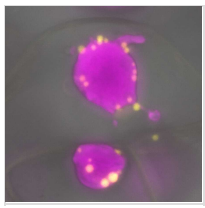

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Anthoceros agrestis Oxford (hornwort) transformation v02

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Hornworts

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ABSTRACT

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MATERIALS TEXT

KNOP recipe:

Stock 1
25g/L KH_2PO_4
Stock 2
25g/L KCl
Stock 3
25g/L $MgSO_4$ $7H_2O$
Stock 4
100g/L $Ca(NO_3)_2$ $4H_2O$
autoclave and store at RT or 4°C

KNOP solid working solution:

In 600 mL of water add:
10ml Stock 1
10ml Stock 2
10ml Stock 3
10ml Stock 4
12.5mg $FeSO_4 7H_2O$

pH to 5.8 with KOH

top up water to 1L after adjusting pH

add 7 gr of Gelzan - G1910 - CAS Number71010-52-1

KNOP liquid working solution:

In 600 mL of water add:
10ml Stock 1
10ml Stock 2
10ml Stock 3
10ml Stock 4
12.5mg $FeSO_4 7H_2O$
10 gr of sucrose (1% w/v final concentration, 2% also fine)

40mM MES (*very important*)

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pH to 5.5 with KOH

top up water to 1L after adjusting pH

Filter sterile (do not autoclave), aliquot into 50mL falcon tubes and store at -20°C.

Sterile disposable scalpels (#0501, Swann Morton)

Razor blades (#11904325, Fisher Scientific)

100 µm cell strainer (#352360, CORNING),

6-well plate (#140675, ThermoFisher)

3',5'-dimethoxy-4'-hydroxyacetophenone (acetosyringone) (#115540050, Acros Organics, dissolved in dimethyl sulfoxide (DMSO) (#D8418, SIGMA))

Cefotaxime (#BIC0111, Apollo Scientific)

Hygromycin (#10687010, Invitrogen)

Corning Disposable Vacuum Filter/Storage Systems (#430767)

BEFORE STARTING

Pray (not needed anymore)

1 ⚠

IMPORTANT: The light intensity used to cultivate *A. agrestis* tissue is a very critical factor for successful transformation. Tissue should be grown under low light intensity (3-5 µmol $m^{-2}s^{-1}$) and should have a morphology similar to the tissue in **Figure 1**

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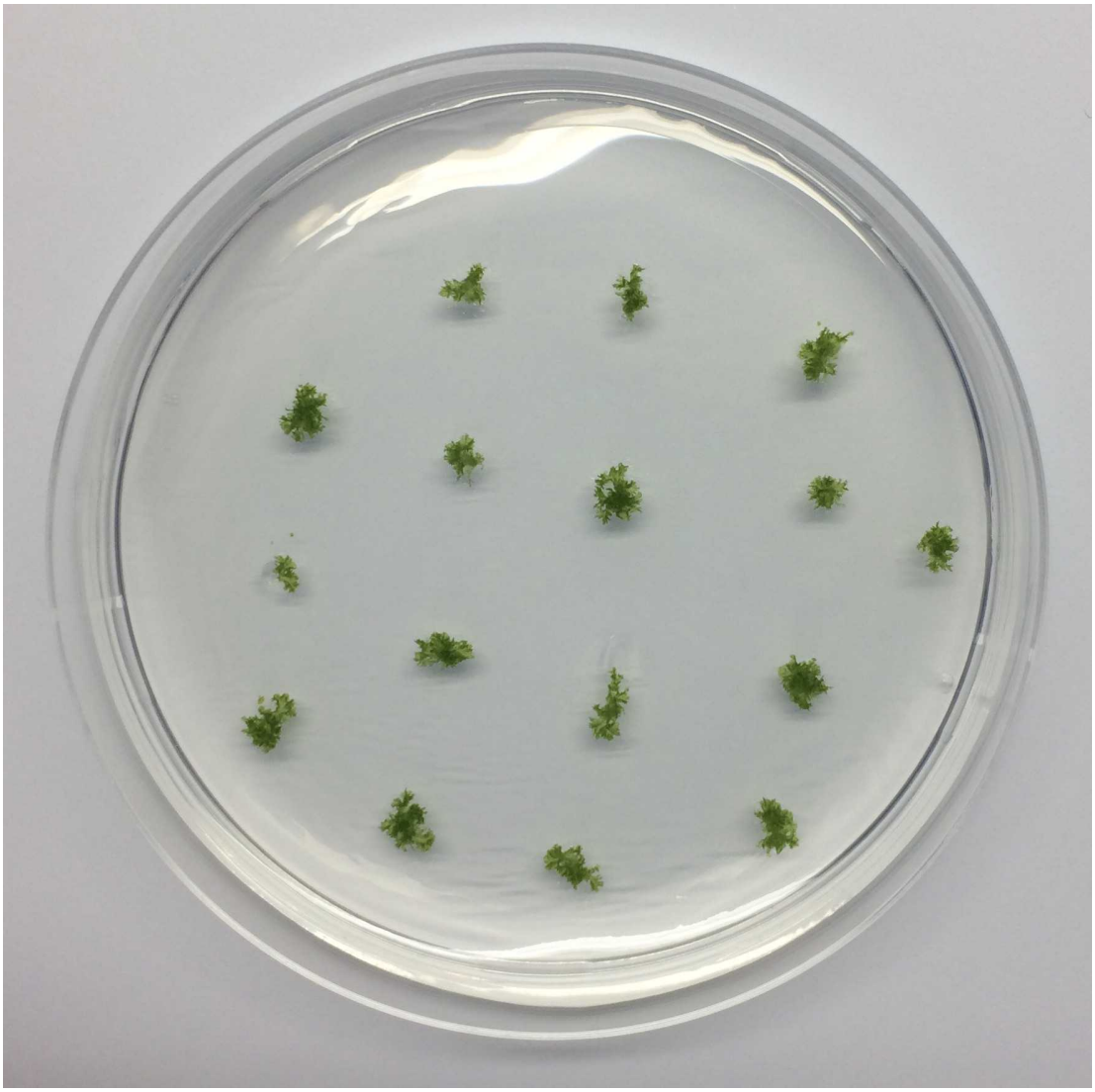

