Short Communication

Effects of nutrients, cell density and culture techniques on protoplast regeneration and early protonema development in a moss, *Physcomitrella patens*

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Summary

To regenerate auxotrophic mutants of *Physcomitrella patens*, two media of increasing complexity were developed. The survival rate of protoplasts was around 30 % higher on full medium when compared to standard minimal medium. Protoplast survival was higher in a medium containing 2.5 mmol/L ammonium tartrate compared to a medium with 5 mmol/L of this compound.

Solid medium had a positive effect on protoplast survival compared to either liquid medium or solid medium overlaid with cellophane; the maximum survival rate being 31.6 %. However, the number of surviving protoplasts without any cell division during the first ten days increased on solid medium.

Density and survival rate of protoplasts were positively correlated, but the formation of long protonema filaments decreased markedly. The effect of different protoplast densities could be explained partly by physiologically active compounds excreted into the medium.

Key words: auxotrophic mutants – full medium – functional genomics – moss – protoplast density – protoplast regeneration

Introduction

To date, the moss *Physcomitrella patens* (Hedw.) B.S.G. is the only land plant showing a high rate of homologous recombination in its nuclear DNA (Schaefer and Zryd 1997, Reski 1998 a), thereby facilitating gene/function-analyses by targeted gene knockout and subsequent analysis of the knockout-mutants (e.g. Strepp et al. 1998, Girke et al. 1998). This approach is especially straightforward as the dominating phase in mosses is the haploid gametophyte (review Reski 1998 b), making loss-of-function mutations readily screenable. As we are interested in a functional genomics approach based on a random mutant collection of targeted knockout

plants (Egener et al. 2002), we developed a complex medium to complement knockouts in essential pathways.

Additionally, different culture techniques for protoplast regeneration had to be analysed according to their effect on protoplast survival, regeneration velocity, and their ability for a high-throughput working platform.

Material and Methods

Physcomitrella patens was grown in a bioreactor according to Hohe et al. (2002 a) in modified Knop medium (1.84 mmol/L KH_2PO_4, 3.36 mmol/L $MgSO_4 \times 7H_2O$, 1.02 mmol/L KCl, 4.24 mmol/L $Ca(NO_3)_2 \times 4H_2O$, 0.045 mmol/L $FeSO_4 \times 7H_2O$, pH 5.8) supplemented with either 5.0 or 2.5 mmol/L ammonium tartrate. After protoplast isolation, mock-transformation was performed according to Hohe et al. (2002 b), but without adding DNA. Protoplasts were regenerated in three different media:

Medium A: modified Knop medium (without ammonium tartrate), supplemented with 50 g L^{-1} glucose, adjusted to 540 mOs by adding mannitol, pH 5.8.

Medium B: medium A supplemented with 50 µmol/L H_3BO_3, 50 µmol/L $MnSO_4 \times H_2O$, 15 µmol/L $ZnSO_4 \times 7H_2O$, 2.5 µmol/L KJ, 0.5 µmol/L $Na_2MoO_4 \times 2H_2O$, 0.05 µmol/L $CuSO_4 \times 5H_2O$, 0.05 µmol/L $CoCl_2 \times 6H_2O$, 50 µmol/L adenine, 22 µmol/L myo-inositol, 20 µmol/L choline chloride, 13.8 µmol/L Na-palmitic acid, 8 µmol/L nicotinic acid, 4 µmol/L Ca-D-pantothenate, 1.8 µmol/L p-aminobenzoic acid, 1.5 µmol/L thiamine-HCl, 1.2 µmol/L pyridoxine-HCl, 0.04 µmol/L biotin, 0.04 µmol/L riboflavine, 0.25 g L^{-1} peptone and either 2.5 mmol/L or 5 mmol/L ammonium tartrate, 540 mOs, pH 5.8.

Medium C: all components included in medium B and additionally 1 mmol/L glycerol, 1 mmol/L ascorbic acid, 100 µmol/L D-ribose, 100 µmol/L glutathione, 10 µmol/L hemin, 2 µmol/L lipoic acid, 2 µmol/L folic acid, 0.25 g L^{-1} amicase instead of peptone and either 2.5 mmol/L or 5 mmol/L ammonium tartrate, 540 mOs, pH 5.8.

