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Fruiting body productivity of protoplast-derived clones in *Lentinula edodes**

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More than 100 dikaryotic clones (protoclones) derived from mycelial protoplasts of a *Lentinula edodes* dikaryon were examined for their mycelial growth and fruiting body productivity. These protoclones exhibited a variety of vegetative mycelial growth rates, but no apparent difference in colonial morphology compared with the original (parental) dikaryon. Protoclones were cultivated on wood logs under natural conditions, and they exhibited a very wide range of fruiting body yields. Of the 134 protoclones, four were selected that produced a 30–40% increase in dry weight of fruiting body yield over that of the original dikaryon. This high productivity of fruiting bodies was maintained for at least several years. The present results suggest that *L. edodes* protoclones can be practically used in strain improvement to increase the capability of fruiting body formation.

Key Words——fruiting body productivity; *Lentinula edodes*; protoclones.

Introduction

The shiitake, *Lentinula edodes* (Berk.) Pegler [=*Lentinus edodes* (Berk.) Sing.], is an excellent edible mushroom cultivated most widely using logs of *Quercus* trees as substrate in Japan. Commercial production of shiitake has taken an essential place in agriculture. Therefore, the development of new strains of this mushroom with qualitatively and quantitatively superior traits in fruiting body formation has practical importance.

Secor and Shepard (1981) studied biological properties of protoclones regenerated from the leaf protoplasts of potato, *Solanum tuberosum* L., and reported that wide variation was observed in morphology, yield, and other agronomic characteristics. Likewise, Magae et al. (1985) reported evidence that randomly selected protoclones of *Pleurotus ostreatus* (Jacq.: Fr.) Kummer, another important cultivated mushroom, yielded more fruiting bodies than the original dikaryon did. These studies suggest that protocloning may be a useful technique for strain improvement of these mushrooms. In the present study, we examined the biological properties with special reference to the fruiting body productivity of protoclones derived from mycelial protoplasts of an *L. edodes* dikaryon.

Materials and Methods

Preparation and culture of protoplasts A dikaryon TMI-563 of *Lentinula edodes* (Berk.) Pegler from the Culture Collection of the Tottori Mycological Institute was used in this study. To prepare mycelium for protoplast production, the dikaryon was grown in MYG liquid medium (2% malt extract, 0.2% yeast extract, and 2% glucose) at 25°C for 2 weeks, then fragmented with a Waring blender at a speed of 10,000 rpm for 15 sec. Portions of 2 ml of the mycelial suspension were inoculated into 100-ml Erlenmeyer flasks containing 15 ml of MYG medium. The flasks were incubated statically at 25°C for 3 days. After the incubation, the mycelium was harvested, washed once with sterilized water and 0.6 M mannitol solution (pH 6.0) by centrifugation at $800 \times g$ for 10 min, then incubated in the enzyme mixture of 0.5% (w/v) Novozym 234 (Novo Biolabs, Denmark) and 0.25% (w/v) chitinase (Sigma, USA) dissolved in the 0.6 M mannitol solution at 28°C for 2 hr with shaking (90 strokes/min). The mixture of protoplasts and undigested hyphal debris was passed through a 3G2 glass filter to remove hyphal debris. The protoplasts were collected and washed twice with the 0.6 M mannitol solution by centrifugation at $600 \times g$ for 6 min. The resulting protoplasts (approx. 2×10^7 protoplasts/100 mg fresh weight mycelium) were suspended in the 0.6 M mannitol solution at a concentration of 5×10^4 protoplasts/ml. One hundred μl of the protoplast suspension was plated on MYG medium (pH 6.0) supplemented with 0.5 M sucrose and 2% agar in a 9-cm petri dish, and immediately overlaid with 5 ml of the MYG medium containing 0.5 M sucrose and 0.5% agar cooled to 41°C. The plates were incubated at 25°C for 7 days. The percentage of protoplast regeneration was calculated based on the ratio of the number of colonies developing on regeneration medium in 7 days of incubation to the total number of protoplasts inoculated.

