

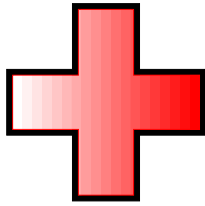


Plant Tissue Culture

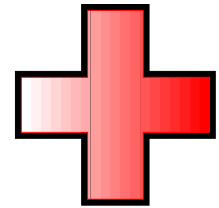
for the Serious Hobbyist, Teacher, Nurseryman and All Plant Lovers

- Plant tissue culture involves the sterile growth of plants in containers for the purpose of mass production.
- Through the use of plant hormones and other growth regulators, small plant parts can be induced to produce hundreds of small "plantlets" which can be further developed and grown in greenhouses or as house plants.
- Using a microwave oven or a pressure cooker, supplies found in your kitchen, plus supplies provided at this workshop, you can mass propagate hundreds of your favorite plants in your kitchen or classroom.
- In this workshop you will make your own media, disinfect and culture plant leaves, axillary buds and seeds, and discuss trouble shooting and internet resources.

Tentative Workshop Schedule	
9:00	<ul style="list-style-type: none"> ▶ Introductions ▶ Fill out information sheets ▶ Discuss what everyone wants to accomplish ▶ Safety and food.....PAGE 2 ▶ Location of aprons, restrooms, vending machines, building exits ▶ Please turn off cell phones and remove hats
9:15	Plant Tissue Culture for Hobbyists, Teachers and "All Plant Lovers" PPT1
10:15	"Coffee Break"
10:30	Media preparation using a microwave: Instructor will assist students in preparation of media using a microwave and "kitchen" vs. scientific methods PAGE 3
11:30	Assemble PVC boxes, prepare areas, discuss aseptic technique.... PAGE 7
12:00	Lunch
1:00	Plant Tissue Culture for Hobbyists, Teachers and "All Plant Lovers" PPT2
2:15	Demo of disinfection and culture of <ul style="list-style-type: none"> ▶ African violet leaves.....PAGE 8-10 ▶ Axillary buds (node sections).....PAGE 11 ▶ Orchid seed (dry).....PAGE 12 ▶ Subculture of established culturesPAGE 13
2:30	Hands-on disinfection and culture of African violet leaves, axillary buds (node sections), orchid seed (dry), and subculture of established culture and work on plant material that you brought
4:00	Discuss problems, trouble shooting, Clean up and return supplies for travel.



SAFETY RECOMMENDATIONS



Tissue culture techniques normally used in a scientific laboratory can be dangerous without proper training and supervision. Instruction from a qualified plant tissue culture specialist is NECESSARY before using this manual. Methods included here have been modified to maximize safety of novice tissue culturists. Material Safety Data Sheets (MSDS) provide information on the safe handling of chemicals. An MSDS (in PDF format) for each chemical involved here is located on the MSDS diskette. Read about each chemical that is unfamiliar to you before working with it. Follow safety recommendations.

- Bleach solutions will discolor clothing and can be harmful to the skin and eyes. Wear protective clothing including gloves, goggles, apron, and shoes. **Plastic aprons are provided; some goggles are available if you do not wear glasses.**
- Alcohol is flammable. Smoking and open flames should not be permitted in the area. A fire extinguisher and running water should be available.
- Sterile technique must be used to minimize contamination of cultures.
 - A **plastic lined box or plastic "re-cycle type" container** can be used as a clean area. The container should be wiped or sprayed down with 70% alcohol before starting work.
 - All items that are put into the clean area should be sprayed or wiped with 70% alcohol.
 - **Hands** should be washed, and then wiped with 70% alcohol.
 - If you have to sneeze, leave work area immediately.
 - **Long hair** should be tied back to minimize contamination.
 - Tools should be positioned in the clean area to minimize passing hands over sterile areas.
 - The 70% alcohol that is used for dipping instruments (forceps, knives) should be positioned to the far right or far left. **Use fresh alcohol daily.**
 - Do not use an alcohol burner in a plastic lined "clean box".
 - **Sterile work surfaces** are needed for cutting plant tissues. A small salad plate or a paper towel, sprayed with 70% alcohol, will provide a sterile surface.
 - Media and water should be processed in a pressure cooker or microwave.
 - Food and drink should be kept away from the tissue culture area.

MEDIA PREPARATION

The instructor will demonstrate how to make plant tissue culture media using household supplies and items provided in today's workshop.

