Claudia Girnth and Anne Heister

Solrød Gymnasium
Solrød Strand, Denmark

Cloning a plant

Culturing African violets using the callus method

Plant tissue culture is an important technique in modern plant biotechnology. Cultured plants may be stored in gene banks where many different types of one species are deposited to conserve genes for plant breeding programmes. Tissue culture methods may also be used to create *clones* — that is, many identical copies of a plant with particularly good traits. This method is widely-used in commercial horticulture, *e.g.,* for houseplants and for producing stocks for agriculture where conventional methods are not possible or have serious practical drawbacks. For example, an oil palm must grow for many years before it is possible to see its characteristics. Since palms do not reproduce vegetatively, tissue culture is used to multiply selected individuals. The same technique applies whether you use oil palms or African violets *(Saintpaulia ionatha).*

Aim

This practical activity will give the students knowledge of the development of a callus and that it starts from an individual cell in the leaf. The cells divide rapidly and a lump of undifferentiated cells, a callus, is created. The cells will differentiate after some time and shots and roots develop depending on the type and concentration of plant growth substances ('hormones') in the culture medium. Small plants will develop in two to three months. This work will also allow students to become familiar with aseptic techniques.

CORRESPONDENCE TO
Solrød Gymnasium
Solrød Center 2
DK 2680 Solrød Strand
Denmark
Claudia.Girnth@newmail.dk

Equipment and materials
Needed by each person or group:

- African violet leaf, 1 per student (this is a minimum requirement, since some cultures are likely to become infected)
- Pair of metal forceps or around 12 pairs of pre-sterilised plastic forceps
- Scalpel with a new blade
- Permanent marker pen
- Small bottles with lids or beakers with aluminium foil for covering, a minimum of 3. *To be used for 70% ethanol, sterilizing solution and sterile water.*
- Sterile Petri dish
- Bunsen burner or nightlight to sterilise forceps and scalpel by flaming
- Sterile glass containers containing growth medium, 2 per leaf (baby food bottles are ideal)
- Beaker for waste
- 70% ethanol (**not** denaturated) for sterilising and de-waxing leaves
- 10% chlorine (or 3% NaOCl) with detergent or *Tween 20* added, for surface-sterilising plant tissue
- Sterile water, ~500 ml for rinsing sterile leaves

Procedure
A. To sterilise the plant material

N.B. This work must be done in a sterile area and under aseptic conditions

1. Take young leaves (about 2–4 cm long) from healthy plants. Dip them into 70% ethanol with the help of a forceps for 30 seconds. *Ethanol both sterilises the leaf surface and removes the waxy cuticle.*

2. Put the leaves into 10% chlorine solution (or 3% NaOCl) for 10 minutes. Stir carefully with a sterile forceps or similar every minute. *Chlorine or sodium hypochlorite kills both bacteria and fungi.*

3. Put the leaves into sterile water in a sterile beaker. *Cover the beaker to avoid airborne contamination*.

4. Pour off the water and add fresh sterile water to the

Fig. 1

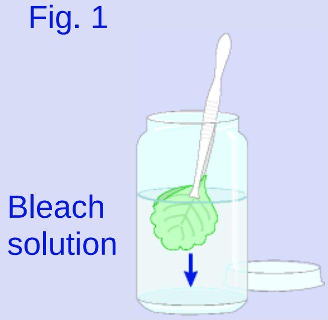
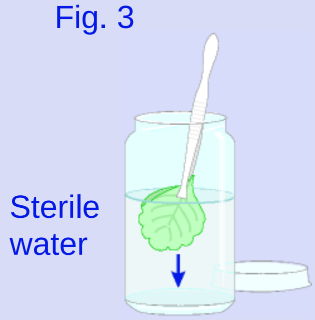


Fig. 2

Fig. 3



beaker. This should be done three more times *(i.e., four times in total)* to wash away all traces of the chlorine solution. *Leaves can be stored in sterile water for up to four hours.*

