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Propagation of Miniature Roses by Plant Tissue Culture

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Abstract: Tissue culture is a propagation technique widely used in modern agriculture because it allows production of many clonal plants from relatively little starting material. During this lab exercise, miniature roses are propagated by simple plant tissue culture. Students learn the different steps involved in the tissue culture procedure, practice aseptic technique when handling explants, and observe the effects of different hormones and nutrient levels on explant development.

Key words: plant tissue culture, plant propagation

Contents:

Introduction

This lab exercise was designed for a one-semester non-majors botany class. Students taking this course generally have a very minimal background in science. The primary objective of this lab is to give the students hands-on experience using plant tissue culture to propagate plants which they will eventually take home at the end of the exercise. As it is presented here, the exercise is not investigative, but it could easily be converted to an investigative format more appropriate to an upper level course. The lab exercise requires three lab sessions over a 6-8 week period to complete. The first lab requires approximately 90 minutes and the subsequent lab periods only require 10-15 minutes each. At Georgia Perimeter, this lab exercise is performed with two lab sections of 24 students each. Performing this lab with a large number of students or a large number of lab sections would be problematic due to the amount of preparation required for the media and solutions, as well as the need for bench space to house the plants as they develop. The culture protocol outlined here is similar to rose culture protocols from Carolina Biological (kit cat.#19-1192), Hasegawa (1980), Hyndman *et al.* (1982), and Kane (1991). This protocol reflects minor changes to the sterilization and explant handling procedures, as well as significant changes to the equipment suggested by previous authors.

Materials

- Rose shoot initiation medium
- Plastic backed paper
- Brown single-fold paper towels
- Rose root initiation medium
- Sterile petri dishes
- Vermiculite or potting soil
- Gel-Rite gellan gum
- Aluminum foil
- 14 qt. plastic bins
- Sucrose
- Bleach
- Magenta GA7 growth chambers
- 1 M NaOH
- Stopwatches
- Self-filling repetitive syringe
- 1 M HCl
- Miniature rose plants
- 0.22 um Sterivex filtration units
- Ethanol
- Small disposable cups
- Small plastic spray bottles
- L(+)-Ascorbic acid
- Forceps (4”)
- Transfer boxes
- Citric acid monohydrate
- Transfer baskets
- Lighting unit
- Labels
- 4 oz. polypropylene jars
- Stem washer
- Autoclave tape
- Scalpel handles
- Parafilm
- Scalpel blades

Student Outline

Plant tissue culture is a propagation technique widely used in modern agriculture that allows a complete plant to be grown from a single plant cell. Tissue culture is considered an asexual propagation technique since it only involves the cells from a single parent plant. Asexual propagation techniques produce plants that are genetically identical to the parent plant and to each other. This is important if a particular trait needs to be present in all of the offspring. The ability to produce an entire plant from a single cell is also an important part of genetic engineering. When a plant is genetically engineered, one or more foreign genes are inserted into the nucleus of a single plant cell. Tissue culture is then used to produce a complete plant from that single engineered cell. Each cell in the new plant will now contain and express the inserted gene. It is common in modern agriculture to insert genes for traits such as herbicide resistance or to produce compounds which are insecticidal.

Producing a plant from a single cell in the laboratory is not a simple process. Plants are complex, multicellular organisms consisting of thousands, or even millions, of specialized cells. A plant normally begins its life as a single-celled zygote that resulted from the union of the egg and sperm from the two parent plants. The zygote divides repeatedly which gradually increases the number of cells in the plant. At some point, the cells of the young plant will begin to become specialized for specific functions. For example, the outermost layer of cells will become specialized for protection, while other cells inside the stems and roots will specialize to transport food and water around the plant. Under most circumstances, once a cell has become specialized for a specific function, it can't change to perform a different function.

Eventually, most of the new growth in the plant becomes restricted to specific areas at the tips of the stems and roots called meristems. The cells that compose the meristems (meristematic cells) are relatively unspecialized and retain the ability to become any of the specialized cells in the plant. This property is referred to as totipotency. The only other type of cells that remain totipotent are the parenchyma cells that compose much of the interior of the stems, roots, and leaves. Most tissue culture procedures utilize either meristematic cells or parenchyma cells as the starting material. Meristematic cells are usually the preferred cell to initiate new plants since they begin to develop into stems and leaves very quickly. If parenchyma cells are used, they must first be allowed to grow in culture for several weeks until they form a mass of unspecialized cells called callus. The cells that compose the callus will then behave much the same way as meristematic cells. In this exercise we will use meristematic cells from a part of the plant called an axillary bud.

All of the different cells in a plant must develop and work together in a coordinated manner in order to carry out the various processes necessary for the plant to live. During normal development, the specialized cells within the plant are produced at the proper times in response to growth stimulating and regulating chemicals called hormones. During tissue culture, the hormones must be supplied artificially to the plant at the proper time. Two important classes of hormones used in tissue culture are cytokinins and auxins. These hormones promote division and specialization of cells, and later development of stems, leaves, and roots. It is often necessary to treat the developing plant with different hormones at different times because a hormone that promotes stem and leaf development may inhibit root formation.

In addition to supplying the plant with the proper hormones at the proper time, it is also necessary to supply the plant with all of the nutrients necessary for its development. Water and mineral nutrients such as nitrogen, phosphorous, and potassium are needed by all plants for proper growth. Some plants also benefit from the application of certain vitamins during the early stages of development. A simple sugar such as sucrose is often used as an energy source until the plant develops the ability to photosynthesize

and produce its own sugar. All of these chemicals are incorporated into a growth medium that is made semisolid by the addition of agar. Agar is a chemical harvested from certain types of brown algae (seaweed), and is similar in consistency to gelatin. There are dozens (possibly hundreds) of different tissue culture media formulations for the various plants that are grown in tissue culture today.

The last requirement for successful tissue culture is to prevent bacteria or fungi from attacking the developing plant. Microorganisms, such as bacteria and fungi, are everywhere in the environment, and under normal conditions a plant has defenses against most bacteria and fungi. However, a plant in the early stages of tissue culture is very susceptible to attack by these microorganisms, so tissue culture must be carried out under sterile conditions if it is to succeed. Everything that will be used in the tissue culture procedure must be sterilized, including the plant that the starting cells are taken from. The instruments, containers, tissue culture media, and solutions are sterilized by heat in an autoclave, which is like a large pressure cooker. The plant cells that will be used to start the process, referred to as explants, must be sterilized by soaking them in ethanol and bleach. The cells must be soaked long enough to kill any bacterial or fungal cells, but not so long that all of the plant cells are killed.