Students will break up into 2 groups and each group will make a liter of media. Recipes are below:



Materials Needed:

- | | |
|--|---|
| <ul style="list-style-type: none">• MS Medium packets (1 L)• sucrose (table sugar)• agar• BAP and PPM• vinegar and baking soda (for pH adjustment)• water (distilled or filtered)• food coloring | <ul style="list-style-type: none">• measuring spoons• “smidgen” spoons• transfer pipettes• pH papers or pH meter• container or microwave beaker (1 liter)• baby food jars with plastic covers or plastic test tubes or Unicorn vessels |
|--|---|

Axillary Bud or Orchid Seed Medium (Green) Half Strength MS Medium with No Hormones:

- _____ 1/2 packet MS Medium with Vitamins = 1/2 teaspoon
- _____ 1 ml PPM
- _____ 1 tablespoon sugar
- _____ 2 drops **green** coloring

Dispense into Unicorn vessels (30 ml or 2 tablespoons each)

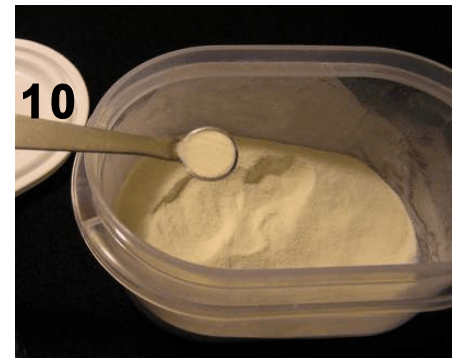
Shoot Inducing Medium (Blue) MS medium with 1 mg BAP (benzylaminopurine)

- _____ 1 packet MS Medium with Vitamins
- _____ 1 ml PPM
- _____ 1 ml BAP (cytokinin that induces shoot development)
- _____ 2 tablespoons sugar
- _____ 2 drops **blue** coloring

Dispense into 40 baby food jars (45 ml or 3 tablespoons each or prepare test tubes (500 ml media + 11 pinch agar; melt and pour 10 ml into tube)

Media preparation using a microwave and baby food jars

1. Fill jar with about 3 cups distilled water or Brita filtered water. Add the powdered medium, sugar, and PPM. Mix well with a long handled spoon. **ADD ENOUGH WATER TO BRING VOLUME TO almost one LITER.**
2. Test the pH of the solution by dipping the edge of a piece of **wide range** (pH 1-14) pH paper into the solution. A pH of 5 to 6 is preferred. Compare the color of the wet pH paper to the pH color chart.
3. If the pH is too low ("acidic"), add a pinch of baking soda to the solution. Mix well and test the pH again.
4. If the pH is too high ("basic"), add a few drops to a few milliliters of vinegar. Stir to mix and test again.
5. Continue this process until the pH is between 5 and 6.
6. To better adjust the pH to 5.6-5.8, dip the edge of the **narrow range** ("pHydrion Rain Survey Kit pH 3-6") into the solution. Compare the color to the chart.
7. Follow the steps above using vinegar and baking soda to adjust the pH to 5.6 - 5.8.
8. **ALTERNATIVELY** test the pH using a handheld pH meter. The instructor will demonstrate this.
9. Add 3 tablespoons of liquid medium to each baby food jar using a plastic measuring tablespoon or 45 ml using a Tupperware turkey baster. Add 2 tablespoons liquid media to each Unicorn vessel.
10. Add one level "pinch" spoon of agar to each baby food jar or one level "smidgen" spoon of agar to each Unicorn vessel.
11. Place the polypropylene caps on the jars loosely.
12. Place 4-5 jars in the microwave oven.
13. Microwave for about 3 - 4 minutes. Time will vary with individual microwaves. Watch the liquid and when it starts to boil, continue microwaving for **60 seconds**.
14. While wearing hot pad gloves, or using hot pads, remove the jars and sit them on a stable surface. **PUSH CAPS ON TIGHTLY.**
15. Swirl each jar briefly to mix the media and the agar. Do not hold the jar by the plastic cap since they can readily come loose.
16. Allow to cool.



