Conditions for efficient protoplast release in moss (*Physcomitrella patens*)

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Abstract

Effect of culture conditions on protoplast formation was examined in the moss, *Physcomitrella patens*. The yield of protoplast was increased by static culture using shallow liquid medium, as compared to the culture using solid medium. Moss cultured for 2 weeks efficiently produced protoplasts. Phenolic compounds which are possibly released during this culture period seem to have no inhibitory influence on the yield, but the yield was slightly increased by low concentration of salicylic acid. A short (20 to 30 min) treatment with driselase is suitable to obtain a high quality of protoplasts.

Introduction

Mosses have been recognized as a promising model plant for genetic and physiological analysis. Their morphology is simple and *in vitro* maintenance and handling of these tiny plants is easy. Similar responses in moss and higher plants can be observed to environmental and growth factors (1, 2, 10, 14). The developmental processes is possible to be followed at the cellular level (4). The predominance of the haplophase in the life cycle allows efficient genetic analysis (5, 13). Recent studies indicate that the moss (*Physcomitrella patens* (Hedw.) Bruch & Schimp.) is a highly promising model system for analysis of gene function because of the remarkably high efficiency of homologous recombination and, consequently, possibility to apply gene knockout techniques (6, 8, 9, 16, 18).

Techniques for gene transfer to *P. patens* have been established (15, 17). Since *Agrobacteruim* system is applicable for broad range of organisms like yeast (3) and a liverwort (12), it may also be applicable for moss. However, this method may be unsuitable for gene knockout experiments because of the chance of random insertion of foreign gene in the genome. The most efficient methods for gene knockout in moss are micro-projectile bombardment (15) and polyethylene glycol (PEG) mediated transformation (17). Especially, the PEG method is thought to be promising since this method is easy and special equipment is not required.

One of an important factor for successful PEG transformation is the yield of high quality of protoplasts. Efficient protoplast induction, isolation and regeneration has already been reported (7), however, effect of culture conditions on the yield of protoplast is not fully understood. In this study, we aimed to find out the optimal conditions for obtaining moss protoplasts. Our results are valuable for efficient induction of the protoplast but also for maintenance of

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cultures with a high regeneration potential.

Materials and methods

Culture conditions

Protonema tissue of *P. patens* was cultured on modified Murashige and Skoog medium (11) composed of 1/2 strength of MS salts and vitamins (ICN, USA), fumaric acid (2 g/l), L-malic acid (40mg/l), rhamnose (5 mg/l) and glucose (20g/l). Agar (8 g/l; Sigma, USA) was used to solidify the medium. Polyvinyl poly pyrrolidone (PVPP) was added at 10g/l before autoclaving. Gametophyte tissue was induced on 1/10 strength of MS solid medium without sugar. The moss cultures were grown at 22℃ with a 16h light regime.

Protoplast formation

Plant material was treated with 1 % (w/v) of filter sterilized driselase (Sigma, USA) dissolved in 8 % (w/v) of mannitol (pH=5.6) and incubated under 28℃. The protoplasts were centrifuged (100 to 200 x g, 3 min) and washed twice with sterilized 8 % (w/v) of mannitol. After washing, the protoplasts were suspended using 1 - 3 ml of 8 % (w/v) of mannitol and the number was counted from at least 6 replicate samples using a hemacytometer. For regeneration, suspended protoplasts were poured and cultured on a plate of modified MS solid medium containing 8 % (w/v) of mannitol as osmoticum. Protoplasts were cultured at 22℃ with a 16h light regime.

Results and Discussion

The yield of protoplast of *P. patens* was significantly influenced by culture conditions. Approx. 0.46×10^6 protoplasts per gram of tissue were released from *P. patens* cultured for 8 days on solidified medium. The yield was increased to 1.0×10^6/g by static culture using liquid medium (7 ml/60mm petri dish). However, cell walls of *P. patens* grown in liquid medium with shaking were hardly digested and the yield was significantly decreased to approx. 1.2×10^2/g. This moss may sensitively response to shaking, which may alter the composition of the cell wall and/or growth properties.