Once the plant tissue has been sterilized, it is important that only sterile instruments be used to touch it. Also, the piece of plant tissue can only be placed into sterile solutions or onto sterile surfaces. This close attention to maintaining sterile conditions is called aseptic technique. Observing proper aseptic technique will be one of the most important factors determining the success of this lab.

The Basics of Aseptic Technique

The primary goal of aseptic technique is to reduce contamination by bacteria and fungi. The most important thing to remember is to touch a sterile item only with another sterile item. Anything that has been treated with heat or **soaked** in bleach or ethanol to kill bacteria can be considered sterile. An item is no longer sterile if it has been touched by an unsterile item.

1. Wipe your lab table down with disinfectant and keep the table clear of everything except your lab manual and the tissue culture equipment and supplies.
2. Wash your hands gently with waterless hand cleaner before working with any sterile items. Do not touch your face, hair, or clothing while you are working with sterile items.
3. All work with sterile items must be performed inside of a transfer box. The transfer box will keep you from breathing or coughing on any sterile items. It also prevents contamination by airborne dust particles falling into a sterile area.
4. Long sleeves should be pushed up beyond the elbow. Do not allow clothing to enter the transfer box.
5. All items that you put into the transfer box should be wiped down with a sterile towel that has been sprayed with 70% ethanol before being placed into the box. Spraying with ethanol will remove some, but not all, of the bacteria from an item, so it is **NOT** sterile.
6. All instruments, such as forceps and scalpels, have been wrapped in foil and autoclaved to sterilize them. After the instruments are unwrapped, they must be kept in 70% ethanol to keep them sterile. Do not let the instruments touch any unsterile items.
7. Read and follow directions exactly. Know what you are going to do in advance so that you can work quickly and expose items to air for a minimum amount of time.

Miniature Rose Tissue Culture

During this lab period, you will begin the process of cloning a miniature rose plant by tissue culture. This process may take as long as eight weeks to give fully formed rose plants that are ready for you to take home. The tissue culture process can be divided into 4 main stages (Fig. 1). Today you will complete Stage 1 and begin Stage 2. Stages 3 and 4 will be started during two lab periods later in the semester. There is not an exact schedule for the timing of Stages 3 and 4. They will be done based on how quickly the plants develop.

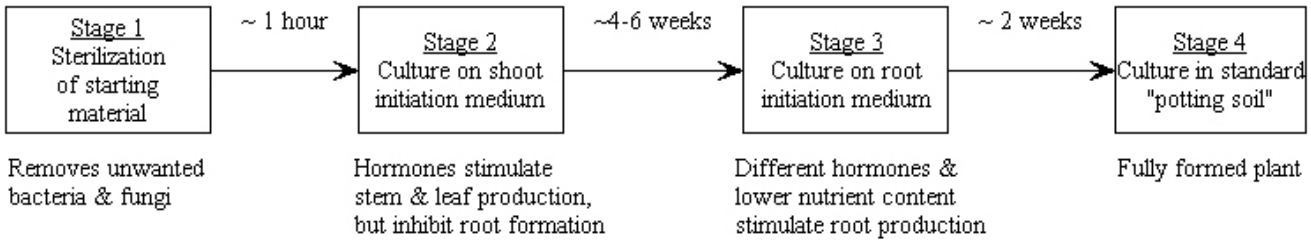

Fig. 1. Flowchart for rose tissue culture

Culture media

The compositions of the two culture media used in this protocol are outlined below:

Shoot initiation medium	Root initiation medium
Indole acetic acid (IAA)	Naphthalene acetic acid (NAA)
Benzyladenine (BA)	
Mineral nutrients	Mineral nutrients (1/4 strength)
Citric & ascorbic acid	
Sucrose	Sucrose
Vitamins	Vitamins
Agar	Agar

The media differ primarily in the hormones they contain. The shoot initiation medium contains the hormones IAA (an auxin) and BA (a cytokinin). Both of these hormones promote the formation of stems and leaves, but inhibit the formation of roots. The root initiation medium contains only one hormone, the auxin NAA, which will promote the formation of roots.

The two media have the same composition of mineral nutrients, but at different concentrations. The rooting medium has one fourth the amount of mineral nutrients, which appears to promote more rapid formation of roots in roses (Hyndeman *et al.*). The shoot initiation medium contains citric and ascorbic acid which act as antioxidants. Both media contain equal amounts of sucrose (carbon/energy source), vitamins, and agar (a solidifying agent).

Stage 1: Sterilization of starting material

The starting material for Stage 1 will consist of 1 cm pieces of rose stem. These pieces were cut from a large mother plant kept in the prep room. The regions on the stem where leaves attach are called nodes. Each node contains an area of meristematic tissue called an axillary bud. This is what will give rise to our cloned plants. The primary objective of Stage 1 is to sterilize these stem pieces.

Your instructor has cut the long stems into 1 cm sections containing a single axillary bud and an attached leaf. The small leaflets have been removed (Fig. 2), leaving the stem-like portion of the leaf called the petiole. The stems were then washed under running water for approximately one hour.

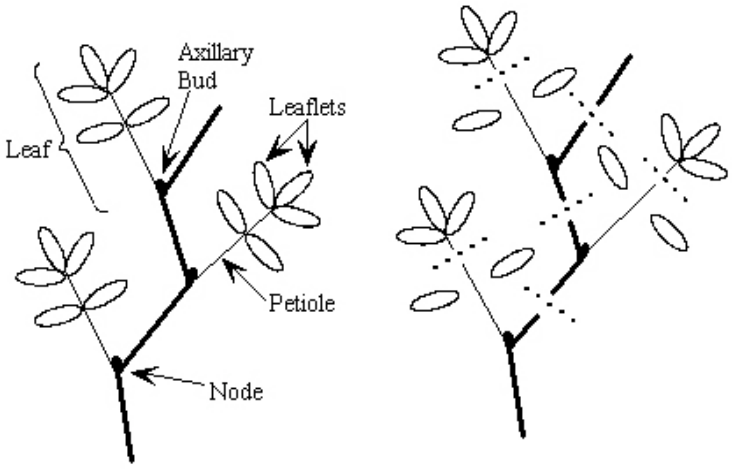

Fig. 2. Rose stems were prepared by removing leaflets and cutting the stems into 1cm pieces.

