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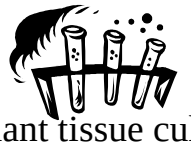
PLANT TISSUE CULTURE



INTRODUCTION

Plant tissue culture is the propagation of plants through "cloning" an asexual method of reproduction. A portion (explant) of a desired plant is cultured *in vitro* ("glass") on a defined medium, which promotes rapid multiplication of cells. The new plants are removed from the culture and transferred to a standard potting medium. Tissue culture is based on the theory of totipotency; that is, the genetically based ability of a nonembryonic organ or cell to develop along a pathway similar to that of a zygote, leading to the formation of a new entire plant identical to the original.

Currently, tissue culture is being used in both research and commercial applications. Tissue culture not only provides a method of mass propagation, but also makes possible the production of disease-free plants, mutants, and secondary plant products. A new and important use is in the genetic engineering of plants. A single plant can be genetically modified and grown into a mature plant or plants having new characteristics.

In this lab you will use a portion of African violet leaf to observe the four stages of plant tissue culture.

Stage I: Initiation stage. Takes the plant from *in vivo* ("life") to *in vitro*. This means simply that the explant is taken from its normal relationships to the other plant parts and is placed under "test-tube" culture conditions.

Stage II: Multiplication stage. The explant undergoes rapid tissue or shoot multiplication. This process can be repeated several times, depending upon how many plants are ultimately desired.

Stage III: Rooting stage. A different growth medium is used to induce root formation from Stage II plants**.**

Stage IV: Acclimatization stage. Transfer of the plants to potting medium.

STAGE 1

PREPARATION OF EXPLANT

(Sterile technique is **NOT** required...This process may be completed by the teacher to save time.)

1.) Select the younger leaves near the center of the plant, as their cells will be more likely to have retained their totipotency. Remove the young leaves, leaving a length of petiole (leaf stalk) attached to each.

2.) Disinfestation process: Sterilize the leaves by immersing them into the **10% bleach** (sodium hypochlorite) solution to which **one drop** of dish washing **detergent** has been added. Put the top on and **shake** the solution with the leaves in it for **10 minutes**. (The detergent acts as a wetting agent and allows the entire surface of the leaf will be exposed to the sodium hypochlorite. This process will remove surface contaminants such as: exterior bacteria, fungi, spores, mites, or small insects.)

3.) Make sure all items that will be needed are sprayed with ethanol and placed in the sterile area.

4.) Loosen the lids of media but do not remove them.

PREPARATION CONTINUED

(Sterile technique **REQUIRED**)

5.) Wash your hands and forearms with antibacterial soap and dry thoroughly with paper towels. Put on gloves and mask if you have one.

6.) Go to sterile area. Using the **70% ethanol or isopropyl alcohol**, spray your hands, forearms and the opening to the isolation box. Spray down the interior of the box.

7.) Spray down every object before placing it inside of the box. (Remember you **MUST** spray your hands and forearms **every time** you remove them from the box).

8.) Remove one leaf at a time and place in a jar of sterile water until you are ready to cut and culture them**. RINSE** each leaf in three separate washes of sterile water.

9.)

10.) With **sterile** forceps, remove the leaves from the jar and place each leaf, **underside up**, in a separate **sterile** petri dish; replacing each lid.

11.) Return the forceps to **ethanol**.
12.) Remove the scalpel and forceps from ethanol and dip in **sterile water**.
13.) Open a petri dish and set its cover (**sterile side up**) on the sterile work surface. While holding the leaf by its petiole with the forceps, cut off both sides and ends (petiole end last) with the scalpel, leaving a rectangle of leaf with the midvein running through it.
14.) Cut the rectangle into sections perpendicular to the midvein and about 1 cm wide.
15.)
16.) Replace the cover on the dish. Wipe any debris from the instruments and return them to the **ethanol.**

CULTURING

(Sterile technique **REQUIRED)**

17.) Remove the lid from one test tube. **Be certain to not contaminate the lid.**
18.) Rinse the forceps in the **sterile water** then use them to place leaf sections in the medium surface so the midvein is **perpendicular** to the surface and half the section is sticking into the medium. Place no more than two sections in each test tube.
19.)
20.) **Do not touch the rim of the test tube.** Carefully replace the lid onto the test tube and seal with parafilm.

21.) Repeat steps 10-12 with the 2nd test tube.
22.) Return the forceps to the **ethanol.**
23.) Remove the petri dish with the trimmings from the isolation box.
24.) Spray hands again before entering the box to remove both test tubes. Place both test tubes in the test tube racks. Using the wax pencil write your name and date near the top of each test tube.
25.) Put your test tubes in the rack that is on the counter.
26.) Avoid direct sunlight, low light for about 16 hours daily and constant temperature of about 25C is best.

