J Wood Sci (1998) 44:469–472

ORIGINAL ARTICLE

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Variation of fruiting body productivity in protoplast fusants between compatible monokaryons of *Lentinula edodes*

Received: January 28, 1998 / Accepted: June 12, 1998

Abstract Mycelial protoplasts of two compatible monokaryons of *Lentinula edodes* were prepared, and electrofusion was undertaken. Sixteen dikaryons isolated after the electrofusion were tested for their fruiting abilities in comparison to the two dikaryons obtained by reciprocal mycelial mating. There were significant differences in fruiting body yield among the fusants, and some of them produced more fruiting bodies than did normally mated dikaryons. Although these dikaryons were divided into five genetically different groups by isozyme analyses and a cluster analysis, we could not find clear differences among the groups in terms of fruiting body yield, mycelial growth rate, or degree of wood decay. It appeared that fruiting body yield correlated positively with the mycelial growth rate but negatively with the degree of wood decay.

Key words Fruiting body productivity · *Lentinula edodes* · Breeding · Protoplast-fusion · Isozyme analysis

Introduction

Breeding of shiitake, *Lentinula edodes* (Berk.) Pegler, has been done commonly with mating between compatible monokaryons. Regeneration and cell fusion of protoplasts are new techniques developed for the purpose of breeding some edible mushrooms such as *Pleurotus*[1–9] and others.[10]

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Variable fruiting body productivity among protoplast-derived dikaryons was reported, for *L. edodes*,[11] suggesting that protoplast formation and its regeneration may cause genetic variation. In the present study, we prepared dikaryons by protoplast fusion between compatible monokaryons and showed that these fusants had genetic variation. Moreover, some of them produced more fruiting bodies than did normally mated dikaryons.

Materials and methods

Preparation of dikaryons with electrofusion of protoplasts

Two monokaryotic mycelia of *L. edodes*, TMIC182-54 and TMIC332-11, were used. Mating factors (A and B) of the two monokaryons were different. By reciprocal mating on agar medium, two dikaryons, 182-54 × (332-11) and 332-11 × (182-54), were obtained.[12] The monokaryon in parentheses showed the nuclear donor parent for dikaryosis. Preparation of mycelial protoplasts of the two monokaryons and their electrofusion were undertaken by the method previously reported.[12] The protoplast suspension after electrofusion was plated onto MYG agar medium (2% malt extract, 0.2% yeast extract, 2% glucose, 1.5% agar, pH 6.0) containing 0.5 M sucrose and incubated at 25°C for 7 days. Regenerated mycelial colonies were transferred to PDA medium (Difco). Enlargement of the mycelial colonies on PDA medium at 25°C from the 4th day to the 8th day of incubation was used as the mycelial growth rate.

Wood decay test

Sawdust medium (9 g of air-dried sawdust of *Fagus crenata* Blume and 21 ml of 0.1% ammonium sulfate) was packed in a 50-ml glass bottle (35 mm diameter, 60 mm hight) with a ventilative silicon cap. The water content of the medium after autoclaving was about 70%. The sterilized medium was inoculated with a test mycelium. After incubation at 23°C in darkness for 60 days, each bottle was dried at 100°C

for 3 days and weighed. The weight loss was used to indicate the decay ability of a given dikaryon.

Isozyme analysis

Preparation and gel electrophoretic analysis of isozymes were performed according to the method previously reported.[13] Mycelium was grown on WGC medium (20 g glucose, 0.2 g casamino acid per liter of hot water extract from 100 g dried wood powder of *Quercus serrata* Thunb.) at 23°C for 14 days. The acetone powder of the each mycelium was extracted with distilled water, and the supernatant was used for electrophoresis. Esterase, malate dehydrogenase, and laccase were separated by isoelectric focusing in 5% polyacrylamide gel according to the procedure by Vesterberg[14] using pH 2.5–8.0 carrier ampholytes with 2000 V at 5°C. Isozymes of esterase and malate dehydrogenase were stained by the method of Allendorf et al.[15] Laccase bands were stained with 5 mM *p*-phenylenediamine. Using the banding patterns of the three enzymes, similarity coefficients between the strains (number of common bands/number of discordant bands) were calculated, and a cluster analysis based on the similarity coefficients was undertaken.

Fruiting trial

Logs of *Quercus serrata* (8–12 cm diameter, 100 cm long) were inoculated with *Lentinula* spawns, cylindrical wood pieces colonized by dikaryotic mycelium, using 15 logs for each dikaryon. The bedlogs thus inoculated were placed under the shade of trees for development of mycelium and for production of fruiting bodies. All mature fruiting bodies produced on the bedlogs for 4 years after inoculation were harvested, and the number and dry weight were recorded.