Protoplasts were cultured at 25 °C in the dark for 24 hours and subsequently exposed to 70 µmol m^{-2} s^{-1} (fluorescent tubes Philips TLD 25), 16 h per day.

After 10 days at least 500 protoplasts were counted per petri-dish using an inverse microscope (magnification 400).

Results and Discussion

Development of complex media

Physcomitrella protoplasts are normally either regenerated on Knop medium supplemented with a carbon source (Batra and Abel 1981) or supplemented additionally with 5 mmol/L ammonium tartrate and Hoagland's A–Z solution (Ashton and Cove 1977, Jenkins and Cove 1983, Nishiyama et al. 2000). Ashton and Cove (1977) developed a full medium to germinate spores after chemical mutagenesis and evaluated the effect on growth parameters. We developed a full medium to regenerate auxotrophic mutants after protoplast transformation and tested the effect of different media supplements and two different concentrations of ammonium tartrate on the protoplast survival rate. Microelements (based on Murashige and

Skoog 1962), vitamins and supplements used in standard cell culture media were applied, as well as choline chloride, p-aminobenzoic acid and riboflavine according to Ashton and Cove (1977) and Na-palmitic acid, D-ribose, glycerol, lipoic acid, hemin and ascorbic acid. The medium B is dependent on peptone as a source for amino acids, whereas in medium C all amino acids are present as single compounds (amicase, Sigma). In addition, in medium C a higher number of coenzymes is present.

Amounts of regenerating protoplasts were generally higher in the newly developed complex media. The survival rates for medium B were 129±43 % (5 mmol/L ammonium tartrate) and 141±52 % (2.5 mmol/L) and for medium C 94±36 % (5 mmol/L ammonium tartrate) and 130±43 % (2.5 mmol/L) compared to medium A (100 %). The inhibitory effect of 5 mmol/L ammonium tartrate, when compared to 2.5 mmol/L, might be due to the increase of total nitrogen, an altered relation of ammonium to nitrate, or an indirect effect of pH shifts in the medium depending on the NH_4^+/NO_3^- relation. The latter reason is unlikely to explain our results, because in another experiment the pH-value remained almost the same during the first ten days of regeneration, indicating that during this phase alteration of the pH due to nitrogen nutrition hardly occurred. Further experiments are necessary to determine whether the nitrogen content or different NH_4^+/NO_3^- ratios are affecting protoplast survival in *Physcomitrella.*

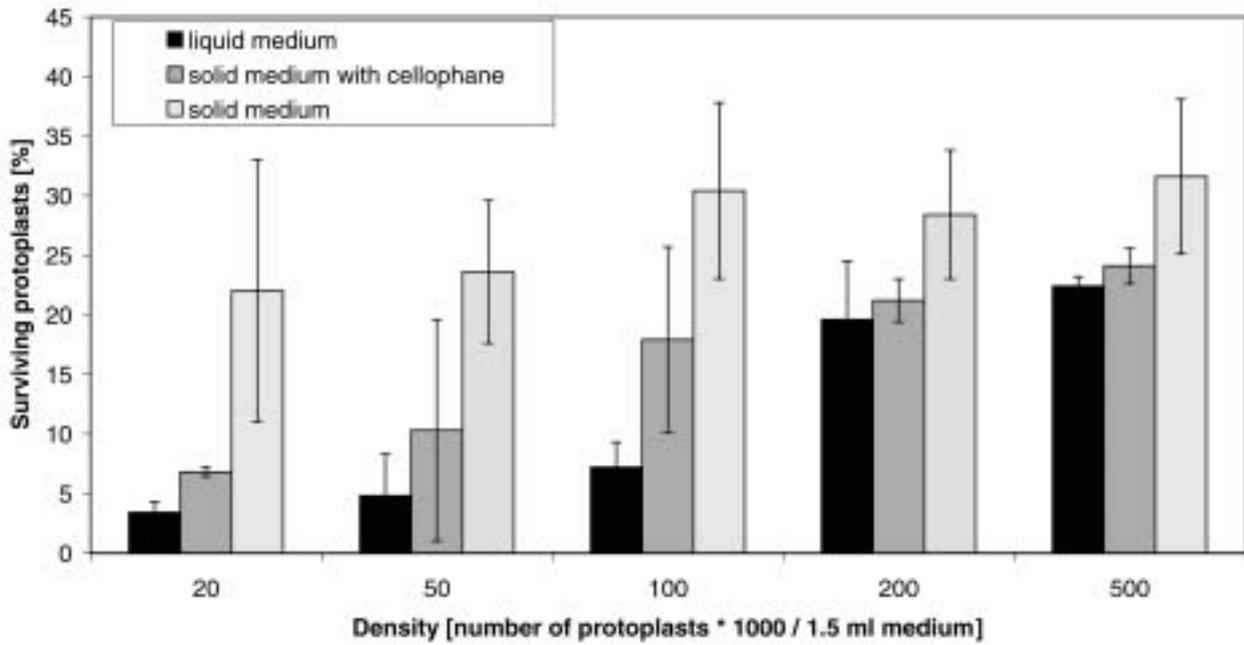## Protoplast density and regeneration method

