**76. Isolation and Regeneration of Protoplasts from
Cultures derived from the Spermatogonial State
of *Gymnosporangium asiaticum*^{*)}**

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Since isolation of protoplasts from an yeast was reported by Eddy and Williamson,⁶⁾ protoplasts have been obtained from a number of fungi.^{5),11)} Recently, fungal protoplasts have been used for physiological, biochemical and genetic studies.^{3),8)} Studies on axenic culture of rust fungi are now in progress,^{9),12)} but isolation and regeneration of protoplasts from cultures of rust fungi have not been reported yet. This is the first report on isolation and regeneration of protoplasts from axenic cultures of a rust fungus.

Materials and methods. Four cultures derived from the spermatogonial state of *Gymnosporangium asiaticum* Miyabe ex Yamada were obtained from rust-infected leaves of *Pyrus pyrifolia* var. *culta*.¹³⁾ The cultures were maintained on test tube slants holding 10 ml of "Medium IV" containing per liter, NH_4NO_3 , 412.5 mg; H_3BO_3 , 1.55 mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 5.575 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 110 mg; KI, 0.2075 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 92.5 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.00625 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.00625 mg; $\text{Na}_2\text{-EDTA}$, 93.25 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0625 mg; KH_2PO_4 , 42.5 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 69.5 mg; $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$, 2.15 mg; Mioinositol, 25 mg; Pyridoxine HCl, 0.125 mg; Glycine, 0.5 mg; Thiamine HCl, 0.025 mg; Nicotinic acid, 0.025 mg; Evans peptone, 1 g; Difco yeast extract, 1 g; Sucrose, 30 g; 2,4-D, 1 mg; Kinetin, 0.1 mg and Agar, 10 g.

About one gram (fresh weight) of mycelia grown at 20°C in the dark for 15 to 60 days on Medium IV were harvested with a sterilized forceps on a steel sieve (35 mesh), washed with sterilized distilled water to remove agar, and then suspended in 10 ml of a combined enzyme solution in a 100 ml Erlenmeyer flask. The combined enzyme solution consisted of macerozyme R-10 (5 mg/ml), cellulase "Onozuka" R-10 (20 mg/ml), driselase (10 mg/ml) and β -glucuronidase (0.06 mg/ml) in 0.6 M mannitol (pH 5.2). The pH of the solution was adjusted to pH 5.2 with 0.1 N KOH after ad-

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dition of the enzymes. The flask was placed in an incubator at 20–23°C for 1.5–4.0 hr. The enzyme solution with the mycelia was filtered through the steel sieve to remove mycelial fragments, and the filtrate was centrifuged at least twice at 2500 rpm for 5 min to remove the enzyme. Purification of protoplasts was carried out by the liquid-liquid two phase system consisting of 0.6 M sucrose and 0.6 M mannitol described by Hashiba and Yamada.⁷⁾ After purification, appropriately diluted suspensions of protoplasts (ca. 2000/plate) were incubated on a regeneration medium at 20°C in the dark. The regeneration medium consisted of the components of Medium IV in 0.6 M mannitol instead of distilled water and adjusted to pH 5.7 with 1 N KOH or 1 N HCl before autoclaving at 121°C for 10 min.

Pieces of rust mycelium derived from protoplasts and grown on the regeneration medium were observed by light and scanning electron microscopes, using the methods described by Naito and Onoe,¹⁰⁾ and Ando and Katsuya,¹¹⁾ respectively.

Results. Mycelia from cultures of different ages were examined for protoplast production. Yields of the protoplasts were high (3.0×10^5 /g, f. wt.) when 15- to 30-day-old colonies were used,

Table I. Culture conditions to release protoplasts from mycelia of *Gymnosporangium asiaticum*

Isolate	Culture age (days)	Incubation time (hr)	Incubation temperature (C)	Yield (Protoplasts/g***)	
Gyf*	11	15	2.0	23	3.0×10^5
Gyf	16	"	"	"	1.0×10^5
Gyf	11	25	"	20	4.5×10^4
Gyf	16	"	"	"	7.5×10^3
Gyf	16	30	1.5	"	5.0×10^4
Gyf	11	"	4.0	"	0
Gyf	16	"	"	"	0
Gyw**	13	"	2.5	"	1.0×10^5
Gyw	82	"	2.0	"	1.8×10^4
Gyf	16	60	1.5	"	0
Gyf	16	"	2.0	"	0

* Fluffy colony. ** Water-soaked colony. *** Fresh weight of mycelium.

although the yields showed variation (Table I). On the other hand, no protoplasts resulted from the 60-day-old colonies.

