PLANT MICROPROPAGATION USING AFRICAN VIOLET LEAVES

Through the use of biotechnology, desirable genetic traits can be transferred from one organism to another by transfer of DNA. In plants, the DNA of interest is transferred into the new plant by using *Agrobacterium tumefaciens*, a bacterium that can infect plant tissues and incorporate part of its DNA into the DNA of the host plant. Alternatively, a particle gun can be used to directly "shoot" the DNA into plant cells. In both methods, the target for the foreign DNA is a small piece of plant tissue or a small mass of plant cells. Once the DNA has been transferred, new plants must be regenerated from the small pieces of transformed plant tissue using micropropagation (tissue culture) techniques.

Micropropagation is the aseptic culture of cells, pieces of tissue, or organs. It is possible to regenerate new plants from small pieces of plant tissue because each cell of a given plant has the same genetic makeup and is totipotent, that is, capable of developing along a "programmed" pathway leading to the formation of an entire plant that is identical to the plant from which it was derived. In addition to its biotechnological applications, micropropagation is used commercially to asexually propagate plants. Using micropropagation, millions of new plants can be derived from a single plant. This rapid multiplication allows breeders and growers to introduce new cultivars much earlier than they could by using conventional propagation techniques, such as cuttings. Micropropagation also can be used to establish and maintain virus-free plant stock. This is done by culturing the plant's apical meristem, which typically is not virus-infected, even though the remainder of the plant may be. Once new plants are developed from the apical meristem, they can be maintained and sold as virus-free plants.

Micropropagation differs from all other conventional propagation methods in that aseptic conditions are essential to achieve success. The process of micropropagation can be divided into four stages:

1. Initiation stage. A piece of plant tissue (called an explant) is (a) cut from the plant, (b) disinfested (removal of surface contaminants), and (c) placed on a medium. A medium typically contains mineral salts, sucrose, and a solidifying agent such as agar. The objective of this stage is to achieve an aseptic culture. An aseptic culture is one without contaminating bacteria or fungi.
2. Multiplication stage. A growing explant can be induced to produce vegetative shoots by including a cytokinin in the medium. A cytokinin is a plant growth regulator that promotes shoot formation from growing plant cells.
3. Rooting or preplant stage. Growing shoots can be induced to produce adventitious roots by including an auxin in the medium. Auxins are plant growth regulators that promote root formation. For easily rooted plants, an auxin is usually not necessary and many commercial labs will skip this step.
4. Acclimatization. A growing, rooted shoot can be removed from tissue culture and placed in soil. When this is done, the humidity must be gradually reduced over time because tissue-cultured plants are extremely susceptible to wilting.

TEACHER PREPARATION AND INSTRUCTION GUIDE

Preparation for the plant micropropagation exercise should begin at least 24 hours in advance of the laboratory.

The following supplies can be provided to the class in groups of two students:

- 2 fresh African Violet leaves
- 4 culture medium plates
- aseptic work surface/hood
- 1 aluminum foil packet containing 1 sterile forceps
- 1 aluminum foil packet containing 2 sterile razor blades (or 1 sterile #3 scalpel handle with sterile #10 blade attached)

- 2 sterile petri dishes
- 1 liter sterile water
- 1 500-ml or 1-liter jar with screw-cap lid (for disinfesting the leaf)
- alcohol or Bunsen burner
- spray bottle containing 70% ethanol (a spray bottle of the type used to mist plants or spray window cleaning solution works very well)

The following supplies can be shared by four students:

- *500-ml bottle (for sterilizing medium)
- *500-ml or 1-liter plastic or glass beaker (for preparing medium)

The teacher should have available for the entire class:

- commercially-purchased water for medium preparation
- 70% ethanol (dilute 700 ml of 95% ethanol with 250 ml distilled water)
- 0.1% detergent (add 1 ml detergent to 1 liter of water)
- 10% bleach solution plus a few drops of dishwashing detergent
- pH meter or pH paper
- a few mls of the following solutions for adjusting pH:
    - 1.0 M NaOH
    - 1.0 M HCl
    - 0.1 M NaOH
    - 0.1 M HCl
- parafilm for wrapping plates
- scissors
- Sharpie marking pens
- 5-10 extra sterile scalpel blades
- 5-10 extra sterile petri plates
- dishwashing detergent

I. PREPARATION OF PLANT TISSUE CULTURE MEDIUM

The culture medium is formulated so that it provides all of the compounds needed for plant growth and development, including certain compounds that can be made by an intact plant, but not by an isolated piece of plant tissue. Typically, the medium contains mineral nutrients, organic compounds such as sucrose and vitamins, and plant growth regulators (plant hormones). Agar is added if a solid medium is desired.

