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908

Introduction to Plant Cell Culture

Store experiment in the refrigerator.

EXPERIMENT OBJECTIVE:

In this experiment, students will learn how to establish plant cell cultures from a plant tissue to generate whole plants. In accomplishing this, students learn how to prepare plant tissue for culture, practice sterile technique, and learn about the components of plant culture media.

Table of Contents

Safety Data Sheets can be found on our website:
www.edvotek.com/safety-data-sheets

Experiment Components

Store entire experiment in the refrigerator.

This experiment is designed for 10 laboratory groups.

A Shoot Initiation Growth Medium
B Shoot Elongation Growth Medium
C Tween

- Sterile Petri Plates (60 mm and 100 mm)
- Sterile 10 ml Pipets
- Parafilm
- Plant Growth Container
- Peat Pellets

Requirements

- Healthy Leaf Tissue from *Saintpaulia ionantha*, African Violet
- Sterile Distilled Water (autoclaved or filter sterilized through 0.2 µ filter)
- 70% Ethanol
- 95% Ethanol
- 100% Isopropanol
- Bleach
- Forceps (15-18 cm long)
- Scalpels (15-18 cm long) and Blades
- Bunsen Burner or Alcohol Lamp (optional)
- Laminar Flow Hood or Sterile Transfer Hood
- Antibacterial or Germicidal Soap
- Latex or Vinyl Gloves
- Cool White Fluorescent Light Source
- Autoclave (optional)
- Sterile 600 ml Glass Beakers
- Aluminum Foil
- Pipet Pump
- Test Tubes (10-11 cm long) and Racks
- Spray Bottle

Background Information

INTRODUCTION TO PLANT CELL CULTURE

The applications of plant tissue culture biotechnology are quite important. Regeneration of plants from cell culture offers a practical strategy for plant cloning, since most regenerants from a culture are genetically identical. For commercially valuable plants that are difficult, costly or inefficient to propagate by cuttings or other asexual means, cell culture may offer the only practical means of propagation. Most hybrid orchids, for example, are propagated by the tissue culture method of "meristemming" or "mericloning". Plant tissue cultures are also being investigated as sources of valuable plant products like drugs, flavors and fragrances.

Many strategies for genetic engineering of plants rely on plant tissue culture. Plant cells in culture can be genetically transformed by a number of techniques. Explants can be transformed directly by *Agrobacterium tumefaciens* or by bombardment with DNA-coated particles from a particle gun. Protoplasts (plant cells with their cell walls removed) are the targets of microinjection and electroporation (DNA uptake mediated by an electric field). Plant cell culture is central to these techniques in that cell culture allows transformants to proliferate and, sometimes, regenerate into genetically identical clones.

While many formulations for plant tissue culture media have been developed, all contain these same basic types of ingredients:

- Inorganic Salts (Macro and Micronutrients)
- Vitamins
- Organic Carbon Source

In addition to these components, most formulations contain plant growth regulators (plant hormones), auxin and/or cytokinin. Plant tissue culture media are generally solidified by the addition of a gelling agent. The medium utilized in this experiment contains an agar substitute which forms a clear, colorless, strong gel that aids in the detection of microbial contamination. (Plant cultures are sometimes maintained as a suspension of cells in liquid medium, but this type of culture will not be included in this laboratory experiment.)

The medium is an MS medium which is a formulation developed by Murashige and Skoog and reported in the now classic 1962 article, "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures." (1962, Physiologia Plantarum 15:473-497.)

Background Information

MS medium is one of the most commonly used of all plant culture media. The components of MS media are:

Compound	Concentration in medium, mg/L
NH_4NO_3	1650
KNO_3	1900
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
KI	0.83
H_3BO_3	6.20
$MnSO_4 \cdot 4H_2O$	22.30
$ZnSO_4 \cdot 7H_2O$	8.6
Myo-inositol	100.00
$CaCl_2 \cdot 2H_2O$	440.00
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.3
$CuSO_4 \cdot 5H_2O$	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Glycine	2.0
Sucrose	30 g/L

In this experiment, students will culture leaf tissue from *Saintpaulia ionantha*, African Violet to initiate shoots. The plant material used to start a culture is called an explant. Shoots formed on explants will be transferred to medium which contains plant hormones that allow shoots to elongate and form roots. Finally, whole shoots are transferred to soil where mature plants will develop.

