several aromatic compounds, and copper affected the expression of a phenol-oxidizing enzyme in the white-rot basidiomycete, *Lentinula edodes*. Compared to liquid media that had not been supplemented with sawdust extract (MYPG), MYPG containing low (MYPG-S_{100}) or high (MYPG-S_{500}) concentrations of sawdust extract had a marked effect on the promotion of mycelial growth. No manganese peroxidase (MnP) production was observed in either MYPG or MYPG-S_{100} media until 35 days after inoculation. However, MnP production was enhanced by culture in MYPG-S_{500}, with a marked increase observed suddenly at 14 days after inoculation. Northern blot analysis revealed that the transcription of the *lemnp2* gene coding extracellular MnP was initially observed at detectable levels at day 10 after the initial inoculation of MYPG-S_{500}, increasing gradually thereafter until days 22-25. However, laccase (Lcc) production was not observed in any of the media until 35 days after inoculation. Addition of 10 mM aromatic compounds – 1,2-benzenediol, 2-methoxyphenol, hydroquinone, and 4-anisidine – into the MYPG-S_{500} medium completely inhibited MnP production and did not enhance any Lcc production. While the addition of 1 or 2 mM Cu^{2+} ($CuSO_4 \cdot 5H_2O$) to MYPG-S_{500} medium completely inhibited MnP production, this Cu^{2+} addition caused a marked increase in Lcc production at 17 and 6 days after the addition, respectively.

Key words：Copper / Laccase / Manganese peroxidase / Sawdust extract / Shiitake.

INTRODUCTION

The shiitake mushroom, *Lentinula edodes* (Berk.) Pegler, a white-rot basidiomycete, is one of the most valuable edible mushrooms cultured in the world (Chang and Miles, 1989). This fungus has traditionally been cultivated on Fagaceae logs or, in recent decades, on sawdust-based media. White-rot basidiomycetes are capable of degrading whole wood components including lignin and are frequently among the primary colonizers of decaying wood in nature (Shortle and Cowling, 1978; Tanesaka et al., 1993; Tanesaka, 2012). In addition, these mushrooms have been reported to cause serious economic damage to timber (Dickinson, 1982). Although *L. edodes* secretes the phenol-oxidizing enzymes involved in lignin-degradation, laccase (Lcc, EC 1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13), when cultivated on sawdust-based media (Tokimoto et al., 1987; Makker et al., 2001; Sakamoto et al., 2009; Nagai et al., 2009), it does not typically secrete these enzymes in liquid media (Ikegaya et al., 1993; Buswell et al., 1995; Nagai et al., 2002; Cavallazzi et al., 2005; Saeki et al., 2011). Lcc production in white-rot basidiomycetes including *L. edodes* can also be promoted by the addition of various metals, particularly Cu^{2+} (Collins et al., 1997; Palmieri et al., 2000; Galhaup et al., 2001, 2002; Soden et al., 2001, 2003; Faraco et al., 2003), or by aromatic compounds (Fahraeus, 1962; Scheel et al., 2000).

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Wood meal spread on the surface of agar media has been used for preparing ligninolytic culture conditions for the white- and brown-rot basidiomycetes and the soft-rot deuteromycetes (Enoki et al., 1989; Tanaka et al., 1999, 2000). Similarly, a marked increase in Lcc production has been reported in *Pleurotus ostreatus* cultured together with cotton stalk extract (Ardon et al., 1996).

