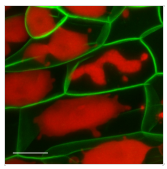

Jan 10, 2021

Anthoceros agrestis (hornwort) transformation v01

Eftychis Frangedakis[1]
[1]University of Cambridge

1 Works for me dx.doi.org/10.17504/protocols.io.6izhcf6

Eftychis Frangedakis
University of Cambridge, Plant Sciences

ABSTRACT

Anthoceros agrestis (hornwort) transformation

pre-print:
https://biorxiv.org/cgi/content/short/2021.01.07.425778v1

This protocol works better for the Oxford strain

DOI

dx.doi.org/10.17504/protocols.io.6izhcf6

PROTOCOL CITATION

Eftychis Frangedakis 2021. Anthoceros agrestis (hornwort) transformation v01. **protocols.io** https://dx.doi.org/10.17504/protocols.io.6izhcf6

CREATED

Aug 14, 2019

LAST MODIFIED

Jan 10, 2021

PROTOCOL INTEGER ID

26937

GUIDELINES

Be patient...

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 $FeSO_47H_2O$

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 $FeSO_47H_2O$
20 gr of sucrose (2% w/v final concentration)

pH to 5.8 with KOH

top up water to 1L after adjusting pH

Sterile disposable scalpels (#0501, Swann Morton)

Homogenizer (#727407, IKA Ultra-Turrax T25 S7 Homogenizer)

Dispensing tools (#10442743, IKA Dispersing Element)

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)

ABSTRACT

Anthoceros agrestis (hornwort) transformation

pre-print:
https://biorxiv.org/cgi/content/short/2021.01.07.425778v1

This protocol works better for the Oxford strain

Citation: Eftychis Frangedakis (01/10/2021). Anthoceros agrestis (hornwort) transformation v01. https://dx.doi.org/10.17504/protocols.io.6izhcf6

BEFORE STARTING

Pray

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** bottom left image.