Results and discussion

Among a total of 400 colonies obtained by protoplast fusion, 24 were shown to be dikaryons with clamp connections. These dikaryons were regarded as protoplast fusants because no colonies with clamp connection were recovered from the mixture of protoplasts before fusion treatment.

The 24 dikaryons obtained by the protoplast fusion, 2 dikaryons from usual reciprocal mating, and 2 parental monokaryons were tested for mycelial growth rate at 25°C for 4 days on PDA medium (Table 1). The growth rates in

Table 1. Characters of dikaryons of *Lentinula edodes* obtained by protoplast fusion

Isolate	Mycelial growth speed (mm)[a]	Degree of wood decay (%)[a]	Isozyme pattern			Electrophoretic phenotype
			Esterase	Malate dehydrogenase	Laccase	
Dikaryon produced by protoplast-fusion						
A	17.3	9.88	c	c	c	3
B	17.3	10.09	c	c	e	4
C	15.2	11.97	e	d	f	8
D	17.4	11.23	c	d	e	7
E	17.7	9.75	c	d	d	6
F	16.2	10.44	c	d	e	7
G	16.0	9.87	c	e	e	9
H	16.9	9.58	c	e	e	9
I	16.9	9.92	d	e	e	12
J	12.0	12.26	c	e	g	10
K	17.2	9.64	c	d	c	5
L	17.4	9.66	c	d	e	7
M	15.5	10.44	e	d	f	8
N	17.2	9.63	c	d	e	7
O	16.6	10.00	c	d	e	7
P	15.6	9.82	c	d	e	7
Q	16.7	9.60	c	e	e	9
R	14.6	10.21	e	d	f	8
S	17.5	11.21	c	d	e	7
T	16.0	10.45	c	e	e	9
U	15.9	10.09	e	d	f	8
V	17.7	9.76	c	d	e	7
W	15.7	11.92	c	d	e	7
X	16.2	9.69	e	d	f	8
Dikaryon obtained by mating[b]						
Y	19.1	9.46	f	g	h	13
Z	16.1	9.81	c	f	c	11
Parental monokaryon						
182-54	11.5	15.17	a	a	a	1
332-11	12.6	9.01	b	b	b	2

[a] Mean of two repetitions.
[b] Y: 182-54 × (332-11), Z: 332-11 × (182-54); see text for mating method

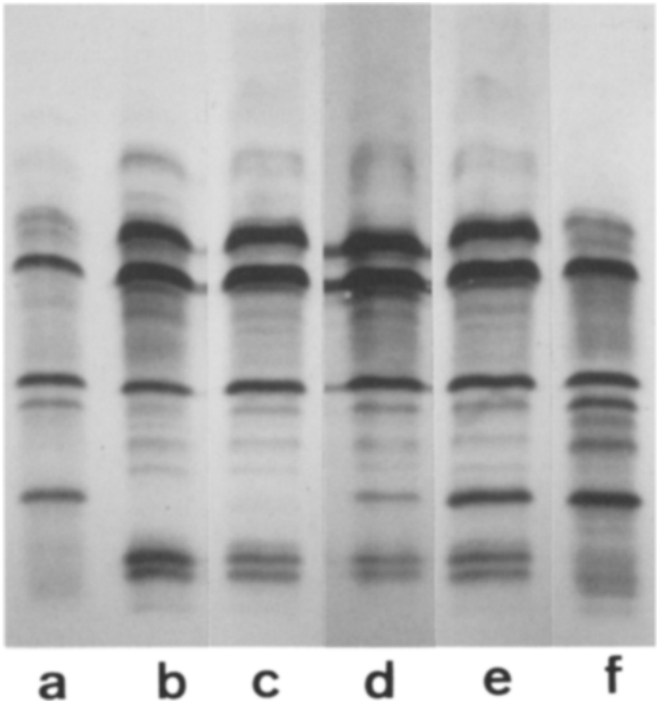

Fig. 1. Gel electrophoresis of esterase isozymes in *Lentinula edodes* mycelia. *a–f*, isozyme patterns (see Table 1 and Fig. 2)

dikaryons ranged from 12.0 to 19.1 mm, and the coefficient of variation (standard deviation/mean) was 8.8%. The two parental monokaryons showed 11.5 and 12.6 mm growth, respectively. The wood decay test for the dikaryons showed a coefficient of variation of 7.7%, which was similar to that of mycelial growth rate.

