

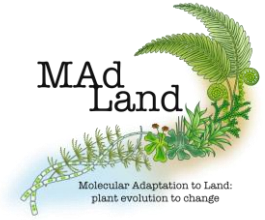
**MadLand coordination:**

madland-coord@plantcode-mail.biologie.uni-marburg.de

**Protocol setup and contact person:**

Rabea Meyberg

rabea.meyberg@biologie.uni-marburg.de



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## Vegetative and sexual reproduction of *Physcomitrium patens*

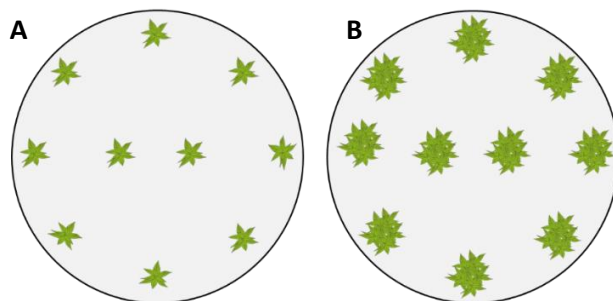
- *P. patens* Reute2020 (Re20, collected 2006 by M. Lüdt (Hiss *et al.*, 2017))
- Inoculation of the plants for standard analysis is performed on solid minimal medium as e.g. Knop/BCD (s. *Anthoceros agrestis* cultivation protocol) in 9cm petri dishes with vent
- For standardised and synchronised growth, always the same amount of the same tissue should be used (e.g. one gametophore, one-half gametophore)
- Inoculation is performed under sterile conditions (e.g. clean bench) using flame sterilized forceps

### Protonemal vegetative reproduction

To e.g. generate large amounts of tissue for DNA extraction or to analyse the protonemal stage of *P. patens*, filamentous cultures can be grown either in liquid or on solid medium. Filamentous cultures can be started from any tissue. To start a solid grown protonema culture, plant material will be cut by grinding for two minutes at 14 000 rpm in e.g. 10ml sterile MilliQ/tap water with a disperser. Medium of choice (e.g. Knop) should be overlayed with cellophane foil for easy harvesting of the material but can affect growth (important, when used for experiments). Per plate, ~1.5ml of ground material is distributed evenly on the cellophane placed on the medium. For fast growth, plates can be closed with a micropore tape and grown for 6-7 days under long day (LD) conditions: 70µmol/m<sup>2</sup>/s, 16h light, 8h dark, 22°C. Additional supplementation of ammonium-nitrate increases the tissue yield, but does not allow sexual reproduction.

### Gametophore inoculation

To generate gametophore tissue, 10 gametophores are inoculated per petri dish, following the pattern in Fig. 1A. The plates are enclosed by using parafilm, for fast growth 1-2 weeks of growth with a micropore tape is feasible. Plants (defined as one inoculum including newly developed gametophores) are grown under LD conditions. During this time, each inoculum reproduces vegetatively and gives rise to ~4-8 new gametophores (Fig. 1B). For sporophyte development, plates are enclosed with parafilm and grown for 5 weeks.

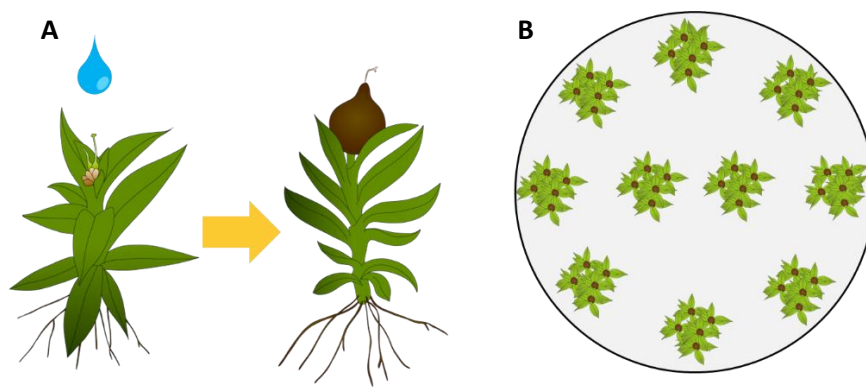


**Figure 1: Inoculation of *P. patens* on solid minimal medium in 9cm petri dishes.** A: Inoculation pattern. B: Expected vegetative reproduction after five weeks of growth under LD conditions.

### Gametangia induction

After five weeks of LD growth, the cultures can be transferred to short day (SD) conditions: 20 $\mu$ mol/m<sup>2</sup>/s, 8h light, 16h dark, 15°C. Tip: Note the date of the transfer on the lid to track timing properly, since it is important for following developmental steps and analyses putatively performed.

Antheridia emerge and mature first, followed by archegonia. In our conditions, mature gametangia (at least one archegonium shows an open tip cell and antheridia already released spermatozooids and/or have swollen tip cells) on nearly all apices are found at day 21 (needs to be adjusted lab wise and highly depends on the climate chamber setup). At this day, watering is performed to synchronize fertilization (Landberg *et al.*, 2013; Meyberg *et al.*, 2020).



