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Production of phenol-oxidizing enzymes in the interaction between white-rot fungi

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Abstract The role of the phenol-oxidizing enzymes, laccase and peroxidase, was examined in the fungus-to-fungus interaction in dual cultures. Among five white-rot fungi, the following predominance in competition was observed: *Pleurotus ostreatus* > *Trametes versicolor* ≧ *Pycnoporus coccineus* > *Ganoderma applanatum* > *Schizophyllum commune*. Both phenol-oxidizing enzyme activities were detected markedly at the confrontation region, and under the mycelia growing over other colonies more than in other areas of the dual culture. This property was most notably observed in the *P. ostreatus* cultures. The fungi that produce superior active phenol-oxidizing enzymes were predominant in the competition between confronting fungi, indicating that phenol-oxidizing enzymes relate to fungus-to-fungus interaction.

Key words Fungus-to-fungus interaction · Laccase · Peroxidase · White-rot fungi

Phenol-oxidizing enzymes such as laccase and peroxidase that are produced by white-rot fungi are known to play a role in lignin degradation. On the other hand, they also are confirmed to participate in detoxification of xenobic compounds such as antifungicides. White and Boddy (1992) reported the relation of phenol-oxidizing enzymes to fungus-to-fungus interaction in dual cultures of white-rot fungi and assumed the role of the enzymes is to detoxify unidentified antifungicides. Production and localization of phenol-oxidizing enzymes were specific in each dual culture; however, the results differed depending on the pairing of confronted fungi, and no predominance was observed because not all pairings were checked. Here, we used five white-rot fungi that are commonly seen in Japan and examined all pairings of fungus-to-fungus interaction, in accordance with the procedures described by White and Boddy (1992).

Five strains of white-rot fungi that are preferential to angiospermous wood were tested: *Ganoderma applanatum* (Pers.) Karst. (KPUF 0694), *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (KPUF 2494), *Pycnoporus coccineus* (Fr.) Bond. et Sing. (KPUF 2094), *Schizophyllum commune* Fr.: Fr. (KPUF 8805), and *Trametes versicolor* (L.: Fr.) Quel. (IFO 30340). Dual culture tests of fungus pairs were performed with initial seeding at 5-cm distance on MA media (malt extract, 2%; agar 1.5%) at 28°C. MA medium was used to experiment with the interactive behavior of wood-rotting fungi because the behavior in this culture correlates well with their patterns of occurrence in the field (Griffith and Boddy 1991). After the predominance in confrontation was observed, the localities of laccase and peroxidase were determined by pouring substrate solutions (1.44% α-naphtol in 96% aqueous ethanol, and a mixture of 1% pyrogarol and 0.4% H_2O_2, respectively) on the culture plates in accordance with White and Boddy (1992).

Figure 1 shows a result of the dual culture with *P. ostreatus* and *S. commune*. After confrontation occurred between them, the *P. ostreatus* colony grew over *S. commune* and then stopped after expanding into the colony a few millimeters. In most cases, one fungus grew over the other after confrontation and invaded to about 1–2 cm in depth. However, mycelia stopped growing in all cases except for the dual culture of *T. versicolor* and *S. commune*, where the former overgrew the latter. In the competition between *T. versicolor* and *P. coccineus*, both fungi stopped growing within a few millimeters of the confrontation regions, and mycelia of both became hypertrophic. These observations are summarized in Table 1. Porter (1924) classified the interaction between two fungi into five types.