The substrates used for mushroom cultivation are typically dependent on the availability of agricultural wastes (Smith et al., 1988; Hadar et al., 1992) or the natural resources prevalent in a particular region. Japanese chinquapin, *Castanopsis cuspidata* (Thunb. ex Murray) Schottky, a member of the Fagaceae, is abundant in the warm, temperate, broad-leaved forests of Korea and Japan, and is commonly used as a component in sawdust-based media for *L. edodes* cultivation. We recently reported that the production of the primary MnP isozyme by *L. edodes* cultured on sawdust-based media, LeMnP2 (Sakamoto et al., 2009), was strongly promoted during liquid cultures supplemented with *C. cuspidata* sawdust extract (Saeki et al., 2011). In addition, the expression of the Lcc isozyme, Lcc1 (Nagai et al., 2002), was strongly promoted by the addition of 2 mM Cu^{2+} seven days after the copper was added to the same media (Saeki et al., 2001). Liquid cultures supplemented with sawdust extract made it easy to collect mycelia separately from the culture liquid compared with cultures supplemented with solid sawdust or wood powder. However, when cultured in liquid medium supplemented with the sawdust extract, *L. edodes* produced either MnP or Lcc, but not both. These observations suggest that the expression of MnP and/or Lcc in *L. edodes* is controlled by a negative feedback interaction, i.e., where MnP production inhibits Lcc production and MnP-inhibition enhances Lcc production (Tanesaka et al., 2012).

This study therefore examined the expression of phenol-oxidizing enzymes in *L. edodes* under liquid culture conditions in response to the addition of varying combinations of the following putative inducers of phenol-oxidizing enzymes: different concentrations of sawdust extract, 10 mM aromatic compounds, and 1 or 2 mM Cu^{2+}. Implications of the results in the regulation of phenol-oxidizing enzyme expression employed by this fungus are also discussed.

MATERIALS AND METHODS

Fungal stock and culture conditions

The *L. edodes* variety, 'Hokken 600' (Hokken Co., Ltd., Tochigi, Japan), which is well suited to sawdust cultivation, was used in this study. To enhance the expression of phenol-oxidizing enzymes in liquid media, mycelia were cultured in MYPG liquid medium (2.5 g malt extract; 1.0 g yeast extract; 1.0 g peptone; 5.0 g glucose in 1,000 ml distilled water) supplemented with sawdust extract (MYPG-S). The sawdust extract was prepared by adding 1 g of sawdust (*C. cuspidata*) to 30 ml of distilled water and then autoclaving the mixture at 121℃ for 15 min before filtering it through filter paper (No. 1, Advantec, Tokyo) and collecting the extract. Two concentrations of MYPG-S liquid media were then prepared by supplementing MYPG liquid medium with either a 1/10 or 1/2 volume of sawdust extract instead of distilled water, which gave MYPG-S containing extracts from 100 mg (MYPG-S_{100}) or 500 mg (MYPG-S_{500}) of sawdust in 30 ml media, respectively. Mycelia were then sub-cultured at 25℃ on MYPG 1% agar plates for 14 days. Three mycelial disks measuring 3 mm in diameter were then harvested from the plates and used to inoculate 30 ml liquid media in a 100 ml flask, which was then statically cultured at 25℃. All media treatments were performed in triplicate, and measurements of mycelial dry weight and enzyme activity were performed as described below.

Dry weight of mycelia

Mycelia samples were collected by filtration through a double nylon stocking mesh at 3-, 4- or 7-day intervals until sampling ended at day 35 of incubation. After washing the mycelia twice with distilled water, excess water was removed by blotting with a paper towel. The mycelia were then dried overnight at 60℃ and then at 105℃ for 1 h before being weighed.

Enzyme assay

The phenol-oxidizing enzymes were assayed as described previously (Saeki et al., 2011; Tanesaka et al., 2012). Briefly, a 100 μl aliquot of culture medium was collected during culture and centrifuged at 13,000 rpm for 10 min. The obtained supernatant was used as the crude enzyme solution, and was assayed for Lcc, peroxidases (Per, EC 1.11.1.x), and MnP in identical 5 ml test tubes containing an Lcc assay mixture consisting of 0.1 mM *o*-dianisidine in 0.1 M sodium tartrate buffer (pH 5.0) and the addition of H_2O_2 (final concentration 0.1 mM) to assess Per activity. $MnSO_4 \cdot 5H_2O$ (final concentration 0.1 mM) was then added to the Per assay mixture to assess MnP activity. Aliquots (20 μl) of crude enzyme solution were added to test tubes containing 980 μl of each reaction mixture, and after incubating these test tubes at 37℃ for 10 min, the reactions were stopped by the addition of 50 μl of 40 mM NaN_3. To inactivate the enzymes in the control tubes, sodium azide was added to tubes that contained the Lcc assay mixture before incubation.