Summary

Mix “ingredients” together as described on page 3



Adjust pH to 5.5 – 5.9



Dispense 3 tablespoons liquid media per baby food jar
with measuring spoon



OR



Dispense 45 ml liquid media with
turkey baster



Add ONE level pinch spoon of agar
to each jar



Place cap on jar loosely



Place 4-5 jars in microwave



Microwave baby food jars about 3 minutes
watching for the first signs of boiling



Count 60 seconds and then stop



CAREFULLY remove jar wearing hot pad
glove and swirl



Look for clear specks of unmelted agar



Microwave another 30 seconds or until
all unmelted agar has disappeared

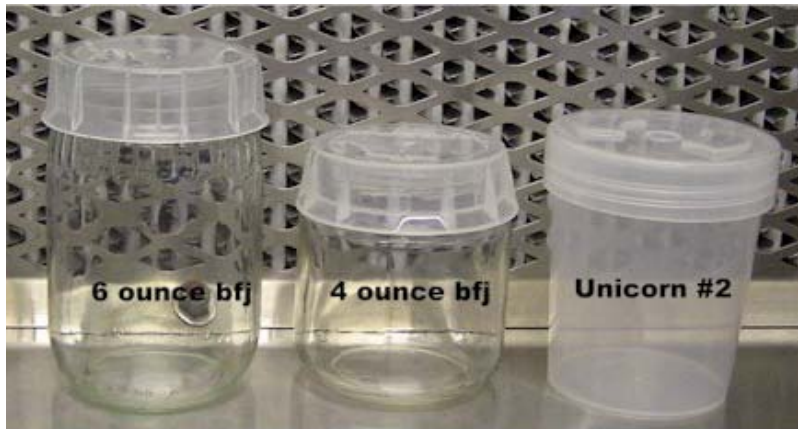


CAREFULLY remove jar wearing hot pad
glove and swirl. Press cap to tighten.

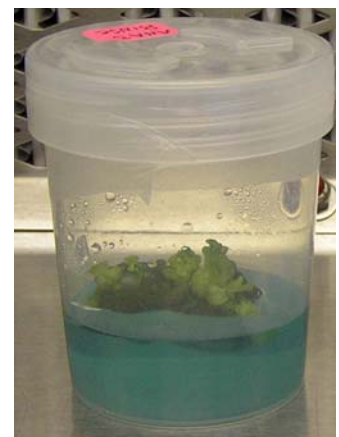
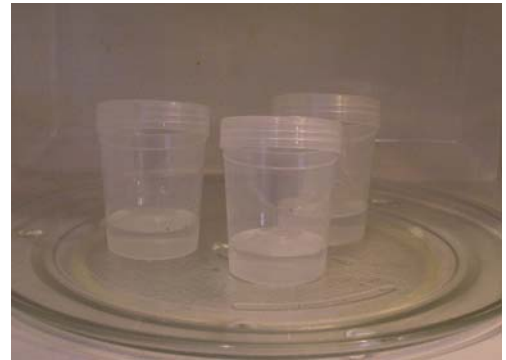


Allow to cool.

Media preparation using a microwave and new “Unicorn vessels”



- A. Prepare medium as described in previous pages.
- B. Add 2 tablespoons (30 ml) of liquid medium to each Unicorn. A Tupperware turkey baster can also be used.
- C. Add one level “smidgen” spoon of **agar** to each vessel containing 30 ml of liquid media.
- D. Place the polypropylene **Unicorn vessel cover #2** on the jar loosely.
- E. Place 4-5 jars in the microwave oven
- F. Set microwave for 2 minutes. Time will vary with individual microwaves. Watch the liquid and when it starts to boil, continue microwaving for **60 seconds**.
- G. While wearing hot pad gloves, or using hot pads or a jar holder, remove the jars and sit them on a stable surface. **PUSH CAPS ON** completely.
- H. Swirl each jar briefly to mix the media and the agar. Do not hold the jar by the plastic cap since they can readily come loose.
- I. Allow to cool. Store in plastic container or in zip lock bags on a tray.



Media preparation using a PRESSURE COOKER and “Unicorn vessels”

- Follow steps A - C above.
- Place Unicorn vessel cover on each jar.
- Place jars in pressure cooker following manufacturer’s advice. Process 15 minutes at 15 p.s.i.

- Allow pressure cooker to cool completely and pressure to go down to ZERO. Open carefully and remove jars using canning tongs or jar holder and place on solid surface..



- While wearing hot pad gloves, or using hot pads, Swirl each jar briefly to mix the media and the agar. Do not hold the jar by the plastic cap since they can readily come loose.

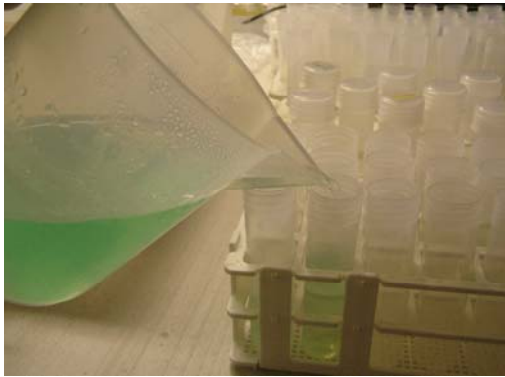
- Allow to cool. Store in plastic container or in ziplock bags on a tray to minimize media drying out.



