



**FACULTY OF AGRICULTURE SCIENCES AND
ALLIED INDUSTRIES**

(Principle of Biotechnology)

For

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Course Instructor

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PLANT TISSUE CULTURE

Plant research often involves growing new plants in a controlled environment. These may be plants that we have genetically altered in some way or may be plants of which we need many copies all exactly alike. These things can be accomplished through tissue culture of small tissue pieces from the plant of interest. These small pieces may come from a single mother plant or they may be the result of genetic transformation of single plant cells which are then encouraged to grow and to ultimately develop into a whole plant. Tissue culture techniques are often used for commercial production of plants as well as for plant research.

Tissue culture involves the use of small pieces of plant tissue (**explants**) which are cultured in a nutrient medium under sterile conditions. Using the appropriate growing conditions for each explant type, plants can be induced to rapidly produce new shoots, and, with the addition of suitable hormones new roots. These plantlets can also be divided, usually at the shoot stage, to produce large numbers of new plantlets. The new plants can then be placed in soil and grown in the normal manner.

Many types of plants are suitable for use in the classroom. Cauliflower, rose cuttings, African violet leaves and carnation stems will all easily produce clones (exact genetic copies) through tissue culture. Cauliflower florets in particular give excellent results since they can be grown into a complete plant in the basic tissue culture media, without the need for additional growth or root hormones. Green shoots are generally observable within three weeks, and roots develop within six weeks.

The most important part of this activity, however, is to maintain as sterile an environment as possible. Even one fungal spore or bacterial cell that comes into contact with the growth media will rapidly reproduce and soon completely overwhelm the small plant piece that you are trying to clone.

Objectives

1. To understand a procedure that is often used to propagate many plants of the same genetic background.
2. To understand the importance of sterile techniques.

Materials

- 1 Vial of Murashige Skoog (MS) media. (If you wish to make up your own growing medium you could use the recipe for the Murashige medium given at the end of this section.)
- 1 L sterile distilled water
- 10 g of agar/L
- 30 g sucrose/L
- 1.5 L or 2 L container in which to prepare the growth medium
- small amounts of 1M NaOH and 1M HCl to adjust the pH of the media
- 60 flat bottom culture tubes with closures.
- Glass aquarium or box lined with plastic
- Plastic sheet to cover the top of the aquarium
- Adhesive tape
- 10% Bleach in a spray bottle
- 70% alcohol in a spray bottle
- Forceps or tweezers
- Gloves
- Cutting equipment such as a scalpel blade or razor blade
- 2 bottles of sterile distilled water (*purchase at the grocery store*)

- Pressure cooker
- Your chosen plant (cauliflower, rose, African violet or carnation)
- paper towel for cutting on or sterile petri dishes if available
- Beaker or jar in which to wash the plant material
- Detergent-water mixture - 1ml detergent per liter of water
- Bleach sterilizing solution - dilute commercial bleach (5-6% sodium hypochlorite) to a final concentration of 1-2% sodium hypochlorite in distilled water in a large beaker or jar.
- 2 or 3 beakers or jars of sterile water
- A well-lit area away from direct sunlight or use full-spectrum gro-lights
- Hormones such as BAP (benzylaminopurine) and NAA (naphthalene acetic acid) to stimulate growth and root development, respectively. (Commercial rooting hormone solutions and powders are also available from hardware stores.)

**Murashige Minimal Organics Medium recipe
(MMOM)**

<u>Inorganic salts</u>	<u>mg/L</u>
NH ₄ NO ₃	1,650.00
KNO ₃	1,900.00
CaCl ₂ (anhydrous)	332.20
MgSO ₄ (anhydrous)	180.70
KH ₂ PO ₄	170.00
Na ₂ EDTA	37.25
FeSO ₄ .7H ₂ O	27.80
H ₃ BO ₃	6.20
MnSO ₄ .H ₂ O	16.90
ZnSO ₄ .H ₂ O	5.37
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ (anhydrous)	0.016
CoCl ₂ (anhydrous)	0.014
Sucrose	30,000.00
i-Inositol	100.00
<u>Thiamine.HCl</u>	<u>0.40</u>

The pH is adjusted to 5.7 using 0.1 M HCl or NaOH.

Procedure

Preparation and sterilization of growing medium (when not provided pre-poured)

These steps will make 1 L of growth medium which is enough to prepare about 65 growing tubes.

1. Dissolve the MS mixture in about 800 ml of distilled water. Stir the water continuously while adding the salt mixture. Add 30 g sugar and stir to dissolve. Adjust pH to 5.8 using 1M NaOH or 1M HCl as necessary while gently stirring. Add distilled water to make the total volume up to 1 L.
2. Weigh out 10 grams of agar and add it to the MS solution. Heat the solution gently while stirring until all the agar has dissolved.
3. Pour the still warm medium into the polycarbonate tubes to a depth of about 4 cm which will use about 15ml of media per tube.
4. Place the tubes (with lids sitting on the tubes but not tightened) in a pressure cooker and sterilize for 20 minutes. Cool the pressure cooker, then remove the tubes and tighten the lids. Alternatively the tubes can be placed in boiling water for 30 minutes, but make sure that none of the water is able to enter the tubes.

