National Centre for Biotechnology Education

University of Reading

Cloning Cauliflower

1.1 Teacher's and technician's notes

Introduction

Plant tissue culture is widely used in commercial horticulture and scientific research. It involves growing individual plant cells or tissues in aseptic conditions. In theory, almost any part of a plant can be used as the source of cells (the 'explant') from which tissue is cultured. In practice, however, young, actively-dividing (meristematic) cells give the best results. When placed on a sterile growth medium containing the right blend of vitamins, minerals and plant hormones (plant growth regulators), plus sucrose as a source of energy, these cells can be triggered into growing and dividing. Under the influence of light and various plant growth substances, they can produce shoots and roots, then will eventually develop into small plants.

Plant tissue culture methods were first devised by scientists in the 1950s and are now used routinely to study biochemistry, cell biology and genetics.

Tissue culture is applied in a commercial context too — micropropagation techniques enable the production of large numbers of disease-free, genetically-identical plants (clones) for sale. Increasingly, the technique is also used to rescue critically-endangered species, by growing many plants from a small amount of starting material so that they can be re-introduced into habitats from which they have almost become extinct.

Conservation Biotechnology at the Royal Botanic Gardens, Kew, specialises in saving plant species on the verge of extinction. Tissue culture forms a vital part of their strategy. For instance, *Cylindrocline lorencei* is a small tree which was native to Mauritius. It became extinct in the wild in 1990, when the last-known specimen died. Although some seeds had been collected from the tree, these could not be germinated. Using tissue culture methods, however, scientists at Brest Botanic Gardens (in Brittany) managed to culture plants from the embryos in the seeds. Back-up cultures were sent to Kew for further micropropagation. Fifteen rooted plants were eventually sent from Kew to Mauritius for potential re-introduction.

Educational context

In the brief period after the introduction of the GCSE examination in England and Wales, but before the National Curriculum came fully into force, biotechnology education flourished. Practical experience of plant tissue culture was called for in many biology and in some science syllabuses — notably in the first incarnation of the Suffolk Science GCSE course.

A landmark publication triggered this interest: *Plant tissue culture* by Tony Storr was published by the ASE in 1985 (see *Further information*, page 8). The plant most frequently used for such work in schools and colleges was the cauliflower. Carrots were sometimes tried, but it was difficult to isolate the actively-dividing meristematic tissue from the tap root. Cauliflower, in contrast, proved ideal for school use because it was readily-available, easy to isolate the meristematic tissue from (the tips of the white curd) and robust enough to withstand the rigours of being handled by students. Cauliflower tissue also grows rapidly: growth can be seen after 10 days and plantlets are ready for transplantation into soil within 12 weeks. There was one persistent problem, however: maintaining aseptic conditions.

With conventional plant tissue culture methods, you have to surface sterilise the isolated pieces of plant tissue (explants) in bleach solution. This must then be removed by several successive washes with sterile water. Not only is this a lot to prepare, but students rarely achieve success. Too often, they see no growth at all (the plants are killed by the bleach) or black fungus grows over everything (due to incomplete surface sterilisation or poor aseptic technique).

Several solutions have been proposed. Antimicrobial additives such as silver nitrate, antibiotics and even an extract of rowan berries (*Sorbus* sp.) have been added to plant growth media with varying degrees of success. None of these methods have overcome the need for autoclaving the growth media and rinsing water however, and all have added significant expense.

The method used in this kit is different. It was devised by the Conservation Biotechnology team at the Royal Botanic Gardens, Kew. It was developed for use in situations where laboratory facilities are limited, but it is also excellent for use in schools. The materials are easy to prepare, the procedure is straightforward and it gives good results. No autoclaving of media or rinsing water is required — the growth medium simply needs to be microwaved. The aseptic techniques are safe and reliable — no Bunsen burners are required and neither does disinfectant have to be rinsed from the plant tissue.

