Short Communication

Regeneration and Fusion of Mycelial Protoplasts of *Tricholoma matsutake*

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The basidiomycete *Tricholoma matsutake* is a mushroom which has been eaten since old times in Japan. Ogawa and Hamada reported that the fruiting body primordium of *T. matsutake* could be developed by an artificial procedure. However, it is generally recognized that the artificial production of a perfect fruiting body is very difficult with *T. matsutake*. This report deals with the regeneration and the fusion of protoplasts from *T. matsutake*.

The organism used in this study was a dikaryotic strain of *T. matsutake* IFO 6916. Liquid medium (LM) for the culture contained (per liter) glucose (20 g), yeast extract (1.5 g) and Bacto-Soytone (Difco Laboratories; 1.5 g). The same medium supplemented with 0.6 M mannitol was used as regeneration medium (RM). Both media were adjusted to pH 5.0 before autoclaving. All the cultures in this study were performed at 23°C.

Twenty ml of LM in an Erlenmeyer flask (100 ml) was inoculated with 1.0 ml of a mycelial suspension of a culture grown in LM homogenized with a homogenizer (Nippon Seiki Co.) at 12,000 rpm for 15 sec, and incubated for 15~25 days without shaking. The cultured mycelium was harvested by filtration and washed several times with sterile water. About 2.0 g of the damp mycelium was incubated in 15 ml of 0.05 M maleic acid–NaOH buffer (pH 5.6) containing 0.6 M MgSO₄⋅7H₂O, 0.5% cellulase Onozuka R-10 (Kinki Yakult Mfg. Co.), 0.5% Zymolyase 5000 (Kirin Brewery Co.) and 1.5% β-glucuronidase (Sigma Co.), at room temperature (about 25°C) for 3 hr with occasional shaking.

After the incubation, the crude protoplast suspension was passed through a 3G2 glass filter (Shibata Chemical App. Mfg. Co.) without pressure to remove mycelial debris. Fifteen ml of 0.7 M NaCl solution was added to the filtrate containing protoplasts, and the filtrate was centrifuged at 3000 rpm for 4 min. This procedure was repeated once more to obtain the protoplast precipitate. The resulting precipitate was used as a protoplast preparation for the experiments on regeneration and fusion.

The mycelium cultured for 15~25 days was suitable for the release of protoplasts. Released protoplasts were observed after 30~60 min incubation with the enzyme solution, and the number of released protoplasts reached the maximum in 3~4 hr. The diameter of the protoplasts was in the range of 2~10 μm (Fig. 1A).

Regeneration with a solid medium was performed according to the method of Ferenczy et al. with a slight modification. The protoplasts suspended in 1.0 M mannitol solution were plated on RM containing 2.0% agar in a culture tube and covered with a thin layer of RM containing 0.5% agar with a Komagome pipette. The covering agar solution was previously warmed at 34°C in a water bath. The thickness of the covering layer was about 0.5 mm. RM was also used for regeneration with a liquid medium. One ml of RM in a culture tube was inoculated with the protoplasts suspended in 1.0 M mannitol solution.

The protoplasts began to regenerate after 3~4 days of incubation in the solid and liquid media. The percentage of regenerated protoplasts was about 10%. Two basic patterns of regeneration were observed (Fig. 1B, C). In the first, the spherical protoplasts directly produced one or more germ tubes (Fig. 1B). In the second, the protoplasts developed into bud-like structures of cells, and finally the cells produced germ tubes (Fig. 1C). The second type was observed only in the solid medium.
In order to fuse the protoplasts, 0.05M glycine-NaOH buffer (pH 8.0) containing 0.1M CaCl₂ and 0.55M NaCl was added to isolated protoplasts of *T. matsutake*, and the suspension was centrifuged at 3000 rpm for 4 min. The precipitated protoplasts were resuspended in small amounts of the same buffer and observed under a microscope (Olympus Co.).

When the protoplasts were treated with 0.05M glycine-NaOH buffer (pH 8.0) containing 0.1M CaCl₂ and 0.55M NaCl, protoplast fusion was more frequently observed and the frequency was 1~10%. The protoplast fusion usually occurred between two cells (Fig. 2). Two protoplasts which adhered to each other gradually became one cell with the lapse of time resulting in the formation of a larger one. The protoplast fusion of *T. matsutake* was enhanced by calcium ions at a high pH like the case of the fungus reported by Binding and Weber, but its treatment with polyethylene glycol solution resulted in rupturing of the protoplasts. The protoplasts of *T. matsutake* may be easily damaged by polyethylene glycol.

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**REFERENCES**

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**Fig. 2.** The Fusing Process of *T. matsutake* Protoplasts (scale line = 5 μm).

A. At the start of the fusion.
B. After 30 min had elapsed.
C. After 60 min had elapsed.