Mycoscience 40: 433–436, 1999

Short Communication

Preparation and regeneration of protoplasts of three fungi of Boletaceae

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Accepted for publication 7 June 1999

Protoplasts of three fungi of Boletaceae, *Suillus luteus*, *S. grevillei*, and *Boletinus cavipes*, were prepared with yields of 45, 8.0, and 1.8×10^7/g fresh mycelia under the optimal conditions, respectively. Nucleate protoplasts accounted for 42% of the whole preparation of *S. luteus* and 32% of that of *S. grevillei*, and 21% of the nucleate protoplasts of *S. luteus* and 35% of those of *S. grevillei* possesed two nuclei. Regeneration efficiency of protoplasts was 0.4% for *S. luteus* and 0.05% for *S. grevillei*. The regeneration of *B. cavipes* protoplasts was also confirmed. Optimal conditions for regeneration were determined. Addition of gellan gum instead of agar to the medium and activated charcoal treatment of agar medium increased the regeneration efficiency significantly.

Key Words——*Boletinus cavipes*; protoplast formation; protoplast regeneration; *Suillus grevillei*; *Suillus luteus*.

Protoplast production and regeneration have been studied for several mycorrhizal fungi (Abe et al., 1984; Kropp and Fortin, 1986; Hebraud and Fevre, 1988; Barrett et al., 1989; Anunciacao et al., 1990; Chen and Hampp, 1993; Stülten et al., 1995; Dias et al., 1996; Hampp et al., 1998). In general, the growth rate of mycorrhizal fungi is very low, and protoplast formation is difficult. The efficiencies of protoplast production and regeneration vary widely among mycorrhizal fungi, probably because physiological characters such as growth rate differ greatly even among species of the same genus.

In this study, we investigated the conditions for protoplast preparation and regeneration of three fungi of Boletaceae, *Suillus luteus* (L.:Fr.) S. F. Gray, *S. grevillei* (Klotz.) Sing., and *Boletinus cavipes* (Opat.) Kalchbr. Here we report for the first time the preparation of nucleated protoplasts and the regeneration of protoplasts of *S. luteus*, and the first successful preparation and regeneration of protoplasts of the other two fungi.

The strains used in this study, *S. luteus* SA-51, *S. grevillei* SA-53, and *B. cavipes* SA-286, were stock cultures of our laboratory. They were maintained on potato sucrose agar (PSA) slant medium or on Ohta agar medium (Ohta, 1990) with the following composition (per liter): glucose, 10 g; citric acid, 1 g; ammonium tartrate, 1 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 1 g; $CaCl_2 \cdot 2H_2O$, 50 mg; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 7 g; $FeCl_3$, 50 mg; $MnSO_4 \cdot 4\text{-}6H_2O$, 0.5 mg; $ZnSO_4 \cdot 7H_2O$, 3 mg; $CoSO_4 \cdot 7H_2O$, 0.5 mg; $CuSO_4 \cdot 5H_2O$, 1 mg; $NiSO_4 \cdot 6H_2O$, 2 mg; acetylacetone, 30 μl; thiamine HCl, 3 mg; nicotinic acid, 50 μg; folic acid, 30 μg; biotin, 50 μg; pyridoxine HCl, 5 μg; cartinine chloride, 10 μg; adenine $H_2SO_4 \cdot 2H_2O$, 30 μg; choline chloride, 30 μg; pH 5.0–5.1. For the preparation of protoplasts, mycelia were precultured in the dark in liquid Ohta medium for 14–20 d, depending on the strain, at 25°C. They were then fragmented in a homogenizer (Nihon Seiki, Ace homogenizer AM-8) for 10 s, and a portion of the mycelial suspension was inoculated into a 100-ml Erlenmeyer flask containing fresh liquid Ohta medium and cultured in the dark for 6 d at 25°C (second culture). Cultured mycelia were collected by centrifugation (2,200 × g, 10 min), washed once with distilled water and once with buffer A (0.6 M mannitol in 0.05 M maleic acid-NaOH buffer, pH 5.0), then incubated in the lytic enzyme solution in buffer A (50 mg of wet mycelia per ml) for 2 h at 28°C with shaking at 90 strokes/min. As the lytic enzyme, Novozym 234 (NovoBioLabs, Denmark), Cellulase Onozuka RS (Yakult Honsha), and Chitinase (Sigma Chemical) were used singly or in combination. Protoplast suspension was filtered through a glass filter (Sibata Scient. Technol., 3G2), washed twice in buffer A by centrifugation (800 × g, 6 min), and suspended in the same buffer. The number of protoplasts was determined with a Thoma hemacytometer.