Media preparation using a microwave and screw cap test tubes



- a. Prepare liquid medium as described above.
- b. Pour 500 ml into a 1 liter microwave beaker with a spout and handle. **Add 11 level pinch spoons of agar.**
- c. Microwave until the solution boils. Stir.
- d. Microwave more until all agar is dissolved. No floating clear specs should be left in solution. Do not allow to boil over.



- e. Pour about 10 ml into each plastic test tube (about 1 inch). Put screw cap on tube loosely. NOTE: YOU COULD ALSO POUR MEDIA INTO BABY FOOD JARS OR OTHER MICROWAVE-PROOF VESSELS.



- f. Place tubes in microwave-proof test tube rack and microwave for 15 seconds. Push OFF button.
- g. Microwave 15 seconds more. Watch tubes closely and push OFF button if you see media boil. Continue this for a total of 120 seconds or until you see all tubes begin to gently boil.



- h. Remove rack of tubes from microwave. Check media to see if melted. Mix or “rock” the tubes to mix.
- i. Cool rack on a slant to increase surface area of medium and to allow moisture to flow down away from plant piece.

Preparation of "sterile" water using microwave and PPM



Assemble materials:

- Brita filtered water or distilled water
- PPM
- transfer pipette
- microwave proof containers
- one liter container



In the one liter container, add one liter water and 1-2 ml PPM. Mix.

Add about 1-2 inches of water to each microwave-proof container.

Place covers on loosely.

Microwave for about 3 - 4 minutes. Time will vary with individual microwaves. Watch the liquid and when it starts to boil, continue microwaving for **60 seconds**.

While wearing hot pad gloves, or using hot pads, remove the jars and sit them on a stable surface.

Allow to cool. Tighten caps.



Building Clean boxes

The purpose of the clean area is to limit the number of particles that fall into your tissue culture jar. These airborne particles carry bacteria and fungi, and can kill your plant tissues because they grow faster than the plants.

Break into pairs and assemble a clean box. We have three kinds: PVC, CPVC and our new “tinker toy” boxes.



PVC



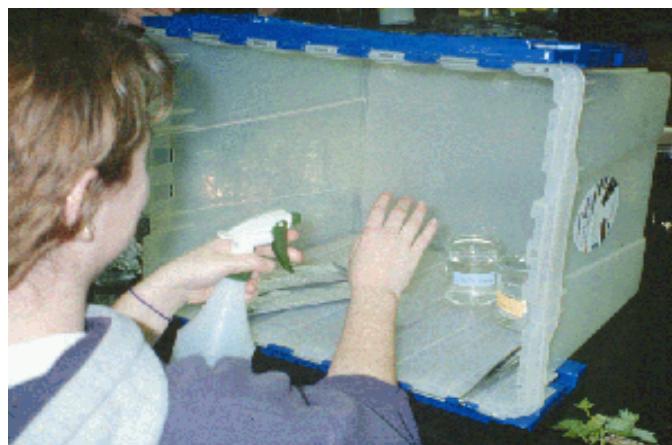
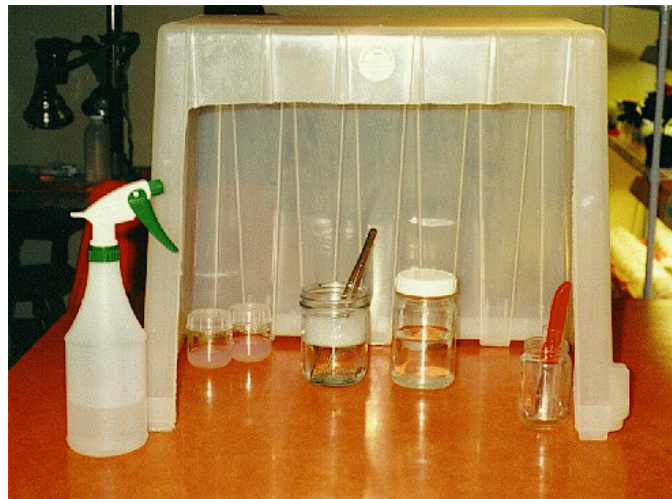
CPVC



“Tinker Toy”