1. Wash your hands with the waterless hand cleaner, clean your bench top with disinfectant, and clear the top of your lab table if you have not already done so.

2. Get a supply bin and a plexiglass transfer chamber from the supply bench. Your supply bin should contain the following:

   1 spray bottle of 70% ethanol
   1 jar of 70% ethanol
   1 jar of 50% ethanol
   4 jars of sterile water
   1 jar of 20% bleach
   1 jar of sterile antioxidant solution
   2 sterile forceps wrapped in foil (marked "FF")
   1 sterile transfer basket wrapped in foil (marked "B")
   1 pack of sterile towels wrapped in foil (marked "T")

3. Organize your workspace as indicated below (Fig. 3). Keep all unused items to the left of the transfer box. Once an item has been used, place it in the empty supply bin to the right of the transfer box.

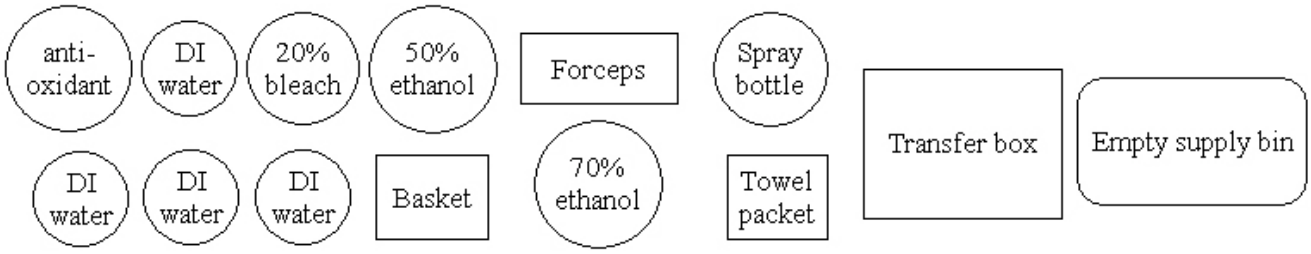

Fig. 3. Bench layout diagram. Keep all unused items to the left of the transfer box and all used items to the right.

4. Open the pack of sterile towels. This is the only item that you will open outside of the transfer box. Leave the towels loosely wrapped in the foil.

5. Lightly spray the floor of the plexiglass transfer box with 70% ethanol and wipe down the inside of the box with one of the sterile towels

6. Lightly spray a towel with ethanol and use it to wipe down the jar of 70% ethanol and the pack of forceps, then place them into the transfer box.

7. Working inside the transfer box, open the jar of 70% ethanol. Grasp the package of sterile forceps by the taped end and tear the foil off of the other end of the package. Hold each pair of forceps by their exposed handles only and place the tips of the forceps down into the jar of 70% ethanol.
6. Wipe down the packet containing the transfer basket with ethanol, and place into the transfer box.
7. Carefully unfold the foil package containing the transfer basket. Be careful not to touch the inside of the foil or the basket. Leave the basket on the inner surface of the sterile foil. The foil will provide a sterile barrier between the basket and the floor of the transfer chamber.
8. Remove the forceps from the ethanol and use them to carefully open the transfer basket by grasping the small tabs along the front of the basket. Return the forceps to the 70% ethanol.
9. Your instructor will bring a beaker around containing washed rose stem pieces. Use a pair of the forceps to remove two stem pieces, place them into the transfer basket, and close the basket.
10. You will now transfer the stem pieces through a series of sterilizing soaks and water rinses. Follow the flow chart (Fig. 4) and observe the times closely.

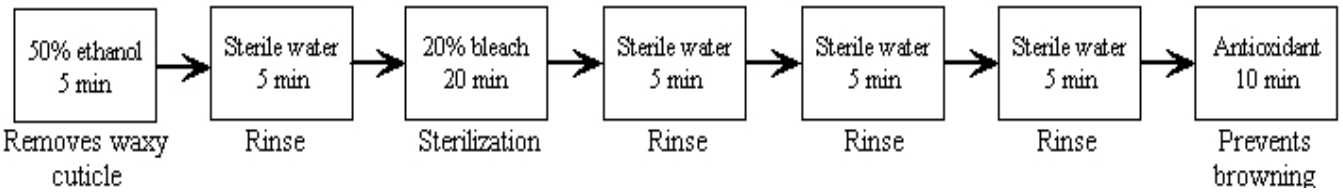

Fig. 4. Flow chart for Stage 1 of the rose tissue culture protocol

11. Please remember the following points:
    - When you open the jars, be very careful not to touch the inside of the jars or the lids with your fingers.
    - Use the forceps to transfer the basket between each of the soaking solutions.
    - After each transfer, close the lid of the jar tightly, and agitate the jar gently 2-3 times during each of the soaking intervals.
    - Remove each jar from the transfer box after it has been used, then wipe down the next jar and place it into the transfer chamber.
    - Keep the last sterile water in the transfer box

12. After you transfer the rose stems to the antioxidant rinse, go to Stage 2: Shoot initiation.

Stage 2: Shoot initiation

After the explants have been properly sterilized, they need to be placed into a sterile growth chamber containing the shoot initiation medium. The meristematic cells in the axillary buds should begin to grow within a few days. The hormones in the medium (BA and IAA) will promote the formation and growth of stems and leaves. Within a few days you will notice that the axillary bud has swollen and growth of new leaves and stem tissue should be visible within the first week. However, it may take four to six weeks for the stems to be mature enough to move to the next stage.

1. After you move the stems into the antioxidant soak, get a Magenta GA-7 growth chamber (Fig. 5) containing shoot initiation medium and an empty sterile petri dish from your instructor.
2. Wipe down the growth chamber and the petri dish with 70% ethanol and place them into the transfer box.
3. If the top of the chamber is wrapped with Parafilm, remove the film.
4. Open the petri dish and place it on the bottom of the transfer box. Remove the transfer basket from the antioxidant solution and place it in the sterile petri dish.
5. Carefully open the transfer basket using the forceps. **DO NOT** touch the basket or the explants with your fingers.
6. Open the growth chamber and place the explants into the agar using Fig. 6 as a guide. You should grasp the explant carefully and be extremely careful that you do not touch the inside of the growth chamber. Immediately recap the chamber once the stems are inserted into the medium.
7. Wrap the top of the growth chamber with a strip of Parafilm where the lid and the chamber meet.
8. Write your names on a label and place it on the side (not on the lid) of your growth chamber. Place your chamber under the grow lights on the back bench.
9. Summarize what you did today in your observation log.

