



# Protonema induction, transient transformation, and protoplast regeneration in the peat moss *Sphagnum papillosum*

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## Abstract

*Sphagnum* mosses are important carbon sequesters and emerging model organisms. However, induction and long-term cultivation of thalloid protonema in several species was not achievable so far. Here, we provide protocols for a set of new tools relevant for *Sphagnum* molecular biology: a new way for *Sphagnum* protoplast isolation and regeneration, and a first protocol for transient protoplast transformation. Together, these protocols will support the emerging *Sphagnum* research community in basic and applied science.

## Key message

Advancing molecular research in climate-critical plants.

**Keywords** Biotechnology · Carbon storage · Cell culture techniques · Peat mosses

## Abbreviations

|           |                                              |
|-----------|----------------------------------------------|
| DNA       | Deoxyribonucleic acid                        |
| G         | Relative centrifugal force                   |
| GFP       | Green fluorescent protein                    |
| ME        | Micro elements                               |
| MES       | 2-( <i>N</i> -Morpholino)ethanesulfonic acid |
| mOsm      | Milli osmole                                 |
| PEG       | Polyethylen glycol                           |
| PIM       | Protoplast isolation medium                  |
| RM        | Regeneration medium                          |
| rpm       | Rounds per minute                            |
| <i>S.</i> | <i>Sphagnum</i>                              |
| TM        | Transformation medium                        |
| YFP       | Yellow fluorescent protein                   |

## Introduction

Peat mosses of the genus *Sphagnum* comprise most of the biomass in northern peatlands, storing over 500 Gt of carbon, while covering only 3% of the earth's land mass (Gorham 1991; Clymo et al. 1998; Yu et al. 2021). As climate-critical plants, *Sphagnum* mosses have gained interest in recent years (Beike et al. 2015; Weston et al. 2018). While there are a number of axenic *Sphagnum* laboratory strains (Heck et al. 2021a) and cultivation techniques available (Batra et al. 2003; Beike et al. 2015; Zhao et al. 2019; Heck et al. 2021b), the toolbox for working with *Sphagnum* mosses under axenic conditions is still small. While *Sphagnum* gametophores are easy to propagate (Beike et al. 2015; Heck et al. 2021a, b), juvenile protonema tissue, from which the gametophores arise, is naturally formed only after spore germination (Clymo and Hayward 1982; Beike et al. 2015). Upon wounding, gametophores will eventually form thalloid protonemata, a method described in Zhao et al. (2019), where the protonemata of *S. squarrosum* had to be sub-cultivated regularly to prevent gametophore formation. So far, protoplasts were isolated from gametophore apices of *S. fallax* (Batra et al. 2003). This is laborious and contrary to other mosses, where protoplasts are isolated from protonemata as an “easier to handle” material, as protonemata are grown in suspension culture in different volumes (Hohe and Reski 2002;

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Schween et al. 2003; Hohe et al. 2004). So far, regeneration of *Sphagnum* protoplasts has been achieved only with a chlorophyll-free *Solanum* hybrid clone as feeder (Batra et al. 2003) which hampers subsequent analyses. Here, we report on an efficient method for protonema induction, which works in different *Sphagnum* species, as well as long-term cultivation, preventing differentiation of gametophores, and allowing growth of homogenous, thalloid protonemata in suspension. Moreover, we provide protocols for protoplast isolation from protonemata, the first protocol for transformation of *Sphagnum* cells and protoplast regeneration without feeder cells.

## Materials and methods

### Protocols

#### Media, moss lines and cultivation

- Axenic *Sphagnum* strains are available from the International Moss Stock Center ([www.moss-stock-center.org](http://www.moss-stock-center.org)) and published in Heck et al. (2021a).
- All cultivation techniques used for *Sphagnum* gametophores are described in Beike et al. (2015) and Heck et al. (2021a).
- Suspension culture for protonema induction and pre-culture prior to protoplast isolation were performed in 50 mL and 200 mL of media.
- Media:
  - (1) Knop ME pH 5.4: standard moss medium, described in Beike et al. (2015).
  - (2) Protonema medium (PM): Knop ME pH 5.4 with 30 mM ammonium tartrate.
  - (3) Knop ME pH 4.5: Knop ME medium, 0.1% MES, pH 4.5, cultivation at 22 °C, 16 h light/8 h dark, light intensity  $70 \pm 5 \mu\text{mol/s}$ .
  - (4) Protoplast isolation medium (PIM): 80 g/L mannitol, pH 5.7, 450 mOsm/L.
  - (5) Transformation medium (TM): 3.045 g/L  $\text{MgCl}_2$ , 1 g/L MES, 70 g/L mannitol, pH 5.6, 450 mOsm/L.
  - (6) Regeneration medium (RM): Knop ME medium with 50 g/L glucose monohydrate, 20 g/L mannitol, pH 5.5, 440 mOsm/L.

