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Extracellular laccase production during hyphal interactions between *Trichoderma* sp. and Shiitake, *Lentinula edodes*

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Abstract *Lentinula edodes* (Berk.) Pegler was cultivated in liquid media containing malt and yeast extract. Extracellular laccase activity, measured in the culture fluids, was 5–18 times higher in cultures incubated for 29 days than in cultures incubated for 24 days. The addition of water-soluble lignin derivatives or *Trichoderma* sp. in cultures of *L. edodes* incubated for 11 days increased laccase activity 3- to 20 fold. The higher response was obtained with live mycelium of *Trichoderma* sp., but cell-free culture fluids of *Trichoderma* sp. in pure cultures were also effective. *Trichoderma* sp. induced changes in the laccase isoenzyme pattern as a result of the alteration of laccases secreted by *L. edodes* and not the induction of new isoforms.

Introduction

Lentinula edodes (Berk.) Pegler, the popular Japanese shiitake, is the second most important mushroom among the industrially cultivated species. This important Asian mushroom is a white rotting fungus, which is cultivated throughout the world on sterilised or pasteurised agricultural and forest by-products (Royse et al. 1985; Delpech and Olivier 1991; Levanon et al. 1993). The quantity and the quality of the lignin and soluble phenolic compounds are among the major components varying with lignocellulosic substrates. As a consequence, the varying ability to produce polyphenol oxidases (EC 1.10.3.2) is probably an important factor in the adaptation of different strains of *L. edodes* to different cultivation substrates (Mata et al. 1997).

Whatever the cultivation substrate used, one of the largest production problems is the competition with other fungi (Badham 1991). Different species of *Trichoderma* commonly cause injury to mycelial growth and fruit body formation of *L. edodes* (Tokimoto and Komatsu 1979). The antagonistic action of *Trichoderma* hyphae on those of *L. edodes* correlates with fungal cell-wall lytic enzyme activities and ether-soluble neutral antifungal compounds produced by *Trichoderma* spp. (Ishikawa et al. 1980; Tokimoto 1982). However, strains of *L. edodes* are able to reject the attack by *Trichoderma* under temperature and nutritive conditions favourable to them (Ishikawa et al. 1980; Kawamura et al. 1980; Tokimoto 1982; Badham 1991). It is commonly observed, both in laboratory cultures and in commercial substrates, that there is production of a brownish pigment in the contact zone between the hyphae when *L. edodes* reject the attack of *Trichoderma*. High levels of polyphenol oxidase activity in the browning area of interaction have been reported by Tokimoto (Tokimoto 1980, 1982) and significant increases in laccase activity were observed in wheat-straw cultivation substrates contaminated by *Trichoderma* sp. (Savoie and Mata, unpublished data). A better understanding of the mechanism of rejection of *Trichoderma* sp. by *L. edodes* would be interesting to improve both strains and cultivation techniques. In addition *L. edodes* is a potential source of laccases for biotechnological applications (Crestini et al. 1996).

The production of laccases by *L. edodes* in axenic cultures and during the antagonism with *Trichoderma* sp. was studied with four strains of *L. edodes*. The aims of this study were to define the effects of the antagonism on laccase production and to determine the origin of the increases in the laccase activities associated with the reaction of *L. edodes* to *Trichoderma* sp.

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Materials and methods

Strains

The strains are deposited in the Research Culture Collection of Institut National de la Recherche Agronomique, Bordeaux,

France. A strain of *Trichoderma* was isolated from a naturally contaminated shiitake cultivation substrate. It was identified as *T. harzianum* as defined by Rifai (1969) but, in the absence of a confirmed identification, the strain was called *Trichoderma* sp (Bxc 20048). Four strains of *L. edodes* were used in this study. M115 (Bxc 22011) and S610 (Bxc22010) were cultivars of shiitake commercialised respectively by Lambert Spawn (USA) and France Mycelium (France); A9 (Bxc 22013) was a hybrid produced at the INRA laboratory (France). IE105 (Bxc22012), was from the Instituto de Ecologia's collection (Mexico).

