43. *Polyphenoloxidase Activation of Lentinus edodes* (Berk.) Sing. Induced by *Trichoderma* Invasion^{*)}

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(Communicated by Naohide HIRATSUKA, M. J. A., April 12, 1980)

Species aggregates of *Trichoderma*, such as *T. viride* Pers. ex S. F. Gray, *T. harzianum* Rifai, *T. polysporum* (Link ex Pers.) Rifai, etc., are of major injurious inhabitants in cultivation of *Lentinus edodes* (Berk.) Sing. on bed-logs (Komatsu 1976). However, the mode of interaction between the host and the parasite varies with the nutritional milieu of media (Tokimoto and Komatsu 1977, 1979a). For example, on medium containing glucose, *L. edodes* develops the ability to reject *Trichoderma* attack, whereas on medium with xylose, the former is invaded by the latter continuously. Also, the rejecting reaction of *L. edodes* has been positively correlated with brownish pigmentation of the mycelium at the contact zone with *Trichoderma*. The present investigation focused on polyphenoloxidase (PPO) activity of *L. edodes* during antagonistic reaction with *Trichoderma* in relation to the brownish pigmentation. A part of the works has been published in the abstract (Tokimoto and Komatsu 1978).

Materials and methods. Equally to the previous paper (Tokimoto and Komatsu 1979), *L. edodes*, TMI-655, and three species aggregates of *Trichoderma* were used. Glucose medium used in this study contained (per liter of distilled water): D-glucose 20 g, casamino acids (vitamine free) 2.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, KH_2PO_4 1.0 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.9 mg, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.8 mg, H_3BO_3 1.0 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.15 mg, $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ 0.1 mg, $(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 0.2 mg, adenine-HCl 5.0 mg, and thiamine-HCl 0.1 mg. Xylose medium which contained xylose in place of glucose was also prepared. A disk of mycelium (5 mm in diameter) which was cut off from the plate culture was floated on a liquid medium (10 ml in 100 ml-flask), and then incubated stationarily at 25°C in the dark. For the study of antagonistic reaction, a disk of the plate culture of *Trichoderma*, 5 mm in diameter, was placed on a *L. edodes* mycelium colony which had been grown previously for 20 days in a liquid medium. Crude enzyme was prepared as follows: Mycelial mat and culture

^{*)} Contribution from the Tottori Mycological Institute, no. 140.

filtrate were separated by filtration. The mycelium was homogenized in 0.001 M citric acid-phosphate buffer, pH 4.8, with a Waring Blender for a total of 5 min (1 min of blending with 1 min intervals). The cell debris was centrifuged for obtaining the supernatant solution. Ammonium sulfate was added to the solution obtained or culture filtrate to bring it 0.6 saturation. After centrifugation (15,000×g, 20 min), the precipitate was resuspended in 0.001 M citric acid-phosphate buffer (pH 4.8) and dialyzed against the same buffer. These procedures were performed below 5°C. PPO activity was determined using L-dihydroxyphenylalanine as a substrate, and dopachrome formation at 30°C was measured colorimetrically at 475 nm. The reaction mixture contained 5 mM of L-dihydroxyphenylalanine in 2.0 ml of 0.03 M citric acid-phosphate buffer, pH 4.8, and 1.0 ml of crude enzyme. An enzyme unit was defined as an absorbancy change of 0.001 per min. Enzyme activity was expressed in enzyme units per flask or mg protein. Protein concentration was determined by the method of Lowry *et al.* (1951) using crystallized bovine albumin as a standard. Degree of brownish pigmentation of culture medium was estimated for convenience by determining directly the optical density at 420 nm. The zero point was adjusted with distilled water. Absorbance of 0.001 was used for a unit of pigment concentration.

Results and discussion. Similarly to the previous investigation using agar media (Tokimoto and Komatsu 1977, 1979a), rejecting reaction of *L. edodes* to *Trichoderma* was observed in glucose medium, but not in xylose one. In xylose medium, *L. edodes* mycelium was invaded completely by *Trichoderma* after *ca.* 5 days' incubation. However, in glucose medium, *L. edodes* rejected *Trichoderma* invasion at least within 8 days' interaction. The rejection of *L. edodes* coincided with brownish pigmentation of the mycelium at the contact zone with *Trichoderma*. Figs. 1–4 show variations of PPO activity during the antagonistic reactions. The enzyme activity of mycelium increased just after the inoculation of *Trichoderma*. Though the maximum points of the activity existed for periods of 1–4 days after *Trichoderma* inoculation, remarkable reductions occurred immediately. PPO activation in culture filtrate started 1–2 days later than that in the mycelium one. *Trichoderma* species produce mycolytic enzymes which lysis *L. edodes* mycelium, and the activity in TMI-60622 is higher than that in TMI-60147 (Tokimoto and Komatsu 1979b). It is supposed that the remarkable PPO increase in culture medium is the result of mycelium lysis of *L. edodes* by the action of *Trichoderma* enzymes. High PPO activities after *Trichoderma* inoculation are probably attributed to *L. edodes*, because no PPO activity was detected in the single cultures of *Trichoderma*. Without *Trichoderma* inoculation, PPO activity of