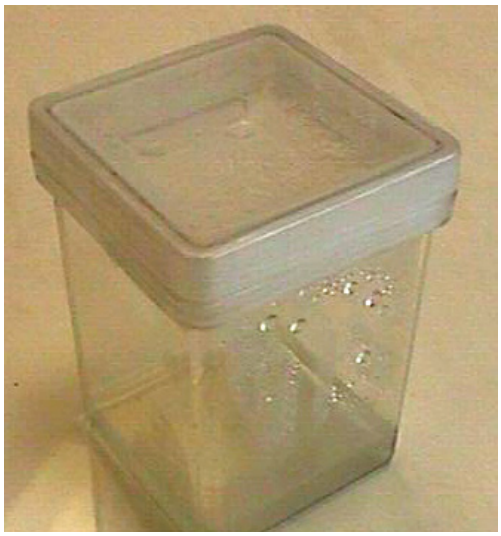

Fig. 5. Magenta GA-7 growth chamber

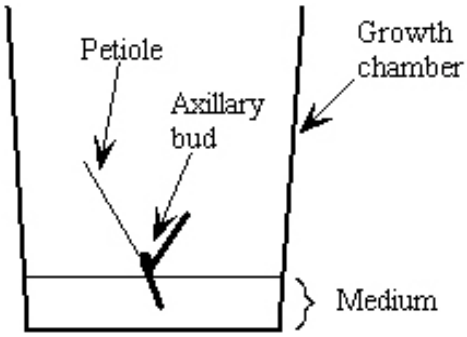

Fig. 6. Proper placement of rose stem in shoot initiation medium. Note that the petiole and axillary bud must be facing up.

Stage 3: Root initiation

At this point, each of the rose explants that you put into the shoot initiation medium should have produced one or more stems. However, the hormones that promoted the stem formation, have inhibited the proper formation of roots. During Stage 3, you will put two of the stems onto rooting medium so that they will form proper roots. Your instructor will have a transfer box and all of the needed supplies at the instructor's bench. Each group will need to go to the instructor's bench sometime during the lab period to transfer their rose plants to the next type of agar. After 7-10 days on the root initiation medium, you should notice small white bumps forming along the part of the stem in the medium. These will elongate and become the new roots for your rose plants.

1. Wash your hands with the waterless hand cleaner before you work with your rose explants.

2. Get the growth chamber containing your rose explants from the back bench. Wipe down the growth chamber with 70% ethanol and place it into the transfer box. Remove the Parafilm from the lid of the chamber.
3. Get a new growth chamber containing root initiation medium and an empty sterile petri dish. Wipe down the box and the petri dish lightly with 70% ethanol and place them in the transfer box.
4. Remove the Parafilm from the lids of both growth chambers.

*** The following steps must be done quickly or your rose plants will dry out and die.***

5. Open both growth chambers and the sterile petri dish. Do not touch the insides of the chambers or the petri dish with your fingers.
6. Remove the forceps from the jar of 70% ethanol and dip them in the jar of sterile water to remove any excess ethanol.
7. Using the sterile forceps, remove one of the rose plants from the shoot initiation medium and place it in the sterile petri dish.
8. Remove the scalpel from the ethanol and dip it in sterile water. Carefully cut two of the new stems from old stem piece and place the new stems into the growth chamber containing the root initiation medium. Return the forceps and scalpel to the jar of ethanol.
9. Recap the growth chamber and remove them from the transfer box. Label your growth chamber, wrap the lid with Parafilm, and place it back under the grow lights.
10. Summarize what you did today in your observation log.

Stage 4: Transfer to soil

Hopefully you have made it to this stage with at least one fully formed rose plant. Your rose plants are now ready to be transferred out of sterile culture and into soil. Your instructor will have all of the needed supplies at the instructor's bench. Each group will need to go to the instructor's bench sometime during the lab period to transfer their rose plants to a small pot of soil or vermiculite. This stage does not need to be done in a transfer box, since the medium you are going to transfer your rose plants to is not sterile.

1. Get the growth chamber containing your rose plants from the back bench. You will also need an empty growth chamber or ziploc bag, a small pot with soil or vermiculite, and a pair of forceps. These should be at the instructor's bench.
2. Fill the pot with soil or vermiculite. The medium should be slightly packed down so that it is firm. Use the forceps to create a small hole in the soil for your plant.
3. Open the growth box containing your rose plants and remove one of them with the forceps.
4. Place your rose plant into the hole in the soil. Be careful that you do not break or damage the plants roots.
5. Place the pot into an empty growth box or a ziploc bag. Label the box or bag and put it back under the grow lights.

6. Your rose plant needs to be gradually acclimated to the lower humidity environment of the class room. It should be ready to go home within about 2 weeks.
7. Summarize what you did today in your observation log.

Observation log

Observe your rose plants every week and record your observations. Also note when you perform any transfers to other media. (Note to readers of this manuscript: the following log has been shortened for space, but I leave enough space for students to write observations and notes at 7 stages.)

Date:
Stage ___
Observations & notes:

Date:
Stage ___
Observations & notes:

Date:
Stage ___
Observations & notes:

Date:
Stage ___
Observations & notes:

Date:
Stage ___
Observations & notes:

Instructor Notes

Contamination

Bacterial or fungal contamination is a major concern with any tissue culture exercise. One of the most important tools to prevent contamination is to have the students perform the exercise inside a protected transfer box. A laminar flow hood offers the best protection from contamination, but these are expensive and bulky. The transfer boxes used in this protocol (see App. B) do not have any air filtration or air circulation, but do provide a barrier to dust and students breathing on sterile items. In our botany course, we observed a 50% decrease in contamination with the introduction of the transfer boxes to the protocol.

Another way to decrease contamination is to minimize how much the students have to touch and manipulate items during the procedure. Currently, sterile solutions are dispensed into small 120 ml (4oz.) polypropylene jars. In a previous version of this lab, the students were given larger bottles of sterile solutions and sterile foil covered beakers. The students had to pour the solutions into the sterile beakers and use these to soak their explants. Predispensing the solutions into smaller containers is more time consuming to prepare, but we saw an additional 25% decrease in our contamination with this change.