Kinetics of laccase production

The kinetics of laccase production was studied by cultivating the four strains in 50 ml YME (2% malt extract, 0.2% yeast extract) medium for 29 days at 25 °C in darkness. Three replicates of each strain were inoculated with mycelial colonies on cellophane membranes obtained after cultivation for 6 days on a YME/agar medium (YMEA). At each sampling time, 1 ml cell-free culture fluid was collected and replaced with 1 ml YME medium. The oxidation of 2,2′-azino-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) was determined by measuring the absorbence at 436 nm to estimate laccase activity (Niku-Paavola et al. 1990). The incubation mixture contained 3 mg ABTS in a total volume of 1.5 ml 0.1 M acetate buffer pH 5.0 and incubation was at 30 °C. One unit of laccase activity was 1 μmol ABTS oxidised/min. It was calculated by using $\varepsilon = 29,300\ M^{-1}\ cm^{-1}$ (Niku-Paavola et al. 1990). Measurements of laccase activities were completed three times for each sample.

Induction of laccase activity

Liquid cultures were studied to determine the effect of the antagonism on extracellular laccase production. *L. edodes* and *Trichoderma* sp. were pre-cultivated on cellophane membranes placed on YME agar media for 5 and 2 days respectively. Each colonised membrane was used to inoculate 50 ml YME liquid medium. Liquid cultures of *L. edodes* were incubated for 11 days at 25 °C and the three following treatments were performed. In controls, the incubation was prolonged for 19 days. In the treatment with water-soluble lignin derivatives, a solution of water-soluble lignin derivatives was added to 50 ml of culture medium to obtain a final concentration of 2 mM phenols. Water-soluble lignin derivatives were obtained as previously (Mata et al. 1997) from Indulin AT (Sigma). Treatments with *Trichoderma* sp. were obtained by placing a mycelial colony of *Trichoderma* sp. at the margin of each *L. edodes* mycelial colony in the liquid culture medium. After incubation the mycelial biomass was collected, freeze-dried and weighed. Laccase activity was measured in culture fluids with ABTS; five repetitions per strain were performed.

The same experiment was duplicated with the strain S610, and controls without active mycelium of *L. edodes* were included. In these controls, the mycelium of *L. edodes* was removed after 11 days of cultivation before application of the two types of treatment to the cell-free culture fluids and incubation for 5 days. Laccase activity was measured in culture fluids collected 1 day and 5 days after the treatment.

To determine the effects of extracellular metabolites from *Trichoderma* sp. on laccase production by *L. edodes,* cell-free culture fluids were collected by filtration after incubation of *Trichoderma* sp. for 4 days in pure cultures. The culture fluids were then inoculated with mycelial colonies of *L. edodes* on cellophane and incubated at 25 °C for 22 days (L/T treatment) with five replicates for each of the two strains used (A9, IE105). Control cultures of *L. edodes* were performed with no change of culture medium. Every day, 1 ml culture fluid was collected and replaced by 1 ml culture medium in both controls and L/T treatments. Extracellular laccase activity was measured in each sample of culture fluid, with ABTS as substrate. The ratio of laccase activities in the L/T treatment to laccase activity in the control was calculated. Otherwise *Trichoderma* sp. was cultivated in *L. edodes* culture fluid (T/L treatment). The four strains of *L. edodes* were cultivated for 6 days in YME, the mycelial colonies were removed and the culture fluid collected by filtration. *Trichoderma* sp. mycelial colonies on cellophane were inoculated in these cell-free culture fluids and laccase activity was measured every day with ABTS as substrate.

Native polyacrylamide gel electrophoresis (PAGE)

Native PAGE was performed on culture fluids concentrated (1000×) by ultrafiltration (10 000 Da). An acrylamide\2 × bisacrylamide 37.5/1 mixture (Bioprobe) was used to prepare 10% gels, 155 mm long and buffered at pH 4.3, with a 5% upper stacking gel zone of 45 mm and pH 6.8. The gels were 3 mm thick and a constant current of 150 mA was applied for 30 h in β-alanine buffer pH 4.5 (Hames 1990). Following PAGE, 4-chloro-1-naphthol (0.09%) in 0.1 M acetate pH 5 was used to reveal laccase isoenzymes in the gel by a stable blue precipitate (Robène-Soustrade and Lung-Escarmant 1997).