B. Preparation of the plant material for culturing

5. Take a sterile Petri dish and, without opening it, mark the bottom of the dish with an arrow, showing how the leaf should be oriented (tip of arrow = leaf stem). If you are right-handed the tip should point to the left. *The stem transports the water and hormones from the growth medium into the cells of the leaf; therefore the leaf's stem should face down towards the medium. Without careful orientation at this stage it will not later be possible to tell the correct orientation from small pieces of leaf.*

6. Open the marked Petri dish and place the leaf on the base in the correct position. Use the lid of the Petri dish as a cover as you do this — a second person can help holding the lid while you perform operations. *Note: Avoid falling contamination from objects or air movements, especially from people moving nearby.*

Fig. 4

Fig. 5

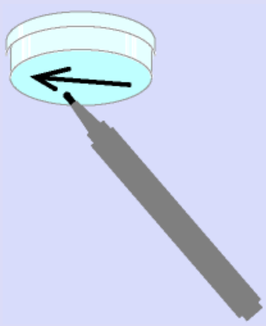


Fig. 6

7. Dip a scalpel in 70 % ethanol and set light to the alcohol, allowing it to burn off. **CAUTION! Keep naked flames away from the beaker of ethanol.** With this sterile scalpel, cut out a piece about 15 x 15 mm from the middle of the leaf. *The main vein of the leaf must be in the middle of this piece.* Now cut the piece into two, perpendicular to the middle vein (see Fig. 7). The rest of the leaf may be thrown away.

8. Transfer the leaf pieces onto the growth medium as follows:

   Open the culture bottle with the growth medium and push the leaf piece in vertically so that 1/3 of it is in the jelly. *Be careful to put the part that faced the stem down into the agar (i.e., in the direction of the arrow-head you drew).* Seal the culture bottle again. *If you* *use aluminium foil as a lid you must put tape around to avoid both contamination and evaporation of water. The bottles can be closed tightly to maintain a high humidity within.*

C. Plant material is growing

9. Put the bottle(s) in a light place, but avoid direct sunshine. If the medium starts to dry out, add some sterile water. During a period of 6–9 weeks a callus will develop, which looks like a small, light green (or brown) irregular clump of cells.

Fig. 7

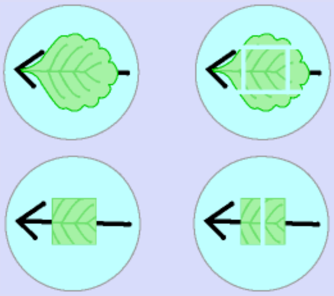
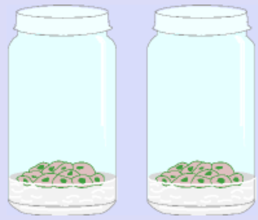


Fig. 8

Fig. 9



10. After several weeks the callus culture will spotaneously regenerate small leaflets and roots.

11. When the plants are about 20 mm tall they can be transferred to soil in a pot. *Note: Cover each pot with a plastic bag so that the plants do not dry out as they will at first have no leaf cuticle to prevent moisture loss. The plantlets are very delicate when they are so small.*

Fig. 10

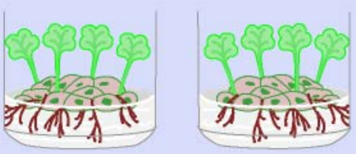


Fig. 11

Fig. 12

Results

The plants must be tended at least once a week. Changes can be photographed or/and recorded in a notebook. For example:

Plant material:
Date of treatment:
Type of treatment:
Name of observer:

Description of changes	Date of observation	Remarks

Expected development

This schedule outlined below (under 'timing') can be used as the basis for a record of the development either for one culture or as a summary of the general development of several cultures.

Safety

Ethanol

Be very careful with ethanol and burners. Keep the ethanol on one side of the table and the burner/flame on the opposite side – far away from each other.