STAGE II

MULTIPLICATION

(Sterile conditions **REQUIRED**)

In a few weeks small pimplelike buds should appear on or near cut surfaces. You may need a microscope to see these structures as they begin to grow, but after 5-6 weeks they should be visible to the unaided eye. If a sterile environment was not maintained, contamination will be obvious within 3-4 days. Materials contaminated by fungus will have a fuzzy growth on them. Materials contaminated by bacteria will have a slimy growth on them. If you note any contamination in your test-tubes, you must let me know so that I can properly discard them so other test-tubes are not contaminated.

STAGE THREE:

ROOTING

Once a sufficient number of shoots have been generated, portions of explant that contain one or more shoots could be transferred to a medium that contains a higher concentration of the hormone auxin, resulting in root production.

1) In the transfer box, under sterile conditions, remove the para-film and cap from the test-tube and use the forceps to carefully remove the explant from the medium. Place the explant inside a sterile petri dish. Remove the old test tube from the box.
2) Using a sterile scalpel carefully remove or cut plantlets away from the explant.
3) While maintaining sterile conditions, carefully remove the cap off of the new test-tube. Using sterile forceps rinsed in sterile water, carefully place plantlet into the medium.
4) Carefully cap your test tube, write your initials and date on the cap and return them to the rack for 2- 4 weeks under low lights.

During this time, the shoots will continue to grow, however, most of the plants energy will be focused into producing roots.

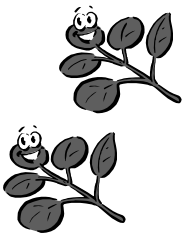
STAGE FOUR:

ACCLIMATIZATION

Once roots are visible, plantlets need to be moved from the medium to soil.

1.) Gently wash all medium from the leaves and roots with distilled water.
2.) Transplant plantlets into small clean pots of African Violet planting soil.

Plants grown in vitro are very sensitive to change and are accustomed to specific conditions. To maintain the high humidity the plantlets are used to in the capped tubes, put the small pots in a larger container. Add 1-2cm of water in the bottom of the large container, and cover it with cellophane wrap. Each day fold back more and more of the cover. In 1-2 weeks, the plants will acclimated to room conditions.



PLANT TISSUE CULTURE

STERILE TECHNIQUE

WHAT IS IT? A method used to remove contaminants from a given area.

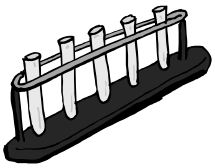
WHY? In plant tissue culture, small pieces of plant tissue are placed on or in a medium rich in nutrients and agar. If bacteria or fungi come in contact with the plant tissue or medium, the culture becomes contaminated. The contaminants will use nutrients from the medium and the plant, which will result in quickly destroying the plant tissue.

AIM: To surface sterilize the plant tissue and put it on a sterile growth medium without any bacteria of fungi getting on the plant or medium. This is not easy because bacteria and fungal spores are in the air, on us, in us, and under us!

HOW DO WE DO THIS? By following the precautions below, we can (*hopefully*) reduce the number of contaminants in our environment.

1. Use a transfer box that has been surface sterilized by spraying all surfaces with 70% ethanol.
2. Doors and windows must be closed to limit airflow.
3. Have spray bottles filled with 70% ethanol. Spray **EVERYTHING** going into the sterile area/box.
4. Sterilize instruments needed inside the box. (This will be demonstrated)
5. Sterile petri dishes are used inside the box as a place to trim, cut, and divide the sterile tissue. Spray the bag of presterilized petri dishes before you open it. Remove the desired petri dishes and spray the outside only before placing them into the box. Each petri dish has two sterile sides, the inside top and the inside bottom. When ready to use, remove the top and turn sterile surface up and set it down behind the bottom.
6. Long hair should be tied back. Hands should be washed not scrubbed-(scrubbing dries hands and creates flakes of skin that have bacteria). Latex gloves are strongly suggested. Hands/gloves and forearms should be sprayed before entering the box. Masks also provide extra protection, especially if you need to talk while performing this lab.
7. Only one set of hands should be in the box at a time.
8. Do not lean over your work. Try to work with your back and arms straight. This position may be uncomfortable but it is necessary to reduce the risk of contamination. Reach around rather than over your work. Do not pass nonsterile items into the sterile area. Your movements should be slow—you don't want to disturb the air more than is necessary. Work quickly, the longer it takes to manipulate the tissues, the greater the chance of contamination.





List of Websites

- http://ns2.d20.co.edu/kadets/lundberg/violets.html
- http://www.jmu.edu/biology/biofac/facfro/cloning/cloning.htm
- http://www.kitchenculturekit.com/cleanbox.htm
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http://aggie-horticulture.tamu.edu/tisscult/microprop/facilities/microlab.html