Nuclei of plotoplasts were fixed and stained for observation by the method of Kuroiwa and Suzuki (1980)

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using DAPI (4'-6-diamidino-2-phenylindole), and observed with a fluorescence microscope (Olympus Model BH2-RFK).

For regeneration of protoplasts in plate culture, 100 μl of protoplast suspension of the appropriate concentration in buffer A was pipetted onto 12 ml of Ohta solid medium containing 0.5 M mannitol or 0.5 M sucrose and 1.5% agar (extra pure grade, Nacalai Tesque) or 0.5% gellan gum (Wako Pure Chemical Industries) in a 9-cm Petri dish. Then 5 ml of molten Ohta media containing 0.5% agar with low gelling temperature (Nacalai Tesque) that had been kept at 33°C or 0.25% gellan gum that had been kept at 37°C was poured over the suspension and mixed well. The Petri dish was incubated at 25°C in the dark for 10 d, then protoplast regeneration was checked by use of an inverted microscope (Olympus Model CK2-TRC-2) or by observation of colony formation. As a control, the protoplast suspension diluted 5- to 10-fold with distilled water to burst protoplasts was plated in the same way. The number of colonies regenerated for the protoplast suspension was corrected using the result of control experiments.

For regeneration in liquid culture, 100 μl of protoplast suspension diluted with buffer A to appropriate concentration was pipetted into 10 ml of Ohta liquid media containing 0.5 M mannitol in a 25 mm diam test-tube and incubated for 10 d at 25°C in darkness, the contents of the test-tube was transferred into a Petri dish, and the number of regenerated colonies was counted.

To determine the optimum conditions for protoplast preparation, we examined the effects of 1) composition of lytic enzymes, 2) pH, 3) the osmotic stabilizer in the lytic enzyme solution, 4) period of enzyme treatment, 5) period of preculture, and 6) period of second culture. The results are shown in Table 1.

The following four compositions of lytic enzyme solution, were used in this experiment: A, 0.5% Novozym 234; B, 0.5% Novozym 234+0.5% Cellulase Onozuka RS; C, 0.5% Novozym 234+0.1% Chitinase; D, 0.5% Cellulase Onozuka RS+0.1% Chitinase.
The pH of the lytic enzyme solution was varied between 4.0 and 7.0 for *S. luteus* SA-51 and *S. grevillei* SA-53, and between 3.0 and 7.0 for *B. cavipes* SA-286. For *B. cavipes*, very acidic condition pH 3.0 gave best yield of protoplasts, as shown in Table 1, but the reason is not clear at present. As osmotic stabilizer of the enzyme solution, we examined 0.6 M mannitol, 0.6 M sucrose, 0.6 M $MgSO_4$, and 0.3 M KCl. The period of the lytic enzyme treatment was varied between 1 and 4 h. Preculture periods of 10, 15, 20, and 30 d were tested for each of the three strains. The period of second culture was varied between 3 and 9 d.

In the following experiments, protoplasts were prepared under the conditions listed in Table 1 unless otherwise noted. Under these conditions, about 4.5×10^8 protoplasts/g fresh mycelia were obtained for *S. luteus* SA-51, 8.0×10^7/g fresh mycelia for *S. grevillei* SA-53, and 1.8×10^7/g fresh mycelia for *B. cavipes* SA-286.