Equipment and materials

Each student or working group needs

Straight from the kit

- a copy of the Student's guide [1]
- a 7 mL bijou bottle with cap
- a pair of plastic forceps [2]

Prepared in advance by the teacher or technician

(see pages 4–5 of this guide for instructions)

- *Virkon*® solution, for sterilising the work surface, made by dissolving each tablet in 500 mL of tap water.
- 80 mL of *Milton*® solution, made using half a 4 g *Milton*® tablet in each 80 mL of water, for sterilising the forceps and the cauliflower tissue [3]
- a vial containing ~10 mL of growth medium

Not in the kit: supplied by you

- a permanent marker pen
- a scalpel
- a non-sterile Petri dish lid or base, cutting board or tile
- a small (*e.g.*, 100 mL) beaker for waste
- a small floret from a cauliflower
- paper towels
- sunny windowsill or lights to illuminate the plant cultures

Notes

1. Supporting *PowerPoint* and *Keynote* presentations are available on the NCBE's web site: **www.ncbe.reading.ac.uk/ptc**
2. Metal will be damaged by prolonged immersion in *Milton*® solution.
3. Note that only the tablet form of *Milton*® is suitable. The liquid form of the product is another chemical entirely.

Preparing the growth medium

For 500 mL of growth medium you will need

Equipment

- Eye protection
- Heat-proof gloves
- Microwave oven
- Balance
- 250 mL measuring cylinder
- 1 mL syringe for the kinetin stock solution
- 10 mL syringe for the *Milton*® solution
- 1 L beaker (or plastic jug), to fit inside microwave
- Glass stirring rod

Materials

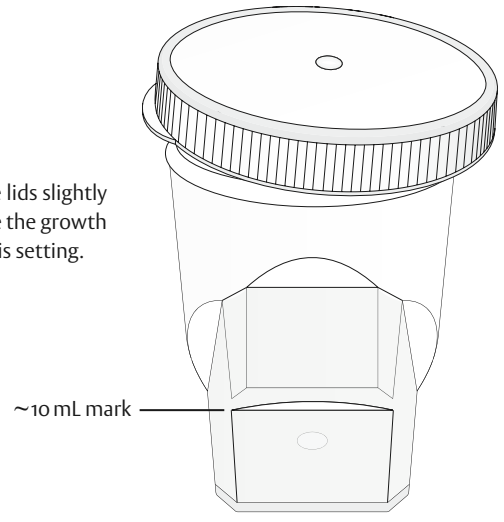
- 6 g Murashige and Skoog (MS) agar powder
- 10 g (4 sachets) granulated cane sugar [1]
- 1.25 mL kinetin solution (containing 0.001 g of kinetin per millilitre of 70% ethanol) [2]
- *Milton*® stock solution (that is, one 4 g *Milton*® tablet dissolved in 100 mL of distilled or deinonised water) [3]
- Sterile vials with caps [4]
- Clingfilm
- *Virkon*® disinfectant solution or 70% ethanol and paper towels for wiping down the bench surface

Notes

1. Cane sugar is purer than that produced from sugar beet, and is therefore preferable here.
2. If you wish, eventually, to grow the plants in soil (see page 6), add 0.65 mL of kinetin solution to half of the growth medium at Step 6, and do not add any kinetin to the other half. If you wish, you can colour-code the different media by adding 0.5 mL of food colouring to each 250 mL batch of medium.
3. You *must* use *Milton*® tablets, not *Milton*® liquid, which is a totally different chemical.
4. The vials that are supplied in the kit are manufactured under sterile conditions and should be handled carefully (as if they were Petri dishes) to ensure that they remain sterile.

Note

Leave the lids slightly ajar while the growth medium is setting.