Catalytic products of the reaction were spectro-

photometrically assayed using *o*-dianisidine as a substrate, and the activity of enzyme products was estimated by subtracting the respective absorbance values at 460 nm (Szklarz et al., 1989): i.e., Lcc activity = Lcc assay minus the control assay; Per activity = Per assay minus the Lcc assay; and MnP activity = MnP assay minus the Per assay. One unit (U) of enzyme activity was defined as the amount of enzyme required to catalyze 1 μmol of *o*-dianisidine in 1 min (ε_{460} = 29,400 $M^{-1}cm^{-1}$) (Paszczynski et al., 1988). Lignin peroxidase (ligninase, LiP, EC 1.11.1.14) was assayed in the reaction mixture using veratryl alcohol as a substrate at a final concentration of 2 mM with 0.1 mM H_2O_2 in 0.1 M sodium tartrate buffer. This mixture was incubated at 37℃ for 10 min and the absorbance of veratryl aldehyde was measured at 310 nm (Tien and Kirk, 1984).

To examine the effects of aromatic compounds on phenol-oxidizing enzyme production, the following aromatic compounds were used: 1,2-benzenediol (catechol), hydroquinone, 2-methoxyphenol (guaiacol), and 4-anisidine. Specifically, each substance was added to MYPG or MYPG-S_{500} culture media to make 10 mM at day 10 after the initial inoculation, and enzyme activities were assayed daily, or at 2- to 3-day intervals thereafter. In addition, the effect of 1 or 2 mM Cu^{2+} ($CuSO_4 \cdot 5H_2O$) on phenol-oxidizing enzyme production was also examined. All substance-addition treatments were conducted in triplicate, except for the 1 and 2 mM copper-addition treatments which were performed in duplicate.

RNA isolation and Northern blot analysis

Total RNA was extracted from mycelia using TRIzol Reagent (Invitrogen, CA) after varying incubation periods on MYPG-S_{500}. cDNA was synthesized from total RNA using an RNA PCR Kit Ver.3.0 (Takara Bio, Japan), and amplified using the primer set LeMnP2En5f (5'-TCCGACAGTGTCAATGACCTCGCTC) and LeMnP2En13r (5'-GTCAGTGGTGAGATTTGGG AAGGGC), which were designed based on the highly conserved *lemnp2* region (DDBJ Acc. No. AB306944; Sakamoto et al., 2009). A fragment measuring approximately 700 bp was then excised from the 1% agarose—formaldehyde gel, purified, and sequenced using an ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems, CA) according to the manufacturer's instructions. Fragments with sequences matching *lemnp2* were then labeled using a PCR-based digoxigenin (DIG)-dUTP labeling kit (PCR DIG Probe Synthesis Kit; Roche Diagnostics, Germany) according to the manufacturer's instructions. The resulting DIG-labeled probe, *lemnp2*, was then used for Northern hybridization after blotting 10 μg of total RNA onto a Hybond-N^+ membrane (GE Healthcare, Switzerland) using an established protocol (Sambrook et al., 1989).

Data analysis

Statistical analysis was performed using the *STATISTICA* statistical package (ver.6, StatSoft, Tulsa, OK). Analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed to compare the dry weight of mycelia and enzyme activities between treatments.

RESULTS

Promotion of growth and enzyme production by sawdust extract

Compared to the MYPG medium (with no sawdust extract), the media supplemented with sawdust extract (MYPG-S_{100} and MYPG-S_{500}) had a marked effect on promoting mycelial growth (FIG. 1). Indeed, significant differences in mycelial growth were observed among the three media 7 days after the initial inoculation ($F = 10.05$, $P = 0.012$). Mycelial growth in MYPG-S_{100} and MYPG-S_{500} liquid media was four-to-five-fold higher than that observed in MYPG alone ($P < 0.001$ at day 35, Tukey's test).

