

***Physcomitrella* cell culture conditions**

1 Overview

Physcomitrella plants can be cultivated either on solidified medium or in liquid culture (Fig. 1). Upon cultivation on solidified medium the plants undergo normal developmental progression resulting in the formation of leafy gametophores. Starting from protonema tissue gametophore development is initiated by the formation of buds consisting of a three-faced cell. Plant tissue cultures of gametophores can be maintained by sub-culturing the gametophores monthly. Liquid cultures can be started from gametophores by inoculating liquid medium with gametophore tissue. Mechanical disruption of the gametophores using an Ultra-Turrax leads to predominant growth of protonema tissue in the liquid medium. Liquid cultures can be maintained in Erlenmeyer flasks in small volumes or plants are grown in semi-continuous bioreactor cultures for large-scale production of moss material. At any stage moss material from liquid cultures can be used to set up cultures on solidified medium. Upon regular sub-culturing the protonema tissue will undergo normal developmental progression resulting in the formation of leafy gametophores. For routine use of *Physcomitrella* plants it is recommended to keep plant cultures on solidified medium as a backup system or to keep spores which can be used to initiate new culture lines. Long-term storage of *Physcomitrella* strains was shown to be reliably working via cryopreservation (Schulte and Reski, 2004).

2 Methods

2.1 Small-scale cultivation of Physcomitrella plants

2.1.1 Reagents

The two media described below are the most commonly used media for standard growth of *Physcomitrella* plants.

Knop medium (Reski and Abel, 1985): Prepare the following stock solutions: 25 g/l KH_2PO_4 , 25 g/l KCl, 25 g/l $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 100 g/l $\text{Ca}(\text{NO}_3)_2$ and sterilize by autoclaving. To make up 1 l Knop medium take 10 ml of each stock solution add 12.5 mg $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$, adjust pH 5.8 with KOH or HCl. For the preparation of solid medium add 1.2% (w/v) agar (Oxoid Ltd., England). Sterilize the medium by autoclaving. Petri dishes containing solidified medium can be stored in a sealed bag up to 4 weeks at room temperature.

BCD medium (Ashton and Cove, 1977): 1 mM MgSO_4 , 10 mM KNO_3 , 45 μM FeSO_4 , 1.8 mM KH_2PO_4 (pH 6.5 adjusted with KOH), 0.22 μM CuSO_4 , 0.19 μM ZnSO_4 , 10 μM H_3BO_3 , 0.1 μM Na_2MoO_4 , 2 μM MnCl_2 , 0.23 μM CoCl_2 , and 0.17 μM KI. For the preparation of solid medium add 0.8% (w/v) agar (Sigma, USA). Sterilize the medium by autoclaving.

For cultivation of metabolic or auxotrophic mutants addition of supplements to the medium might be necessary. An in this respect enriched medium (Egener et al., 2002) contains MS-microelements (Murashige and Skoog, 1962), 4 mg/l myo-inositol, 2.8 mg/l choline chloride, 1 mg/l nicotinic acid, 0.5 mg/l thiamine-HCl, 0.25 mg/l pyridoxine, 0.01 mg/l biotin, 0.25 mg/l p-aminobenzoic acid, 1.9 mg/l Ca-D-pantothenate, 0.015 mg/l riboflavine, 6.76 mg/l adenine, 3.84 mg/l Na-palmitinic acid, 250 mg/l peptone, 920 mg/l ammonium tartrate and 50 g/l glucose.

2.1.2 Method

Physcomitrella plants are grown axenically on solid medium. The plants are cultured in petri dishes in a growth chamber at $25 \pm 1^\circ\text{C}$ under a 16/8 h light/dark photoperiod with a light intensity of $55 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Philips TLD 25). Maintenance of the plants is achieved by monthly sub-culturing the plant tissue onto fresh medium. For small-scale propagation of moss material *Physcomitrella* plants can be cultivated in liquid medium in Erlenmeyer flasks (Fig. 1A). When initiating a primary liquid culture a 100 ml flask containing 30 ml of Knop medium is inoculated with two to three *Physcomitrella* gametophores (approximately 50-100 mg fresh weight). The plants are disrupted for 60 sec at 19.000 rpm using an Ultra-Turrax device. To promote fast growth of the moss material 1% (w/v) sucrose can be added. Cultivation of plants in the presence of sucrose for long-time periods should be avoided. Starting from this culture 500 ml Erlenmeyer flasks containing 200 ml Knop medium without sucrose can be inoculated with 100 mg/l dry weight. Disintegration of the protonema filaments has to be carried out weekly using an Ultra-Turrax. These cultures can be kept as long term suspension cultures by regular subculture every two weeks with an inoculation density of 100 mg/l dry weight. The determination of dry weight is carried out by drying three 10 ml samples of the cultures for 2 h at 105°C .

2.2 Large-scale cultivation of *Physcomitrella* plants in bioreactors

Physcomitrella bioreactor cultures are useful for scale-up and to establish highly standardized growth conditions, because environmental parameters may affect growth kinetics, gene-expression patterns and differentiation. The cultivation of *Physcomitrella* in bioreactors has been reported previously (Reutter and Reski, 1996; Cove et al., 1997). We developed a semi-continuous, long-term bioreactor for the cultivation of *Physcomitrella* plants (Hohe et al., 2002).

2.2.1 Method

For the growth of *Physcomitrella* in bioreactors we use standard Knop medium. The cultures are carried out in stirred tank glass bioreactors (Applikon, Schiedam, The Netherlands; Fig.