Prepare 250 ml per group of four students. In this instruction guide, a prepackaged African violet medium from Carolina Biological Supply Company that includes all necessary components except agar and sucrose, will be used (Carolina Biological Supply Company, 2700 York Road, Burlington, NC 27215-3398, Phone 1-800-334-5551, Fax 1-800-222-7112). The instructions that follow are for making 250 ml of medium. Modifications of the procedure needed to make 1 liter are included in brackets ([ ]) in **bold**.

Step 1. Add about 100 ml [**500 ml**] of deionized or distilled water to a 500-ml or 1-liter [**2-liter**] beaker.

Step 2. Add 1.16 grams [**one package**] of the prepackaged mix to the beaker.

Step 3. Add 7.5 grams [**30 grams**] of sucrose to the beaker. (Table sugar purchased from a supermarket can be used if reagent-grade sucrose is not available.)

Step 4. Bring the solution up to about 200 ml [**800 ml**] by adding deionized or distilled water.

Step 5. Stir the solution until all components are dissolved.

Step 6.
Adjust the pH to about 5.6 to 5.8 with a pH meter, using NaOH and HCl as necessary. If a pH meter is not available, use pH indicating paper that can detect pH 6.0 ± 0.5 pH units. It is preferable to have the pH at slightly less than 6.0 rather than at slightly greater than 6.0.

At pH < 5.0, the ability of the explant to take up nutrients will be adversely affected. At pH > 7.0, iron will precipitate out of the medium, becoming unavailable for uptake. (When using the prepackaged mix and commercial distilled water, it will take approximately 3 drops [**13 drops**] of 1.0 NaOH and 6 drops [**4 drops**] of 0.1 NaOH to raise the pH of 250 ml [**1 liter**] of medium to 5.6 to 5.8.)

Step 7. Bring the solution up to 250 ml [**1 liter**] by adding deionized or distilled water, and pour into a 500-ml bottle [**1.5 to 2.0-liter bottle**] to autoclave.

Step 8. Add 2.0 grams [**8.0 grams**] agar. (Note: the agar will not dissolve until the medium is heated.)

Step 9.
KEEP THE CAP LOOSE ON THE VESSEL CONTAINING THE MEDIUM WHILE STERILIZING. Sterilize the bottle of medium by placing it in one of the following:

a) boiling water for 30 minutes;
b) a pressure cooker at 15 pounds for 15 minutes; or
c) an autoclave for 15 minutes at 121ƒ C (250ƒ F) at 1.1 kg/cm^2 (15 psi).

Step 10.
After removal from the water bath, pressure cooker, or autoclave, gently swirl the medium to mix the agar. When the agar is completely dissolved and mixed, the medium should appear clear and not turbid.

Step 11.
If pouring plates, cool the medium to 45-55ƒ C (110ƒ F), then pour plates on a clean work surface (spray down with 70% alcohol), working near a small Bunsen burner or alcohol lamp. Use about 25 ml per plate (about one-third to one-half full). If plates are not being used immediately, put them back into the plastic sleeve, tape the sleeve shut, and store plates at 4ƒ C (39ƒ F).

II. STERILIZATION OF SUPPLIES

1. Sterilization of packets of packets of forceps and razor blades can be accomplished by wrapping each item in aluminum foil, labeling the contents with a marking pen, and:

a) baking them in an oven at 350ƒ F for 15 minutes;
b) putting them in a pressure cooker at 15 pounds for 15 minutes; or
c) placing them in an autoclave for 15 minutes.

The pressure cooker and autoclave should be at the desired pressure for the 15-minute period. After the packets have cooled, they should be stored unopened at room temperature. The students should be instructed when opening the packets to touch that part that will not come in contact with the plant material or petri dishes.

2. Sterilization of the sterile water needed for the rinses can be accomplished as described for sterilization of the medium.

NOTE: Teachers may decide for themselves which parts of the procedure can be successfully completed by the student. Middle school students enjoy transferring disinfested explants previously prepared by the instructor to the plates with medium. Older students may be included in parts of the medium preparation and/or the disinfestation

procedure. All students will enjoy seeing the growth of shoots from the leaf surface of the African violet. Seeing this regeneration process will make it easier for the teacher to start a discussion of the totipotency of plant cells and the practical application of plant propagation.

PLANT MICROPROPAGATION

STUDENT INSTRUCTIONS

ASEPTIC TRANSFER OF CULTURES TO FRESH MEDIUM

Micropropagation is the use of tissue culture methods to propagate plants. Micropropagation if a form of clonal propagation that differs from all other conventional propagation methods in one important aspect: aseptic conditions are necessary to achieve success with micropropagation.

In commercial and research laboratories, aseptic transfer is usually done in a hood that excludes particles larger than 3 microns from the work area. Contaminants of plant tissue culture are bacteria that reside on dust particles and fungi, including spores. A hood will effectively filter out all of these contaminants.