How to prepare the growth medium

1. Measure 480 mL of distilled water into a litre beaker.
2. Add the sugar and stir until dissolved.
3. Add the MS agar powder, stirring constantly until it is evenly dispersed in the liquid (the agar will not dissolve fully until the mixture is heated).
4. Cover the beaker with clingfilm, pierced several times to allow steam to escape.
5. Heat the beaker in a microwave oven on high power for 90 seconds, stir, re-cover and heat for another 2 minutes (these times may need to be varied according to the power rating of the oven. The times given are for a 500 mL volume in an 800 W microwave oven). **IMPORTANT: You must watch the liquid constantly as it is heating to ensure that it does not boil over.**
6. When the agar has dissolved completely, and the solution has boiled, take the beaker from the oven and stir 1.25 mL of kinetin solution into the liquid [but see note 2]. **CAUTION: Any solution heated in a microwave oven may become superheated and boil vigorously and suddenly when moved or touched. Take great care when handling the beaker and *ensure* that you wear heat-proof gloves.**
7. Allow the liquid to cool to about 50 °C (that is, still liquid but cool enough to reduce chlorine release in the next step). It will take about 30 minutes for the agar to cool to 50 °C. The agar will begin to set at about 42 °C.
8. In a well-ventilated area or a fume cupboard, add 20 mL of the *Milton*® stock solution to the beaker of heated and cooled growth medium [remember, if you are preparing half of the medium without kinetin, you will need to add 10 mL of the *Milton*® solution to each 250 mL volume of medium]. Stir well, taking care not to breathe in any chlorine fumes.
9. Dispose of the remaining *Milton*® stock solution. **N.B. You must dilute the waste *Milton*® solution with plenty of water as you dispose of it — it is highly toxic to aquatic organisms.**
10. Immediately dispense the medium into the vials as follows:
    - wipe down the work surface with *Virkon*® solution.
    - remove the lids from the vials and place them on the sterilised work surface, inner side downwards.
    - dispense the medium into the vials (~10 mL per vial)
    - leave to cool with the lids on top of the vials but slightly ajar to allow steam to escape (see picture).
    - when the medium has cooled and set (after about 60 minutes), press the caps down to seal the vials.

Storage

Once cool, the vials of growth medium can be stored for about six weeks in a fridge at 3–5 °C until required.

Safety

Virkon®

Virkon® is a wide-spectrum surface disinfectant which is suitable for use in school laboratories. It kills bacteria, fungi and viruses.

Each pink tablet should be dissolved in 500 mL of tap water before use. Wear a lab coat, suitable protective gloves and tightly-fitting safety goggles when making up the solution. Avoid splashes of concentrated solution by adding the tablet to the water (NOT the water to the tablet).

Avoid raising an airborne dust: you should NOT grind the tablets up to encourage them to dissolve (they will dissolve quickly without being powdered).

Once made up, the solution should be used within 5 to 7 days (the pink colour fades when the solution is no longer active). *Virkon*® solution at 1% concentration does not irritate the skin and eyes, therefore eye protection and gloves are not essential. Unlike chlorine-based disinfectants, *Virkon*® does not have a toxic vapour and therefore extra ventilation is not required.

Milton®

Milton® is widely used for disinfecting babies' bottles *etc*. Its main active ingredient is sodium dichloroisocyanurate (SDICN, also known as triclosene sodium). When used at the dilution suggested on the packet, it is very safe to handle. In the current practical activity, however, it is being used at several times the recommended concentration.

Because of this, it is *essential* that eye protection is worn by students undertaking this task. Splashes in the eye should be rinsed with water and medical attention should be sought immediately. Splashes on the skin or work surfaces should be washed with water. Concentrated *Milton*® solution can bleach fabrics, so lab coats should be worn to prevent accidental damage to clothing.

WEAR EYE PROTECTION

WEAR A LAB COAT

As mentioned on page 4, teachers or technicians should ensure that the *Milton*® stock solution is added to the growth medium in a well-ventilated area (ideally in a fume cupboard), as chlorine gas can be released when the disinfect is added to hot, molten agar.

For educational reasons, you may wish to label the *Milton*® solution given to students with the appropriate GHS hazard symbols (the 'Exclamation mark' and 'Environment' pictograms as shown in the Student's guide).