To compensate for material lost to contamination, instructors should start at least two additional culture containers for each lab class.

Rose cultural requirements

Miniature roses work best for this protocol. A non-patented variety should be selected since it is illegal to propagate patented varieties. Most varieties of roses have a chilling requirement in order to flower and will not flower if kept inside year round. If flowering is desired, the roses should acclimated and planted outdoors.

The lighting requirements of the explants during the culture protocol are modest. Six 40-watt bulbs are sufficient for as many as 60 culture vessels. Natural sunlight is not recommended as there is the possibility of overheating the culture vessels. The mother plants which supply the starting material should be grown under brighter light. Direct natural sunlight or a 400-watt metal halide light are good choices.

The mother plants should also be fertilized regularly. Most commercial rose fertilizers are designed to be dosed on large standard roses grown outside and may be difficult to dose on plants grown in the lab. African violet fertilizer has a similar composition to rose fertilizer.

Cultivated roses have several serious pests. Spider mites, scales, mealy bugs, and whiteflies are common pests that can be controlled with regular application of a soap spray made with Safer's insecticidal soap (2.5 ml concentrate, 15 ml isopropyl alcohol, 120 ml water). Frequent applications of this soap spray will control most insects and does not appear to have any negative effects on the plants.

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About the Author

Michael Stone is an Instructor and the Biology Laboratory Coordinator at Georgia Perimeter College – Dunwoody Campus. He received his B.S. in Biology from Virginia Tech and M.S. in Entomology from the University of Georgia. Michael currently teaches a one semester non-majors Botany course and has taught other labs in general biology for majors and non-majors.

Appendix A: Vendors and preparation notes

Vendors

Aquatic Ecosystems (AES) : 2395 Apopka Blvd., Apopka, FL 32703; 1-877-347-4788; www.aquaticeco.com

BioWorld (BW) : P.O. Box 888, Dublin, OH 43017, 1-800-860-9729; www.bio-world.com

Carolina Biological Supply (CBS) : 2700 York Rd., Burlington, NC 27215; 1-800-334-5551; www.carolina.com

Fisher Scientific (FS) : 3970 John's Creek Ct. Suite 500, Atlanta GA 30024; 1-800-766-7000; www.fishersci.com

McMaster-Carr Supply Co. : P.O. Box 740100, Atlanta, GA; 1-404-346-7000; www.mcmaster.com

Consumable supply items

Description	Vendor	Catalog number	
Rose shoot initiation medium	CBS	HT-19-5600	10 envelopes of dry media; each envelope makes 1 liter
Rose root initiation medium	CBS	HT-19-5610	" "
Gel-Rite gellan gum	CBS	HT-19-8210	Solidifying agent
Sucrose	FS		Carbon source in medium
1 N NaOH			Used to adjust pH of medium
1 N HCl			Used to adjust pH of medium
Ethanol	FS	A407	Sterilizing agent
L(+)-Ascorbic acid	FS	AC10502-1000	For antioxidant solution
Citric acid monohydrate	FS	A104-500	For antioxidant solution
0.22 um Sterivex filtration units	FS	SVGP-B10-10	To sterilize antioxidant solution
Autoclave tape	FS	11-889-2	
Parafilm	FS	13-374-12	To seal growth chambers
Labels	FS	11-850D	
Plastic backed paper	FS	14-127-47	To line bench under lighting unit
Sterile disposable petri dishes	FS	08-757-12	Used as cutting surface
Aluminum foil	Grocery		Wrap instruments and towels
Bleach	Grocery		Must be fresh (< 1 month old)
Brown single-fold paper towels			Standard 9.25"x10.5" commercial paper towel
Miniature rose plants	CBS		
Small disposable cups	Grocery		Pots for final stage
Vermiculite or potting soil			Growth medium for final stage

Equipment

Description	Vendor	Catalog number	
Small plastic spray bottles			For ethanol; need 1 per group
4 oz. polypropylene jars (36/cs)	FS	891C	To hold solutions; need 8 per group
Scalpel handles	FS	08-917-5	To cut stem pieces; need 1 per group
Scalpel blades	FS	08-918-5D	To cut stem pieces; need 1 per group
Forceps (4")	FS	08-890	To handle stems; need 2 per group
Transfer baskets	AES	TC678	Listed as "tissue capsules"; need 1 per group
Stopwatch	FS	14-649-11	Need 1 per group
14 qt. plastic bins			To hold supplies; need 1 per group
Magenta GA7 growth chambers	BW	7652000	Need 1 per group
Self-filling repetitive syringe	FS	13-689-50E	To dispense solutions
Transfer boxes			See Appendix B
Lighting unit			See Appendix B
Stem washer			See Appendix B

Media

Prepared, tubed media may be purchased from Carolina Biological. Growth of explants is better and transfer is easier if culture is carried out in a larger container such as a Magenta GA7 growth chamber. Mason jars or baby food jars capped with foil are also possible options. Dry media from Carolina contains all nutrients, vitamins, and hormones. Sucrose and a solidifying agent are the only components that must be added.

To prepare one liter of media:

1. Empty one packet of dry media into a two liter flask. Add 800 ml of deionized water. Use another 200 ml of water to rinse the media packet several times and add the rinse water to the flask..
2. Add 30 grams of sucrose and dissolve with stirring.
3. Adjust pH of media to 5.7 using 1M NaOH or 1M HCl.
4. Add 3.0 grams of Gel-rite and heat to boiling.
5. Dispense 40 ml of media into containers.
6. Cap containers and autoclave at 121ºC for 15 minutes.
7. After the end of the cycle, allow the autoclave to cool without opening the autoclave chamber. Allowing the autoclave to cool completely assures that no room air is drawn into the growth chambers as they cool. This will greatly decrease the likelihood of contamination. When the autoclave is cool, remove the growth chambers, wrap the lids with Parafilm, and store in the refrigerator.
8. If refrigerated, media will keep for several months.

For those interested in preparing the media from scratch, Table 1 is excerpted from Carolina Biological's media formulation booklet (1986).

Table 1. Chemical composition of rose shoot and root media.

