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Isolation of Protoplasts from Fern Prothallia and Their Regeneration to Gametophytes

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Protoplasts were isolated enzymatically from prothallia of *Lygodium japonicum*. The protoplasts grown in a culture medium containing 0.6 M mannitol and 0.05 M sucrose began to divide within 8 days of culture, and after 30 days 10-cell clusters were present. When the cell-clusters were transferred into fresh media followed by sequential reduction of mannitol concentration, they developed rhizoids and protonemata. The reduction of mannitol concentration to 0.3 M resulted in the regeneration of a common gametophyte within 50 days of culture, and subsequently the regenerated gametophytes produced sporophytic leaves and roots.

Key words: Fern gametophyte — *Lygodium* — Prothallium — Protoplast —Regeneration.

Several methods are now available for the isolation of protoplasts from various plant tissues and for the culture of these protoplasts leading to formation of whole plants (Cocking, 1972; Evans and Cocking, 1977). However, only in a few cases have protoplasts been isolated from gametophytes of lower plants such as moss, liverwort and fern (Schieder and Wenzel, 1972; Gay, 1976; Maeda, 1979; Partanen *et al.*, 1980). The gametophytes of these plants which are haploids in nature are of great significance not only in the study of haploid plant development but also in the examination of the differences between the developmental regulation mechanisms of haploid and diploid plants.

This report describes the isolation of protoplast from prothallia of a fern, *Lygodium japonicum*. The isolated protoplasts underwent cell division and formed cell aggregates leading to regeneration of gametophytes. This success shows the possibility of the use of these protoplasts in investigating some of the factors influencing the development of prothallia and ontogeny of fern gametophytes.

Materials and Methods

Materials for protoplast isolation

Gametophytes of a fern, *Lygodium japonicum* were used. For culture of prothallia, spores were sterilized by immersing them in 0.5% Hyamine (a commercial 10% benzethonium chloride, Sankyo Co., Tokyo) for 20 min, and then in 20% Oyalax (a

commercial 5.57% solution of sodium hypochlorite, Oyalax Co., Tokyo) for 5 min, and then rinsed with sterile distilled water. The sterile spores were sowed on a sterilized cellulose membrane (Visking Cellulose Tubing, Visking Co., Ltd.) placed upon 20 m*l* culture medium in 50 m*l* Erlenmeyer flasks. The culture medium contained 0.25 g/*l* KH_2PO_4, 0.12 g/*l* KCl, 1.00 g/*l* Ca $(NO_3)_2 \cdot 4H_2O$, 0.25 g/*l* $MgSO_4 \cdot 7H_2O$, and 0.001 g/*l* $FeCl_3$, and were supplemented with Murashige-Skoog's minor elements (1962), with pH adjusted to 5.8. For spore culture, the medium was solidified with 1% agar. Cultures were kept at 25 C under continuous white light of about 600 lux at the plant level supplied by daylight-fluorescent lamps. Prothallia from 6-week-old *Lygodium* (after germination) were mainly used as material for the protoplast isolation.

Isolation of protoplasts

Prothallia were cut into several pieces with a razor blade, and were incubated in an enzyme solution which contained 1.5% (w/v) Cellulase Onozuka R–10, 0.5% (w/v) Macerozyme R–10 and 0.6 M mannitol in the culture medium, at pH 5.8. Incubation was carried out at 30 C with reciprocal shaking (100 excursions/min), and usually completed within 3 hr. Isolated protoplasts were separated from debris by filtration through a stainless steel mesh 74 μm in pore size and collected by centrifugation at 150 $\times g$ for 3 min. The protoplasts were washed three times with a 0.6 M mannitol solution and resuspended in the liquid culture medium. Experiments were also designed to find suitable conditions for protoplast isolation by using the following enzymes of fungal origin; Driselase (Kyowa Hakko Kogyo Co., Ltd. Tokyo), Sigma's cellulase and pectinase (Sigma Chemical Co., Ltd. St. Louis, MO., USA), Meicelase (Meiji Seika Co., Ltd. Tokyo), Cellulase Onozuka R–10 and Macerozyme R–10 (Kinki Yakult Mfg. Co., Ltd. Nishinomiya).

Culture of protoplasts

The culture medium supplemented with 0.2 to 0.6 M mannitol and 0.05 M sucrose was solidified with agar at a concentration of 1% in 6-cm diameter Petri dishes. A round agar disc, 1 to 1.5 cm in diameter, was removed from the central portion of the solid medium, leaving a small culture well. After about 0.5 m*l* of the liquid culture medium containing 100 to 200 protoplasts was pipetted into the well, the dishes were sealed with Parafilm (American Can Co., Ltd. Neenah, WI., USA). The mannitol concentrations of the liquid media were adjusted to those of surrounding solid media. These cultures were usually kept at 25 C under continuous white light of about 400 lux at the plant level, and periodically observed under an inverted microscope.