Microwave ovens

Because it contains a disinfectant, the growth medium needs only to be microwaved and does not need to be autoclaved.

There are three potential hazards when heating agar media in a microwave oven. Firstly, the medium must be placed in an *open* container so that any gas can escape (heating media in a sealed container risks a dangerous explosion).

Secondly, there is a risk that the viscous medium will bubble over the top of the container. It should therefore be watched constantly and the power should be stopped (usually by opening the door of the microwave oven) at the first sign of overheating.

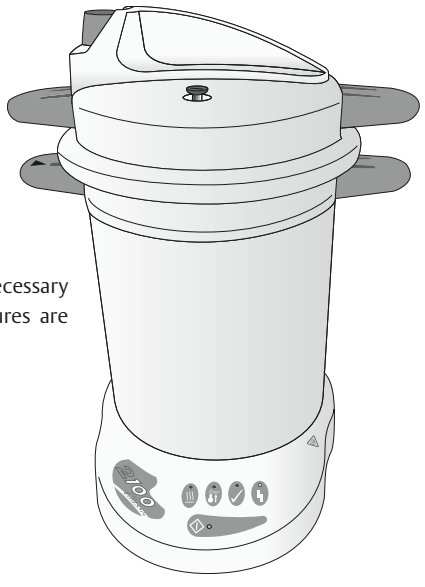
Thirdly, as with any liquid heated in a microwave oven, there is a danger that the agar medium may become superheated and froth up unexpectedly when the container is taken from the oven. For this reason, heat-proof gloves must be worn when handling the beakers of hot liquid.

Scalpels

Care should be taken when using scalpels.

Contaminated cultures

In the unlikely event of any plant tissue cultures becoming contaminated with microorganisms, the vial(s) should not be opened, but instead be placed in an autoclave bag and sterilised by autoclaving.



Note
Autoclaving is necessary only if the cultures are contaminated.

The procedure in words

This can be used in conjunction with the Student's guide

It can be used as a sequencing exercise, where students are given the sentences below in a random order, and have to place them in the correct sequence with the aid of the pictures in the Student's guide.

Put on a lab coat.

Wear eye protection (safety glasses or goggles) throughout the procedure.

Wash your hands with antibacterial soap.

Wipe down the work surface using disinfectant solution and paper towels.

Set out the work area with everything you need: plant growth medium containing kinetin, vitamins and minerals; *Milton*® disinfectant solution; a piece of cauliflower curd; a scalpel; a surface for cutting on, such as a tile or Petri dish; a pair of plastic forceps; a waste container; a bijou bottle; and a marker pen.

Cut some small pieces about 5 mm x 5 mm x 5 mm from the outer part of the cauliflower curd. These will be the explants from which cauliflowers will be cloned.

Pour approximately 5 mL of *Milton*® disinfectant into the bijou bottle.

Use the forceps to place the explants in the bijou bottle of disinfectant.

Tightly cap the bijou bottle.

Place the forceps in the beaker of *Milton*® disinfectant.

Gently shake the bijou bottle so that the explants are fully coated in disinfectant solution.

After 5 minutes, gently shake the explants again.

After 10 minutes, gently shake the explants again.

After 15 minutes, gently shake the explants again.

Take the cap off the bijou bottle and, holding the bottle against the side of the waste container so that the cauliflower explants are not lost, carefully pour off the disinfectant.

Use the now sterile forceps to place the explants on the growth medium. Do this quickly, and do not place the lid of the vial on the bench, to reduce the risk of microbial contamination.

Gently push the explants slightly into the surface of the growth medium so that they are held there.

Snap the lid fully onto the vial.

Label the vial with your initials and the date.

Place the vial in a warm, well-lit place.

Cloning cauliflower

1. Describe how the steps used in this procedure prevent contamination.

2. Explain the purpose of the following ingredients in the plant growth medium: sucrose; kinetin; Murashige and Skoog medium; agar; *Milton*®.