Isolation and growth test of protoclones Colonies regenerated from protoplasts on the MYG medium were

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examined microscopically for the formation of clamp-connections indicative of dikaryons. Dikaryotic regenerants with clamp-connections, namely protoclones, were randomly isolated and maintained on 2% malt extract agar medium. For examination of mycelial growth, the protoclones were grown in 9-cm petri dishes on PDA medium (Difco) at 25°C and 30°C.

Fruiting trial Logs (100 cm long and 7–13 cm in diam) cut from trunks of *Quercus serrata* Thunb. were inoculated with cylindrical pieces of wood overgrown with mycelium of protoclones or the original dikaryon, using five logs for each strain. The bedlogs were then kept in the raising yard for the colonization of mycelium through the wood and for the induction of fruiting bodies. All fruiting bodies produced on the bedlogs over three years after inoculation were harvested, and their total number and dry weight per 10,000 cm^3 of bedlog-wood were measured.

Results and Discussion

Mycelial protoplasts prepared from an *L. edodes* dikaryon TMI-563 started regenerating into hyphae within 3 days and formed visible colonies, varying in size, at a regeneration percentage of about 6% after 7 days of incubation. Microscopical observation revealed that all the colonies were derived from single protoplasts, and that the much larger and denser of these colonies were almost always dikaryotic protoclones with clamp-connections. In this study, we obtained 134 protoclones isolated randomly from these dikaryotic regenerants for further analysis.

Figure 1 shows the mycelial growth rate of 134 protoclones and the original dikaryon when they were incubated on PDA medium at 25°C and 30°C for 7 days. The protoclones exhibited a variety of mycelial growth rates, but they could be classified roughly in two groups based on the rates at both temperatures, i.e., the slow-growth group (mycelial growth rate smaller than 23 mm at 25°C and 10 mm at 30°C) and the normal-growth group. Only seven protoclones belonged to the former group and the rest, 127 protoclones and the original dikaryon, belonged to the latter. No apparent difference was observed in gross morphology of colonies, or color and density of aerial hyphae between protoclones in either group and the original dikaryon (Fig. 2).

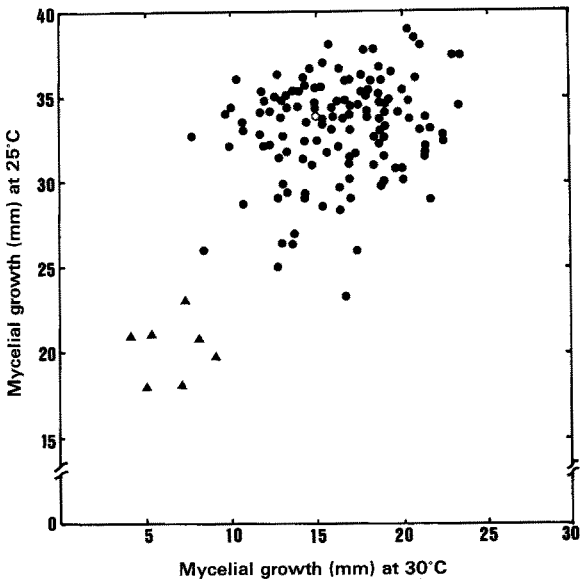

Fig. 1. Mycelial growth of 134 protoclones of *Lentinula edodes* on PDA medium for 7 days at 25°C and 30°C. The open circle and closed triangles indicate the original dikaryon and protoclones showing slow mycelial growth, respectively. Closed circles indicate protoclones showing normal mycelial growth.

Next, the fruiting body productivity of all protoclones

Fig. 2. Mycelial colonies of the *Lentinula edodes* original dikaryon (O), and protoclones with slow (S) and normal (N) growth rate.