**Sterilization** 1.+2. Can be autoclaved, 3.-6. Should be sterilized by filtration with 0.22  $\mu\text{m}$  bottle top filter to assure stability of pH and osmolarity.

**Osmolarity** Osmolarity should be checked using an osmometer with several measurements. For this, a

freezing point depression osmometer was used. Adjust osmolarity via mannitol concentration  $\pm 5 \text{ mOsm}$ .

### General advice

While growth conditions for *Sphagnum* gametophores have been established for many species, induction of protonemata can be difficult. We advise always using more than one sample when trying to induce protonemata or isolate protoplasts. *Sphagnum* protoplasts are very sensitive to mechanical stress; use wide-mouth pipettes, transfer them slowly and be sure to use the correct osmolarity in the desired media. Cultures should be checked for contaminations regularly as described in Heck et al. (2021a). All steps should be performed under axenic conditions with sterilized material, while working in a laminar flow hood.

### Protonema induction and long-term cultivation

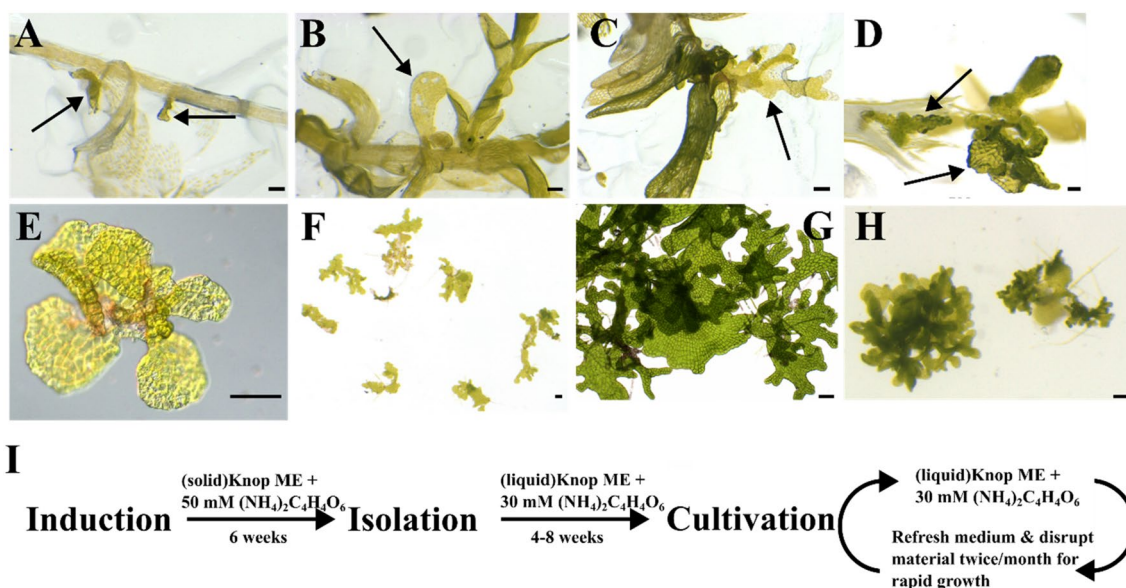
*Sphagnum* gametophores are disrupted into approx. 1 cm long pieces, using sterile forceps and cultivated for 6 weeks on solid Knop ME medium pH 5.4, flooded with PM, i.e. Knop ME pH 5.4 including 50 mM ammonium tartrate (10 mL per 9 cm petri dish). Apices and disrupted stems will form protonemata on wounded tissue (Fig. 1A–D).

This thalloid tissue can be isolated using a stereoscope which was surface-sterilized while working in a laminar flow cabinet. Isolated protonema tissue can be maintained in suspension culture with protonema medium (PM).

Until growth of thalloid tissue is visible, the material should not be transferred to fresh media, to prevent protonema loss during the process.