It is possible to transfer aseptically without a hood when you take all possible precautions to keep dust and spores from falling in the sterile medium. One simple, but fairly effective method for accomplishing this, is to spray a smooth counter top with alcohol and spray all other possible sources of contamination (e.g., hands, instruments, and culture vessels) before transferring. A second method used by plant pathologists is to work around a small Bunsen burner, which creates an updraft to prevent spores and dust from falling into the open dish. Extreme caution is required with this method because of the danger of burns. A third method involves spraying hands, instruments, and vessels with alcohol and working in a large new clear plastic bag. Because newly manufactured plastic is fairly sterile, working inside this sterile bag, which contains still air, will avoid contamination. An old aquarium or cardboard box lined with aluminum foil can be sprayed with alcohol and used to cut down on drafts. With all methods, it is best to keep traffic around the transfer area to a minimum.

Step 1.
Put on a pair of sterile gloves. Clean your gloves with 70% ethanol initially, and reclean them if you touch any nonsterile surface or material.

Step 2. Spray your work surface with 70% ethanol.

Step 3.
Spray vessels and all tool packets (forceps and razor blades) with 70% ethanol before placing them on the work surface.

Step 4.
Arrange the objects on the work surface so that there is a cleared work area for the transferring process. Avoid placing any object "up wind" of your work area.

Step 5.
For African violets, use a fresh, healthy leaf. The disinfestation process (removal of surface contaminants) is as follows:

a) Quickly rinse the leaf under cool tap water, then put the whole leaf into a closed container. A jar with a screw-cap lid is best. Depending on the size of the leaves, either a 500- or 1000-ml jar can be used. Wash the leaf in water with 0.1% detergent for 3-4 minutes. Gently agitate the leaf every 20-30 seconds during this washing step.

b) Rinse off the leaf and rinse out the jar with cool tap water.

c) Gently agitate the leaf in 10% bleach solution for 10 minutes. The jar should be filled three-fourths full with the bleach solution. *(The bleach solution disinfests the plant tissue material, killing most fungal and bacterial organisms.)*

d) Pour off the bleach while keeping the lid loosely in place over the container. (Be careful not to spill bleach solution on clothing because it will leave white spots after laundering.) **Note:** At this point, the leaf is considered sterile. All subsequent rinses should be done with sterile water, and all manipulations of the leaf performed with sterile instruments and supplies. Open one container at a time and never leave the lid off of any container longer than necessary.

e) Take the container to the sterile counter space. Spray the container with 70% ethanol before placing it in the sterile environment. Remove the lid and pour sterile water over the leaves. Fill the jar approximately half-way with the sterile water.

f) Replace the lid and gently agitate for 2-3 minutes to rinse the bleach off the leaves.

g) Pour off the rinse water in the sink, as in Step 5, d.

h) Rinse with sterile water a total of 4 times.

Step 6.
Using the sterile razor blade and forceps, cut off bleach-damaged portions in an empty, sterile petri dish. With the empty dish open, hold the leaf with forceps and cut into strips 1.5-cm x 1.5-cm wide. Place 2 or 3 explants into a petri dish that contains the fresh medium. Gently press on the explants with a forceps to ensure that they make contact with the medium. Do two dishes per student. *(The tissue culture medium contains all the compounds needed for plant growth, including mineral nutrients, sucrose, vitamins, and plant growth regulators that result in shoot or root production.)*

Step 7.
Replace the cover of the petri dish and wrap with parafilm. Use one 1-inch x 4-inch piece per plate. The parafilm will stretch to seal the dish. (Plant tissue cultures require much longer culture times than microbial cultures, so this additional precaution is usually worth the small extra cost and time.)

Step 8.
Place the vessel under lights for 16 hours per day if possible. Keep the temperature between 24 and 26ƒ C (about 75ƒ F). Do not put cultures in direct sunlight, but make sure the area where they are placed is well-lighted by fluorescent bulbs. Growth is slower at lower temperatures.

Step 9.
Look for small green shoots forming on or near cut surfaces. It may take 2-3 weeks or more before any visible evidence of new growth is noticeable. 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 to 4 days. Materials contaminated by fungus will have a fuzzy growth on them, whereas material contaminated by bacteria will have slimy growth on them. Discard contaminated petri dishes promptly to avoid spreading plant diseases to other uncontaminated cultures.

The medium used in this exercise contains a cytokinin, and is specifically formulated to favor the production and multiplication of shoots. Once a sufficient number of shoots has been generated, portions of the explant that contain one or more shoots could be transferred to a medium that contains a higher concentration of an auxin, resulting in root production. Once roots have formed, the plantlets are transferred to pots containing a soil-based or soilless medium, and gradually exposed to conditions of lower humidity and greater light.

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