Results

Kinetics of laccase production

During the cultivation of the four strains in YME liquid media, brown areas appeared on the mycelial colonies on day 21 and extended thereafter. The kinetics of extracellular laccase production showed a peak of activity around day 10 (Fig. 1) but the level of activity was lower than the level in the aged cultures because of important increases in activity after incubation for more than 21 days (Table 1). Significant quantitative differences between the strains were observed however, with M115 having the highest laccase activity and S610 the lowest at the end of the culture. The differences in the extracellular laccase activities at the end of the experiment were not correlated with the differences in mycelial biomass production (Table 1).

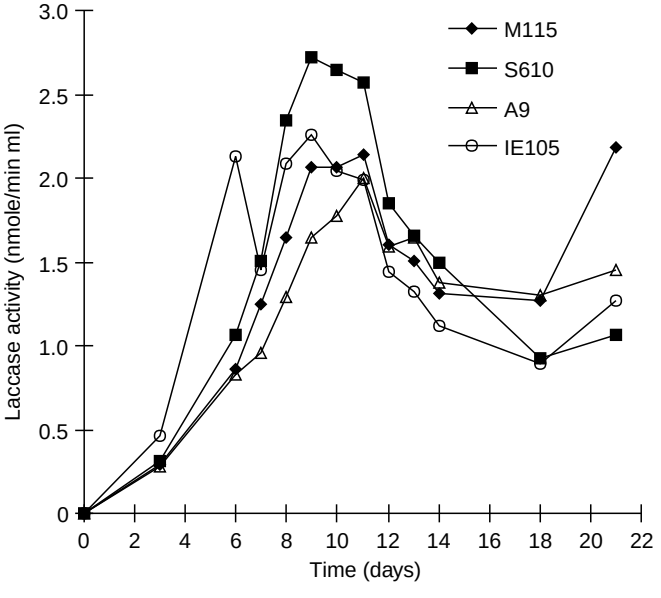

Fig. 1 Changes in laccase activities measured in culture fluids of four strains of *Lentinula edodes*

Table 1 Changes in extracellular laccase activities in the culture fluid of aged mycelia of *Lentinula edodes* and biomass production. Values are means with standard errors in brackets ($n = 5$)

Incubation time (days)	Laccase activities (nmol min^{-1} ml^{-1}) for strains:			
	M115	S610	A9	IE105
11	2.1 (0.28)	2.6 (0.32)	2.0 (0.24)	2.0 (0.27)
24	15.3 (1.64)	3.9 (1.91)	3.2 (2.41)	5.4 (1.17)
29	75.4 (10.80)	22.5 (8.76)	56.6 (10.00)	35.8 (16.44)
Mycelium dry weight (g/50 ml) on day 29	0.30 (0.032)	0.33 (0.024)	0.27 (0.021)	0.30 (0.014)

Effects of addition of *Trichoderma* sp. and water-soluble lignin derivatives

The treatments applied to growing mycelia after culture for 11 days increased the production of extracellular laccase activities for all the strains (Fig 2). The effects of *Trichoderma* treatments were higher than the effects of treatments with water-soluble lignin derivatives. Brown lines appeared at the contact between *Trichoderma* sp. hyphae and *L. edodes* hyphae, and the progression of *Trichoderma* sp. stopped in the *Trichoderma* treatments. When the treatments were applied to the culture fluids of *L. edodes*, strain S610, after the mycelium had been removed, no significant increase in polyphenol oxidase activity was observed (Fig. 2).

The biomass production was higher in the treatment with water-soluble lignin derivatives than in the control without treatment for all the strains except IE105 (Fig 2). However, when the extracellular laccase activity was expressed per gram of mycelium, the treatment with lignin-derivatives still increased the activity 2- to 11-fold.