Electrophoretic analyses of the mycelial enzymes from these dikaryons, including the two parental monokaryons, produced different isozyme patterns: six types (a–f) of esterase, seven types (a–g) of malate dehydrogenase, eight types (a–h) of laccase. When the isozyme patterns of the three enzymes were combined, it appeared that a total of 28 samples, including the 2 parental monokaryons, showed 13 different electrophoretic phenotypes (Figs. 1, 2; Table 1). The two dikaryons from reciprocal mating showed different isozyme patterns. The two parental monokaryons also had their own distinct patterns, different from those of the dikaryons. The 26 dikaryons could be divided into five branches by cluster analysis using the isozyme patterns, showing genetic variation among these dikaryons (Table 2).

The dry weights of fruiting body yields on logs for the randomly selected 18 dikaryons are shown in Fig. 3. Variation analysis and *t*-test revealed significant differences among dikaryons. For example, fusants L and U produced more fruiting bodies than did fusants C, E, F, G, and J and

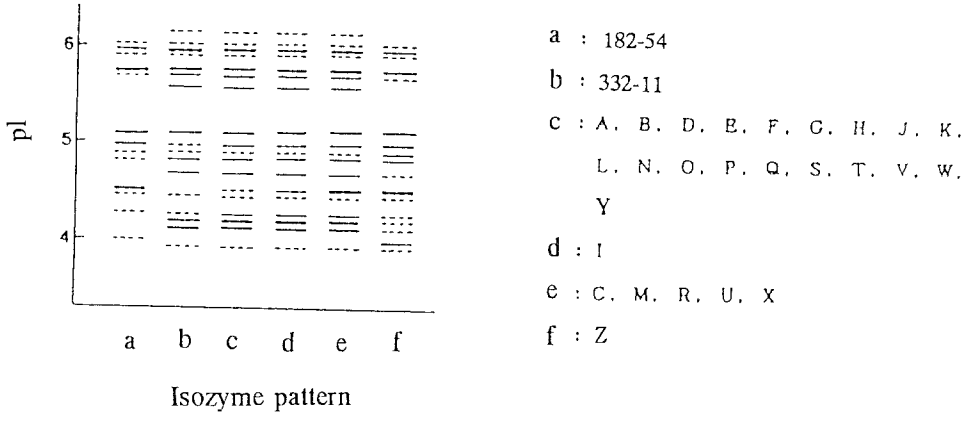

Fig. 2. Isozyme patterns of esterase in *Lentinula edodes* mycelia. Dotted lines show weak bands. See Table 1 for legends

Table 2. Cluster analysis of *Lentinula edodes* dikaryons and comparison of the mycelial characters among the clusters

Cluster name	Dikaryons in the cluster	Mean of dikaryons in the cluster		
		Fruiting body yield (g)	Mycelial growth rate (mm)	Degree of wood decay (%)
I	A, K	71.4	17.3	9.76
II	B, D, F, H, J	53.9	16.0	10.72
III	C, E, G, I, L, M, N, O, P Q, R, S, T, U, V, W, X	69.0	16.4	10.24
IV	Z	56.4	16.1	9.81
V	Y	64.4	19.1	9.46

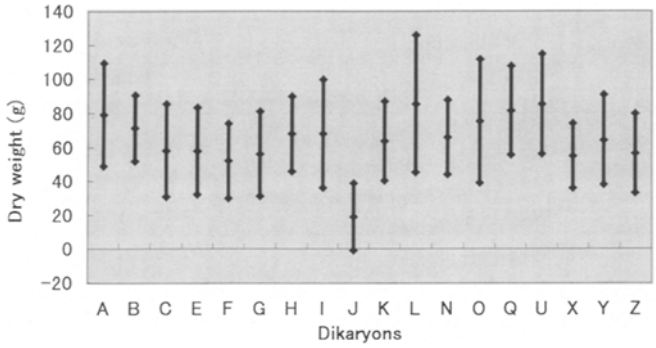

Fig. 3. Comparison of fruiting body yield among dikaryons of *Lentinula edodes*. See Table 1 for legends. Fruiting body yield: dry weight (grams) per 3500 cm^2 of log surface. Each bar indicates the average (center) ± standard deviation (n = 15). The least significant difference (at the 5% level) of the dry weight was 28.1 g

Table 3. Correlation among characteristics of *Lentinula edodes* dikaryons

Parameter	Correlation	
	Mycelial growth speed	Wood-decay degree
Fruiting body yield	0.665*	−0.626*
Mycelial growth speed	–	−0.825*

* Significant at 1%

the normally crossed dikaryons X and Y. In contrast, fusant J fruited more poorly than all the other dikaryons. Similar differences among dikaryons were observed in the number of fruiting bodies (data not shown). Thus we propose that protoplast fusion between compatible monokaryons is a technique for producing genetic variations and for obtaining dikaryons with high fruiting productivity. The coefficients of variation among dikaryons for the number and dry weight of fruiting bodies were 26.4% and 24.7%, respectively. These values were higher than those for mycelial growth rate and wood-decay degree, suggesting that fruiting ability varied more than the vegetative propagation abilities.