Component	Shoot medium (mg/L)	Root medium (mg/L)
NH_4NO_3	1650	412.5
KNO_3	1900	475
$CaCl_2$ (anhydrous)	333	83.25
$MgSO_4$ (anhydrous)	181	45.25
KH_2PO_4	170	42.5
FeNaEDTA	36.7	9.175
H_3BO_3	6.2	1.55
$MnSO_4$ _ H_2O	16.9	4.225
$ZnSO_4$ _ $7H_2O$	8.6	2.150
KI	0.83	.208
$NaMoO_4$ _ 2 H_2O	0.25	.063
$CuSO_4$ _ $5H_2O$	0.025	0.006
$CoCl_2$ _ $6H_2O$	0.025	0.006
Inositol	100	100
Thiamine HCl	0.4	0.4
Nicotinic Acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Glycine	2	2
Benzyladenine	2	0
Indole-3-Acetic Acid	0.3	0
Napthalene Acetic Acid	0	0.03
Citric Acid	50	0
Ascorbic acid	50	0
Sucrose*	30,000	30,000
Gelrite*	3,000	3,000

*These items NOT included when ordering dry media in envelopes

Solutions

The sterilization protocol requires 70% ethanol, 50% ethanol, 20% bleach, sterile water, and sterile antioxidant solution. These solutions are best dispensed to the students in four ounce (120ml) polypropylene jars with screw top lids. The jars may be labeled using a permanent marker or a commercial labeling machine. We have had good luck using a Brother label maker. The labels wrinkle slightly when autoclaved, but hold up fairly well. The following volumes are for 12 groups of four students each (two lab sections). Each group of four students will need four jars of deionized water, one jar of 70% ethanol, one jar of 50% ethanol, one jar of 20% bleach, and one jar of antioxidant solution. It is a good idea to make a few extra jars of each solution. The jars for ethanol, bleach, and the antioxidant solution are autoclaved empty, then filled. Deionized water jars are filled, then autoclaved.

Sterile water: Fill each jar with approximately 60ml (1/2 full) of deionized water.

70% ethanol: Add 1105 ml of ethanol (95%) to a 2 liter flask. Add 395ml of deionized water and stir. Dispense approximately 90ml (3/4 full) into each presterilized jar.

50% ethanol: Add 525 ml of ethanol to a 1-liter flask. Add 475 ml of deionized water and stir. Dispense approximately 60 ml (1/2 full) into each presterilized jar.

20% bleach: Add 200 ml of regular bleach to a 1-liter flask. Add 800 ml of deionized water and stir. Dispense approximately 60 ml (full) into each presterilized jar.

Antioxidant solution: Prepare a stock solution by dissolving 1.5 grams of citric acid and 1.0 grams of ascorbic acid in approximately 50 ml of deionized water. Pour into a 100ml volumetric flask and dilute to 100 ml. Pipet 10ml of stock solution into a 1 liter volumetric flask and dilute to 1 liter.

The antioxidant solution is heat sensitive and must be sterilized by filtration. Dispense approximately 50 ml of antioxidant solution to each presterilized jar using a repetitive syringe and a Sterivex 0.22-um sterilization filter unit.

Preparation for first lab session (Stages 1 and 2)

Two weeks before lab: Prepare instruments and non-perishable solutions

1. Forceps: Wrap pairs of forceps in aluminum foil with the tips of the forceps oriented in the same direction. Place a piece of autoclave tape on the "tip" end of the foil packet. Label the autoclave tape "FF" to indicate two sets of forceps. Prepare 1 pair of forceps for each group of students plus several extra. Autoclave at 121°C for 15 minutes.
2. Towels: Separate individual single-fold brown paper towels. These towels are typically folded to form a rectangle. Fold each towel again to form a square. Stack 10 folded towels together and fold the entire packet in half. Place the packet on a large square of aluminum foil and fold the foil over to seal. Tape with a small piece of autoclave tape and label "T". Prepare a packet of towels for each group plus several extra packets. Autoclave at 121°C for 15 minutes.
3. Transfer baskets: Shut each basket and place on a 6"x 6" square of foil. Fold the foil over the basket and secure with a piece of autoclave tape labeled "B". The foil should be folded in such a way that the students can easily open the packet without touching the inside of the packet or the basket. Prepare a basket for each group plus several extra packets. Autoclave at 121°C for 15 minutes.
4. Fill required number of jars (four per group) with deionized water and loosely cap with screw lids. Autoclave at 121°C for 15 minutes.
5. Jars for 70% ethanol, 50% ethanol, 20% bleach, and the antioxidant solution should be assembled, but **not** filled. Prepare one jar of each solution for each group. Autoclave **empty jars** at 121°C for 15 minutes.
6. Fill the autoclaved 70% ethanol or 50% ethanol jars with the appropriate concentration of ethanol as required. Leave the bleach and antioxidant jars empty until the day before lab.

One week before lab: Prepare medium

1. Prepare shoot initiation medium, dose into appropriate containers, cap, and autoclave at 121°C for 15 minutes.
2. Allow to cool in autoclave overnight.
3. Remove cooled medium, wrap caps with Parafilm, and refrigerate until needed.

One day before lab: Prepare bleach, antioxidant solution, and supply bins

1. Prepare 20% bleach solution and dispense into appropriate jars.
2. Prepare antioxidant solution and dispense into appropriate sterile jars using a syringe and Sterivex 0.22 um sterilization filter unit. Refrigerate antioxidant solution until needed.
3. Assemble supply bins for students. Each bins should contain the following:

1 spray bottle of 70% ethanol	1 jar of sterile antioxidant solution
1 jar of 70% ethanol	2 sterile forceps wrapped in foil (marked "FF")
1 jar of 50% ethanol	1 sterile transfer basket wrapped in foil (marked "B")
4 jars of sterile water	1 pack of sterile towels wrapped in foil (marked "T")
1 jar of 20% bleach	

Day of lab

1. Bring shoot medium and antioxidant solution to room temperature.
2. Cut rose stems into approximately 2cm pieces containing one node each.
3. Place in stem washer and rinse under running water for one hour.

Preparation for second lab session (stage 3)

Two weeks before lab: Prepare instruments and non-perishable solutions

1. Forceps and scalpels: Wrap a single pair of forceps and a single scalpel in aluminum foil with the tips of the forceps and scalpel blade oriented in the same direction. Place a piece of autoclave tape on the "tip" end of the foil packet. Label the autoclave tape "FS" to indicate a set of forceps and scalpel. Prepare 1 pair of forceps for each group of students plus several extra. Autoclave at 121°C for 15 minutes.
2. Towels: Prepare 3-4 packets of towels for each lab section.
3. Prepare three jars of deionized water for each lab section and loosely cap with screw lids. Autoclave at 121°C for 15 minutes.
4. Three jars for 70% ethanol for each lab section should be assembled, but **not** filled. Autoclave **empty jars** at 121°C for 15 minutes.
5. Fill the autoclaved ethanol jars with 70% ethanol.