Release of numerous protoplasts was observed after incubation in the combined enzyme solution for 1.5–2.5 hr. However, prolonged incubation (4 hr) did not produce protoplasts.

The size of purified protoplasts was 5.8–12.5 μ m (average: 9.0 μ m) in diam (Fig. 1). The protoplasts were incubated on the

regeneration medium in 21 petri dishes at 20°C in the dark. After six months 19 of the petri dishes were contaminated. One of the non-contaminated dishes had four rust colonies 5.3–7.5 mm in diam that were derived from the protoplasts (Fig. 2). The other dish had no organismal growth. The rust colonies consisted of filamentous and swollen hyphal layers (Fig. 3). The filamentous hyphal cells were 3.5–5.5 μm in diam, but the swollen cells had considerable variation in diameter ranging from 7.5 to 25.8 μm . One nucleus was always observed in the filamentous and swollen cells (Figs. 4, 5). It was identical with the nucleus of the hyphal cells of the parent culture. In the swollen hyphal layer, short chains of swollen cells were observed and an unknown short isthmus-like structure was present between the cells (Fig. 6). A collar-like structure was occasionally observed on the basal cell of a chain of swollen cells (Fig. 7). A collar-like structure was occasionally observed on the basal cell of a chain of swollen cells (Fig. 7).

When regenerated colonies were transferred onto fresh Medium IV, hyphal strands occurred on the surface of the colonies about 1 month after transfer (Fig. 8). The hyphal strands consisted of filamentous and swollen hyphae in the upper and the basal parts, respectively. During successive transfers, the colonies (Fig. 9)

Figs. 1–10. 1: Protoplasts isolated from rust mycelia. 2: Colonies regenerated from protoplasts, 7 months after incubation. 3: Vertical section of the regenerated colony consisted of filamentous (FL) and swollen (SL) hyphal layers. 4: Filamentous hyphae with single nucleus. 5: Swollen cells with single nucleus. 6: Short chains of swollen cells. 7: A collar-like structure (arrow) observed in the basal cell of the chained swollen cells. 8: Hyphal strand. 9: Regenerated colony. 10: Parental fluffy colony.

changed into the same fluffy type as the parental culture (Fig. 10).

Discussion. Age of mycelia and incubation time in a combined enzyme solution strongly affected protoplast isolation from cultures of *Gymnosporangium asiaticum*. These effects have been reported previously on *Rhizoctonia solani*⁷⁾ and on *Schizophyllum commune*.⁴⁾ The results showed that 15- to 30-day-old colonies and about 2 hr incubation in the combined enzyme solution are suitable to release protoplasts from the rust mycelium.

Hashiba and Yamada⁷⁾ used a combined enzyme solution for releasing protoplasts from mycelia of *R. solani* with high yield (6.6×10^7 protoplasts/g). Using the same enzyme solution, we obtained a tolerable yield (3.0×10^5 /g) of protoplasts from mycelia of *G. asiaticum*. For obtaining a more suitable enzyme system to release protoplasts of rust fungi, it will be necessary to examine various kinds of enzyme and combinations of enzymes.

Usually, colonies of rust fungi grow very slowly on artificial media²⁾ as compared with other fungi. Equally, it took a long time to regenerate protoplasts of *G. asiaticum*. To improve such studies, we need to find better media for regeneration of protoplasts from rust cultures.

G. asiaticum has two mating types, + and - in the spermogonial state. Protoplasts obtained from the different mating types may be useful for studies of cell fusion and sexual reproduction.

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