Results

The first attempt was made to find suitable combinations for the isolation of protoplasts. For this purpose the effects of several enzymes, which were shown in "Methods", were compared. Of all these treatments the mixture of Cellulase Onozuka R–10 (1.5%) and Macerozyme R–10 (0.5%) proved to be the best in terms of total yield.

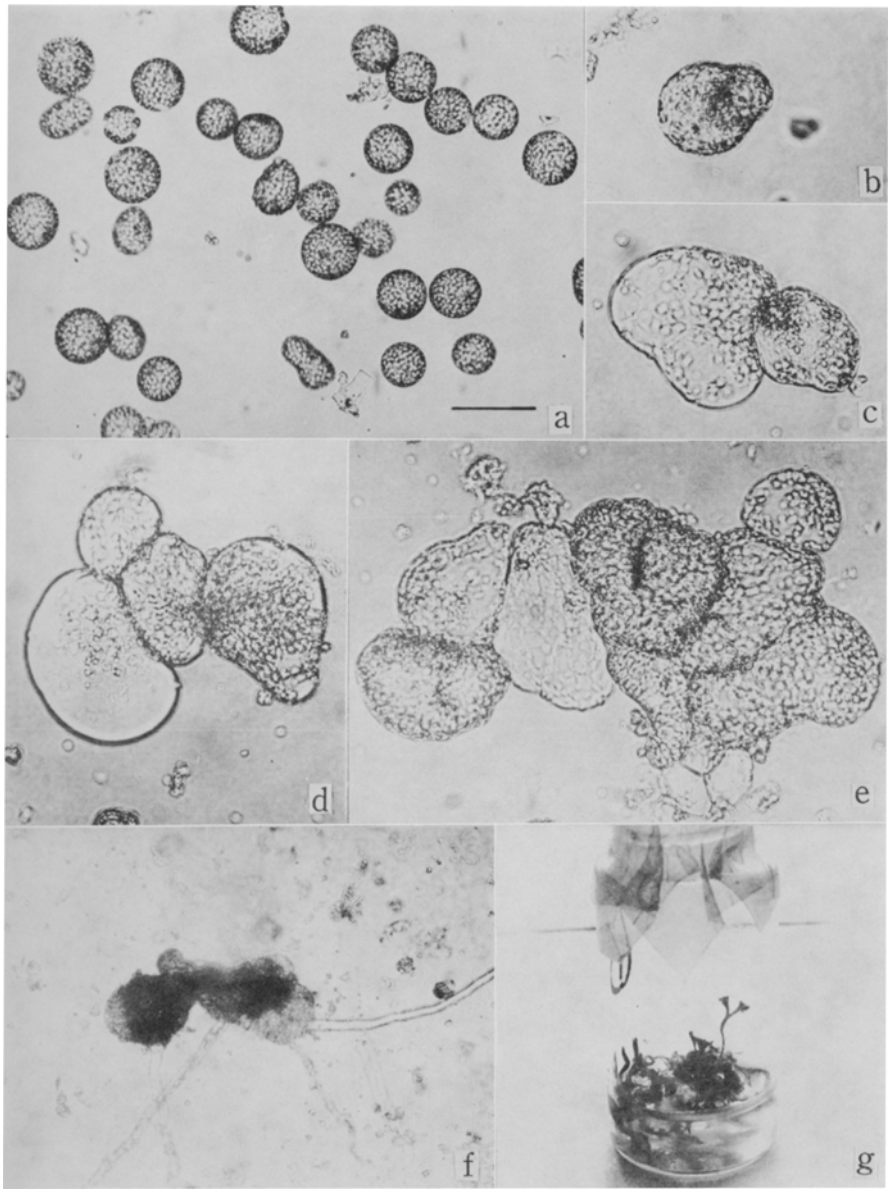

Fig. 1. Protoplasts from prothallia of *Lygodium japonicum* and regeneration from these protoplasts. a: freshly isolated protoplasts. b: cell wall regenerated, 3 days after inoculation in a culture medium supplemented with 0.6 M mannitol and 0.05 M sucrose. c: the first cell division, 8 days after. d: a cell aggregate originated from a single protoplast, 17 days after. e: 30 days after. f: developing rhizoids from an aggregate, 10 days after transfer in a culture medium supplemented with 0.3 M mannitol and 0.05 M sucrose. g: regenerated prothallia and sporophyte from protoplasts, 50 days after the transfer. The size bar in a is 50 μm.