Regular disruption using a dispersion tool (IKA T25 digital ULTRA-TURRAX®, IKA® Werke GmbH & Co. KG, Staufen, Germany; 1 min 15,000 rpm), at the start of inoculation with material from a plate once a month is sufficient. To obtain homogenous material, we recommend disruption and sub-cultivation of protonema material every 2–4 weeks (Fig. 1E, F). For rapid proliferation, weekly disruption and sub-cultivation is advised.

Long-term storage of protonemata in PM is possible (Fig. 1G, H), but culture densities should be kept below 1 g/L dry weight to prevent gametophore formation. Dry weight is estimated as described in Heck et al. (2021a), using  $3 \times 10 \text{ mL}$  protonema suspension culture from a 200 mL culture (200–800 mg/L dry weight). It was possible to recover gametophores from over 1-year old protonema cultures without sub-cultivation after switching to Knop ME pH 5.4.



**Fig. 1** Protonema induction and cultivation. **A–D** Induction of protonema on plates. **A** *Sphagnum fimbriatum* clone 1.1. **B** *Sphagnum fallax* clone 4.5. **C** *Sphagnum centrale* clone 10.2. **D** *Sphagnum squarrosum* clone 5.2. **E–H** Protonema suspension culture of *Sphagnum papillosum* clone 6.1. **E** Thalloid protonema 4 weeks after last

sub-cultivation. **F** Thalloid protonema after biweekly disruption and sub-cultivation. **G, H** large thalloid protonema pieces after 3 months without sub-cultivation. Scale bars = 100  $\mu$ m. **I** Steps of protonema induction including important media and time scale for the single steps

## Protoplast isolation

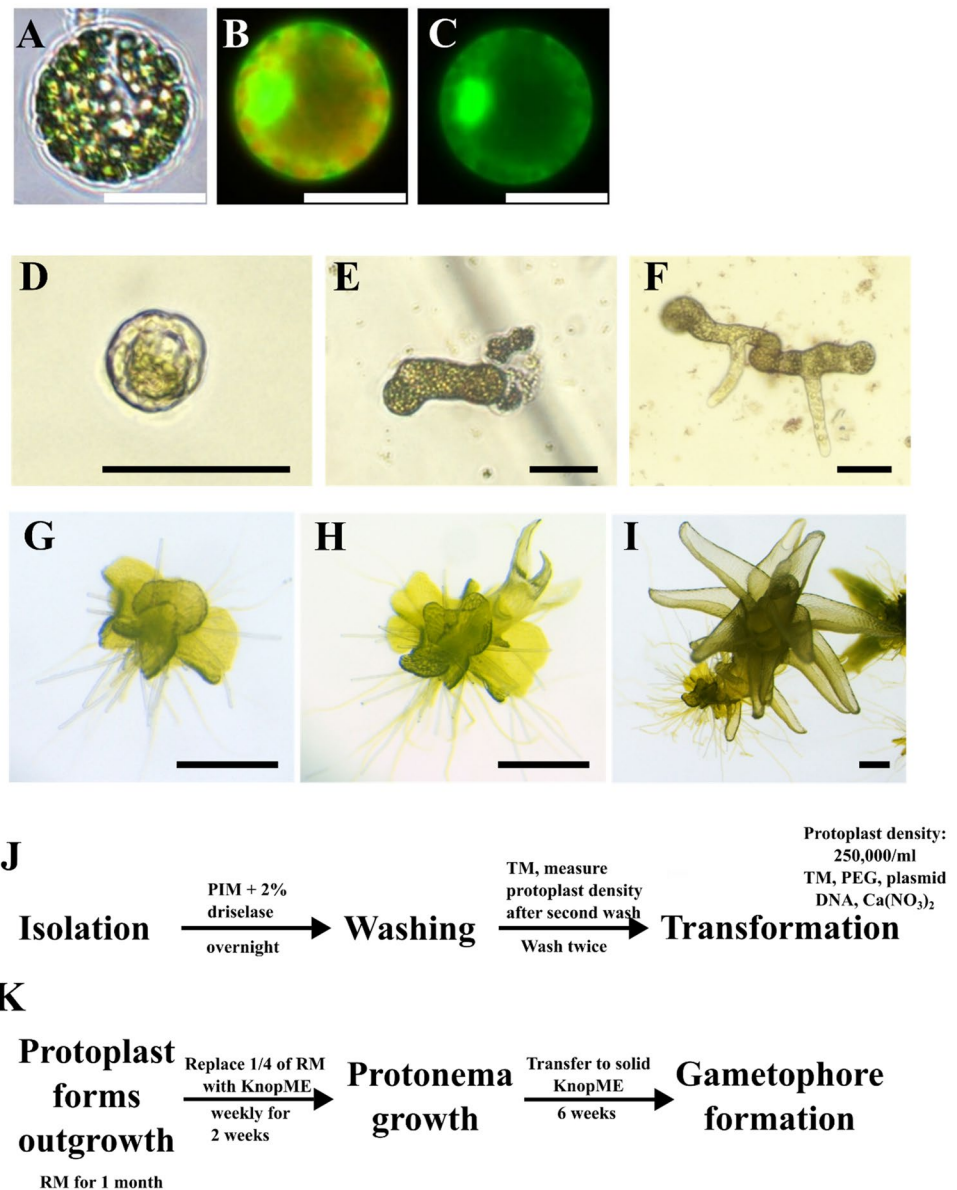
- (1) Pre-cultivation of protonemata 7 days prior to protoplast isolation, density 100 mg/L dry weight, in Knop ME pH 4.5 200 mL.
- (2) Transfer protonemata to a petri dish (in our experience small plate diameters work better, e.g. 3–6 cm) and mix carefully with a 2% Driselase solution (5 mL for 6 cm petri dish), dissolved in PIM (see Hohe et al. 2004). Place in darkness on a tumble shaker at low speed for 18 h. All further steps follow the protocol described in Hohe et al. (2004) and have been adapted considering protoplast density and regeneration.
- (3) After 18 h, filtrate protoplasts by using a sieve with first 100  $\mu$ m, then 50  $\mu$ m pore size. Wash with PIM (without Driselase) (10 mL).
- (4) Sediment protoplasts by centrifugation (50 G max., slow brake/acceleration, 10 min) and wash by replacing supernatant with PIM (10 mL).
- (5) Repeat step 4 twice.
- (6) After the last washing step, concentrate all protoplasts in one centrifugation tube to assess protoplast density using a Fuchs-Rosenthal counting chamber. Expect low yields (5000–10,000/mL), with many dead cells being present.
- (7) Set protoplast density to 250,000/mL in TM for transformation. In our experience around 150,000 proto-