Fig. 1 shows the influence of culture period on the yield. In this experiment, moss was cultured by static condition using shallow liquid medium. The yield was drastically decreased after 3 weeks of culture. In other exeperiments we observed that the release of protoplasts from gametophore tissue is poor (data was not shown). Prolonged growth may generate the gametophore tissue but no gametophores were formed during this experiment. The yield from 2 -wk old cultures was higher than from 1 -wk old cultures. Therefore, moss cultured for 2 weeks may be most suitable for protoplast induction.

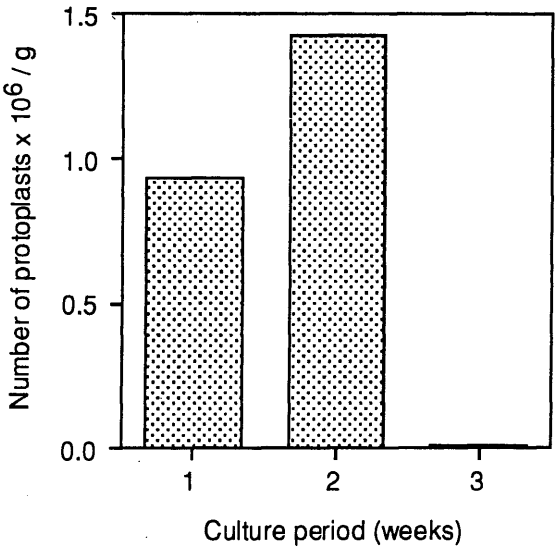

Fig. 1 Influence of culture period on yield of protoplast of *P. patens*. Driselase (1 %) treatment for 30 min was used.

In long-term cultures, the medium was stained brown by products that might be a mixture of phenolic compounds released from the moss. Since such compounds may influence the yield, effect of PVPP as an efficient absorber of phenolics was tested. Growth of *P. patens* was not inhibited on PVPP containing solid medium. However, the protoplast yield was clearly decreased by PVPP application. This result suggests that phenolics may not influence on the cell wall digestion of this moss, whereas PVPP itself might have an inhibitory effect. Effect of phenolics was also examined using salicylic acid (SA) as a model compound which is water soluble and stress related. Fig. 2 shows the effect of SA on growth and protoplast yield of *P. patens* in static liquid culture. Interestingly, the growth was stimulated by a low concentration (10 μM) of SA. Yield of protoplasts was also increased by a low concentration of SA. These result may suggest that some phenolic compound may stimulate cell growth and division, and the number of freshly developed cells may be increased. However, the effect of SA on morphological and developmental changes in moss remain to be studied in more detail. Similarly, further research is required on the responses of *P. patens* to phenolic compounds.

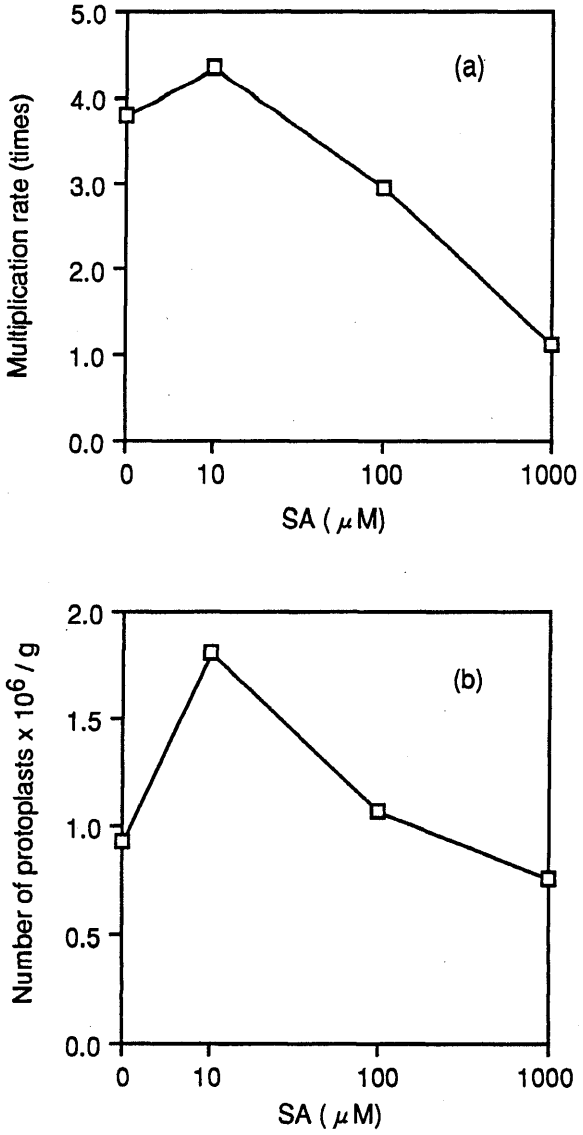

Fig. 2 Effect of SA on growth of *P. patens* in static liquid culture (a) and on the protoplast yield (b). Driselase (1 %) treatment for 30 min was used.