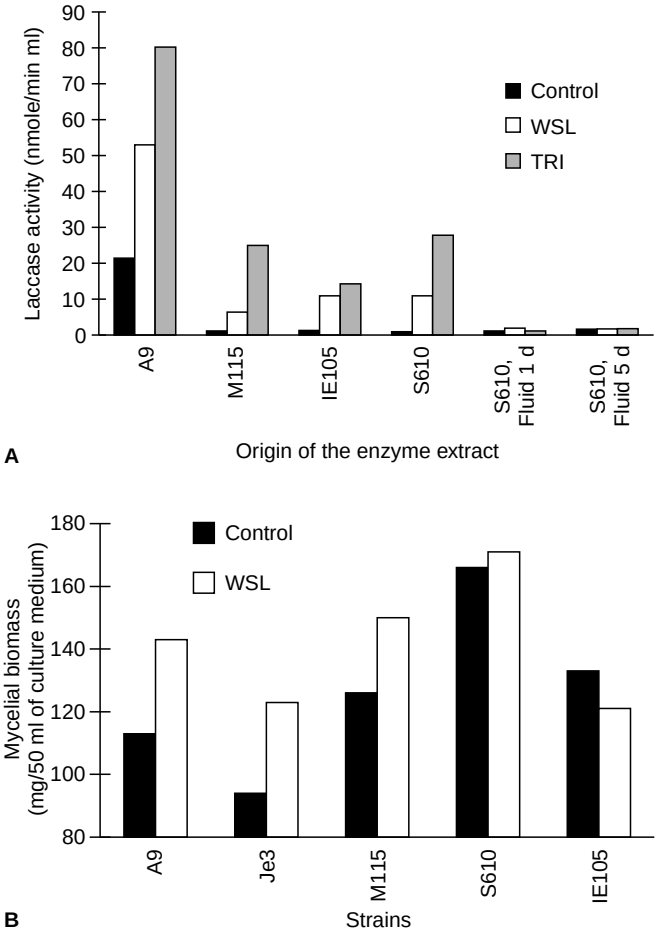

Fig. 2A, B Addition of water-soluble lignin derivatives (*WSL*) and of *Trichoderma* sp. mycelium (TRI) to liquid cultures and cell-free culture fluids of *L. edodes*. Effects on extracellular laccase activities (**A**) and on biomass production (**B**)

Effects of extracellular metabolites

The effects of extracellular metabolites produced by *Trichoderma* sp. were tested with two strains of *L. edodes* in the L/T treatments (Fig. 3). The induction of laccase activity was significant 1 day after the change of culture medium for A9. The increase of activity was slower for IE105, but significant. With both A9 and IE105, brown areas appeared on mycelia in contact with culture media on day 14, i.e. 8 days after the change of culture medium. No laccase activity was found in the culture fluids of *Trichoderma* sp.

In T/L treatments, *Trichoderma* sp. was able to grow in cell-free culture fluids of *L. edodes* without any brown line appearing. Otherwise, laccase activities present at the beginning decreased by 20% for A9 and remained constant for M115. With the strain S610, laccase activities were 1.1 (0.29) and 1.1 (0.04) nmol min^{-1} ml^{-1} in T/L-treated cultures and in controls of cell-free culture fluids of *L. edodes* without addition of *Trichoderma* sp. respectively, 1 day after removal of *L. edodes* mycelium;