Chlorine solutions

Be careful with the chlorine or sodium hypochlorite (bleach) solutions since they are toxic and can irritate the skin, eyes and upper respiratory tract. They can liberate dangerous gases if mixed with acids or ammonia.

During use, avoid contact with eyes, skin and clothing. Protective clothing and eye protection should be worn. In case of eye contact flush with plenty of water. Wash thoroughly after handling.

Plant growth substances (hormones)

Note: The teacher, not the students, must prepare the solutions. The growth-hormones IBA (Indole-3-Butyric Acid) and BAP (6BenzylAminoPurine) are itrritating to the skin, eyes and upper respiratory tract. Use protective glasses, clothing and gloves while handling these chemicals.

In case of eye contact immediately flush with plenty of water for at least 15 minutes. Seek medical attention, with skin contact wash immediately with plenty of soap and water for at least 15 minutes. If inhaled or swallowed wash out the mouth and seek medical attention.

Timing

The experiment takes about 2 hours for a group of 4 students. The development of the culture takes up to 3 months.

Swelling of plant material	1–2 weeks
Visible callus	2–3 weeks
Roots and root hairs visible	3–5 weeks
Green shoots visible	4–6 weeks
First sign of leaflets	6–7 weeks
Transfer to soil	8–10 weeks
The first new leaf	12–14 weeks

Troubleshooting

Plant tissue culture media contain large amounts of sucrose as a carbon source. Such media are therefore an excellent substrate for contaminating microorganisms and consequently the most common cause of failure is inadequate aseptic technique. It is therefore essential that students should know how to carry out aseptic procedures.

The work area should be swabbed down before starting. Doors and windows should be closed. Movement should be kept to a minimum and all required items should be within easy reach. Forceps must be flamed before use by dipping them in 70% ethanol and igniting the alcohol so that it burns off. Beakers must be sterilised by heating them in an oven for 2 hours at 160 °C covered by aluminium foil. The lid of a sterile Petri dish serves as a cover — one student can help with this, while the other student performs the manipulation of the leaves. This procedure helps to prevent micro-organisms from falling into the material.

Further investigations

Ideas for quantitative work

This can only be done when there is enough material — a minimum 20 cultures growing on the same kind of medium.

- **Diagram showing stage of growth (y-axis) against time (x-axis).** Record when the first sign of a stage, as described above, occurs.
- **Bar chart showing stage of growth (y-axis) against time (x-axis).** It is possible to record a single stage or a few easily-recognisable stages *e.g.,* the emergence of the first leaf.

- **Graph showing the level of contamination.** Count dead or infected cultures at different times (for example once a week) and draw a diagram with the time as x-axis and non-contaminated cultures (as a percentage of the total) on the y-axis.
- **Diagram showing the number of leaves / shoots / roots.** The number of organs present at different times are recorded and displayed as a diagram (number on the y-axis and time on the x-axis).

Culturing other species

Other species can be cultured *e.g.,* tobacco or cauliflower (see 'Further reading'). The students can of course try all sorts of leaves with the hormone mixture for African violet, but usually this does not work very well. This can provide a start for discussions and further investigations.

Suppliers

Chemicals for the tissue culture media and plant growth substances can be obtained from suppliers such as Sigma-Aldrich. Although it is possible to make up growth media from scratch (see 'Recipes'), it is often cheaper and more convenient to buy ready-made media to which macro-nutrients (sucrose) and growth substances are added. Several of the major school science suppliers also sell kits for plant tissue culture.

Innovative kits for home and educational use that contain a biocide, dramatically reducing the level of contamination, can be purchased from 'Kitchen culture kits' in the USA: http://www.kitchenculturekit.com

Further reading

Hartmann, H.T., Kester, D.E., Davies, F.T. and Geneve, R.L (2001) *Hartmann and Kester's Plant propagation: principles and practices,* 7th edition. Prentice Hall. ISBN: 0 13 679235 9.