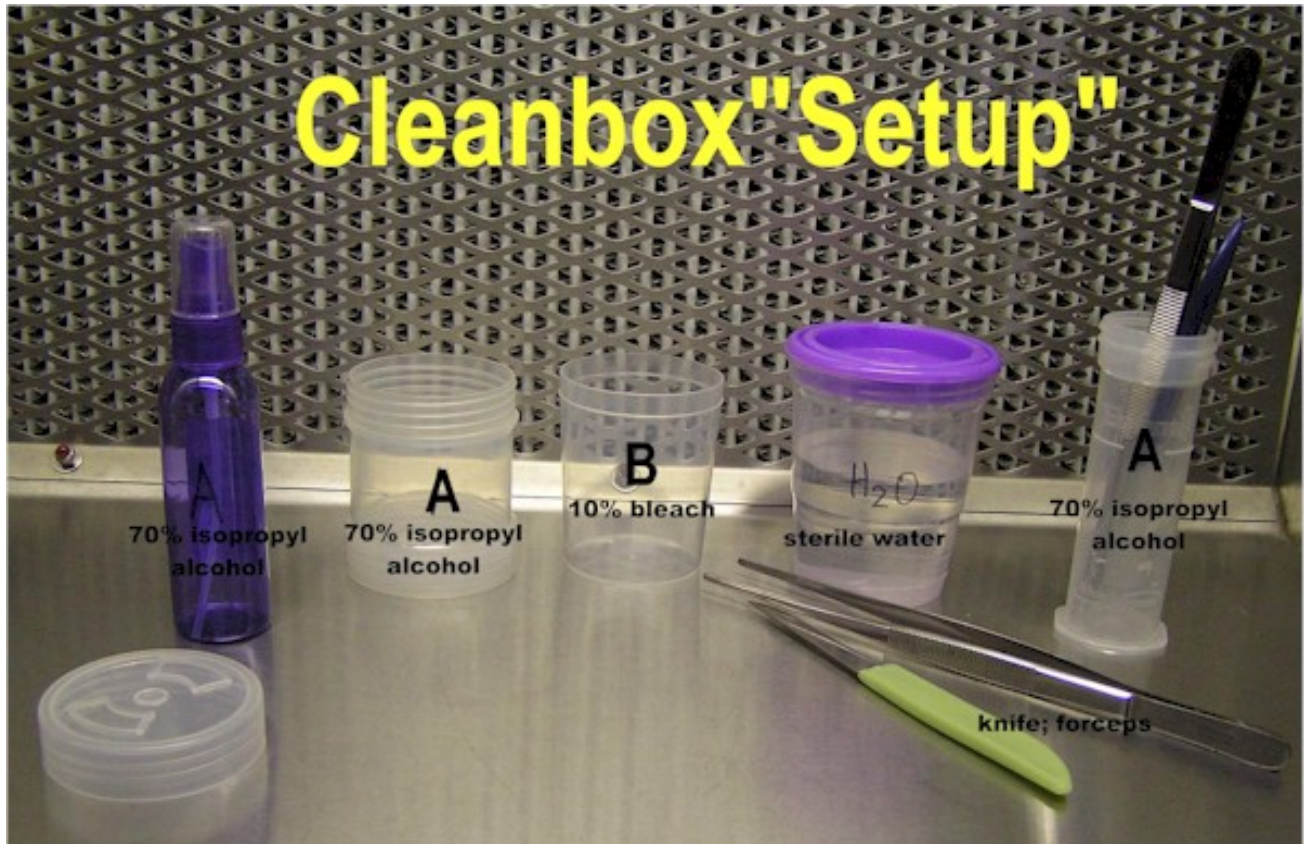
Preparing a clean area

- a. The inside of the clean box and the surface of the clean area should be wiped down, or sprayed, with 70% isopropyl alcohol.
- b. All items that are put into the clean area (media jars, bleach container, sterile water jar, “dipping” alcohol) need to be wiped down, or sprayed, with 70% alcohol.
- c. Hands should be washed in soap and water for at least 20 seconds, and then wiped with 70% alcohol. Do not use the alcohol on your hands if you have sensitive skin. You can also use the hand sanitizers with ethanol. Vinyl gloves are OK. These need to be sprayed with alcohol.
- d. Dip or soak instruments in 70% alcohol. A test tube in a tall baby food jars works well as does a tall “shot glass”, a short bud vase or an olive jar.