Figure 2 shows the activities of the phenol-oxidizing enzyme in different culture media. Lcc production was not observed in any of the media until 35 days after inoculation. Although Per activity was detected after 25 days of culture in MYPG medium and after 14 days of culture in MYPG-S_{500} medium, the activities at day 35 were not significantly different between the two types of media. No obvious MnP-production was observed when mycelia were cultured in either MYPG or MYPG-S_{100} media. Conversely, a marked increase in MnP activity was observed from day 14 on the

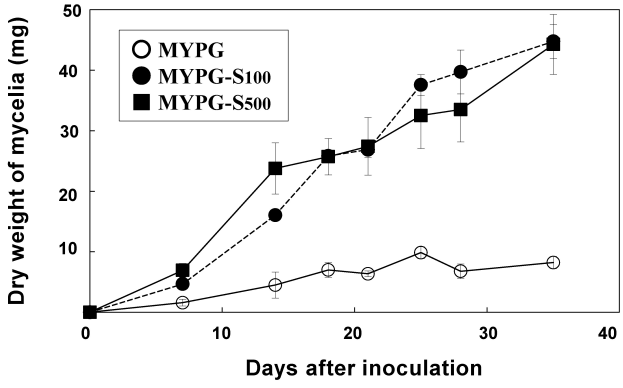

FIG. 1. Mycelial growth of *L. edodes* in different liquid culture media; without sawdust extract (MYPG), in culture media supplemented with low-concentration sawdust extract (MYPG-S_{100}), and in high-concentration sawdust extract (MYPG-S_{500}), respectively. Values are means with standard errors (vertical bars) for cultures grown in triplicate.

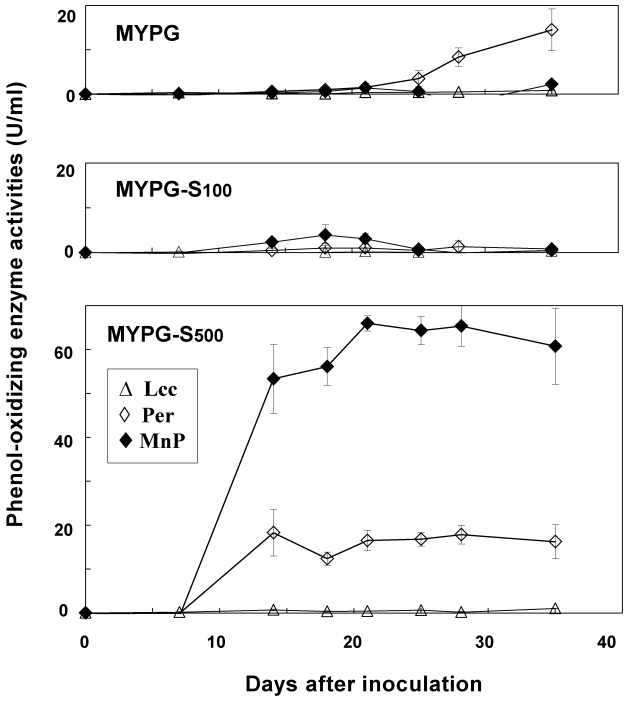

FIG. 2. Phenol-oxidizing activities of Lcc, Per and MnP from *L. edodes* cultured in liquid culture media containing different sawdust-extract concentrations. Symbols indicating the different enzymes are shown in the legend of the bottom graph. Values are means with standard errors (vertical bars) for cultures grown in triplicate.

MYPG-S_{500} medium, with the activity remaining above 60 U/ml until day 35. MnP activities after 14 days of culture in the MYPG-S_{500} medium were significantly higher than the MnP activities obtained from the other two media ($P < 0.001$). Lip activity was not detected in any of the culture media (data not shown).