Plant tissue culture by Tony Storr (1985) Hatfield: Association for Science Education. ISBN: 0 86357 031 3.

This booklet is out-of-print. However, a facsimile of the original 1985 publication can be downloaded from the NCBE Web site: *http://www.ncbe.reading.ac.uk/NCBE/PROTOCOLS/* planttissue.html

Recipes

Sterilising solution

1000 ml 3% sodium hypochlorite with 2 drops of *Tween 20* or other detergent added.

Growth medium for African violet

To 1000 ml of Murashige and Skoog (M&S) medium add 8-10 gram agar, 5 ml IBA and 15 ml BAP stock solutions (see below). M&S medium can be stored up to 3 months, if reheated before using it.

Note: When adding M&S medium to the culture bottles the layer should be at least 20 mm deep. This is because cultures are usually kept for prolonged periods, and thinner layers will dry out and provide insufficient rooting space.

Stock plant growth substance solutions and culture medium for callus

I. All the chemicals and their concentration are listed in the table.

II. Make up solutions nr 1-6 and 8-14, by weighing the amount as written in the row “stem-solution g/100 ml” and dissolve each of them in 100 ml of water. Some solutions (No. 1 and 2) are very concentrated, which means it takes time to solve them even during constant shaking or stirring.

100 ml of solutions nr 1-6 lasts for 10 preparations, while 100 ml of solutions nr 8-14 lasts for 40 preparations.

Solution nr 14 should be kept in a brown bottle.

III. Stem solutions nr 11-14 are micronutrients and therefore the amount of the different chemicals is very small.

Please note the remarks, marked with asterisks (“**”) below the table.

IV. Stem solutions nr 15 - 18 are organic compounds used by the plant culture as vitamins. These solutions cannot be kept at room temperature. You can make them fresh each time, or you can freeze adequate proportions for future use. Adequate proportions are for example: 1 ml of nr 15 + 1 ml of nr 16 + 1ml of nr 17 + 1 ml of nr 18; all together frozen in one flask. This portion can be added to 1 L of MS, when preparing for the experiment.

V. The hormones used in these experiments can tolerate to be heated during autoclave procedure. Please do not use other hormones, since various hormones differ in dose.

Note: The hormone stem-solutions must be prepared by the teacher and not the students, since they are hazardous. See also under “Security”.

Stem solution nr 22: 20 mg IBA should be dissolved in about 1 ml absolute alkohol in a small beaker. The liquid should be sucked with a pasteur pipette and be dropped into 100 ml deionised water during stirring. The solution can be kept in a refrigerator for some weeks. During a longer period (1-2 years) the solution can be deep-frozen in smaller parts. The solution has to be shaken carefully before using, so re-crystallized hormone is dissolved.

Stem solution nr 23: 20 mg BAP should be dissolved in about 1 ml 1 M NaOH in a small beaker. The liquid should be sucked with a pasteur pipette and be dropped into 100 ml deionised water during stirring. The solution can be kept in a refrigerator for some weeks. During a longer period (1-2 years) the solution can be deep-frozen in smaller parts. The solution has to be shaken carefully before using, so re-crystallized hormone is dissolved.

VI. All other chemicals (nr 7, 19, 20, 21) are added as solids.

Production of MS-medium when stem solutions are being used:

The medium should be prepared in a 1 L glass bottle with thick walls and a cap that can be autoclaved.

1. Add 10 ml each of the solutions nr 1-6.
2. Add 2.5 ml each of the solutions nr 8-14.
3. Add water to 1000 ml and shake the bottle.
4. Add 1 ml each of the solutions nr 15-18 (freshly made or thawed, if kept in a freezer, see IV).
5. Substances nr 7 and 19 are weighed and added as solids. Shake the bottle to dissolve them properly.
6. Add the hormones nr 22 and 23 according to the plant material, that you want to cultivate.
7. Adjust pH to 6-7 with 1.0 or 0.1 M HCl or NaOH.
8. Add nr 20 sucrose and nr 21 agar to the solution and shake.
9. Autoclave at $120^{\circ}C$ for 20 minutes.
10. Let the solution cool a little (though not below $60^{\circ}C$) and pour the medium into sterile flasks or other sterile containers. If you do not use all medium, you can use it later. The medium can be melted 2-3 times, if kept sterile. The medium should be about 2 cm thick in the bottom of the culture glass.