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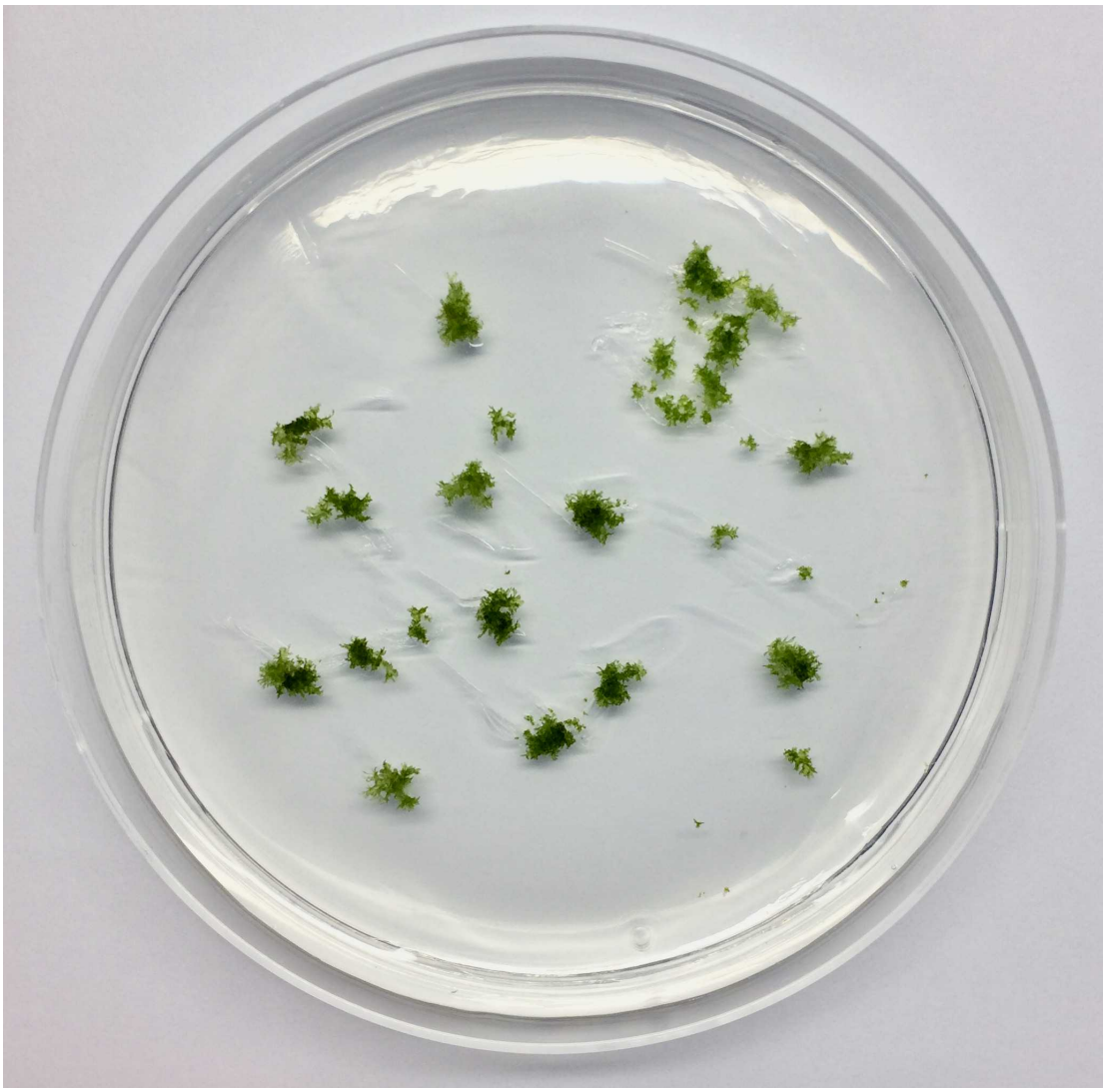