Cleanbox "Setup"

Each cleanbox needs these items:



- **2 forceps
- **2 knives
- **alcohol spray bottle
- **alcohol container (Enfamil bottle) for soaking forceps and knives
- **container for dipping explants in alcohol (marked "A")
- **container for bleach solution (marked "B")
- **Saran wrap or Austraseal for wrapping jars
- **sterile water container (with purple cover)

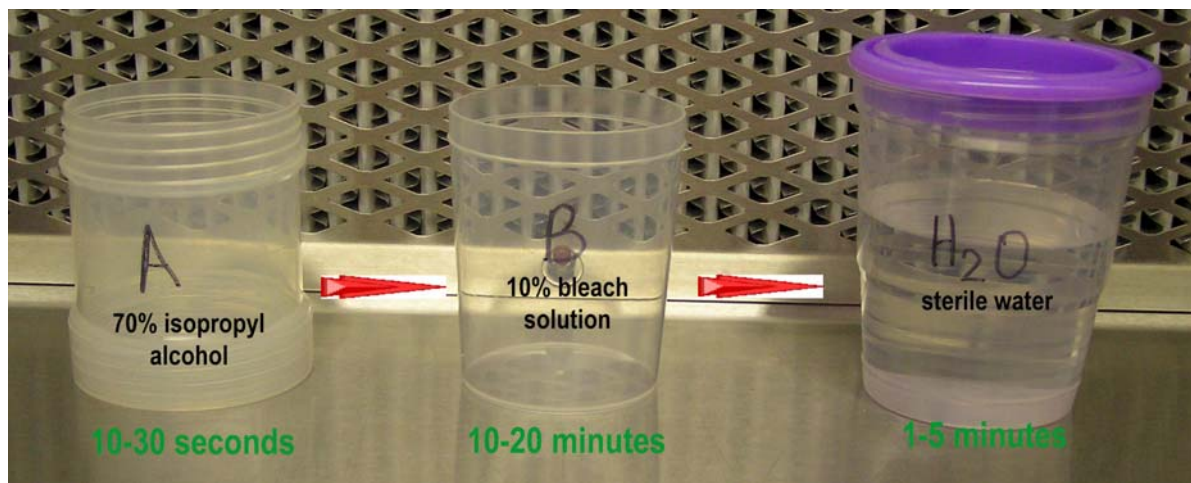
Hands-On Culture

African Violet Leaves

Materials needed for the culture of African violet leaves

- African violet leaves (1 leaf per person).
- African violet medium - BLUE.
- 70% alcohol (about 1 inch deep) for rinsing leaves.
- 10% bleach solution (1/3 cup bleach + 3 cups water + a few drops of detergent)
- Sterile water for rinsing leaves.
- Paper toweling to serve as sterile cutting surface
- Forceps and small kitchen knife.
- Florists' tape, Saran wrap, or Austraseal to wrap jar or tube.

Cleaning the plant material



OUTSIDE OF THE CLEAN AREA:

1. Pick up leaf with a forceps and dip into the 70% isopropyl or ethyl alcohol for a few seconds. This will remove some debris and wax.
2. Place leaf in 10% bleach solution and allow to soak for 10 minutes. Stir occasionally so the solution gets in contact with all of the plant surfaces.

INSIDE THE CLEAN AREA:

3. Move the bottle with leaves to the clean area.
4. Spray area, media bottles, and other containers in the clean area with 70% alcohol.
5. Transfer leaves to sterile water using the forceps that was soaking in the bleach/leaf solution. Allow the leaves to soak for 1-5 minutes in the water.

Culturing the leaves

6. Spray a piece of paper toweling with 70% alcohol.
7. Dip the forceps in 70% alcohol and transfer one leaf to the toweling.
8. Dip the kitchen knife in 70% alcohol and shake off excess alcohol.
9. Holding the petiole end with the forceps, cut the edges of the leaf away. This seems to stimulate more shoot growth. Cut off the petiole and then cut the leaf in half.
10. TO MINIMIZE POTENTIAL CONTAMINATION, DO NOT CUT THE EDGES OF THE LEAF. YOU CAN CULTURE A SMALL LEAF WHOLE RATHER THAN CUT IN HALF.
11. Loosen the caps on baby food jars without holding your hands over the cut plant pieces.
12. Dip the forceps in 70% alcohol and shake them to remove excess. Pick up one leaf piece.
13. With your other hand, pick up the cover of the media jar just enough to allow space to place the leaf piece in the jar. The leaf can be right side up, up side down, or sideways. Quickly replace the cover. *Our goal is to limit the time that the cap is open and the media is exposed to the open air.*
14. Repeat this process for all leaf pieces to be cultured.
15. Wrap florists' tape or Austraseal tape around the outside of the jar. This will help to minimize the debris that gets into the jar and causes contamination of the cultures.
16. Put the cultures in a bright room out of direct sunlight or culture on shelves with cool-white fluorescent lights positioned about 9-12 inches from the shelf below. Lights should be on 16 hours per day.
17. The leaves should start to swell in 2 - 4 weeks, and small bumps and then leaves will appear on the "mother" leaf's surface. The plant growth regulator, BAP, in the growth medium induces shoots to grow from cells in the leaf.