NOTE: If you wish to use plants other than cauliflower you need to prepare two different media which contain plant hormones necessary to stimulate development of differentiated tissues. The first one should contain a cytokinin such as BAP which promotes shoot formation and the second one a rooting hormone such as NAA or store bought rooting hormone. To do this, prepare the mixture up until the end of step 2. Keeping the mixture warm so that it does not solidify, divide it equally into two pre-warmed containers. Each container can be used to prepare 30 or so tubes as above. The first container should have BAP added at the rate of 2.0mg/l. The second container should have the NAA hormone added at the rate of 0.1 mg/L. To do this it is necessary to make concentrated solutions of both BAP (2.0mg/ml) and NAA (1.0mg/ml) and filter sterilize them. Add 1ml of the concentrated BAP stock or 100 μ l of the NAA concentrated stock to each 1 liter of media that you prepare. If you use rooting hormone that is purchased from your local hardware or nursery supply store instead of NAA then just follow the directions before adding to your media.

Preparation of a sterile transfer chamber and equipment

A classroom transfer chamber can be made from a clean glass aquarium turned on its side. Scrub the aquarium thoroughly with a 30% bleach solution, making

sure that you wear gloves and do not inhale the fumes. Rinse with sterile distilled water, turn upside down on a clean counter or paper towels and allow to dry. Cut holes in a clean plastic sheet to allow arms to reach into the chamber and reinforce the cut edges with tape if necessary. Tape the clean plastic sheet over the open side of the aquarium making sure that the arm holes are located at a convenient height. Plastic sleeves could also be fitted to these holes if you wish to make it easier to prevent the entry of airborne spores into the chamber. The finished aquarium chamber can be sterilized by spraying with 10% chlorox bleach just prior to each use and drying with sterile paper towel.

Wrap the forceps, scalpels, razor blades, paper towel and gloves (rubber or surgical) in aluminum foil, seal with tape and sterilize by processing them in a pressure cooker for twenty minutes. These items can also be sterilized by placing in an oven at 350°F for 15 minutes. You can wrap each item separately or put together a "kit" so that each student will have their own sterile equipment to use.

Alternatively the forceps and blades can be sterilized by dipping in 10% bleach and then rinsing in sterile water, or dipping in alcohol and then placing in a flame, although this is not recommended for use in crowded classrooms. If you choose to dip in bleach and rinse in sterile water, it is best if fresh solutions are available for each 3-4 students since the water can easily be contaminated if care is not used. These liquid containers should only be opened once they are inside of the sterile chamber.

Plant preparation

Your plant material must first be surface sterilized to remove any bacteria or fungal spores that are present. We aim to kill all microorganisms, but at the same time not cause any adverse damage to the plant material.

1. Cauliflower should be cut into small sections of florets about 1 cm across. If using a rose or other cuttings, cut the shoots into about 5 to 7 cm lengths. Whole African violet leaves can also be used.
2. Wash the prepared plant material in a detergent-water mixture for about 20 minutes. If trying hairy plant material scrub with a soft brush (toothbrush). This will help remove fungi etc., and the detergent will help wet the material and remove air bubbles that may be trapped between tiny hairs on a plant.
3. Transfer the washed plant material to the sterilizing chlorox solution. Shake the mixture for 1 minute and then leave to soak for 10-20 minutes. Carefully pour off the bleach solution using the lid to keep the plant tissue from coming out and then carefully cap the container.

Note 1: At this point, the tissue is considered sterile. All subsequent rinses should be done with sterile water, and all manipulations of the tissue performed with sterile instruments and supplies. Open one container at a time and never leave the lid off of any container longer than necessary.

Note 2: Many students will not fully appreciate the importance of carefully sterilizing explants and so there will be some cultures that become infected with bacterial or fungal growth. If you do not wish to emphasize this aspect of the laboratory students can be provided with plant materials that the instructor has already sterilized prior to use by the class.

Transfer of plant material to tissue culture medium

Use the sterile gloves and equipment for all of these steps.