Figure 1:
Top: 4 week old *A. agrestis* Oxford thallus
Bottom: 7 week old *A. agrestis* Oxford thallus (this tissue is also good for transformation)

3 Axenic cultures of *A. agrestis* gametophytes can be routinely propagated by monthly sub-culturing as shown in **Figure 2.**

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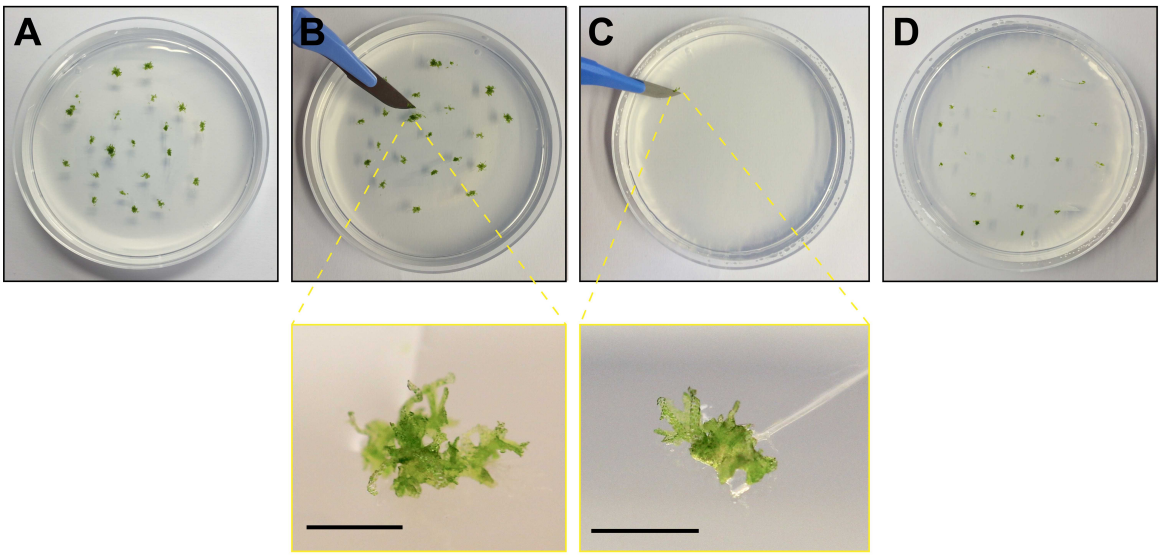

Figure 2: *A. agrestis* Oxford tissue culturing

For sub-culturing, a small piece of thallus tissue is cut using sterile disposable scalpels and placed on plates containing fresh growth medium. Scale bars: 2 mm. Petri dish dimensions: 92 x16 mm.