Fig. 3 shows changes in protoplast density during the enzyme treatment on protonema cultured grown for 8 days. Protoplasts were induced by a short treatment (less than 20 min) and the number of protoplasts was gradually increased during 40 min of incubation. Debris (particles other than protoplasts) were also increased by time, and even more so after 50 min of treatment. The particles looked like broken cells, organelles and fragments of membranes, which may also mean decrease in the quality of the protoplasts. Efficiency of protoplast regeneration was clearly decreased when cells were digested for 40 min or longer. Based on these results, it is suggested that enzyme treatment

should be terminated at 30 min.

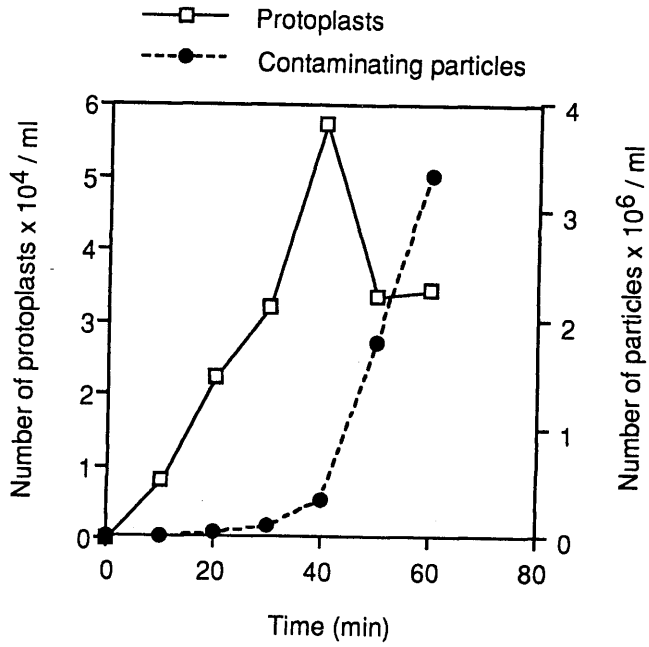

Fig. 3 Density of protoplasts and appearance of contaminating particles during enzyme treatment. Protonema tissue (2.8g, 8 days cultured) was digested in 10ml of enzyme solution. The number of protoplasts and contaminating particles were counted from 1 ml samples of culture.

Results described above suggest several conditions for efficient induction of protoplasts in *P. patens*. *In vitro* cultured *P. patens* should be used within 2 weeks after transfer to fresh liquid medium. The moss is sensitive to physical stress and the yield is significantly decreased by shaking. The optimum length of time of the enzyme (driselase) treatment is 20 to 30 min, as previously reported (7). Phenolic compounds released from the moss during culture might not be a significant factor affecting protoplast yield under these conditions. Further investigations are needed to establish conditions for efficient regeneration of protoplasts, especially after gene transformation using PEG treatment.

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和文抄録

ヒメツリガネゴケ（*Physcomitrella patens*）のプロトプラスト誘導条件に関する検討

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ヒメツリガネゴケ（*Physcomitrella patens*）を用い、プロトプラストの誘導に適当な培養条件について検討した。シャーレ内に浅く満たした液体培地中でコケを静置培養した場合、固形培地上で培養したときよりも、プロトプラストの収率が高まった。培養を開始して2週間以内の材料をプロトプラスト誘導に用いるべきであることがわかった。培地中のフェノール性の化合物は、プロトプラスト収率に影響しないか、あるいは、低濃度で生育とプロトプラストの収率とを高める可能性があることがわかった。プロトプラストの誘導には、20分間から30分間の酵素処理が適当であった。