2

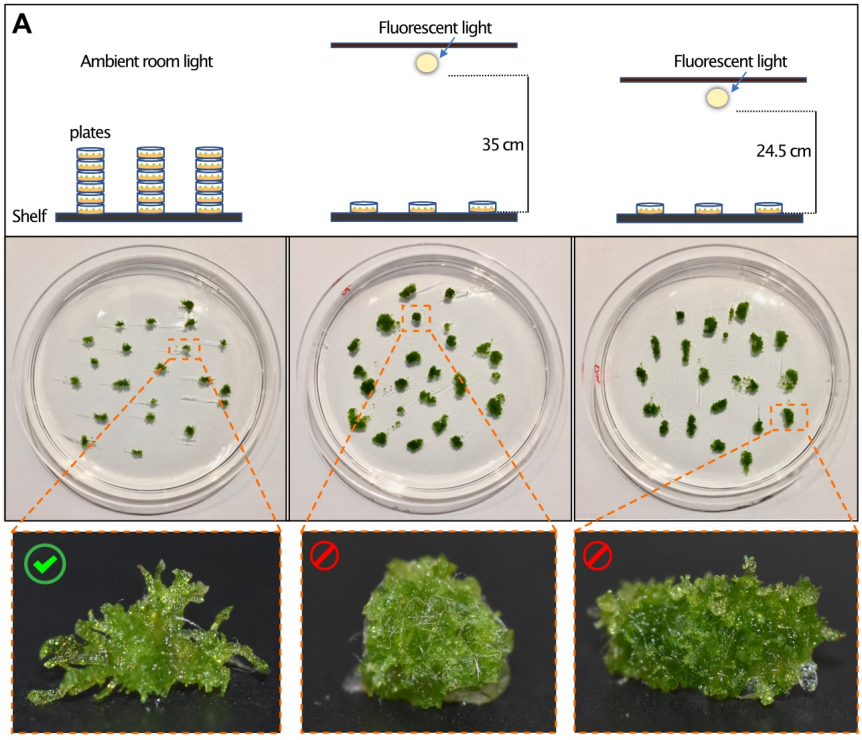

Figure 1: Effect of light on *A. agrestis* growth

Examples of *A. agrestis* tissue grown under different light regimes. From left to right: i) tissue morphology when plates were stacked in the laboratory under ambient room light (3-5 µmol $m^{-2}s^{-1}$), ii) tissue morphology under light supplemented by fluorescent tubes, 35 µmol $m^{-2}s^{-1}$, light intensity (PHILIPS, TL-D58W/835) and iii) tissue morphology under light supplemented by fluorescent tubes, 20 µmol $m^{-2}s^{-1}$light intensity (PHILIPS, TL-D 36W/840).

Tissue similar to (i) is optimal for transformation. Use of tissue similar to (ii) and (iii) should be avoided.

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

4

Citation: Eftychis Frangedakis (01/10/2021). Anthoceros agrestis (hornwort) transformation v01. https://dx.doi.org/10.17504/protocols.io.6izhcf6