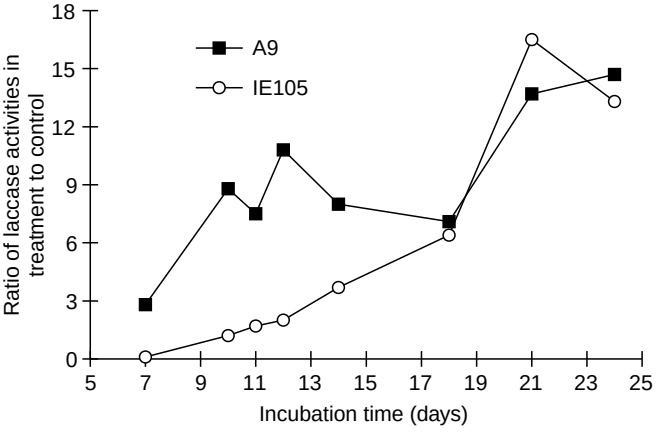

Fig. 3 Effects of extracellular metabolites from *Trichoderma* sp. on extracellular laccase production by two strains of *L. edodes*. Ratio of the activities in treatments where 6-day-old mycelial colonies of *L. edodes* were placed in cell-free fluid from *Trichoderma* sp. cultures, to the activities in controls of *L. edodes* without change of culture medium

4 days later the activities were1.6 (0.08) and 1.8 (0.18) nmol min^{-1} ml^{-1}.

Native PAGE

The native PAGE results for the strain S610 are presented in Fig. 4. One major and three minor bands were observed in controls (lanes 1–4). *Trichoderma* treatments modified the isoenzyme pattern of the culture fluid (lane 8) whereas treatment with water-soluble lignin derivatives had no effect except to change the intensity of the major bands (lane 5). In the banding pattern of the *Trichoderma* treatment the two upper bands were not visible but active bands with high R_F values were observed. Similar banding patterns were obtained when the treatments were performed both in the presence and absence of mycelium of *L. edodes*. However, differences in the stain intensity were observed. For example, changes in the banding pattern following *Trichoderma* treatment, by comparison with the controls (lanes 1–4), were observed both when mycelium of *L. edodes* was present (lane 8) and in enzymes produced by *L. edodes* before *Trichoderma* treatment (lanes 9–10).

Discussion

In *L. edodes*, laccase activities have been associated with rapid cell growth in aerial mycelium, pigmentation and development of primordia (Leatham and Stahmann 1981). In the present study, an interesting variability in extracellular laccase production per unit of biomass was observed. This could be used to select strains adapted to different lignocellulosic substrates (Mata et al. 1997). Peaks of secretion of laccase activity into the log matrix or other substrates had been reported as being related to the early mycelial growth of *L. edodes* (Leatham 1985; Okeke et al. 1994; Mata and Savoie 1998). Equivalent peaks of laccase activity were observed here and in other investigations (Crestini et al. 1996) in liquid media. However, the levels of activity were very low by comparison with activities measured in the culture fluid of browning mycelia. Such browning mycelia were in a physiological state probably corresponding to the stroma formation at the surface of the blocks before primordiation, under commercial cultivation conditions. To continue incubation until this physiological state, is a means for improving the quantity of extracellular laccases obtained from liquid cultures of *L. edodes*.