Manganese peroxidase gene transcription

The sequence of the fragment amplified from cDNA, which was prepared from a mixed pool of total RNA obtained from the mycelia of 10-, 15- and 18-day-old cultures cultured in MYPG-S_{500}, was identical to that of *lemnp2*, which is known to code for extracellular MnP (LeMnP2), but different from *lemnp1*, which is known to code for intracellular MnP (data not shown). The finding indicated that sawdust extract induced the secretion of the same isozyme, LeMnP2, which is a major MnP isozyme that is secreted into sawdust media (Sakamoto et al., 2009). The results of the Northern blotting analysis with a probe *lemnp2* are shown in Figure 3. During culture on MYPG-S_{500}, a detectable amount of *lemnp2* mRNA was present at days 10-19 during the initial stage of culture, with the transcription increasing from day 22 to day 25 and then decreasing at day 28. These changes in *lemnp2* transcription

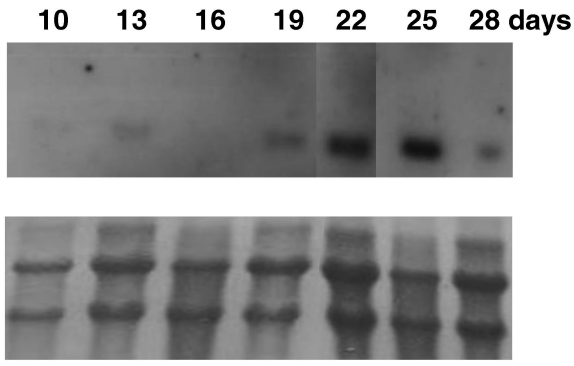

FIG. 3. (top) Northern blot analysis of *lemnp2* gene transcript from *L. edodes* grown in MYPG-S_{500} liquid media. (bottom) 10 μg of total RNA used as a loading control. Days after inoculation are shown above each lane. Adapted from Tanesaka et al., 2012.

coincided approximately with the changes observed in MnP activities in the liquid culture medium.

Effects of aromatic compound and copper addition on enzyme activities

The effect of adding either 10 mM of aromatic compounds or Cu^{2+} (1 and 2 mM) on phenol-oxidizing enzyme activities 10 days after the initial inoculation in MYPG-S_{500} medium are summarized in TABLE 1. In this experiment, MnP activity in the control samples (no additives) exceeded 20 U/ml 20 days after inoculation, which was relatively lower than activities exceeding 50 U/ml that were observed after 14 days in a previous experiment (FIG. 2). This disparity in the timing of MnP production between experiments may have been due to slight differences in the preparation of the sawdust extract or the physiological condition of the mycelia in the culture flasks. Even so, at approximately 60 U/m, MnP activity at 30 days after inoculation was similar between experiments. Compared to the control samples, the addition of all substances – catechol, hydroquinone, guaiacol, anisidine, 1 and 2 mM Cu^{2+} – completely inhibited MnP production (TABLE 1). Instead of inhibiting MnP, the addition of 1 mM Cu^{2+} enhanced Lcc production after 17 days, with activities exceeding 45 U/ml by day 31. Similarly, the addition of 2 mM Cu^{2+} enhanced Lcc production after 6 days, with an activity exceeding 100 U/ml by day 31. Consequently, the increase in Lcc production observed after the addition of 2 mM Cu^{2+} was quicker and there was a higher activity than that observed after the addition of 1 mM Cu^{2+}. Conversely, no noticeable increase in Lcc production was observed in the MYPG liquid medium following the addition of any of the aromatic substances or Cu^{2+} (data not shown).

TABLE 1. Addition of copper, phenolic and non-phenolic compounds and their effect on phenol oxidizing activities (U/ml) in *L. edodes* cultured on MYPG+S_{500}.