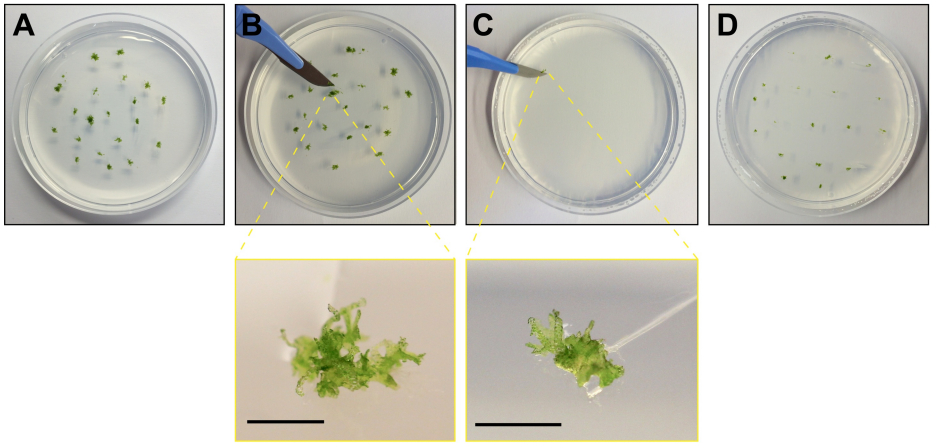

Figure 2: *A. agrestis* growth and tissue culturing

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

5

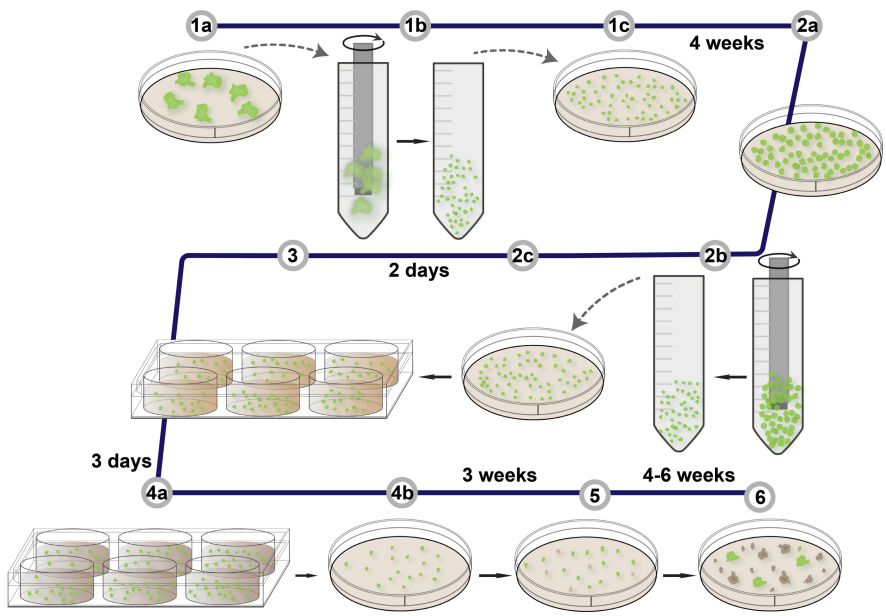

Figure 3: Transformation pipeline outline

1a-c: Tissue is homogenized, transferred on growth medium, and placed under low light conditions. **2a-c**: After 4 weeks, the tissue is homogenized again and grown for two additional days. **3**: The tissue is co-cultivated with *Agrobacterium* for three days and then **4a-b**: spread on appropriate antibiotic-containing growth medium. **5**: After 3 weeks, the tissue is transferred again onto freshly prepared antibiotic-containing growth medium for a second round of selection. **6**: After approximately 4-8 weeks, putative transformants are visible.

6 **Tissue preparation:**

- Collect approximately 2 g of thallus tissue grown for 4 weeks under low light intensity (approximately 0.1 g of tissue per petri dish - 20 petri dishes in total) - **Figure 4.1**
- Split tissue into three parts, transfer each part into a 50 mL falcon tubes containing 15 mL of sterile water and homogenize using a homogenizer and corresponding dispensing tools (for 5 sec, lowest power 8000 rpm) - **Figure**

Citation: Eftychis Frangedakis (01/10/2021). Anthoceros agrestis (hornwort) transformation v01. https://dx.doi.org/10.17504/protocols.io.6izhcf6

4.2-5

- Transfer the homogenized tissue from one falcon tube into a 100 µm cell strainer, wash with 50 mL of sterile water and transfer onto two petri dishes (six plates in total) containing solid KNOP medium add 2-3 mL of sterile water and place at 21°C under 12 hours light and 12 hours dark, light intensity 3-5 µmol $m^{-2}s^{-1}$ - **Figure 4.6-7**
- After 3-4 weeks transfer the tissue from the petri dishes into a falcon tube using sterile scalpels - **Figure 4.8-9**
- Re-homogenize in 15-20 mL of sterile water and filter using a cell strainer - **Figure 4.10-11**
- Spread out again onto 4 petri dishes with solid KNOP medium (if necessary, to facilitate spreading of the tissue, add 2 mL of sterile water to the petri dish) and allow to grow for 2 days at 21°C under continuous light (12 hours light and 12 hours dark also ok) (light intensity 30 µmol $m^{-2}s^{-1}$) - **Figure 4.12**

7 Agrobacterium culture preparation:

- Inoculate 5 mL LB media with a single *Agrobacterium* colony (AGL1 strain), 10 µg/mL rifampicin, 50 µg/mL carbenicillin and the plasmid-specific selection antibiotic.
- Incubate the preculture at 28°C for 2 days at 120 rpm.

8 Co-cultivation:

- Centrifuge 5 mL of 2 d *Agrobacterium* culture (OD: 2.5 - 2.7) for 7 min at 1800 xg.
- Remove supernatant and re-suspend in 5 mL liquid KNOP plus 2% (w/v) sucrose and 100 µM acetosyringone.
- Incubate the culture with shaking (120 rpm) at 28°C for 5 hours.
- Wash the regenerating thallus using a 100 µm cell strainer.
- Transfer ½ of the tissue from one plate into a single well of a 6-well plate containing 4 mL of liquid KNOP medium supplemented with 2% (w/v) sucrose - **Figure 4.13**
- Add 80 µL of *Agrobacterium* culture and 100 µM (final concentration) acetosyringone to the medium.
- Co-cultivate the tissue and *Agrobacterium* for 3 days with shaking at 110 rpm at 21°C with only ambient light from the room (1-3µmol $m^{-2}s^{-1}$) - **Figure 4.14**

9

1st selection:

- Using a sterile plastic pipette transfer the tissue from a single well into a 100 µm cell strainer, drain and then transfer on growth media containing the appropriate antibiotic. To facilitate spreading of the tissue, add 2 mL of sterile water to the petri dish - **Figure 4.15-17**
- Plate the tissue on solid KNOP plates supplemented with 100 µg/mL cefotaxime and 10 µg/mL Hygromycin and grow at 21°C under 12 hours light and 12 hours dark, 35 µmol $m^{-2}s^{-1}$

Citation: Eftychis Frangedakis (01/10/2021). Anthoceros agrestis (hornwort) transformation v01. https://dx.doi.org/10.17504/protocols.io.6izhcf6

10 **2nd selection:**

- After 3 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 $m^{-2}s^{-1}$ - **Figure 4.18**

- After 6-8 weeks successful transformants are visible on the petri dish (successful transformants can be identified using a microscope after 4 weeks selection based on rhizoid production) - **Figure 4.19 and Figure 5 & 6.**

11 **3rd selection:**

To eliminate false positives, transfer surviving tissue fragments again on 100 µg/mL cefotaxime and 10 µg/mL Hygromycin containing growth media. Grow at 21°C under 12 hours light and 12 hours dark, 3-35 µmol $m^{-2}s^{-1}$ - **Figure 7.**

Citation: Eftychis Frangedakis (01/10/2021). Anthoceros agrestis (hornwort) transformation v01. https://dx.doi.org/10.17504/protocols.io.6izhcf6