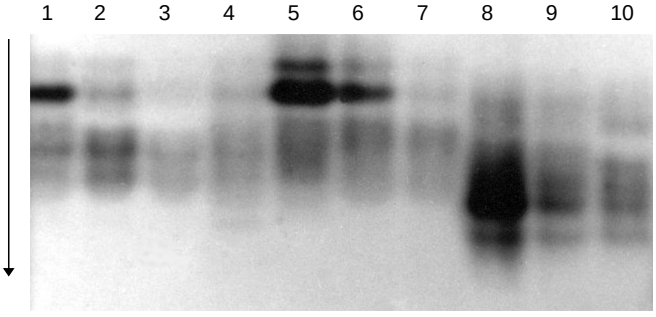

Fig. 4 Detection by native Polyacrylamide gel electrophoresis of *L. edodes* laccase isoenzymes in cell-free media after different treatments. Electrophoresis was performed on 10% polyacrylamide gel and the gel was stained with 4 chloro-1-naphthol. Arrow direction of protein migration. Lanes: *1* control after incubation of *L. edodes* for 11 days; *2* incubation of *L. edodes* for 11 days, removal of mycelium and further incubation for 1 day; *3* as lane 2 but with incubation for 5 days after removal; *4* control after incubation of *L. edodes* for 16 days; *5* incubation of *L. edodes* for 11 days, addition of water-soluble lignin derivatives and further incubation for 5 days; *6* incubation of *L. edodes* for 11 days, removal of mycelium, addition of water-soluble lignin derivatives and further incubation for 1 day; *7* as lane 6 but with incubation for 5 days after addition of water-soluble lignin derivatives; *8* incubation of *L. edodes* for 11 days, addition of *Trichoderma* sp. mycelial colony and further incubation for 5 days; *9* incubation of *L. edodes* for 11 days, removal of mycelium, addition of *Trichoderma* sp. mycelial colony and further incubation for 1 day; *10* as lane 9 but with incubation for 5 days after addition of *Trichoderma* sp.

Laccase production by *L. edodes* has also been associated with the formation of an antagonistic zone in dual cultures (Ohmasa et al. 1991) or when it interacts with competitive *Trichoderma* (Ishikawa et al. 1980; Tokimoto 1982). The antagonistic action of *Trichoderma* spp. hyphae to those of *L. edodes* was correlated with fungal cell wall lytic enzyme activities and ether-soluble neutral antifungal compounds produced by *Trichoderma* (Ishikawa et al. 1980; Tokimoto 1982). Dramatic increases in extracellular laccase activities associated with a brown line formation were observed here when *Trichoderma* sp. hyphae were placed in contact with *L. edodes* hyphae in liquid cultures. This increase was due to *L. edodes* because *Trichoderma* sp. was unable to produce laccases in the present experiment and decreased the level of activities of laccases produced previously by *L. edodes*. Moreover, some similarities between aged hyphae and hyphae attacked by *Trichoderma* sp. were observed, such as brown pigment and increases in extracellular laccase activities. Not only hyphal contact but also extracellular metabolites of *Trichoderma* sp., or transformation of culture medium components by *Trichoderma* sp., efficiently activated this rejection reaction in *L. edodes* mycelia. Production of these compounds did not need an induction by the presence of *L. edodes* mycelium or its extracellular metabolites. Such an enhancement of the production of extracellular laccases could be used to improve some processes of laccase production for biotechnological purposes instead of soluble extracts of lignocellulosic materials which were used efficiently with *L. edodes* (Crestini et al. 1996; Mata et al. 1997). The nature of these non-specific metabolites of *Trichoderma* sp. is not known and has to be determined in future work.

Native PAGE was used to determine whether the increases in laccase activities resulted from the synthesis of new laccase isoenzymes. In previous reports on *L. edodes*, only one major and one minor extracellular laccase isoenzyme were found (Panichajakul et al. 1991; Buswell et al. 1995) and, in a purification run, only one

peak of activity was observed and one laccase was obtained (D'Annibale et al. 1996). With our experimental conditions four bands were observed in the controls. Otherwise *Trichoderma* sp. induced some changes in the isoenzyme pattern. This could not be attributed to the induction of new proteins or to the release of intracellular laccases because the changes in the isoenzyme pattern were observed both in the presence and absence of active mycelium of *L. edodes*, but with quantitative differences in the intensity of band colour. In summary, *Trichoderma* sp. stimulated the production of extracellular laccases by *L. edodes*, but also altered these enzymes, probably through the action of proteases, with weak effects on their activity.

It is proposed that fungistatic compounds produced by *Trichoderma* sp. stimulated the production of extracellular laccases and the formation of brown pigments in *L. edodes* hyphae by activating some general cellular mechanisms of stress resistance. Despite differences in the levels of laccase activity induced by *Trichoderma* sp. with the four strains of *L. edodes* studied, no apparent difference in brown line formation and rejection of *Trichoderma* sp. was observed. Stimulation of laccases is a part of the reaction mechanism, but laccases are probably overproduced and other reactions underlie the ability of *L. edodes* to reject *Trichoderma* attack. This should be studied and could be used to select resistant strains of *L. edodes* (Tokimoto and Komatsu 1995).

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