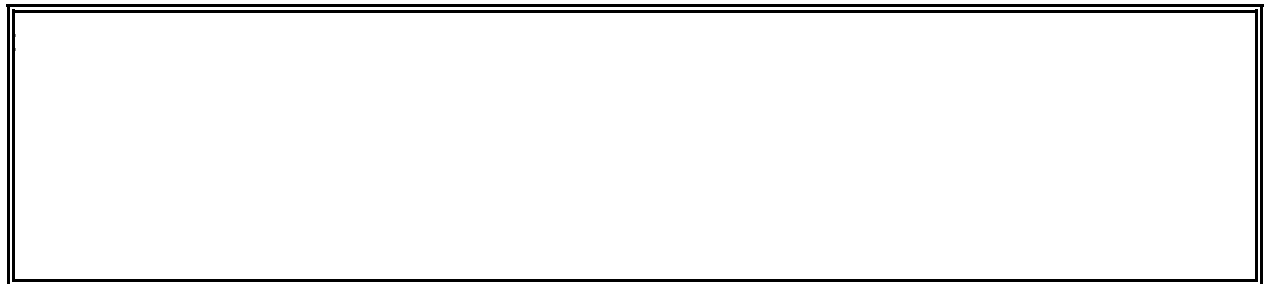
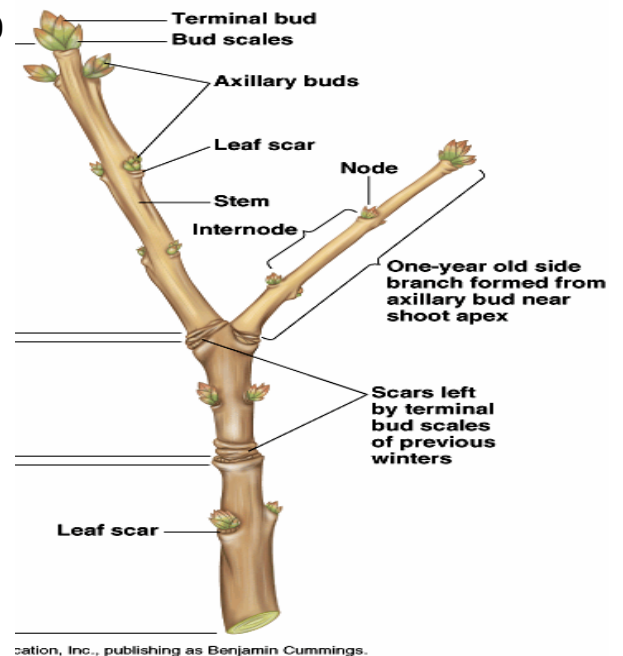


Axillary Bud (Node Section) Culture

Shoots will grow from axillary bud cuttings from many species when cultured on MS medium without hormones or on other species-specific medium.



- A. Cut stems into nodal cuttings with each piece containing an axillary or lateral bud. Stems should be green and vegetative. Remove leaves.
- B. Dip the cuttings in 70% alcohol for about 60 seconds.
- C. Place in 10% commercial bleach and soak 10-15 minutes. Stir occasionally using a long forceps or spatula.
- D. Transfer explants to a jar containing sterile water and soak for 2-3 minutes to rinse off the bleach.
- E. Place one cutting on a sterile plate (wipe off with 70% alcohol).
- F. Slice off the ends where the tissue has turned white using a sterile knife (that had been dipped in 70% alcohol).
- G. Place in a baby food jar containing the proper medium with no hormones. The plant piece can be laid horizontally on the medium surface or stuck in the medium ("up and down"). Cap. Seal.



Orchid Seed (Dry)

You will need these items:

- 5% sucrose with a few drops detergent
- 3% hydrogen peroxide
- dry orchid seeds
- transfer pipettes or small knife or forceps
- small tube to hold seeds
- orchid seed germination medium



1. Place seeds in small test tube or vial.
2. Add 5% sucrose solution (1 teaspoon sugar in 100 ml water with a few drops detergent) and soak for 12 hours at room temperature. This is not sterile. **THIS SHOULD BE DONE THE NIGHT BEFORE THE WORKSHOP. Note: we often omit this step without any problems.**



3. Remove most of the liquid with a non-sterile transfer pipette
4. Add enough hydrogen peroxide to cover the seeds - about 2-3 ml
5. Place cap on tube and tighten. Shake briefly. Loosen cap slightly and allow to sit for 30 minutes at room temperature.
6. In the clean box, sterilize the transfer pipette with alcohol. Shake to remove most of alcohol. **ALTERNATIVELY** sterilize the knife by dipping in alcohol. Shake off excess alcohol.
7. Pipette this solution onto orchid seed germination medium. It will resemble a “lake” on top of the medium. Replace baby food jar cap and seal. Label.
OR
Use the knife as a spatula and scoop the seeds up and tap into the media jar.
8. Your orchid seeds should grow as seen in these photos. Transfer to fresh medium when seedlings are about an inch tall.