Nuclei were observed in 42% of protoplasts prepared from *S. luteus* SA-51 and 32% of those from *S. grevillei* SA-53. Of the nucleate protoplasts, 21% possessed two nucei in the case of *S. luteus* SA-51 and 35% in the case of *S. grevillei* SA-53.

Barrett et al. (1989) reported that, despite the successful regeneration of protoplasts of four other mycorrhizal fungi, they could not regenerate protoplasts of *S. luteus*. They ascribed this failure to the lack of nuclei in the protoplasts. Because we observed nuclei in our protoplasts of *S. luteus* SA-51, we cultured the protoplasts in Ohta agar media and observed their morphological changes. Some surface unevenness appeared on the second day of culture, and some of the protoplasts showed regeneration of hyphae on the sixth day. About a month later, the first colony was observed. The regeneration rate, however, was only 3×10^{-4}%. Because of this low regeneration rate and the slow growth of colonies, we tried other methods of regeneration.

We first tried liquid culture of protoplasts of *S. luteus* SA-51 in Ohta liquid medium with 0.3, 0.5, or 0.7 M mannitol or 0.3, 0.5, or 0.7 M sucrose. In this experiment, we observed uneven surfaces of protoplasts on the second day of culture, regeneration of hyphae on the third day, and colonies on the sixth day. In a similar experiment with *S. grevillei* SA-53, we observed uneven surfaces on the second day of culture, regeneration of hyphae on the third day, and colonies on the eighth day. Liquid culture was better for *S. luteus* than agar culture.

This improvement in the regeneration rate of proto-

Table 1. Optimum conditions for preparation and yields of protoplasts of three fungi of Boletaceae.

Conditions	*Suillus luteus* SA-51	*Suillus grevillei* SA-53	*Boletinus cavipes* SA-286
Composition of lytic enzymes	B[a)]	B	B
pH of enzyme solution	5.0	5.0	3.0
Osmotic stabilizer	0.6 M mannitol	0.6 M mannitol	0.6 M $MgSO_4$
Period of enzyme treatment	2.0 h	1.5 h	3.0 h
Period of preculture	13–15 d	15 d	20 d
Period of second culture	6 d	5 d	7 d
Protoplast yield per g fresh mycelia	4.5×10^8	8.0×10^7	1.8×10^7

a) See text for composition of enzyme solution.

plasts of *S. luteus* SA-51 in liquid medium raised the possibility that the agar medium contains inhibitors of protoplast regeneration. Bjurman (1984) reported that autoclaved agar contained an organic acid that was inhibitory to spore germination of mycorrhizal fungi including *S. luteus* and that it could be removed by treatment with activated charcoal. We treated agar media with activated charcoal and compared it with untreated media for protoplast regeneration. A cellophane sheet was laid over the surface of the Ohta solid medium containing 0.5 M mannitol and 1.5% agar. About 1 g of sterilized activated charcoal (Wako Pure Chemical) was spread over the cellophane sheet, and the plate was incubated at 25°C in the dark for 7 d. The cellophane sheet and activated charcoal were then removed, and protoplasts were regenerated using the treated and untreated agar plates. We found that treatment with activated charcoal clearly increased the regeneration rate. This indicated the presence of inhibitors in the agar medium.

We next compared gellan gum (Ichi et al., 1986) with the agar mainly used in this study as the substrate for regeneration of protoplasts of *S. luteus* SA-51 and *S. grevillei* SA-53. Table 2 shows the results. For SA-51, many more colonies appeared in the gellan gum medium than in the agar medium, while for SA-53, the number of colonies was not significantly different between gellan gum and agar. The growth of colonies was faster in the gellan gum medium for both strains. Since, protoplast regeneration of *S. luteus* SA-51 was difficult with the agar medium, Ohta media with four kinds of agar of different grades and with gellan gum were tested for regeneration of protoplasts. Agars used were extra pure grade agar of Nacalai Tesque which is mainly used in this study, guaranteed grade reagent of Wako Pure Chemical, Bacto Agar of Difco Lab., and purified agar (ash less than 2%) of Sigma Chemical Co. The regeneration rate of protoplasts was highest in the gellan gum medium and lowest in the extra pure grade agar medium.