Tissue similar to the bottom images is optimal for transformation

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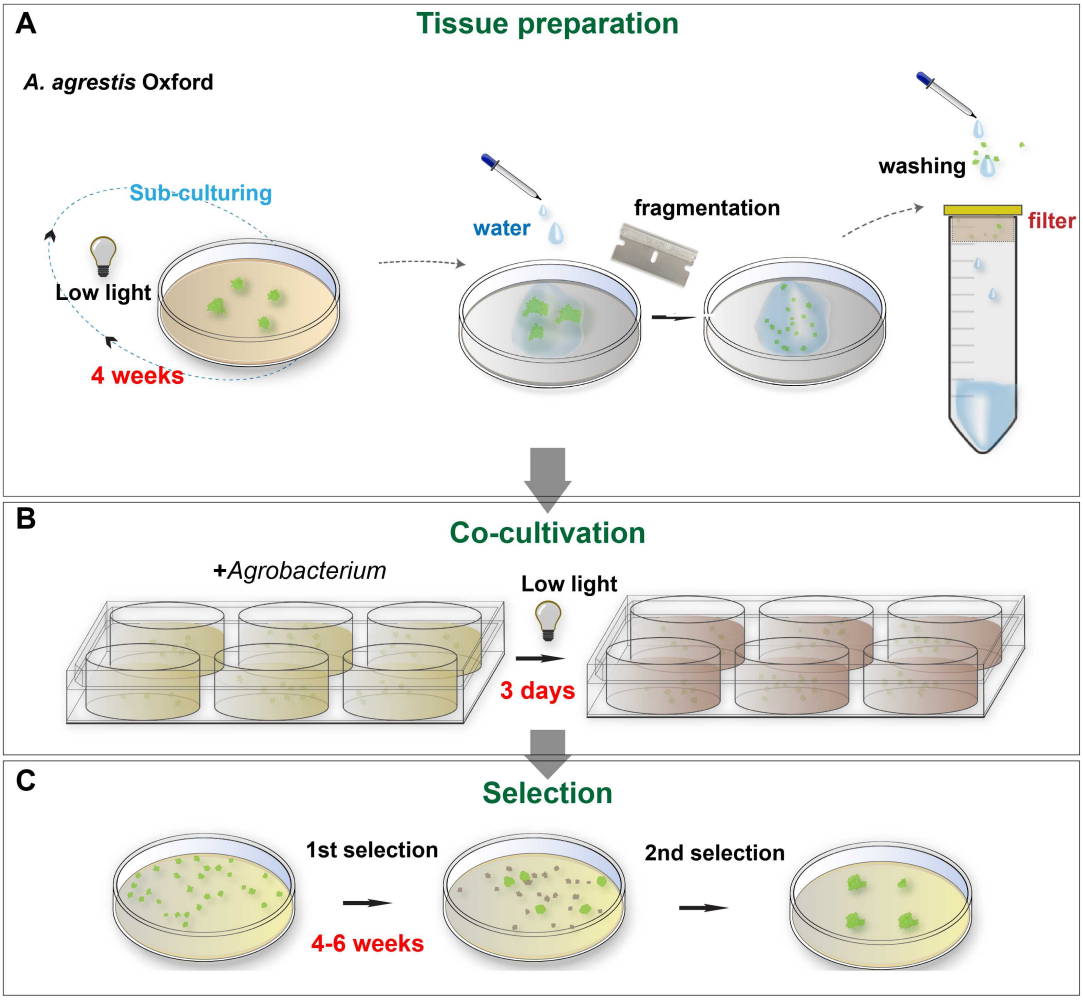

Figure 3: Transformation method outline. A) Thallus tissue is routinely propagated on a monthly basis under low light. 4-6 week old tissue is fragmented with the aid of a razor blade, transferred to a cell strainer, and washed thoroughly with sterile water. B) The tissue is then co-cultivated with *Agrobacterium* for three days (under low light) and C) spread on antibiotic-containing growth medium. After approximately 4-6 weeks, putative transformants are visible. A final round of selection is recommended to eliminate false-positive transformants.

6 **Tissue preparation:**

- Collect approximately 1 g of thallus tissue grown for 4-6 weeks under low light intensity (approximately 0.1 g of tissue per petri dish - 10 petri dishes in total). **Figure1 and Figure 4.1**
- Transfer the tissue into an empty petri dish, add sterile water until the tissue is covered and fragment using a razor blade (it takes approximately 5 mins, similar to Video 1). **Figure 4.2-3**
- Transfer the tissue from the petri dish into a cell strainer positioned on a falcon tube using

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sterile scalpels and wash the tissue using ~100 ml of sterile water or until the flow through was clear. **Figure 4.4-6**

7 Agrobacterium culture preparation:

- Inoculate 5 mL LB media with 3-4 *Agrobacterium* colonies (*AGL1:* 15 µg/mL rifampicin, 50 µg/mL carbenicillin) (*GV3101:* 50 µg/mL rifampicin, 25 µg/mL gentamicin) and the plasmid-specific selection antibiotic.
- Incubate the preculture at 28°C for 2 days at 110 rpm.
- Centrifuge 5 mL of 2 d *Agrobacterium* culture (no need to measure OD) for 7 min at 2000 xg.
- Remove supernatant and re-suspend in 5 mL liquid KNOP plus 1% (w/v) sucrose and 100 µM acetosyringone.
- Incubate the culture with shaking (120 rpm) at 28°C for 3-5 hours.