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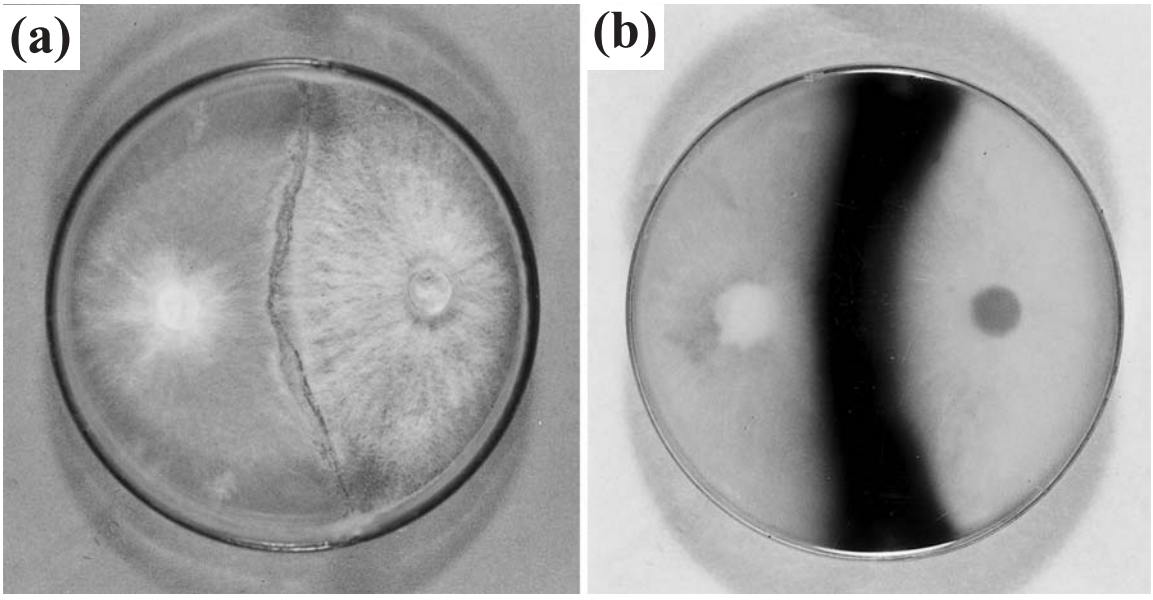

Fig. 1. A dual culture: *Pleurotus ostreatus* (*left*) versus *Schizophyllum commune* (*right*). **a** Confrontation; **b** localization of laccase activity (from underside)

Table 1. Interaction in dual culture of white-rot fungi and mycelial changes

Species 1	Species 2	*Pleurotus ostreatus*	*Trametes versicolor*	*Pycnoporus coccineus*	*Ganoderma applanatum*	*Schizophyllum commune*
Pleurotus ostreatus		–	**B**	**B**	**B**	**B**
Trametes versicolor		(z)	–	**C**	**B**	**A**
Pycnoporus coccineus		(z)	(x)	–	**B**(y)	**B**(x)
Ganoderma applanatum		(x)	(x)	(y)	–	**B**(x)
Schizophyllum commune						–

A, Species 1 overgrew species 2; **B**, species 1 grew over species 2 but stopped after invading to a few centimeters; **C**, both species inhibited at a distance; (x), mycelial hypertrophy was observed near the confrontation region; (y), hypertrophy was observed, but could not be distinguished as to which fungus it occurred in; (z), mycelial malformation or lysis occurred; –, not tested

Based on these classifications, three types were observed in this study: A, overgrowing; B, growing over but stopping after invading a few centimeters; and C, inhibition at a distance. The results of this study showed that *P. ostreatus* grew over all the others whereas *S. commune* allowed other fungi to invade. In the invaded colonies, morphogenetic changes in mycelia were observed near the confrontation region. In many cases, hypertrophy or aggregates occurred in the invaded fungi. A possible explanation for this is that the fungus blocks the invasion by other species (White and Boddy 1992). In some cases of *P. coccineus* and *G. applanatum*, pigmentation and browning of mycelia were observed around the confrontation region, accompanied by hypertrophy. This occurrence would also tend to block invasion (Bull 1970; Kuo and Alexander 1967). The morphogenic changes were sometimes observed in the invading fungi, which could be caused by the resistant action of the invaded fungi in the pairing.

As shown in Table 1, there is a predominance in the fungal interaction among tested fungi, as follows: *P. ostreatus* > *T. versicolor* ≧ *P. coccineus* > *G. applanatum* > *S. commune*. White and Boddy (1992) did not describe such an order of predominance in their report.

To examine the role of phenol-oxidizing enzymes in fungus-to-fungus interaction, localization of laccase and peroxidase was examined in the same dual cultures that had been used for observation of the predominance (Fig. 1, Tables 2, 3). In the case of *T. versicolor* and *P. coccineus*, both enzymes were detected under the whole colonies. *T. versicolor* produced both enzymes in a sole culture, but *P. coccineus* produced only laccase constitutively. However, in all dual cultures with either of these two fungi, strong activi-