Substance	Enzymes	Days after substance was added*						
		0	4	6	10	17	20	31
Control	Lcc	0.3 †	0.0 ab ‡	0.1 a	0.2 a	0.2 a	-0.3 a	0.3 a
	Per	-0.1	0.7	2 a	7.2 b	16.4 c	22.2 c	27.9
	MnP	0.3	2.5	5.7	20.1 c	45.8 b	57.0 b	65.9 b
Catechol	Lcc	0.2	0.3 ab	0.5 a	1.5 a	ne	ne	ne
	Per	0.2	3.2	3.2 a	2.0 a	ne	ne	ne
	MnP	2.3	5.0	4.9	4.1 a	ne	ne	ne
Hydroquinone	Lcc	0.3	-0.3 a	1.0 a	0.0 a	ne	ne	ne
	Per	0.1	1.3	1.0 a	0.8 a	ne	ne	ne
	MnP	0.7	1.0	1.5	0.9 ab	ne	ne	ne
Guaiacol	Lcc	0.3	0.1 ab	-0.3 a	0.5 a	ne	ne	ne
	Per	0.4	0.7	0.5 a	0.7 a	ne	ne	ne
	MnP	0.1	1.2	1.2	1.9 ab	ne	ne	ne
Anisidine	Lcc	0.4	0.3 ab	0.3 a	-0.3 a	ne	ne	ne
	Per	0.6	3.2	3.1 a	1.4 a	ne	ne	ne
	MnP	1.7	8.0	7.7	3.7 ab	ne	ne	ne
1 mM Cu^{2+}	Lcc	0.5	0.5 ab	0.3 a	0.2 a	12.2 b	27.0 b	45.9 b
	Per	0.0	0.4	0.2 a	-0.1 a	-0.9 ab	-0.1 a	4.6
	MnP	0.3	1.4	0.8	0.4 ab	0.6 a	0.7 a	-3.1 a
2 mM Cu^{2+}	Lcc	0.6	0.9 b	23.0 b	63.9 b	98.0 c	95.1 c	109 c
	Per	0.1	4.4	9.1 b	3.6 ab	-4.0 a	2.7 ab	9.2
	MnP	0.5	0.2	0.2	-2.3 a	4.7 a	-0.1 a	3.4 a

*: Each substance was added 10 days after initial inoculation

†: Each value represents the mean of three replicate flasks or two replicate flasks for Cu^{2+}

‡: Values with different lowercase letters are significantly different at the 5% level between treatments. Values with no lowercase letters are not significantly different between any pair of treatments on a given day

ne: not examined

DISCUSSION

The expression of lignin-degrading enzymes in white-rot basidiomycetes has been attributed to the production of secondary metabolites under various growth and culture conditions (Kirk and Fenn, 1982; Cullen and Kersten 2004). Although *L. edodes* has been reported to secrete the lignin-degrading enzymes Lcc and/or MnP when cultivated on sawdust-based media (Tokimoto et al., 1987; Makker et al., 2001; Sakamoto et al., 2009; Nagai et al., 2009), the findings of the present study corroborated our previous studies (Saeki et al., 2011) and showed that extracts produced by autoclaving sawdust in hot water can also promote MnP activity. Compared to mycelial growth on MYPG medium, growth on MYPG-S_{100} and MYPG-S_{500} media was considerably more vigorous. Although the components in the sawdust extract are unclear, the compound(s) responsible for the observed vigorous growth may be flavonoids (Fahraeus, 1962; Ardon et al., 1996). Interestingly, although mycelial growth was promoted in both MYPG-S_{100} and MYPG-S_{500} media, a marked increase in MnP production was only observed in cultures grown in MYPG-S_{500}, and not in MYPG-S_{100}. These observations suggested that MnP is only enhanced by specific, concentration-dependent, functional compounds in the high-concentration sawdust extract (MYPG-S_{500}). In other words, MnP enhancement was not only due to the vigorous growth, which was also observed in the low-concentration sawdust extract (MYPG-S_{100}). Transcription of the *lemnp2* gene, which encodes LeMnP2, was initially observed at detectable levels by Northern blot analysis of cultures grown on MYPG-S_{500} at day 10 after inoculation. Based on these results, the addition of aromatic compounds and copper 10 days after inoculation appeared to coincide, approximately, with the initiation of *lemnp2* gene transcription.