One week before lab: Prepare medium

1. Prepare root initiation medium, dose into appropriate containers, cap, and autoclave at 121°C for 15 minutes.
2. Allow to cool in autoclave overnight.
3. Remove cooled medium, wrap caps with Parafilm, and refrigerate until needed.

Day of lab:

1. Bring root initiation medium to room temperature.
2. Set up two transfer chambers with 70% ethanol, sterile deionized water, a towel packet, and an instrument packet.

Preparation for third lab session (stage 4)

Day of lab

1. Prepare small cups by punching several holes in the bottom of each cup.
2. Place required quantity of vermiculite or potting soil in a shallow pan and wet with water.

Appendix B: Do-It-Yourself Equipment

Stem Washer

The first stage of sterilizing the rose stems requires that the rose stems be washed under running water for one hour. The stem washer is designed fit down into a lab sink and hold the rose stems during the washing phase of the sterilization procedure. It is constructed of PVC pipe and other materials available at any home improvement store (Home Depot, etc). Before beginning the assembly process, cut all pieces of PVC pipe to the proper length as indicated in Table 2. Cuts should be square and smooth. Use sandpaper if need to remove any burs on the cut ends of the pipe pieces.

Table 2. Parts list for stem washer.

Part number	Description
1	3-inch-diameter PVC test cap with inner surface removed
2, 5	Fine mesh window screen, 12 cm x 12 cm
3	3-inch-diameter PVC pipe, 5 cm long
4	3-inch-diameter PVC coupler
6	3-inch-diameter PVC pipe, 1 cm long
7	3-inch-diameter PVC pipe, 15 cm long
8	3-inch-diameter PVC test cap
9	3-inch-diameter PVC toilet flange
10	1/2-inch-diameter PVC street elbow, slip x MPT
11	1/2-inch-diameter PVC pipe 5 cm long
12	1/2-inch x 1-inch PVC reducer bushing
13	1-inch-diameter PVC coupler
14, 18	1-inch-diameter PVC pipe, 5 cm long
15, 17	1-inch-diameter, 45-degree PVC elbow
16	1-inch-diameter PVC pipe, 25 cm long

Construction of stem washer body

1. Drill a 23/32-inch hole in pipe piece #7 centered approximately 5 cm from one end to the pipe. Thread the hole with a 1/2-inch-NPT thread tap.
2. Place a PVC test cap over the end of pipe piece #7 closest to the hole and tap into place with a hammer.
3. Slide pipe piece #7 into the toilet flange.
4. Screw the 1/2-inch PVC elbow (part #10) into the hole in pipe piece #7.
5. Assemble the water supply arm (parts #11 - #18) as outlined in Fig. 7 and attach to the PVC elbow on the washer body. Do not glue any of these pieces together. It will be necessary to move these pieces to adjust the stem washer for use in different size lab sinks.

Construction of stem chamber

1. Place a piece of screen over one end of the 3-inch coupler (part #4). Place pipe piece #6 over the screen and gently tap the piece of pipe down into the coupler until it hits the ridge in the center of the coupler. This should pull the screen tight across the open surface of the coupler. Trim of any excess screen that hangs below pipe piece #6.
2. The stem chamber should fit firmly onto the stem washer body while not being too difficult to remove. If the cap is difficult to remove, sand the outside of the washer body with sand paper to adjust the tightness of the fit.

Construction of stem chamber lid

1. Remove the center portion from one of the PVC test caps by hitting the center of the cap with a hammer. These caps are usually scored around the outer edge and the center portion should snap loose and pop out easily.
2. Place a piece of screen over pipe piece #3 and place the end cap from step 1 on top of the screen.
3. Gently tap the end cap down into the pipe. This should pull the screen tight across the open surface of the test cap. Trim of any excess screen that hangs over the lip of the test cap.
4. The stem chamber lid should fit firmly down into the stem chamber, but it should not be difficult to remove. Sand the outside of the lid to adjust the fit.

Using the stem washer

1. Place the stem washer into a lab sink and adjust the water supply arm extending up from the washer body until it is directly underneath the faucet.
2. Place the stem chamber onto the washer body.
3. Place the trimmed rose stems into the chamber and place the lid down into the chamber.
4. Turn on the faucet and adjust to a slow flow of water through the stem washer. Wash the stems for 1 hour.
5. When stem washing is complete, remove the stem chamber from the washer and place it into a clean pan. Remove the lid and allow the students to take their stem pieces from the chamber.