EXPERIMENT OBJECTIVE:

In this experiment, students will learn how to establish plant cell cultures from a plant tissue to generate whole plants. In accomplishing this, students learn how to prepare plant tissue for culture, practice sterile technique, and learn about the components of plant culture media.

LABORATORY SAFETY

Follow standard laboratory safety practices. Gloves and goggles should be worn routinely as good laboratory practice.

Preparation of sterile utensils/glassware may include the use of an autoclave or pressure cooker. Use extreme caution to avoid serious burns or other injuries.

An alcohol or gas flame may be used to flame utensils during the lab exercise. ***Extreme*** care should be used with open flames. Precautions should be taken to avoid igniting hair or clothing.

Experiment Procedure

Student Experimental Procedures

STERILIZATION OF PLANT TISSUE

REMEMBER:
Scrub hands using antibacterial or germicidal soap before working at the sterile transfer bench/hood. Spray gloved hands with 70% ETOH - do not go near flame until hands are dry.

1. Prepare sterile transfer bench/hood as per your instructor's directions (if not already done). The unit should be wiped with ETOH or other similar agent.

2. Cut one fully expanded healthy African Violet leaf. Avoid using leaf tissue that is scarred or bruised.

3. At the sterile bench, use clean forceps to dip the leaf into the 70% ETOH and swirl for 10 seconds. Immediately transfer the leaf to the beaker with 0.5% bleach solution and periodically swirl for 15 minutes. Place forceps into 95% ETOH.

From this point on, the leaf tissue should be treated as sterile. Take precautions to avoid touching or contaminating the plant tissue, media, and anything that comes into contact with them.

4. Use sterile forceps (flame optionally) to transfer the tissue to the beaker with 1% bleach solution and periodically swirl for 20 minutes. Replace forceps to 95% ETOH. The leaf tissue must be rinsed 4 times with sterile water.

5. Use sterile forceps to transfer the tissue consecutively to each of the 4 beakers of sterile water.

6. After the final rinse in sterile water, aseptically transfer the leaf to a clean, sterile petri dish and replace lid. The closed plate with sterile leaf can be moved carefully to another sterile bench for further manipulation. Proceed immediately to Part A, Explanting.

   NOTE: More than one group may sterile leaf tissue in the same containters (no more than 4 leaves per container).

Student Experimental Procedures

A. EXPLANTING

1. Use sterile forceps and scalpel (flaming is optional) to excise 4-6 pieces (0.5-1.0 cm^2) of sterile leaf tissue. Cut pieces from different areas on the leaf. (You may wish to note their location on the plates of media.) Include some pieces from and near the main vein of the leaf. Replace forceps and scalpel to the tube of ethanol.

2. Use sterile forceps to transfer the pieces to the 2 plates of explant media. Place the pieces in an upright position with 1/4 of the tissue inserted into the media. Replace the lids and label the plates with your initials or lab group number.

3. Wrap the plates with a double strip of parafilm. Place the plate upright on the lab bench. Hold the parafilm strip with your thumb and pull the strip with your other hand to stretch it and wrap it around the whole plate. Overlap at the end and seal with your thumb.

4. Store plates under light in the upright position in the area designated by your instructor.

   Monitor the development of the explants periodically. Adventitious shoots should appear on cut surfaces of the leaf tissue within 3-5 weeks. Expect some contamination to occur, immediately remove and discard any contaminated materials.

B. SHOOT ELONGATION/ROOTING

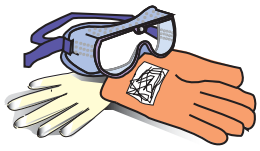
1. Prepare sterile transfer bench/hood as per your instructor's directions.

2. Using sterile forceps and scalpel, cut and transfer 10-20 small shoots to 2 plates of elongation media. Place shoots onto the surface of the media and gently insert, leaving the majority of tissue exposed. Replace the lids.

3. Wrap the plates with parafilm as before.

4. Store plates under light in the upright position in the area designated by your instructor.

   Again, monitor the development of the shoots periodically. Remove plates with contaminants. Shoots are expected to elongate and begin rooting in 4-5 weeks.



Wear Safety Goggles and Gloves

USEFUL HINT:
Periodically wipe the bench surface with 95% ETOH or 100% Isopropanol to minimize contaminants. Keep sterile materials toward the back of the hood near the screen. Do not work overtop of the plant material or media.