When 6-week-old prothallia were treated with the enzymes, removal of the cell walls was observed within 30 min, and in some cases protoplasts were produced within 1 hr. Most of these protoplasts oozed out from the partially digested cell walls, but a few were released by the complete digestion of the cell wall. An enzyme treatment for 3 hr was necessary for the complete release of protoplasts. The proportion of viable protoplasts was usually about 60%. When 30 prothallia were incubated in an Erlenmeyer flask containing 10 m*l* of enzyme solution, viable protoplasts were obtained at a concentration of 300 to 600 protoplasts/m*l*. The age of prothallia was a critical factor in the protoplast production; 6-week-old prothallia were suitable for isolation of protoplasts. Younger prothallia required a prolonged enzyme treatment and the protoplast yield was low. Older prothallia were converted easily into protoplasts, but the released protoplasts tended to burst in the enzyme solution, because of suspension of many chloroplasts derived from cells or protoplasts in the enzyme solution.

Freshly isolated protoplasts were spherical, with an average diameter of 35 μm in the hypertonic enzyme solution, and contained numerous chloroplasts adhering to the plasmalemma (Fig. 1a).

When protoplasts were incubated in the medium containing 0.6 M mannitol and 0.05 M sucrose, their viability was maintained for 1 month. After a 3-day culture most of the protoplasts were no longer spherical, indicating that the cell walls had regenerated (Fig. 1b). Cell division was observed after 8 days of culture, and more than 80% of the cells divided within 10 days after isolation (Fig. 1c). The density of protoplasts in culture was not critical for the induction of cell proliferation, unlike the case of protoplasts from higher plants; cell division was also observed in dishes containing only a few cells. Under hypertonic conditions, sustained cell division produced unorganzied aggregates of dividing cells (Fig. 1d); this is different from the growth of germinated spores from ferns. In the aggregates, two types of cells were observed; some cells being large and vacuolated, and the others being relatively compact with many chloroplasts. Within 1 month of culture, about 10-cell aggregates were formed (Fig. 1e). Such cell aggregates were produced from about 70% of protoplasts, but the growth was quite slow and the formation of rhizoids was not observed. When the cultures were maintained for 2 months, further development of the aggregates was not observed.

Since it was supposed that the hypertonic conditions employed in the culture of protoplasts might prevent the filamentous growth and the development of prothallium, these 1-month-old cultures were transferred into the same culture medium with a lower mannitol concentration, with concentration reductions of 0.05 M at intervals of 3 days. In the medium containing 0.45 M mannitol, the aggregates began to proliferate actively and some of the cells developed rhizoids (Fig. 1f). The growth pattern was the extension of monolayer cells with a meristematic region as it was in the case of the regeneration from pieces of prothallia isolated by mechanical methods (Albaum, 1938). The culture was retained until formation of cordate type gametotphytes occurred. In a medium with a 0.3 M mannitol concentration, some cells began to grow into

filaments, this being followed by the formation of mono-layer cells. In both, mature prothallia were finally formed 50 days after transfer into the media (Fig. 1g). These prothallia normally formed antheridia and archegonia, and subsequently produced sporophytic leaves and roots.

No formation of callus was observed, as is usually observed in cultures of higher plant protoplasts.

Discussion

As natural fern gametophytes are developed from spores, isolated single cells from gametophyte might approximate natural spores. Therefore, the isolation of single cells might be a novel approach for the study of ontogeny and physiology of gametophyte development. Mechanical isolation of single cells was obtained from prothallia of *Pteris vittata* (Ito, 1962), and all of the isolated single cells, except for those from rhizoids, were shown to be capable of regeneration leading to the production of mature gametophytes. The mechanical method is adequate for learning about regenerative ability of particular cells in a prothallium, but it is not sufficient for physiological studies because of the limited quantities involved. In this paper, a method for the isolation and successful culture of protoplasts from fern prothallia is demonstrated. These protoplasts consisting of single cells might provide suitable materials for studies of the mechanism of development in haploid cells. For comparison, the isolation of diploid protoplasts from fern sporophyte is now under way.

Fern protoplasts cultured in the medium containing 0.6 M mannitol formed only cell aggregates and showed no further development. Coincidently in a medium supplemented with much higher sugar concentrations, the spores were shown to be inhibited with regard to growth into filaments, and production of cell colonies resulted (Kato, 1964). When the cell aggregates from the protoplasts were transferred into culture media supplemented with a lower sugar concentration, the cells began to proliferate vigorously. The reason why the hypertonic conditions interfere with the developmental process of fern gametophyte is unclear. Therefore, examination of the range of permissible sugar concentrations in culture media is required.

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