Different regeneration protocols have been applied so far for *Physcomitrella patens* using either liquid culture (Schaefer et al. 1991, Hohe et al. 2002 b) or solid medium with embedding techniques (e.g. Jenkins and Cove 1983, Nishiyama et al. 2000), but no direct comparison of different methods has been published yet.

In order to optimise different culture methods with regard to regeneration efficiencies, a short version of the transformation protocol (without PEG) was applied and protoplasts in five different densities between 20,000 and 500,000 per 1.5 mL were regenerated testing three different methods: liquid medium (regeneration medium A), solid medium (regeneration medium A solidified with 8 g L^{-1} agar, Oxoid Ltd., Basingstoke, Hampshire, England) and solid medium overlaid with cellophane (80 mm, Hans Schütt, Hamburg, Germany), respectively.

A density of 500,000 protoplasts/1.5 mL was most efficient in all three culture methods tested (Fig. 1), while increased densities (750,000 protoplasts/1.5 mL) resulted in total cell debris (data not shown).

At all protoplast densities, solid medium was superior to either liquid medium or to solid medium overlaid with cellophane (Fig. 1).

In contrast to survival rate, the formation of long protonema filaments during the first ten days was strongly stimulated by

Figure 1. Effect of protoplast density and the regeneration method on the survival rate of protoplasts of *Physcomitrella patens*, determined ten days after protoplast isolation. (1,000 protoplasts/variant; mean and standard deviations of two independent experiments).
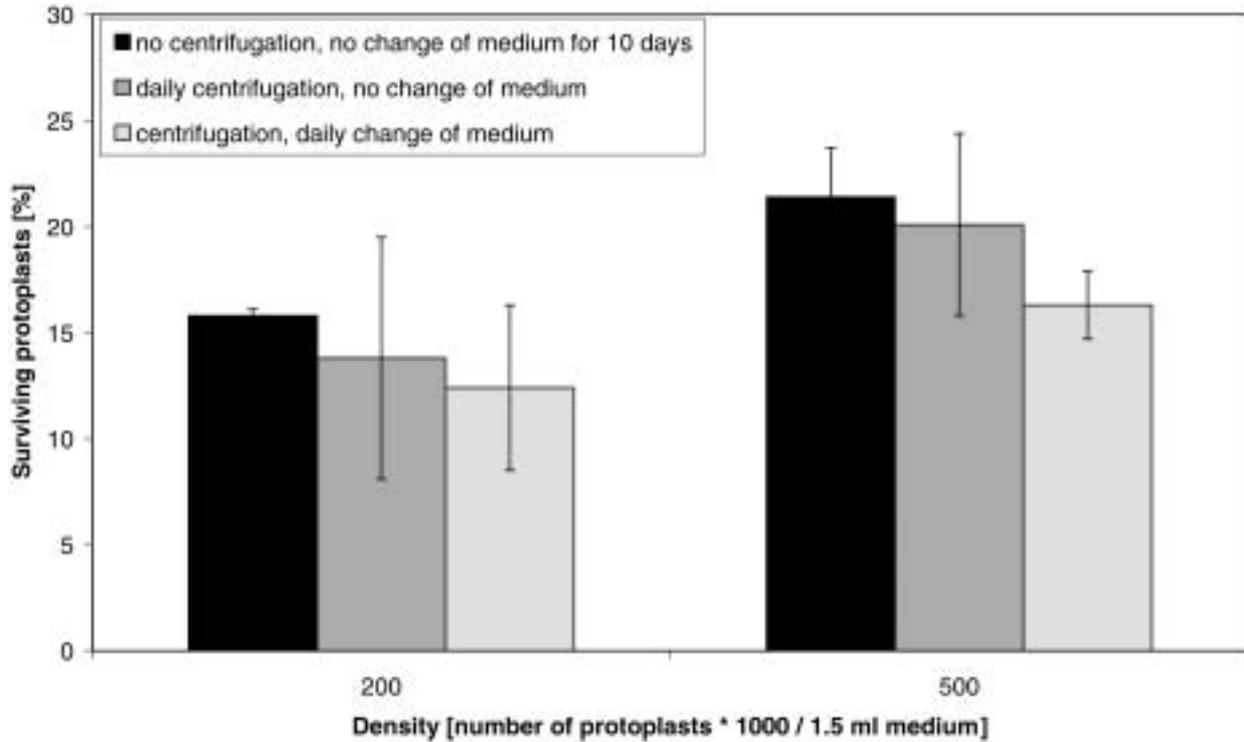
Figure 2. Effect of protoplast density and application of daily medium replacement on the percentage of surviving protoplasts of *Physcomitrella patens*, determined ten days after protoplast isolation. (1,000 protoplasts/variant; mean and standard deviations of two independent experiments).

culture of protoplasts in liquid medium as well as adjustment to low protoplast densities (data not shown). The percentage of viable protoplasts without any cell division varied from 2.9 % at initial densities of 20,000 protoplasts/1.5 mL in liquid medium to 37.5 % at initial densities of 500,000 protoplasts/1.5 mL on solid medium.