1. Place the plant material still in the chlorox bleach sterilizing container, the containers of sterile water, the sterilized forceps and blades, some sterile paper towel to use as a cutting surface and enough tubes containing sterile medium into the sterile aquarium. The outside surfaces of the containers, the capped tubes and the aluminum wrapped supplies should be briefly sprayed with 70% alcohol before moving them into the chamber.
2. The gloves can be sprayed with a 70% alcohol solution and hands rubbed together to spread the alcohol just prior to placing hands into the chamber. Once students have gloves on and sprayed they must not touch anything that is outside of the sterile chamber.
3. Carefully open the container with the plant material and pour in enough sterile water to half fill the container. Replace the lid and gently shake the container to wash tissue pieces (explants) thoroughly for 2-3 minutes to remove the bleach. Pour off the water and repeat the washing process 3 more times.
4. Remove the sterilized plant material from the sterile water, place on the paper towel or sterile petri dish. Cut the cauliflower into smaller pieces about 2 to 3 mm across. If using rose cut a piece of stem about 10 mm in length with an attached bud. The African violet leaf can be cut into small squares about 1-1.5 cm across. Be sure to avoid any tissue that has been damaged by the bleach, which is apparent by its' pale color.
5. Take a prepared section of plant material in sterile forceps and place into the medium in the polycarbonate tube. Cauliflower pieces should be partly submerged in the medium, flower bud facing up. Rose or other cuttings should be placed so that the shoots are level with the medium surface. The African violet leaf pieces should be laid directly onto the medium surface.

6. Replace the cap tightly on the tube.

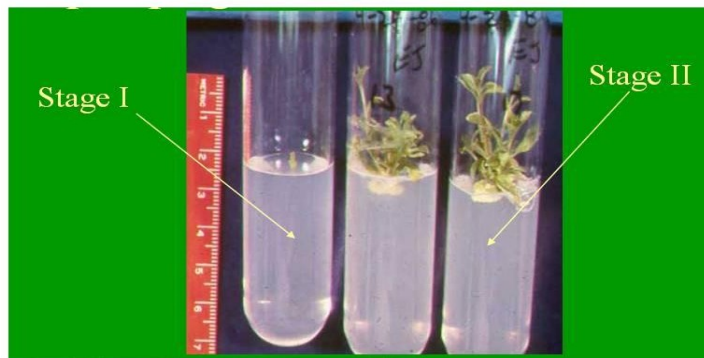


Figure 1: The small explant develops callus which then produces shoots a few weeks after being placed into tissue culture media

Growing the plants

1. The tubes containing plant sections may be placed in a well-lit area of the classroom although not in direct sunlight. The shoots will probably grow more quickly if the explants are placed under fluorescent or grow-lights to provide at least 12 hours of light per day. The aquarium can be used as a growth chamber with the lighting about 8-10" overhead. This will also help maintain a more regular and warm temperature. Ensure that the temperature does not go over 28°C. New shoots should develop within 2 weeks, and should be well advanced in 3 to 4 weeks. Check the tubes daily and discard any that show signs of infection (before discarding first sterilize in the pressure cooker or add bleach into the tube).
2. Roots can appear within 6 weeks on cauliflowers. The roses, African violet and other cuttings will need to be moved into rooting media for roots to properly develop. This transfer to the second, rooting media must be conducted under the same sterile conditions as at the initiation of the culture. All necessary equipment and the aquarium should be set up as before and properly sterilized.
3. Working inside the sterile aquarium chamber, remove the cap from the culture tube. There will usually be several shoots that have arisen from each explant. These shoots should be carefully separated by gently removing the whole explant from the media with sterile forceps and

then separating the shoots by gently pulling them apart using two pairs of forceps. Each shoot should then be placed into a tube of rooting media and the bottom of the shoot pushed into the media so that good contact is made. The cap is replaced and the shoots are then allowed to grow as in step 1 until roots are formed, usually within 2-3 weeks.

Potting the clones

Once roots are well formed the plants are ready to be transferred into soil.



Figure 2: Roots are fully developed prior to moving plants to pots of soil

1. Each plant should be carefully removed from its tube of media and planted into a small pot containing a clean light potting mix. Gently wash off all the agar medium prior to planting. The plants will still need to be protected at this stage since they are not accustomed to the drier air of the classroom when compared to the moist environment of the tube of media.
2. Place all of the pots onto a tray and cover lightly with a plastic dome or tent. Place the plants in an area with 12-16 hours of light (either natural or artificial) but not direct sunlight.
3. After a week the cover can be gradually removed and the plants acclimated to stronger light and drier atmospheric conditions.
4. You now have a collection of plants in your classroom that are genetically exactly the same. You could use these plants to carry out other experimental tests knowing that one of the main variables in the experiment has been eliminated. Some of these tests could include looking at plant responses to low light levels, to drought or to saline soil conditions. (see [activity 7](#))

Student Activity

1. Tissue culture uses a small piece of tissue from a mother plant to grow many new copies of the original plant. What is the term used to refer to this small piece of tissue?
2. What are some of the plants that we might use for tissue culture?
3. Why is tissue culture used for propagation of some plants rather than just planting seeds?
4. What is a sterile environment?
5. Why is a sterile environment important in tissue culture?
6. How did you or your teacher sterilize the instruments that were used in this tissue culture activity?
7. Could we sterilize the plant tissue in the same manner? Why or why not?

8. What happens if you open your sterile plant container when it is not inside a sterile environment?