8 Co-cultivation:

- Transferred the fragmented thallus tissue into a 6-well plate (transfer 1⁄6 of the 1 g tissue into a single well) with 5 mL of liquid KNOP medium supplemented with 1% (w/v) sucrose and **30-40 mM MES (VERY IMPORTANT), pH 5.5**, 80 µL of *Agrobacterium* culture and acetosyringone at final concentration of 100 µM. **Figure 4.7**
- Co-cultivate the tissue with the *Agrobacterium* for 3 days on a shaker at 110 rpm, with only ambient light.
- Using a sterile plastic pipette transfer the tissue of one well into a cell strainer, drain and then transfer on growth media containing the appropriate antibiotic (onto 1 petri dish from one well).To facilitate spreading of the tissue, 1-2 mL of sterile water is added to the petri dish. **Figure 4.8-11**
- After 4-6 weeks successful transformants are visible on the petri dish (successful transformants can be identified using a dissecting scope after 4 weeks selection (sometimes as early as 2 weeks) based on rhizoid production and/or fluorescence if such a marker is present on the construct). **Figure 4.12**
- The emergence of rhizoids is an indication of successful transformation (yellow arrow: transformed thallus fragment, blue arrow: dying thallus fragment). **Figure 5**

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2nd selection (optional):

- To eliminate false positives, after 4 weeks transfer the tissue to fresh growth media containing 100 µg/mL cefotaxime and 10 µg/mL Hygromycin. To facilitate spreading of tissue on the petri dish add 2 mL of sterile water. Grow at 21°C under 12 hours light and 12 hours dark, 35 µmol $m^{-2}s^{-1}$

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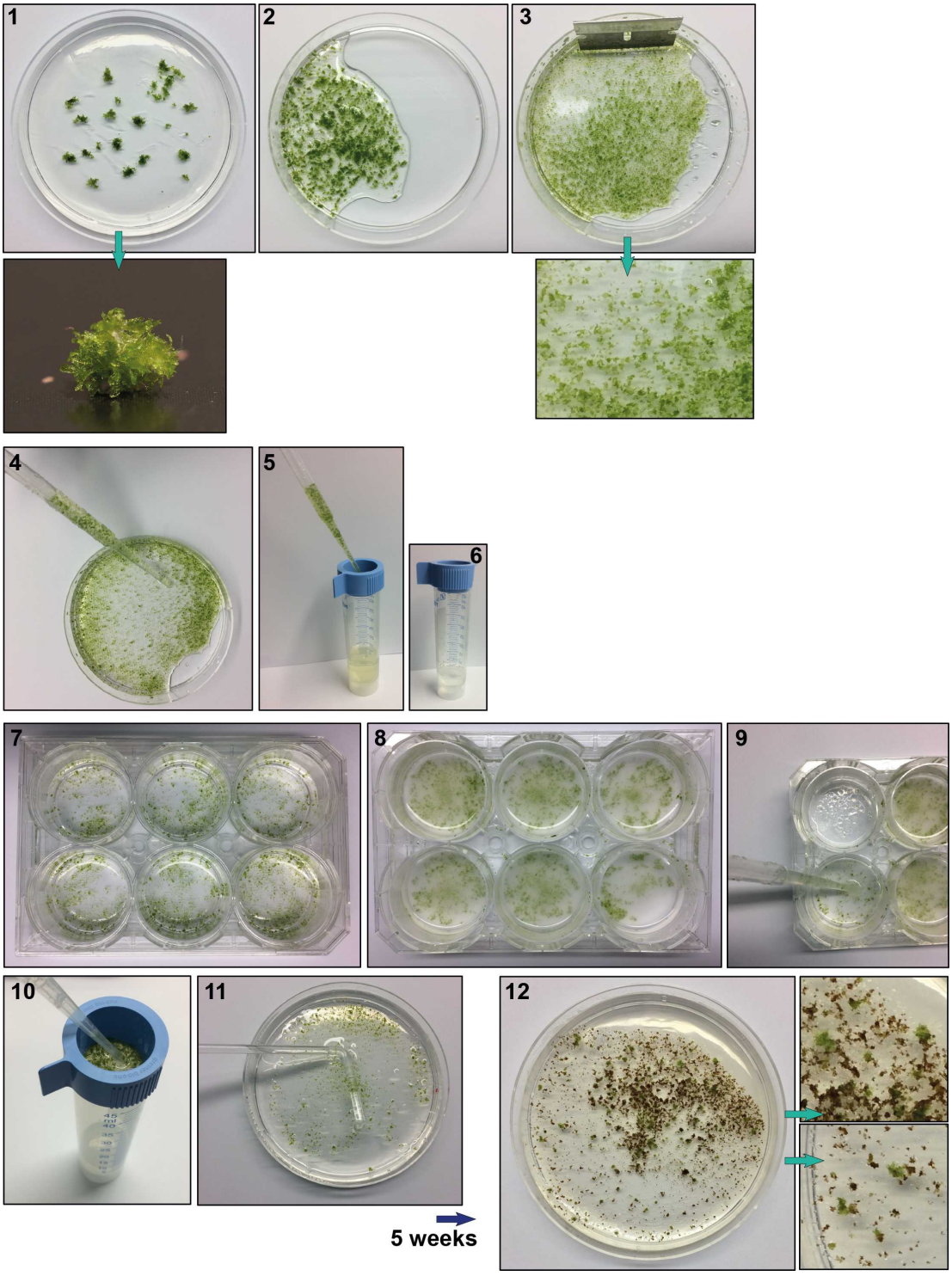