Table 2. Activity and locality of laccase in dual culture of white-rot fungi

Species 1	Species 2	*Pleurotus ostreatus*	*Trametes versicolor*	*Pycnoporus coccineus*	*Ganoderma applanatum*	*Schizophyllum commune*
Pleurotus ostreatus		±	+++[a]	++[a]	±[a]	±[a]
Trametes versicolor		+++	+	+++[c]	±[a]	±[a]
Pycnoporus coccineus		++	++[c]	+	+++[b]	+++
Ganoderma applanatum		+	±	+	±	+[a]
Schizophyllum commune		±[a]	±	+	–	–

Coloration: +++, very strong; ++, strong; +, detected; ±, unclear; –, not colored
[a] Strongest coloration in vicinity of confrontation and under the overgrowing mycelia
[b] Coloration in vicinity of confrontation and under the overgrowing mycelia was almost the same as most other colored regions
[c] Coloration in vicinity of confrontation and under the overgrowing mycelia was weaker than in most other colored regions, but clearly discernible

Table 3. Activity and locality of peroxidase in dual culture of white-rot fungi

Species 1	Species 2	*Pleurotus ostreatus*	*Trametes versicolor*	*Pycnoporus coccineus*	*Ganoderma applanatum*	*Schizophyllum commune*
Pleurotus ostreatus		±	++[a]	±[a]	±[a]	++[a]
Trametes versicolor		++	+	++[a]	++[a]	++[a]
Pycnoporus coccineus		++	++[a]	±	+++[b]	+++
Ganoderma applanatum		±	±	–	+	+[a]
Schizophyllum commune		±[a]	±	+	+	–

Coloration: +++, very strong; ++, strong; +, detected; ±, unclear; –, not colored
[a] Strongest coloration in vicinity of confrontation and under the overgrowing mycelia
[b] Coloration in vicinity of confrontation and under the overgrowing mycelia was almost the same as most other colored regions

ties of phenol-oxidizing enzymes were detected at the confrontation region and under the mycelia growing over the other species. Thus, these fungi produced these enzymes actively in the presence of other fungi. Furthermore, this property was markedly observed in the *P. ostreatus* cultures; enzyme production was detected strongly under the confrontation region and under the mycelia covering over other fungus, although it was observed only slightly under other areas of each colony (Fig. 1b). In the incubation of sole *P. ostreatus*, coloration was slightly determined only at the edge of the colony. For this fungus, the productions of laccases (Palmieri et al. 2000) and manganese peroxidase isozymes (Kamitsuji et al. 2004) were induced by Cu^{2+} and nitrogen sources, respectively. From the results of our study, phenol-oxidizing enzymes were apparently induced by the confrontation with other fungi. *Lentinula edodes* (Shiitake) was reported to produce specific laccase isozymes in the presence of *Trichoderma* sp. (Savoie et al. 1998). In our study, *P. ostreatus* produced the phenol-oxidizing enzymes specifically in the presence of other fungi, similarly to the case of *L. edodes* confronted with *Trichoderma* sp.

To investigate whether a change in pH caused by fungal metabolites might regulate mycelial growth and enzyme activity, pH was observed after pouring a bromothymol blue (BTB) solution into dual cultures. Because local pH change was not determined in any culture, localization of enzyme activity was apparently not affected by pH changes.

White and Boddy (1992) reported that localization of enzyme production changed with increased incubation time after confrontation; however, in our study, the localization had not changed by 2 and 4 weeks after confrontation, and no enzyme production was determined after 2 and 6 months, with a few exceptions. On the other hand, mycelia grew normally after they were picked out from the 4-week incubation colonies, in which growth of both fungi had stopped, and inoculated into new sole cultures. This result indicates that the fungi did not die, but were dormant. As reported previously, mushrooms produce antifungicides and antibacteriocides (Beltran-Garcia et al. 1997). It might be possible that any fungi could secrete such compounds during competition. Interestingly, in the case of *P. ostreatus*, aerial hyphae stopped migrating around a boundary and did not go over the confrontation region, as if an invisible

barrier existed there. This observation suggests that antifungicides could be volatile. In microscopic observation, hyphae of both fungi in dual cultures did not necessarily contact each other and maintained a separation, which also suggests that substances could be produced that deter direct interaction.

The fungus-to-fungus interaction is interesting from the point of view of fungal ecology. In in vitro tests, confronted fungi did not directly attack, but interfered with each other using substances such as antifungicides. Phenol-oxidizing enzymes then play a role in detoxification of the substances, and the fungus that has the superior ability to detoxify is predominant in competition.

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