There is some evidence that protonema development not only depends on internal factors transported from cell to cell, but also on interactions with the substrate during culture (Knoop 1984, Hadeler et al. 1995, Reski 1998 b). Determination of phytohormones in protonema cells and in the medium of an Erlenmeyer flask culture of *Physcomitrella patens* revealed that most of the hormones were extra-cellular (about 65–85 % of the cytokinins were excreted into the medium, 94 % of auxin; Reutter et al. 1998). Differentiation processes in mosses are clearly dependent on the amount of auxin (transition from chloronema to caulonema; Bopp and Atzorn 1992) and cytokinin (formation of buds and subsequent gametophores; Bopp and Atzorn 1992). By daily change of the regeneration medium, we tested whether the observed differences in survival rate and regeneration velocity were an effect of extra-cellular compounds secreted into the medium. At densities of more than 200,000 protoplasts/1.5 mL daily medium replacement had a negative effect on protoplast survival (Fig. 2), demonstrating that the conditioned medium contained compounds that were promoting survival of the protoplasts. Thus extra-cellular compounds do not only stimulate differentiation processes in *Physcomitrella patens* (Reutter et al. 1998), but also survival of protoplasts. An effect on the regeneration velocity afterwards was not observed; the protonema formation during the first ten days was the same (data not shown).

In the next set of experiments, we tried to mimic the effect of protoplast density on their survival rate by transferring conditioned medium of either low-density or high-density feeding cultures. Survival rates of more than 20 % could be obtained by culturing protoplasts at a density of 500,000 protoplasts/1.5 mL, but not by transferring the medium of these cultures to cultures with an initial density of 100,000 proto-

plasts/1.5 mL. Thus, although the medium in protoplast cultures of *Physcomitrella patens* contains extra-cellular compounds stimulating protoplast survival, our results indicate that the density effect is not mediated solely by extra-cellular factors.

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