3. What are the *advantages* of using this method to cultivate new plants?

4. What are the *disadvantages* of using this method to cultivate new plants?

Growing the plants on

After a few weeks, the cauliflower callus tissue will have developed small shoots and leaves, but no proper roots. By transferring the tissue onto a new vial of MS growth medium that contains no kinetin, however, the formation of roots will be encouraged.

Once the small cauliflower plantlets have produced roots, they can be transplanted. The agar should be washed off the roots, then the plantlets can be planted in fine seed compost and grown into full-sized plants.

The leaves of tissue-cultured plants grown in humid conditions lack a fully-developed waxy cuticle; their stomata also respond too slowly to prevent desiccation of the plant. It is therefore essential to keep the plants in a humid environment at first, by securing a clear plastic bag over the plant pot.

After 10 days or so, the plantlets will have acclimatised and can be grown as normal.

Timing (at 26 °C)

Weeks	State of plants
1–2	Curd enlarges and produces chlorophyll
2–3	Leaves produced
3	Shoots produced
3–6	Move tissue to kinetin-free medium to encourage roots
6–12	Rooted plantlets ready for transfer to soil

Further information

General reading

Plants from test tubes. An introduction to micropropagation by Lydiane Kyte *et al* (2013) Timber Press. 4th Edtn. ISBN: 978 1604692068. *An accessible, authoritative and comprehensive guide to plant tissue culture, suitable for domestic and educational practitioners.*

Plant cell culture by H. A. Collin and S. Edwards (1998) Garland Science. ISBN: 978 1872748474. *This book is intended for university students or researchers taking up plant tissue culture for the first time. It describes how to set up and run a tissue culture facility, and includes an overview of many basic tissue culture techniques.*

Growing orchids from seed by Philip Seaton and Margaret Ramsay (2005) Royal Botanic Gardens, Kew. ISBN: 978 184 2460917. *This book, written for the amateur enthusiast, describes the cultivation of orchids by conventional tissue culture methods.*

Plant tissue culture by Tony Storr (1985) Association for Science Education, Hatfield. ISBN: 0 86357 031 3. *This publication describes several educational protocols that are suitable for schools and colleges, using conventional tissue culture methods. It has been out of print for many years, but a facsimile can be downloaded from the NCBE's website:* **www.ncbe.reading.ac.uk/ptc**

Web sites

Science and Plants for Schools (SAPS)
The SAPS website has numerous useful resources for teaching plant science, including educational materials on plant tissue culture: **www.saps.org.uk**

Plant TC Cases Wiki
This collection of open source practical protocols is aimed at schools and amateur growers. The site includes a *Microsoft Excel®* Workbook to convert molar quantities of plant growth substances to masses to assist in the preparation of plant growth media: **www.planttccases.org**

Royal Botanic Gardens, Kew
Conservation Biotechnology at Kew uses tissue culture methods to save critically-endangered plants for reintroduction into the wild:
www.kew.org/science/micropropagation.html

Writhlington School Orchid project
Pupils at Writhlington school use tissue culture techniques in an ambitious and successful orchid cultivation project.
http://wsbeorchids.org.uk

Acknowledgements

The plant tissue culture technique used in this kit is based on one developed by Conservation Biotechnology at the *Royal Botanic Gardens, Kew*. The method is used by Kew scientists in their conservation programmes.

Dan Jenkins at *Science and Plants for Schools* (SAPS) gave advice and encouragement in the development of this kit.

This booklet and the associated Student's guide were written and illustrated by Dean Madden at the NCBE.

National Centre for Biotechnology Education, University of Reading, 2 Earley Gate, Reading RG6 6AU. United Kingdom
Tel: + 44 (0) 118 9873743. Fax: + 44 (0) 118 9750140. eMail: NCBE@reading.ac.uk Web: www.ncbe.reading.ac.uk