plasts per 200 mL protonema culture with 100 mg/L dry weight can be expected.

## Transformation

- (1) 200  $\mu$ L protoplast solution (50,000 protoplasts) per sample is used.
- (2) Mix gently with 100  $\mu$ L DNA solution (0.1 M  $\text{Ca}(\text{NO}_3)_2$ , 65  $\mu$ g of plasmid DNA) and 350  $\mu$ L PEG solution (8 g PEG 4000 dissolved in 20 g TM, filter-sterilized).
- (3) Mix gently every 5 min for 45 min.
- (4) Mix gently by adding first 1 mL, then 2, 3, and 4 mL TM every 5 min.
- (5) Sediment cells by centrifugation (500 rpm, slow brake/acceleration, 10 min) and discard the supernatant.
- (6) See protoplast regeneration step 1 for further cultivation. If you do not want to regenerate and only analyse transient transformation rates, you may use 2 mL of TM instead of RM.
- (7) Fluorescence of protoplasts can be checked 2 days after transformation using an appropriate microscope (Fig. 2A–C).

**Fig. 2** Transient transformation and protoplast regeneration in *Sphagnum papillosum* clone 6.1. Protoplast transformed with *AtUbiq10 promoter-YFP* construct. **A** Brightfield. **B** Fluorescent long-pass channel: green = YFP, red = autofluorescence chloroplasts. **C** Fluorescent bandpass channel: green = YFP fluorescence, scale bar = 20  $\mu$ m. **D–F** Regeneration in suspension culture, scale bars = 50  $\mu$ m. **D** Protoplast 17 days after isolation, forming a circular outgrowth. **E** 29 days after protoplast isolation, multicellular filamentous structures are forming. **F** After 38 days the regenerated *Sphagnum* plant is about to form thalloid protonema. **G, H** Development after plating on solid medium, scale bars = 100  $\mu$ m. **G** 2 weeks after transfer (day 56 after protoplast isolation), thalloid protonema as well as filamentous protonema have formed. **H** 63 days after protoplast isolation, a gametophore develops from thalloid protonema. **I** Fully grown gametophore, 84 days after protoplast regeneration started. **J** Overview of protoplast isolation and transformation. **K** Regeneration of gametophores from protoplasts including time scale and important media changes