Use of readymade MS-medium:

Several companies, among them Sigma, sells portions of MS media, some contain everything except hormones (Sigma M9274). These products can also be used - ask your supplier. But be careful with the type of MS that already contain hormones. The concentration may not be suitable for the plant that you investigate. But these media can be used for testing different plants - you may be surprised what happens.

Culture glasses:

There are basically three types:

- Made of glass, either small marmalade or vegetable glasses (the students have to collect them in time) or buying some at laboratory firms. It is important, that the lid of the glass can take heat of the autoclave, then this type of glass is reusable.
- Sterile plastic cups, usually used for urine- or blood samples. Look in catalogues, but look for the ones that are clear plastic - you want to see the plants inside. These are for one time use only.
- Re-usable plastic cups, can be autoclaved several times, look in catalogues for this type of glasses.

Stem-solutions and chemicals for making of MS-medium

Nr (conc.)	chemical formula	name	stem solution g/100 ml	ml for 1 litre medium	mg/liter
1 (100x)	KNO_3	potassium nitrate	19.0	10	1900.00
2 (100x)	NH_4NO_3	ammonium nitrate	16.5	10	1650.00
3 (100x)	$CaCl_2x2H_2O$	calcium chloride	4.4	10	440.00
4 (100x)	$MgSO_4x7H_2O$	magnesium sulphate	3.7	10	370.00
5 (100x)	KH_2PO_4	potassium dihydrogene phosphate	1.7	10	170.00
6 (100x)	$(EDTA)Na_2$	sodium-EDTA	0.37	10	37.30
7 (solid)	$FeSO_4x7H_2O$	ferrous(II)sulphate	add as solid	*** 10 mg	27.80
8 (400x)	$MnSO_4x\ H_2O$	manganese sulphate	0.68	2.5	17.00
9 (400x)	$ZnSO_4x7H_2O$	zinc sulphate	0.344	2.5	8.60
10 (400x)	H_3BO_4	boric acid	0.248	2.5	0.83
11 (400x)	$CoCl_2x6H_2O$	cobalt(II)chloride	** 10 mg/ 1000 ml	2.5	0.025
12 (400x)	$NaMoO_4x2H_2O$	sodium molybdate	* 10 mg/ 100 ml	2.5	0.25
13 (400x)	$CuSO_4x5H_2O$	copper(II)sulphate	** 10 mg/ 1000 ml	2.5	0.025
14 (400x)	KI	potassium iodide	* 33 mg/ 100 ml	2.5	0.83
15 (conc)		glycine	* 200 mg/ 100 ml	1.0	2.00
16 (conc)		nicotinic acid	* 50 mg/ 100 ml	1.0	0.50
17 (conc)		pyridoxine HCl	* 50 mg/ 100 ml	1.0	0.50
18 (conc)		thiamine HCl	* 10 mg/ 100 ml	1.0	0.10
19 (solid)		m-inositol	add as solid	*** 100 mg	100.00
20 (solid)		sucrose	add as solid	*** 30 g	30000.00
21 (solid)		agar	add as solid	*** 8-10 g	10000.00
22 (conc)		IBA	* 20 mg/ 100 ml	varies	
23 (conc)		BAP	* 20 mg/ 100 ml	varies	

Note the * -marked solutions – the special circumstances are described below:

* The solved amount is small; 10 mg is the smallest amount suitable for weighing.
** Note that the volume is 1000 ml and not 100 ml.
*** No stem-solution is made; the amount written in the table is added directly to 1000 ml MS-medium.