Table 2. Regeneration of protoplasts in agar and gellan gum media.

Substrate	Number of Colonies Regenerated	
	Suillus luteus SA-51	*Suillus grevillei* SA-53
Agar[c]	3.3 (1.5)[b]	2.0 (1.5)
Gellan gum	138.2 (15.1)	2.4 (1.6)

a) 1×10^6 and 1.5×10^5 protoplasts were plated per Petri dish for *Suillus luteus* and *S. grevillei*, respectively.
b) Values in parentheses are standard deviations.
c) Extra pure grade agar was used (see text).

We next examined conditions for protoplast regeneration in gellan gum media, as follows (Table 3). Three media were tested for protoplast regeneration: Ohta medium, Ohta+abietic acid (A), and modified Melin Norkrans (MMN) medium (Aoshima et al., 1983). We tested the effect of abietic acid because Fries et al. (1987) reported it to be an activator of basidiospore germination in the genus *Suillus*. For both *S. luteus* SA-51 and *S. grevillei* SA-53, pH 5.0 yielded the best regeneration rate between 3.0 and 7.0. As the osmotic stabilizer, 0.5 M mannitol, 0.5 M sucrose, and 0.5 M inositol were used. Because 0.5 M $MgSO_4$ inhibited gelation of gellan gum, it could not be tested. The effect of the osmotic stabilizer concentration was determined using mannitol between 0.3 M and 0.9 M. The effect of the lytic enzymes composition was tested for the same four compositions as in the protoplast preparation test. Although enzyme system D was best for regeneration of both fungi, protoplast yield was low with this enzyme system. For *S. luteus*, enzyme system B was more useful, because the regeneration rate of this system was about 80% of that of D and protoplast yield was several times better than that of D. Under the conditions listed in Table 3, regeneration rate of protoplasts was about 0.4% for *S. luteus* SA-51 and 0.05% for *S. grevillei* SA-53.

For *B. cavipes* SA-286, we prepared protoplasts under the conditions shown in Table 1 and cultured them in Ohta medium with 0.5 M mannitol, pH 5.0, and with gellan gum. We confirmed protoplast regeneration.

In general, the regeneration rate in gellan gum culture was better than that in liquid culture for the three fungi tested.

In preliminary experiments, we tested the effect on the regeneration rate of the temperature of protoplast

Table 3. Optimum conditions and rate of protoplast regeneration of *Suillus luteus* SA-51 and *S. grevillei* SA-53 in gellan gum culture.

Factor	*Suillus luteus* SA-51	*Suillus grevillei* SA-53
Medium	Ohta	Ohta+A[a]
pH of medium	5.0	5.0
Osmotic[b] stabilizer	inositol	inositol
Concentration of osmotic stabilizer[c]	0.7 M	0.7 M
Composition of lytic enzymes[d]	D	D
Regeneration rate	0.4%	0.05%

a) Ohta medium + abietic acid (10 mg/l)
b) Concentration of osmotic stabilizer was 0.5 M.
c) Mannitol was used as osmotic stabilizer.
d) See text for composition of enzyme solution.

preparation of *S. luteus* and *S. grevillei* in the range of 22°C to 28°C using enzyme system B. For both fungi, 24°C was best. For *S. luteus*, the regeneration rate more than doubled, and for *S. grevillei* it increased more than 10 times, as the temperature of protoplast preparation was lowered from 28°C to 24°C. Protoplast yield decreased about 35% for *S. luteus* and 50% for *S. grevillei* over this temperature range. This finding indicates that preparation of protoplasts at 24°C is better for protoplast regeneration.

Acknowledgements——This study was supported in part by Research Grant No. 08456078 from the Ministry of Education, Science, Sports and Culture of Japan.

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