### Protoplast regeneration

- (1) Add 2 mL of RM to protoplast pellets and transfer them to a 3 cm petri dish or 6-well plate, cultivating them in standard long-day conditions (16 h light/8 h dark) (Beike et al. 2015; Heck et al. 2021a).
- (2) After 30 days, protoplasts will form an outgrowth from which further cell divisions start (Fig. 2D, E).
- (3) For the next 2 weeks, a quarter of the media should be replaced weekly with Knop ME.
- (4) 6 weeks after protoplast regeneration started (Fig. 2F), half of the supernatant media is discarded, while the remaining solution is plated onto solid Knop ME plates (volume should consider density of regenerating protoplasts and plate size).
- (5) 2 weeks after plating, single protoplasts have regenerated into thalloid protonema structures, with filamentous protonemata growing downwards into the agar (Fig. 2G).
- (6) Around 3 months after protoplast isolation, a single gametophore has grown out of a single protoplast (Fig. 2H, I). In contrast to the low protoplast yield, regeneration of gametophores from protoplasts, once they started growing, works very well and a high percentage (~70%) of regenerants can be expected with the protocols presented here.

## Transient transformation construct

We tested several promoters (Act5, CA200, 35S, AtUbq10) widely used in *Physcomitrella* research (Horstmann et al. 2004; Weise et al. 2006; Orellana-Escobedo et al. 2015; Peramuna et al. 2018) using citrine, GFP, YFP, and the luciferase reporter system. We did not detect any activity using different plasmid DNA concentrations and protoplast densities. The only promoter for which an activity in *Sphagnum* was detectable was the *Arabidopsis*-ubiquitin promoter AtUbq10 fused to YFP (Fig. 2B, C).

## Trouble shooting

| Problem                                                              | Solution                                                                                                                                                                                                 |
|----------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Protoplasts do not form pellets after centrifugation                 | Increase centrifugation time to 30 min (do not increase rpm). Make sure transformation tubes are devoid of any possible PEG residues, as this might lead to insufficient pellet formation of protoplasts |
| Almost all/all protoplasts are dead                                  | Make sure to use wide-mouth pipettes, transfer slowly and make sure that your centrifugation steps are executed at slow acceleration and brake. Also check osmolarity and pH of your protoplast media    |
| Protoplasts form large aggregates during transformation/regeneration | Decrease density of protoplasts to about 50,000 per 2 mL regeneration medium or per transformation approach                                                                                              |
| Differentiation of protonema to gametophores in suspension culture   | Decrease density of gametophores or increase concentration of ammonium tartrate in your suspension medium                                                                                                |

## Discussion

We present protocols which, for the first time, make “easy to take care of” cultivation of protonemata from different *Sphagnum* species possible. All techniques described so far for the cultivation of protonemata and protoplasts from *Sphagnum* plants showed inherent difficulties (Batra et al. 2003; Zhao et al. 2019). While protonema induction is still a difficult and low-yielding process, we improved the cultivation of protonemata, allowing efficient production of homogenous material for protoplast isolation (Hohe and Reski 2002) and long-term storage (Schulte and Reski 2004) in different species. We also provide the first protocol

for the transient transformation of a member of the genus *Sphagnum* together with efficient regeneration of protoplasts without the need for feeder cells or complex mixtures of plant growth stimulants. Stable transformation and appropriate selection protocols are the next steps in our research. With this we provide new tools for the *Sphagnum* scientific community, supplying advanced knowledge on the cultivation of different *Sphagnum* tissues.

**Author contributions** VML, ELD and RR planned and designed the research. VML did the experimental work on protonema induction, protoplast isolation and protoplast regeneration. AK and VML adapted the transformation protocol, while AK tested promoter activity. AP tested osmolarity of different *Sphagnum* species and cloned the expression construct. VML wrote the manuscript with the help of ELD and RR. ELD and RR supervised research. RR acquired funding for this work. All authors read and approved the manuscript.

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**Data Availability** Axenic *Sphagnum* strains are available from the International Moss Stock Center ([www.moss-stock-center.org](http://www.moss-stock-center.org)) and published in Heck et al. (2021a).

## Declarations

**Conflict of interest** All authors declare that they have no competing interests.

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