REMEMBER:
Parafilm will keep out contaminants while allowing for gas exchange to occur.

Experiment Procedure

Student Experimental Procedures

C. TRANSITION TO SOIL

When shoots have elongated and rooted, they are ready for transfer to soil.

1. Place the peat pellets in the growth containers and add warm water (3/4 full). The pellets will absorb the water and swell to approximately 3 cm. After the pellets have swelled completely, remove excess water.

2. Transfer whole shoots to the moistened peat pellets. Insert the stem with roots into the soil. Place under direct flourescent light and keep pellets well watered. Shoots can be transplanted after several weeks to small pots with fresh peat mix. Always water from below.

Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. Plant tissue culture media contain many components. Assuming that all of these are essential, where does the plant get them in nature? Can you assign an essential function to each of the Macronutrients in MS medium?

2. Most plant fertilizers contain three elements: nitrogen, potassium, and phosphorus. Carbon, the most abundant element in MS medium, is not included in fertilizers. Why not?

Instructor's Guide

Notes to the Instructor

If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK® web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800- EDVOTEK (1-800-338-6835).

Safety Data Sheets can be found on our website:
www.edvotek.com/safety-data-sheets

General Information

STERILE TECHNIQUE

The ability of the students to perform sterile technique will be the most important factor in achieving successful results from this experiment. Cleanliness of the lab, workstations, and especially the hands and bodies of the students should be stressed and put into practice. The cleaner the lab and its users, the less likelihood there is for contamination. Contaminants can be bacteria, yeasts, mold or fungal spores, etc. which can enter the tissue cultures via air flow, unsterile utensils, or dirty hands. Most plant tissue culture mediums contain high amounts of sucrose, thus making them ideal for contaminant growth. These microbes tend to overgrow and kill, or severely impair the growth of the tissue cultures.

CAUTION:
If working with fire and ethanol, be extremely careful. Have large glass beakers handy in case a fire starts. Use the beaker to cover the fire and smother the flames.

Some tips that will help to minimize the spread of contamination:

- Wipe down walls, floors, work benches and flow hoods with a disinfectant
- Minimize airflow throughout the lab. Keep doors, windows, and vents shut and try to keep traffic to a minimum.
- On the day of the lab, turn on laminar hood(s) or sterile transfer hood(s) approximately 20 minutes before you wipe them down with isopropanol or 95% ethanol. Make sure the entire inside of the bench is clean, including the ceiling, screen, sides, and bench top.
- Before starting the experiment and before each time the students return to the transfer hood, they should scrupulously scrub their hands and arms, preferably with an antibacterial or germicidal soap.
- Before performing sterile transfers, students should spray gloved hands with 70% ethanol. Use extreme caution when working with fire.

GROWTH CONDITIONS FOR CULTURES

A separate climate-controlled culture room is recommended for plant tissue culturing, but is not necessary for this experiment. A clean area of the lab can be sectioned off and the cultures can be incubated at room temperature, 21-22°C (optimal temperature is 25°C) under continuous cool white fluorescent light set approximately 1-1.5 feet above cultures (100-300 foot candles of light). Alternatively, the light source can be controlled by a 24 hour timer to give 16 hours of light and 8 hours of dark.

SELECTION OF PLANT MATERIAL

The plant material recommended for this exercise is *Saintpaulia ionantha*, African Violet. Students can be encouraged to bring healthy plants from home or you will need to purchase violets from a grocery store or flower shop. Choose plants with leaves free of bruises or blemishes. Each student group will need 1 or 2 healthy African Violet leaves. Other plant material, such as carrot tissue or cauliflower florets can be used, but best results will be obtained with African Violet.

Pre-Lab Preparations

One day to one week before the experiment:

1. Sterilize twelve 600 ml beakers. Wrap with foil and autoclave 121°C, 15 PSI, 20 minutes or bake glass beakers in a 200°C oven for one hour.

2. Sterilize 5 L of distilled water by autoclaving at 121°C, 15 PSI, 20 minutes or by filtering through a sterile 0.2 µ filter and storing in a sterile container.

A. SHOOT INITIATION

One day before the shoot initiation experiment:

- Prepare sterile transfer hood as described in Section V, Notes to the Instructor.