**Figure 2: Synchronized fertilization and sporophyte development in *P. patens*.** A: External application of water leads to synchronized release of the motile biflagellate spermatozooids, which subsequently will fertilize the egg cell. B: Expected sporophyte development after 42 days after watering (daw) showing brown sporophytes.

### Watering

Use sterile tap water to synchronize fertilization by external application of 2-3 ml water by pipetting. Water each plant at least three times, until the tissue is soaked. Leftover water (~2ml) is removed, plates are again enclosed with parafilm and transferred back to SD conditions. Sporophyte development takes now place. Tip: Again note the date of watering on the lid to keep track of your culture.

### Sporophyte observation

To analyse sporophyte development per gametophore, 30 days after watering (daw) sporophytes can be counted. This timepoint is ideal, since a putative second wave of fertilization events did not take place yet and thus, precise sporophyte development analysis can be performed (Hiss *et al.*, 2017).

### Harvesting of mature sporophytes

To generate mature sporophytes, wait at least until 42daw. Harvest dark brown sporophytes from the tallest gametophores under sterile conditions with e.g. two forceps. Using a binocular is recommended. Place sporophytes into reaction tubes and let them dry for at least 2-3 hours before closure of the lid. For germination analysis, harvest each one sporophyte per reaction tube and store it at 4°C for at least two weeks. In our hands, this resulted in a timewise more even distribution of germination (preliminary data).

**Spore germination**

To analyse germination, add 500µl of sterile MilliQ to the previously harvested sterile sporophyte. Open the sporophyte by squashing it against the wall of the reaction tube until spores are released (cloud visible). Analysis can be performed on the one hand, by plating the whole solution or on the other hand, by the inoculation of 4µl drops (at least do triplicates in this case). Suitable medium is e.g. knop or knop+ammonium+microelements with or without cellophane foil, depending on the aim. While handling spores in solution, mix regularly to prevent unequal distribution due to a fast sedimentation of the spores. Inoculums can be grown under LD conditions and germination can be analysed over the next 1-3 weeks (variations possible due to the setup of the climate chamber). For precise measurements, daily counts are recommended (modified after (Vesty *et al.*, 2016)).

**Media and Solutions****Knop minimal medium (Knop, 1868)**

No.	Supplement	Concentration	Comment
1	KH <sub>2</sub> PO <sub>4</sub>	25 g/l	Autoclave and store at 4°C
2	KCl	25 g/l	Autoclave and store at 4°C
3	MgSO <sub>4</sub> x 7 H <sub>2</sub> O	25 g/l	Autoclave and store at 4°C
4	Ca(NO <sub>3</sub> ) <sub>2</sub> x 4 H <sub>2</sub> O	100 g/l	Autoclave and store at 4°C
5	FeSO <sub>4</sub> x 7 H <sub>2</sub> O	250 mg/l	Do not autoclave and store at 4°C

**Preparation of one liter Knop minimal medium:**

- Prepare aprox. 500 ml of VE water in an one liter bottle
- add 10 ml of solution 1-4 and 50 ml of solution 5 and mix inbetween
- adjust pH to 5.8 using 1M KOH
- fill up to 1 liter
- for solid medium add 9g of agar
- autoclave

Pour autoclaved medium into plates with vents: sporophyte development: ~ 30ml per plate, vegetative growth: ~ 20ml per plate

**Moss microelements**

Chemical	Amount per liter
H <sub>3</sub> BO <sub>3</sub>	309mg
MnSO <sub>4</sub> x 1 H <sub>2</sub> O	845mg
ZnSO <sub>4</sub> x 7 H <sub>2</sub> O	431mg
KJ	41,5mg
Na <sub>2</sub> MoO <sub>4</sub> x 2 H <sub>2</sub> O	12,1mg
CuSO <sub>4</sub> x 5 H <sub>2</sub> O	1,25mg
Co(NO <sub>3</sub> ) <sub>2</sub> x 6 H <sub>2</sub> O	1,46mg

- fill up to 1 liter with deionized water, autoclave and store at 4°C

**Preparation of one liter Knop+ammonium+microelements:**

- Prepare approx. 500 ml of VE water in an one liter bottle
- add 10 ml of solution 1-4 and 50 ml of solution 5 and mix inbetween
- for Knop+NH<sub>4</sub>NO<sub>3</sub> and microelements add 920mg NH<sub>4</sub>NO<sub>3</sub> per liter and 10ml of the microelement solution
- adjust pH to 5.8 using 1M KOH
- fill up to 1 liter
- for solid medium add 9g of agar
- autoclave

Pour autoclaved medium into plates with vents: ~ 20ml per plate

**Literature**

**Hiss M, Meyberg R, Westermann J, Haas FB, Schneider L, Schallenberg-Rudinger M, Ullrich KK, Rensing SA. 2017.** Sexual reproduction, sporophyte development and molecular variation in the model moss *Physcomitrella patens*: introducing the ecotype Reute. *Plant J* **90**(3): 606-620.

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**Vesty EF, Saidi Y, Moody LA, Holloway D, Whitbread A, Needs S, Choudhary A, Burns B, McLeod D, Bradshaw SJ, et al. 2016.** The decision to germinate is regulated by divergent molecular networks in spores and seeds. *New Phytol* **211**(3): 952-966.

**How to cite this protocol**

<https://data.uni-marburg.de/handle/dataumr/76>