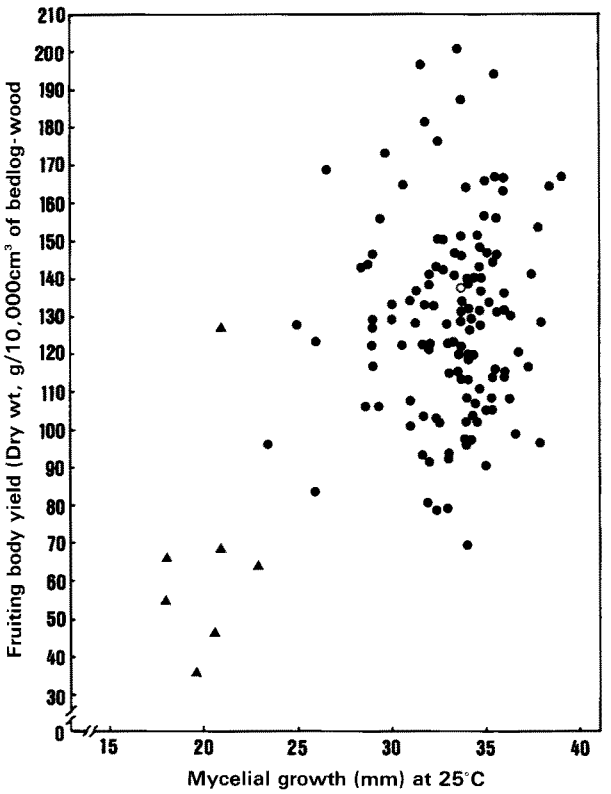

Fig. 3. Fruiting body yields (per 10,000 cm³ of bedlog-wood for full three years of cultivation) and mycelial growth rates (for 7 days at 25°C) of 134 *Lentinula edodes* protoclones. The open circles and closed triangles indicate the original dikaryon and protoclones showing slow mycelial growth, respectively. Closed circles indicate protoclones showing normal mycelial growth.

isolated was compared with that of the original dikaryon by culturing them on wood logs. All fruiting bodies grown out of the protoclones were normal and their morphological characteristics were indistinguishable from those of fruiting bodies from the original dikaryon. However, the protoclones exhibited a very wide range of total fruiting body yields over three years of cultivation (Fig. 3). Nearly all of the slow-growth group produced much smaller yields of fruiting body than the original dikaryon did. For the remaining 127 protoclones of the normal-growth group, there was no significant correlation ($p>0.05$) between their fruiting body yield and vegetative mycelial growth rate at 25°C or 30°C. This suggests that the degree of vegetative mycelial growth on a PDA medium is not always a useful criterion for selecting protoclones with superior fruiting body productivity. Of the 127 protoclones of the normal-growth group, four (P58, P102, P113, and P114)were found to yield 36-46% more dry weight of fruiting bodies than the original dikaryon. This increase in the fruiting body yield resulted apparently from an increase in the number of fruiting bodies (Table 1). A similar increase in fruiting

Table 1. Fruiting body productivity of selected *Lentinula edodes* protoclones.

Protoclone	Fruiting body yield (per 10,000 cm³ of bedlog-wood)			
	First experiment		Second experiment	
	Number (index)	Dry wt, g (index)	Number (index)	Dry wt, g (index)
P58	76.4 (163)	194.7 (141)	27.2 (122)	73.2 (120)
P102	86.3 (184)	187.2 (136)	33.0 (148)	86.1 (141)
P113	68.3 (146)	200.9 (146)	27.8 (125)	73.4 (120)
P114	79.5 (170)	197.0 (142)	30.5 (137)	83.5 (137)
Average	77.6 (165)	195.0 (141)	29.6 (137)	79.1 (130)
TMI-563 (original dikaryon)	46.9 (100)	137.9 (100)	22.3 (100)	61.0 (100)

First and second experiments were carried out from April 1986 to March 1989 and from April 1990 to March 1993, respectively.

body productivity has been reported in another edible mushroom, *Pleurotus ostreatus* (Magae et al., 1985), and detected in protoclones from another dikaryotic strain, TMI-655, of *L. edodes* (unpublished data).

To clarify whether such high ability of fruiting body production in the selected protoclones is stable or transient, we performed a second experiment of bedlog cultivation using the strains preserved for 4 years (twenty-five logs for each strain). Although there was a large difference in fruiting body yield between the first and the second experiment, probably due to unseasonability of climate and/or the physicochemical diversity of wood logs used, we confirmed that these selected protoclones still gave a higher fruiting body yield than the original dikaryon (Table 1). This shows that the selected protoclones maintained their high fruiting body productivity when preserved by agar medium subculture for at least several years. Although the reason for the variation among protoclones is unknown, the present results suggest that it is possible to use the *L. edodes* protoclones in strain improvement aimed at increasing the capability of fruiting body formation.

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