- Prepare 60 mm plates for the initial explant experiment:

    1. Equilibrate a waterbath at 60°C.

    2. Loosen, but do not remove, the cap on Bottle A, Shoot Initiation Growth Medium, to allow for the venting of steam during heating.

    3. Heat the bottle of media by one of the methods subsequently described. The clear solution should appear free of small particles.

        a. Microwave oven method:
        - Heat the bottle on High for two 1 minute intervals.
        - Swirl and heat on High for an additional 25 seconds, repeat until all the media is dissolved.

        b. Hot plate or Bunsen burner method:
        - Place the bottle in a beaker partially filled with water.
        - Heat the beaker to boiling over a hot plate or burner.
        - Using a hot glove, occasionally swirl to expedite melting.

    4. Cool the melted media to approximately 60°C by placing the bottle in a 60°C waterbath to prevent it from prematurely solidifying.

Wear Hot Gloves and Goggles during all steps involving heating.

CAUTION:
Failure to loosen the cap prior to heating or microwaving will cause the media bottle to break.

USEFUL HINT:
When the media reaches approximately 60°C, the bottle will be warm to the touch but not burning hot.

After the Shoot Initiation Growth Media has cooled:

5. Label 20-60 mm sterile petri plates "explant". Place the plates in the sterile hood.

6. Unwrap one of the 10 ml pipets under sterile conditions and use it to dispense 10 ml of media to the 20 sterile petri plates.

7. Allow the media to completely solidify.

8. Store plates in hood or sterile bench until ready to use.

Pre-Lab Preparations

On the day of the lab:

1. Prepare sterile transfer bench/hood as described in Section V, Notes to the Instructor.

2. Prepare bleach solutions for sterilizing plant tissue:

   - 0.5% - Mix 10 ml of bleach with 1990 ml distilled water and 6 drops to Tween (component C). Cover with foil.

   - 1.0% - Mix 20 ml of bleach with 1980 ml distilled water and 6 drops of Tween (component C). Cover with foil.

3. Set up 3 plant sterilization stations in the sterile hood for the student groups to share. Label each beaker with the appropriate contents. Each station requires:

   - 4 sterile 600 ml beakers, each filled with 400 ml sterile water (replace foil)
   - 100 mm sterile petri plates (empty)
   - 200 ml 70% ETOH in a clean beaker
   - 400 ml each of 0.5% and 1% bleach solutions in clean beakers, covered with foil.

For the shoot initiation experiment, each student group requires:

- 2-60 mm plates of sterile explant media
- Healthy Saintpaulia ionantha plant
- 1 pair of forceps, 15-18 cm long
- 1 scalpel with sharp blade, 15-18 cm long
- 1 tube, approximately 10-11 cm long filled with 95% ETOH and placed in a small rack
- Alcohol lamp or bunsen burner (Optional, see NOTE.)
- Gloves
- Spray bottle with 70% ETOH
- 1-100 mm sterile petri plate (empty)

IMPORTANT NOTE:
Alcohol lamps and bunsen burners are used to burn off the alcohol from the utensils and not for sterilization. The combination of alcohol and fire can be extremely dangerous. Use your discretion as to whether or not your students should flame their utensils. Alternatively, the alcohol can be shaken off the utensils and they can be used directly on the plant material or tissue directly without damaging the tissue and compromising the results.

Pre-Lab Preparations

B. SHOOT ELONGATION/ROOTING

Tiny shoots should appear on cut surfaces on the explants within 3-5 weeks. After enough material is available for each group (some groups may need to share with others), the shoot elongation medium can be prepared as follows:

1. Melt and prepare the shoot elongation growth medium as described in Instructor Pre-Lab Preparations for shoot initiation medium. Follow the same procedures for pouring the 60 mm sterile plates, except label the plates "elongation".

For the shoot elongation/rooting experiment, each student group requires:

- 2-60 mm plates of Shoot Elongation Growth Media
- Cultured shoot material initiated from explants
- 1 pair of forceps, 15-18 cm long
- 1 scapel with sharp blade, 15-18 cm long
- 1 tube, approximately 10-11 cm long filled with 95% ETOH and placed in a small rack
- Alcohol lamp or bunsen burner (optional)
- Gloves
- Spray bottle with 70% ETOH

Please refer to the kit insert for the Answers to Study Questions