In several white-rot fungi, Lcc production is promoted by aromatic substances (Fahraeus, 1962; Scheel et al., 2000; Piscitelli et al., 2011). However, in the present study on *L. edodes*, although the sawdust extract likely contained a variety of phenolic and nonphenolic aromatic compounds (e.g. flavonoids), none of the 10 mM aromatic compounds tested in this study enhanced Lcc production. In addition, all of the aromatic compounds

tested completely inhibited MnP production. These findings corroborated those of a similar study on *L. edodes* which found that none of the 1 mM aromatic compounds tested (i.e. gallic acid, catechol, hydroxybenzoic acid, or vanillin) enhanced Lcc production; rather, the aromatic compounds either inhibited or repressed Lcc production (Cavallazzi et al., 2005). In contrast, Ikegaya et al. (1993) reported that the addition of 50 μg/ml (0.33 mM) vanillin in a peptone-glucose liquid medium significantly promoted mycelial growth, laccase production and fruit-body formation by *L. edodes*. The differences observed in Lcc expression between studies may be attributable to differences in the concentrations of substances added, nutritional conditions, or stocks and strains used.

The addition of metallic ions such as Cu^{2+} to media has also been used to induce Lcc production in several fungi, including *L. edodes* (Nagai et al., 2002; Cavallazzi et al., 2005; Saeki et al., 2011). Indeed, Cu^{2+} is considered to be a strong Lcc inducer in the white-rot fungi, *Pleurotus ostreatus* (Palmieri et al., 2000; Faraco et al., 2003), *P. sajor-caju* (Soden et al., 2001, 2003), *Trametes pubescens* (Galhaup et al., 2001, 2002), and *T. versicolor* (Collins et al., 1997). For example, compared to a control treatment (no Cu^{2+} added), a 1.5-fold increase in Lcc production was achieved in a liquid culture of *L. edodes* by the addition of 10 mM Cu^{2+} to the culture medium (Nagai et al., 2002). Similarly, more than a 25-fold increase in Lcc production was achieved by the addition of 250 μM or 2 mM Cu^{2+} to a liquid culture of *L. edodes* (Cavallazzi et al., 2005; Saeki et al., 2011). The promoter region of the gene coding the major Lcc isozyme (LAP2) of *T. pubescens* has copper-sensitive metal responsive elements (MRE), and Lcc gene transcription is induced within 10 h after the addition of 2 mM Cu^{2+} (Galhaup et al., 2001, 2002). Copper regulation of Lcc by MRE consensus sequences has also been reported in *Pleurotus* spp (Faraco et al., 2003; Soden et al., 2003).

The main findings of this study were as follows: i) marked mycelial growth was observed in *L. edodes* cultured in low- and high-concentration sawdust extract produced from *C. cuspidata*. However, MnP production was only enhanced by the high-concentration sawdust extract; ii) all of the aromatic compounds tested completely inhibited MnP production and did not enhance Lcc production; iii) the addition of both 1 and 2 mM Cu^{2+} completely inhibited MnP production, but markedly enhanced Lcc production. Although the simultaneous expression of MnP and Lcc was not observed in this study, the hypothesis of whether a negative feedback interaction exists between MnP and Lcc is still unclear, i.e. whether MnP production initially inhibits Lcc production and MnP inhibition promotes Lcc production. However, in this study, MnP inhibition by the addition of aromatic compounds did not enhance Lcc production. These enzyme expression dynamics are highly sensitive to nutrient nitrogen and manganese (Buswell et al., 1995), as well as the fungal response to Cu^{2+} and aromatic compounds as discussed above. Indeed, the findings presented here do not provide sufficient evidence for even the first part of the proposed negative feedback interaction involving these two enzymes. However, the findings do show that MnP inhibition and Lcc production are affected by Cu^{2+} addition and that this inhibition-production relationship might be functionally independent.

Importantly, the experimental conditions employed in this study, in which sawdust extract was shown to enhance mycelial growth and MnP production, were similar to the conditions employed to cultivate edible mushrooms (e.g., autoclaved sawdust-based media). The liquid-culture media described here could therefore be employed to evaluate the lignin-degrading ability of cultivated mushroom varieties. Further analyses are therefore necessary to examine the functional component(s) in the sawdust extract and the correlation between enzyme expression and regulation using this experimental system.

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