Figure 4

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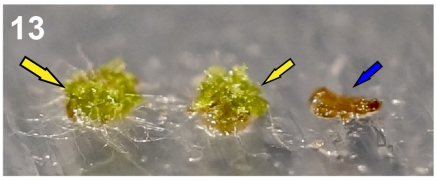

Figure 5: The emergence of rhizoids is an indication of successful transformation (yellow arrows: transformed thallus fragment, blue arrow: dying thallus fragment).

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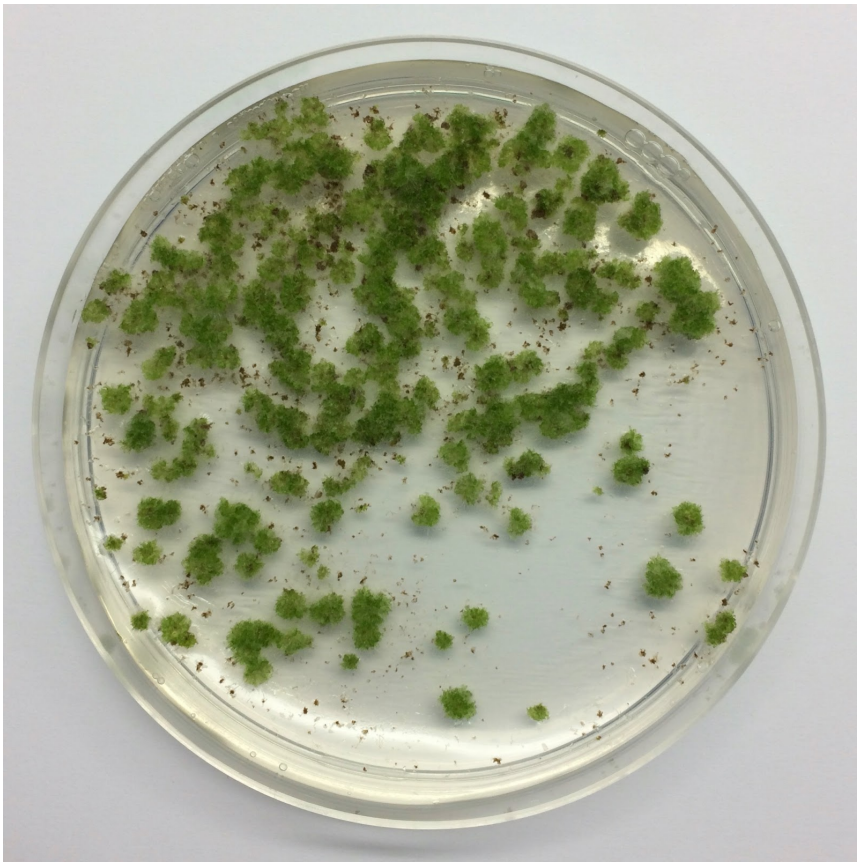

Example of plate with successful transformants 8 weeks after co-cultivation.

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Video 1, Example of tissue fragmentation for *A. agrestis* Bonn. The method is the same for *A. agrestis* Oxford.

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