1B) with working volumes of 5 l or 10 l, respectively, equipped with a marine impeller running at 500 rpm (5 l vessel) or 400 rpm (10 l vessel). The cultures are aerated with 0.3 vvm [(aeration volume)/(medium volume)/min] air and grown at 25°C under a photoperiod regime of 16/8 h (light/dark) with light (Philips TLD 25) supplied at an intensity at the surface of the vessels of either 120 $\mu\text{mol}/\text{m}^2$ per second (5 l vessel) or 190 $\mu\text{mol}/\text{m}^2$ per second (10 l vessel). The cultures can be run semi-continuously e.g. the suspension has to be harvested and replaced by an equal amount of fresh medium daily. The density of the cultures is controlled by determining the dry weight and the daily dilution has to be calculated in order to maintain an average dry weight of 150 mg/l. The determination of the dry weight is performed by drying the cell material of 50 ml samples for 2 h at 105°C. Under these conditions we have successfully run a bioreactor culture for seven weeks. For long-term cultivation of *Physcomitrella* suspension cultures the cell density should be maintained between approximately 150 mg/l and 200 mg/l dry weight. The growth rate of the suspension cultures can be increased by aeration with air that is enriched with 2% CO₂. We have observed that the growth rate can be doubled, but under non-controlled pH conditions the pH value of the culture may decrease markedly.

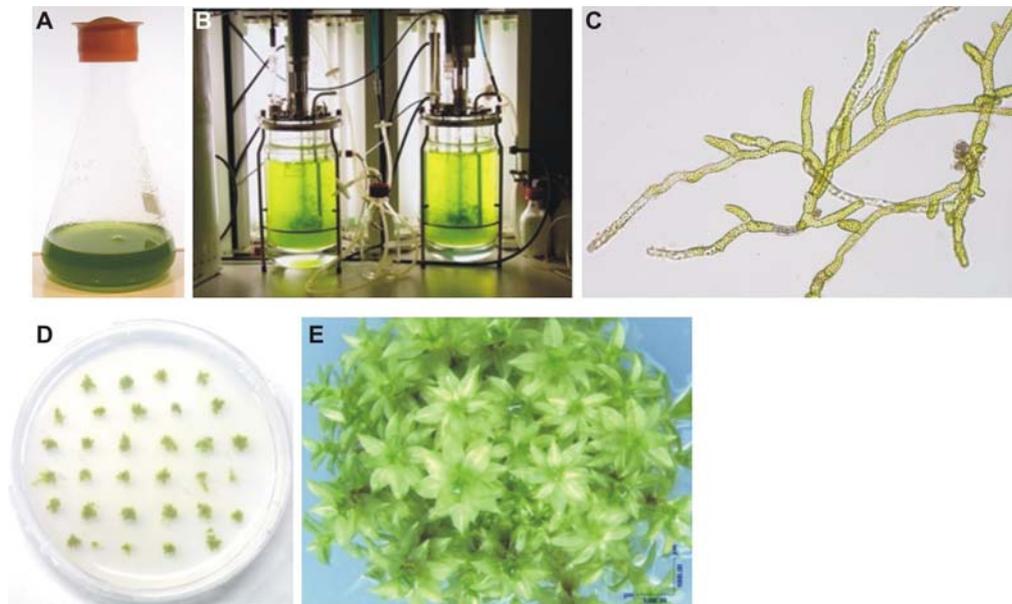


Figure 1:

Cell culture of *Physcomitrella* plants on solidified and in liquid medium. (A) Small-scale liquid culture in a 500 ml Erlenmeyer flask. (B) Bioreactor cultures for highly standardized growth conditions and up-scaling purposes. (C) Protonema filaments representing the predominant tissue type in liquid cultures. (D) *Physcomitrella* plants grown on solidified medium in a petri dish. (E) Close up of a single plant grown on solid medium showing the leafy structures of the gametophores.

References

- Ashton, N. W., and Cove, D. J. (1977) The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants in the moss *Physcomitrella patens*. *Molecular and General Genetics* 154, 87-95.
- Cove, D. J., Knight, C. D., and Lamparter, T. L. (1997) Mosses as model systems. *Trends in Plant Science* 2, 99-105.
- Egener, T., Granado, J., Guitton, M. C., Hohe, A., Holtorf, H., Lucht, J. M., Rensing, S. A., Schlink, K., Schulte, J., Schween, G., Zimmermann, S., Duwenig, E., Rak, B., and Reski, R. (2002) High frequency of phenotypic deviations in *Physcomitrella patens* plants transformed with a gene-disruption library. *BMC Plant Biol* 2, 6.
- Hohe, A., Decker, E. L., Gorr, G., Schween, G., and Reski, R. (2002) Tight control of growth and cell differentiation in photoautotrophically growing moss *Physcomitrella patens* bioreactor cultures. *Plant Cell Reports* 20, 1135-1140.
- Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* 15, 473-497.
- Reski, R., and Abel, W. O. (1985) Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine. *Planta* 165, 354-358.
- Reutter, K., and Reski, R. (1996) Production of a heterologous protein in bioreactor cultures of fully differentiated moss plants. *Plant Tissue Culture and Biotechnology* 2, 142-147.
- Schulte, J., and Reski, R. (2004) High throughput cryopreservation of 140,000 *Physcomitrella patens* mutants. *Plant Biology* 6, 119-127.