Figs. 1-4. PPO activity variations of mycelia and culture media during antagonistic reactions between *Lentinus edodes* (TMI-655) and *Trichoderma* species aggregates. Open mark, mycelium; filled mark, medium. ●: Interaction between *L. edodes* and *Trichoderma*. ■: *L. edodes* single culture. ▲: *Trichoderma* single culture. 1, 2: *T. viride*, TMI-60147, was employed in glucose and xylose media, respectively. 3, 4: *T. harzianum*, TMI-60622, was employed in glucose and xylose media, respectively.

the mycelium and the culture filtrate of *L. edodes* maintained constant values, respectively. PPO activation of *L. edodes* was induced by the ether extracts of *Trichoderma* culture filtrates, though no or a little increase was observed in the case of TMI-60622 (Table I). The ether soluble fractions of *Trichoderma* culture filtrates inhibited mycelial

growth of *L. edodes*, except the TMI-60622 extracts. There were negative correlations between the mycelial growth rate of *L. edodes* and PPO activity in mycelium and culture filtrate (Table I). Brownish

Table I. Effect of ether extracts of *Trichoderma* culture filtrates on growth and PPO activation in *L. edodes*

Ether extract of <i>Tricho- derma</i> *	Mycelial growth of <i>L. edodes</i> **	PPO activity***					Brownish pigment- ation in medium***
		per flask			per mg protein		
		Myce- lium	Medium	Total	Myce- lium	Medium	
60146·G	6	225	92	317	566	113	54
60146·X	7	235	177	412	491	455	60
60147·G	16	307	42	349	514	125	41
60147·X	15	309	52	361	631	222	43
60622·G	22	38	17	55	110	42	6
60622·X	20	102	15	117	205	28	4
Control	18	41	16	57	178	35	

* Each *Trichoderma* species aggregate, TMI-60146, -60147, and -60622, was grown on glucose (G) and xylose (X) media (100 ml in 500 ml-flask) at 25°C for 10 days, respectively. The filtrates were extracted with ethyl ether after adjusting the pH to 3.0, and each extract was dissolved in 5 ml of 50% ethanol.

** To 10 ml of glucose medium, 0.5 ml of the extract in alcohol was added. Mycelial dry weights (mg/flask) of *L. edodes* on those media were determined after 15 days' incubation at 25°C. *** Ether extract, 0.5 ml, was added to each *L. edodes* culture which had been grown previously for 20 days. PPO activity and brownish pigment content of medium were determined after 6 days' incubation. Each pigment content of media was expressed after deducting the one of ether extract added.

pigment of *L. edodes* was formed by enzymatic oxidation of polyphenols (PP), since no pigmentation occurred in the culture containing PPO inhibitor, p-aminobenzoic acid or phenylthiourea. PP content of *L. edodes* also increases after *Trichoderma* inoculation (Tokimoto and Komatsu 1980). PPO activation of *L. edodes* is induced by the mycelial damage which is caused by *Trichoderma* metabolites, e.g. ether soluble antifungal substances (Table I). However, breakdown of *L. edodes* mycelium results in the end of PPO and PP syntheses. In xylose medium, *L. edodes* seems to be damaged too severely by *Trichoderma* to produce the brownish pigment.

Acknowledgements. The writer wishes to express his heartiest thanks to Dr. Naohide Hiratsuka, Director of the Tottori Mycological Institute, and Dr. M. Komatsu, Chief of Breeding and Cultivation of the institute, for their cardinal help and valuable advice. He is also indebted to Prof. S. Nishimura, Tottori University, for reading the manuscript.

References

- Komatsu, M. (1976): Rept. Tottori Mycol. Inst. (Japan), **13**, 1-113.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951): J. Biol. Chem., **193**, 265-275.
- Tokimoto, K., and Komatsu, M. (1977): Abstr. 2nd Intern. Mycol. Congr. (Tampa, Florida, U.S.A.), M-Z, p. 672.
- (1978): Proc. 22nd Annual Meet. of the Mycol. Soc. Japan (Tsukuba, Ibaraki), p. 83.
- (1979a): Ann. Phytopath. Soc. Japan, **45**, 261-264.
- (1979b): Proc. 23rd Annual Meet. of the Mycol. Soc. Japan (Otsu), p. 47.
- (1980): Proc. 1980-Annual Meet. of Phytopath. Soc. Japan (Tokyo), pp. 2-30.