Subculture (transfer) of “plantlets” to fresh medium

The newly developing plantlets will grow better if they are transferred to fresh medium without growth regulators. The growth regulators can inhibit elongation of the shoots and the formation of roots.

This transfer is called “**subculture**”.

- After 4-6 weeks, make fresh medium without hormones.
- Prepare the clean area as you did before. Wipe off the original culture bottles with alcohol and loosen the caps. Loosen the caps on the fresh media jars.
- Wipe a small plate with alcohol to use as your cutting surface or use a paper towel sprayed with alcohol.
- Dip the forceps in 70% alcohol and carefully remove the plant culture from it's jar and place on the plate.
- Cut into sections or pull apart plantlets using sterile forceps and knife. Place each small piece or plantlet into fresh medium. Recap and seal.



For further information see:

www.kitchencultureEducation.org

www.hometissueculture.org

www.kitchenculturekit.com

Contact Carol Stiff at:

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carol@kitchencultureEducation.org

608-302-2750

Media Prepared Before the Workshop [Based on 24 Students]:

African Violet Leaf Medium

MS medium with BAP + **blue** color
[1 liter – 24 Unicorn vessels + 24 test tubes]

Per liter:

- _____ 1 packet MS media packet (Caisson Labs)
- _____ 1 ml PPM
- _____ 1 ml BAP
- _____ 2 tablespoons sugar
- _____ 1 drop **BLUE** food coloring

Prepare media according to instructions in manual: Add ingredients to about 900 ml of water. Mix well. Bring to almost 1 liter. Adjust pH to 5.5. Bring volume to 1 liter.

A: Dispense 3 tablespoons to a baby food jar. Add 1 level “pinch” of agar to each jar

B: Dispense 2 tablespoons to each Unicorn vessel + one level “smidgen” spoon of agar.

C: For test tubes, add 11 level smidgen spoons agar to 500 ml liquid media.

Process in microwave as described in preceding pages.

Axillary Bud Medium/Subculture Medium

MS medium without hormones (no food coloring)
[2 liters - 48 Unicorn + 48 tubes needed for workshop]

Per liter:

- _____ 1 packet MS media packet (Caisson Labs)
- _____ 1 ml PPM
- _____ 2 tablespoons sugar

Prepare as described above.

Orchid Seed Medium

Half Strength MS Medium without hormones - **GREEN** color
[2 liters - 48 Unicorn vessels + 48 tubes needed for workshop]

Per liter:

- _____ ½ packet MS medium (= ½ teaspoon) (Caisson Labs)
- _____ 1 ml PPM
- _____ 1 tablespoons sugar (60 g)
- _____ 1 drop **GREEN** food coloring

Prepare as described above.

Summary of Hands-On Afternoon Activities

EACH **BOX** NEEDS:

- **2 forceps
- **2 knives
- **alcohol spray bottle
- **alcohol container (Enfamil bottle)
for soaking forceps and knives
- **container for dipping explants in alcohol
(marked "A" or alcohol)
- **container for bleach solution (marked "B" or bleach)
- **Saran wrap or Austraseal for wrapping jars
- **pencil

To be handled out in afternoon:

- 1 sterile water (with purple cover)
- 1 TEST TUBE of orchid seeds

EACH **PERSON** NEEDS:

- 1 blue baby food jar for African violet leaf
- 1 blue Unicorn vessel for African violet leaf
- 2 green Unicorn vessels for orchid seeds
- 2 white test tubes for axillary buds

- 1 African violet leaf (or 2 small ones)
- 2 node cuttings (with axillary buds)

Before starting culture of plant parts, spray cleanbox down with 70% alcohol as instructed in class. Any containers entering the cleanbox must also be sprayed with alcohol.

- A. You will culture one African violet leaf and two axillary bud cuttings. These can be disinfected at the same time:
1. Dip leaf and node sections into 70% alcohol about 30 seconds
 2. Transfer these plant pieces to 10% bleach solution.
 3. Inside the cleanbox: After 10 minutes in the bleach solution, transfer the plant pieces to sterile water for about 3 minutes.
 4. Transfer to culture medium.
- B. You will also culture orchid seeds – Carol will demonstrate.
- C. There will also be time for subculture of existing cultures and establishment of cultures from plants you brought to class. Extra media (blue, white, or green) will be available for these.