12

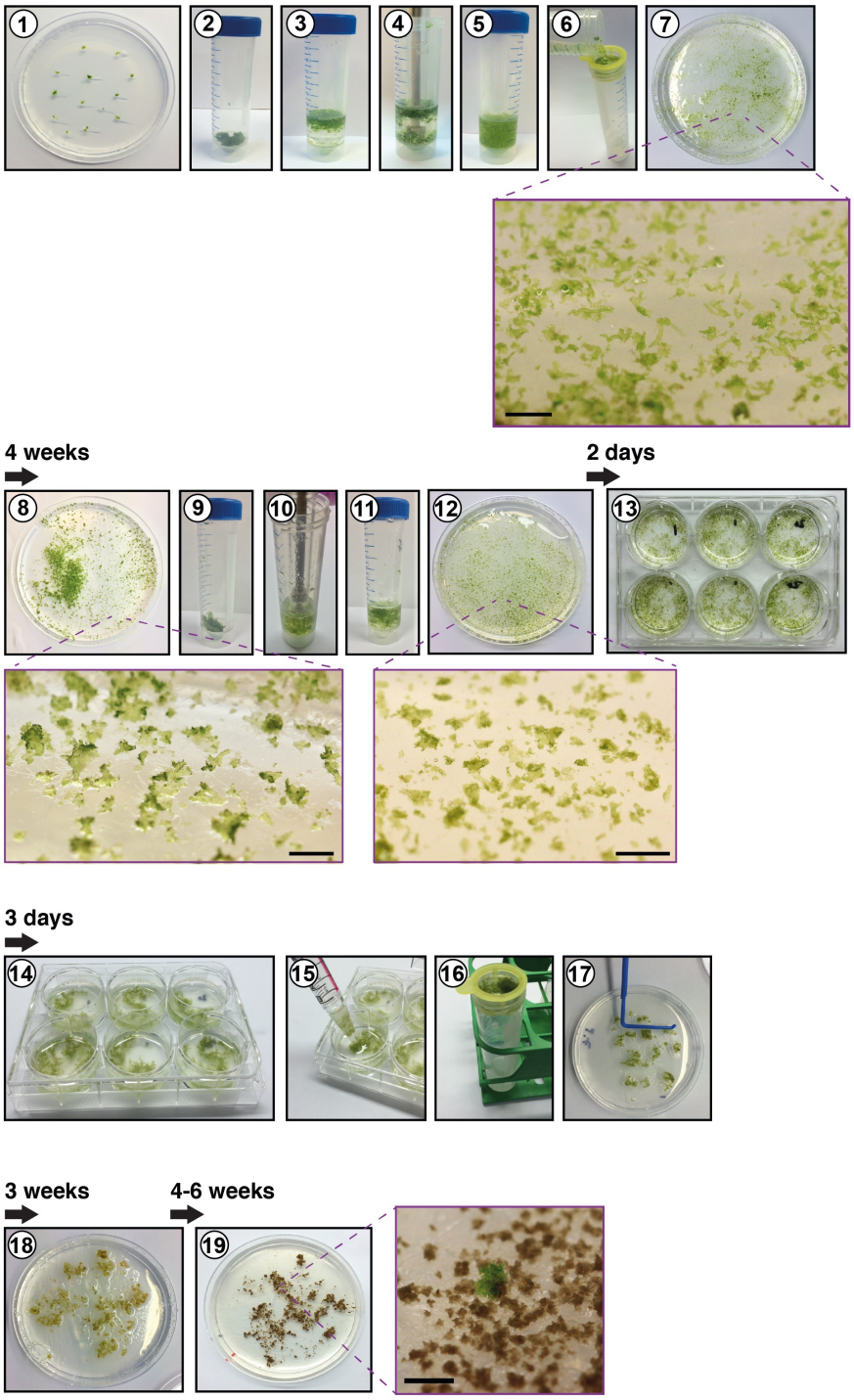

Figure 4: *A. agrestis* Oxford transformation protocol

Petri dish dimensions: 92 x16 mm.

Scale bars: 2 mm.

Citation: Eftychis Frangedakis (01/10/2021). Anthoceros agrestis (hornwort) transformation v01. https://dx.doi.org/10.17504/protocols.io.6izhcf6