As a practical use of isozyme analyses, we tried to compare fruiting body yield, mycelial growth rate, and wood-decay degree among the clusters obtained by isozyme analyses. Contrary to expectation, we could not find clear differences among the clusters (Table 2). Although additional studies are required to clarify the existence of differences among the clusters, isozyme loci in this study probably do not link to the genes that affect fruiting, mycelial growth, and wood decay.

Among all the dikaryons used, the fruiting body yield correlated positively to mycelial growth rate but negatively to wood-decay degree, at the 1% level of significance (Table 3). Depending on these characteristics, it is possible to establish a laboratory method that allows us to select highly productive dikaryons, although further studies are required.

Acknowledgment We thank Dr. Yasuyuki Hiratsuka, Director of the Tottori Mycological Institute, for reading the manuscript.

References

1. Ohmasa M (1986) Intraspecific protoplast fusion of *Pleurotus ostreatus* using auxotrophic mutants. Jpn J Breed 36:429–433
2. Toyomasu T, Matsumoto T, Mori K (1986) Interspecific protoplast fusion between *Pleurotus ostreatus* and *Pleurotus salmoneostramineus*. Agric Biol Chem 50:223–225
3. Toyomasu T, Mori K (1987) Intra- and interspecific protoplast fusion between some *Pleurotus* species. Agric Biol Chem 51:935–937
4. Toyomasu T, Mori K (1987) Fruit body formation of the fusion products obtained on interspecific protoplast fusion between *Pleurotus* species. Agric Biol Chem 51:2037–2040
5. Eguchi F, Higaki M (1992) Production of new species of edible mushrooms by protoplast fusion method. I. Protoplast fusion and fruiting body formation between *Pleurotus ostreatus* and *Agrocybe cylindracea* (in Japanese). Mokuzai Gakkaishi 38:403–410
6. Takehara T, Kumata A, Aono S (1993) Selection of high-yield strains among regenerants from mutagenic protoplasts of *Pleurotus ostreatus* (in Japanese). Mokuzai Gakkaishi 39:967–971
7. Eguchi F, Fukuzumi T, Higaki M (1993) Production of new species of edible mushrooms by protoplasts fusion method. II. Analysis of the mycelia and basidiospores of a fusant between *Pleurotus ostreatus* and *Agrocybe cylindracea*. Mokuzai Gakkaishi 39:825–830
8. Eguchi F, Higaki M (1995) Production of new species of edible mushrooms by protoplasts fusion method. III. Protoplasts fusion and analysis of the fusant between *Pleurotus sajor-caju* and *Mycoleptodonoides aitchisonii*. Mokuzai Gakkaishi 41:342–348
9. Tamai Y, Miura K, Kayama T (1990) Electrical fusion of protoplasts between two *Pleurotus* species. Mokuzai Gakkaishi 36:487–490
10. Sunagawa M, Miura K, Ohmasa M, Yokota S, Yoshizawa N, Idei T (1992) Intraspecific heterokaryon formation by protoplast fusion of auxotrophic mutants of *Auricularia polytricha*. Mokuzai Gakkaishi 38:386–392
11. Fukumasa-Nakai Y, Matsumoto T, Komatsu M (1994) Fruiting body productivity of protoplast-derived clones in *Lentinula edodes*. Mycoscience 35:137–139
12. Fukuda M, Harada Y, Imahori S, Fukumasa-Nakai Y, Hayashi Y (1995) Inheritance of mitochondrial DNA in sexual crosses and protoplast cell fusions in *Lentinula edodes*. Curr Genet 27:550–554
13. Fukuda M, Tokimoto K (1991) Variation of isozyme patterns in the natural population of *Lentinus edodes*. Proc Jp Acad 67B:43–47
14. Vesterberg O (1972) Isoelectric focusing of proteins in polyacrylamide gels. Biochim Biophys Acta 257:11–19
15. Allendorf FW, Mitchell N, Ryman N, Stahl G (1977) Isozyme loci in brown trout (*Salmo trutta* L.): detection and interpretation from population data. Hereditas 86:179–190