13

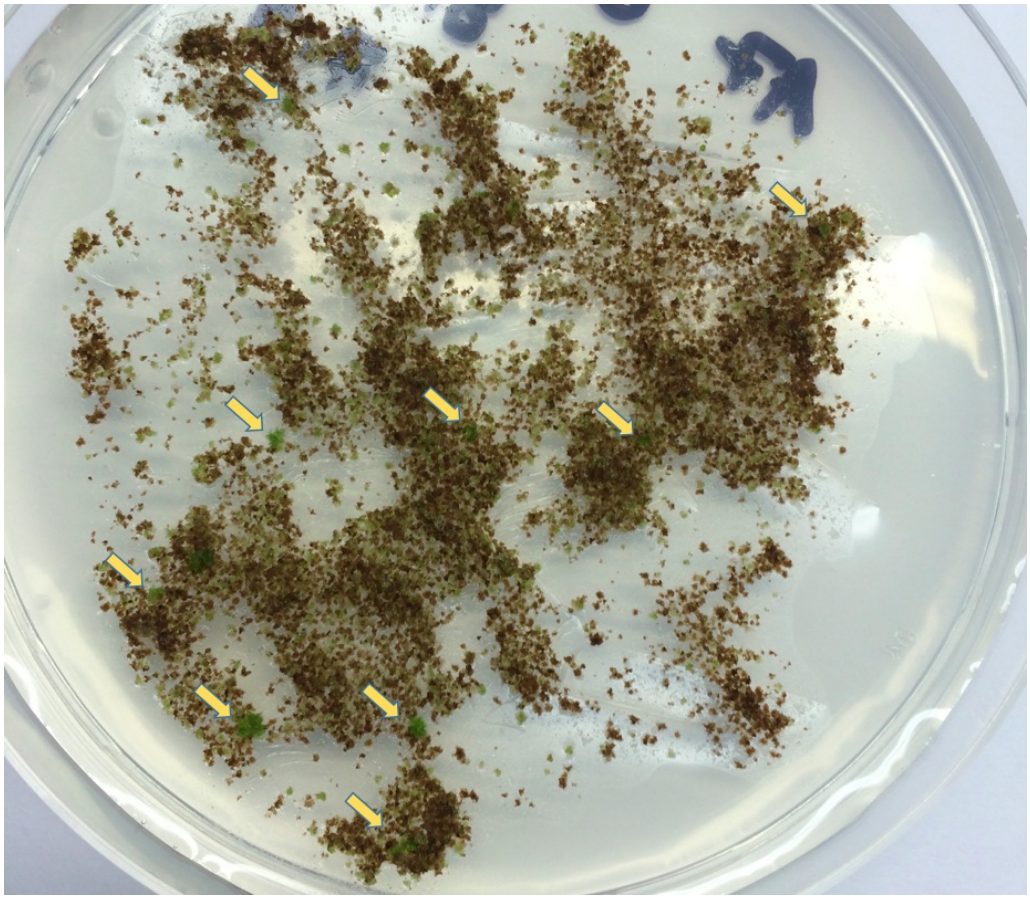

Figure 5: Example of plate with tissue after two months of selection.

Transformants are indicated with arrows
Petri dish dimensions: 92 x16 mm.

14

Figure 6: Transgenic regenerating thallus fragment.
Rhizoid indicated with an arrow head.

Citation: Eftychis Frangedakis (01/10/2021). Anthoceros agrestis (hornwort) transformation v01. https://dx.doi.org/10.17504/protocols.io.6izhcf6

15

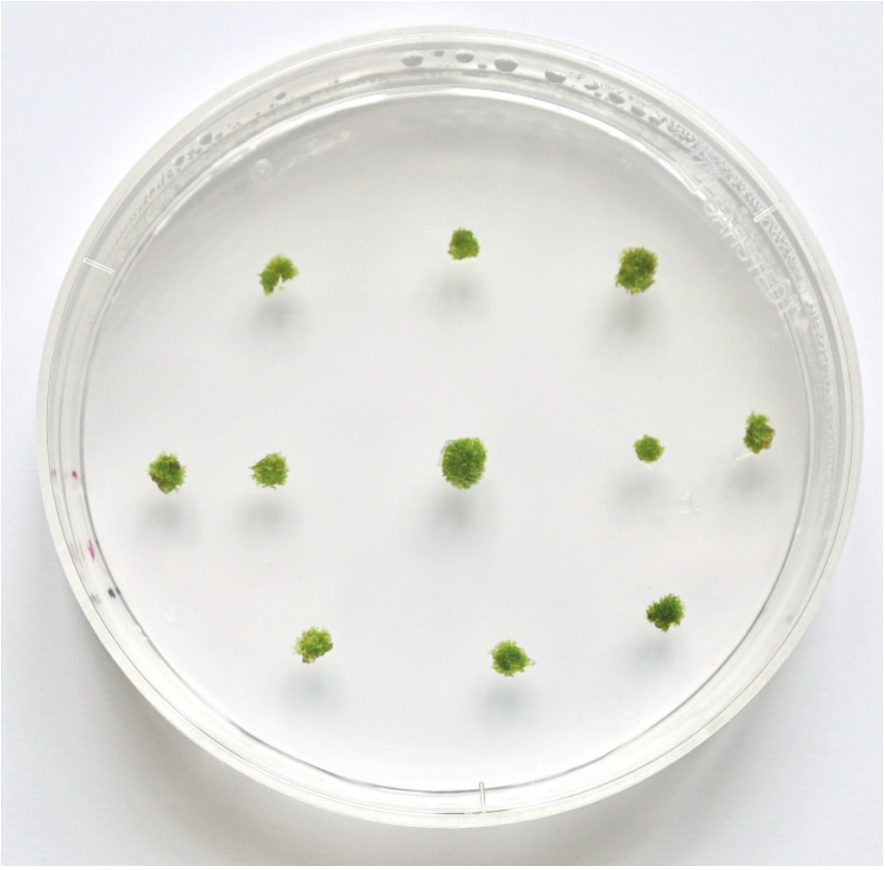

Figure 7: Tissue after 3rd selection

Petri dish dimensions: 92 x16 mm.

Citation: Eftychis Frangedakis (01/10/2021). Anthoceros agrestis (hornwort) transformation v01. https://dx.doi.org/10.17504/protocols.io.6izhcf6