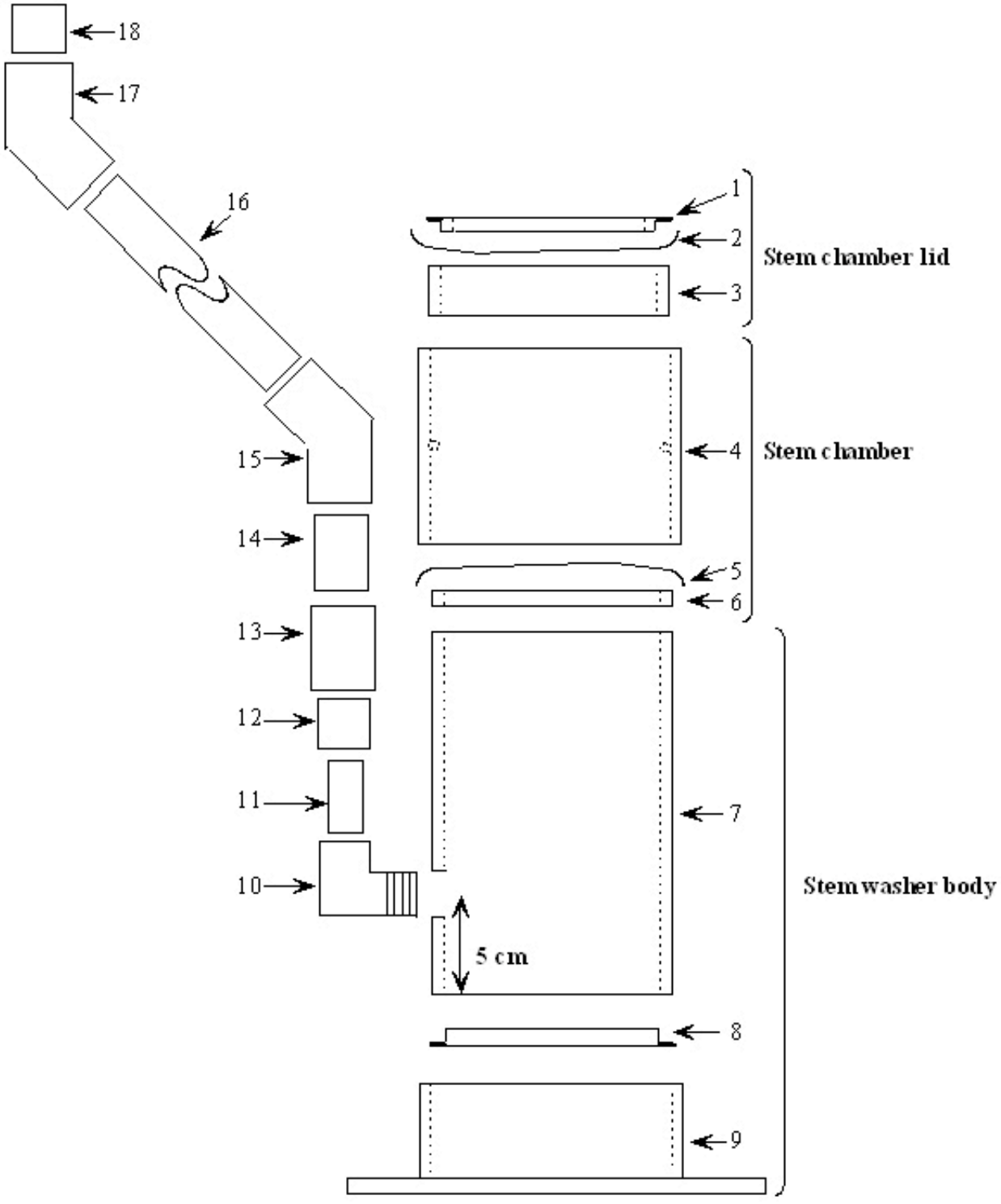

Figure 7. Exploded diagram of stem washer

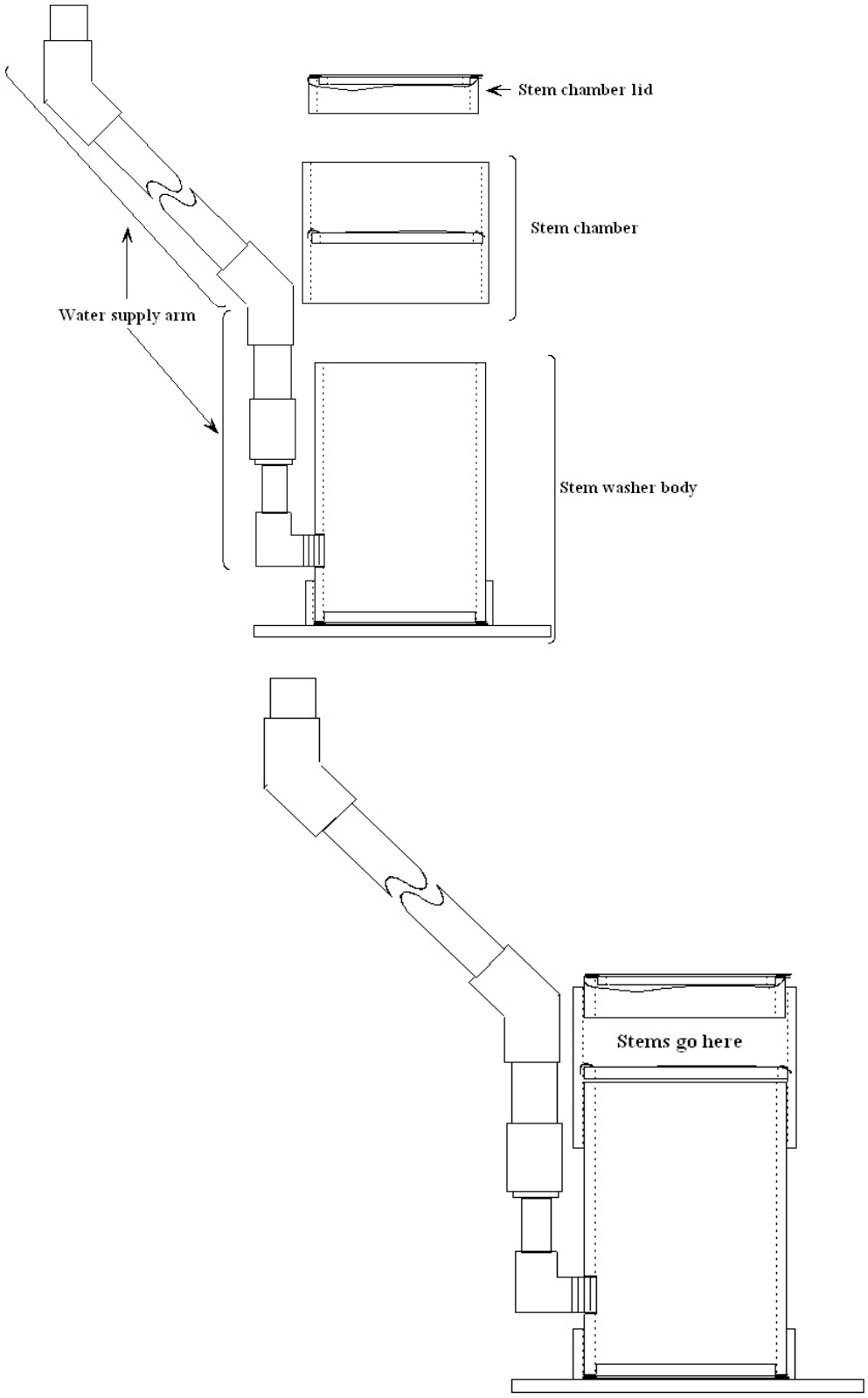

Figure 8. Diagram of stem washer partially (top) and completely assembled (bottom).

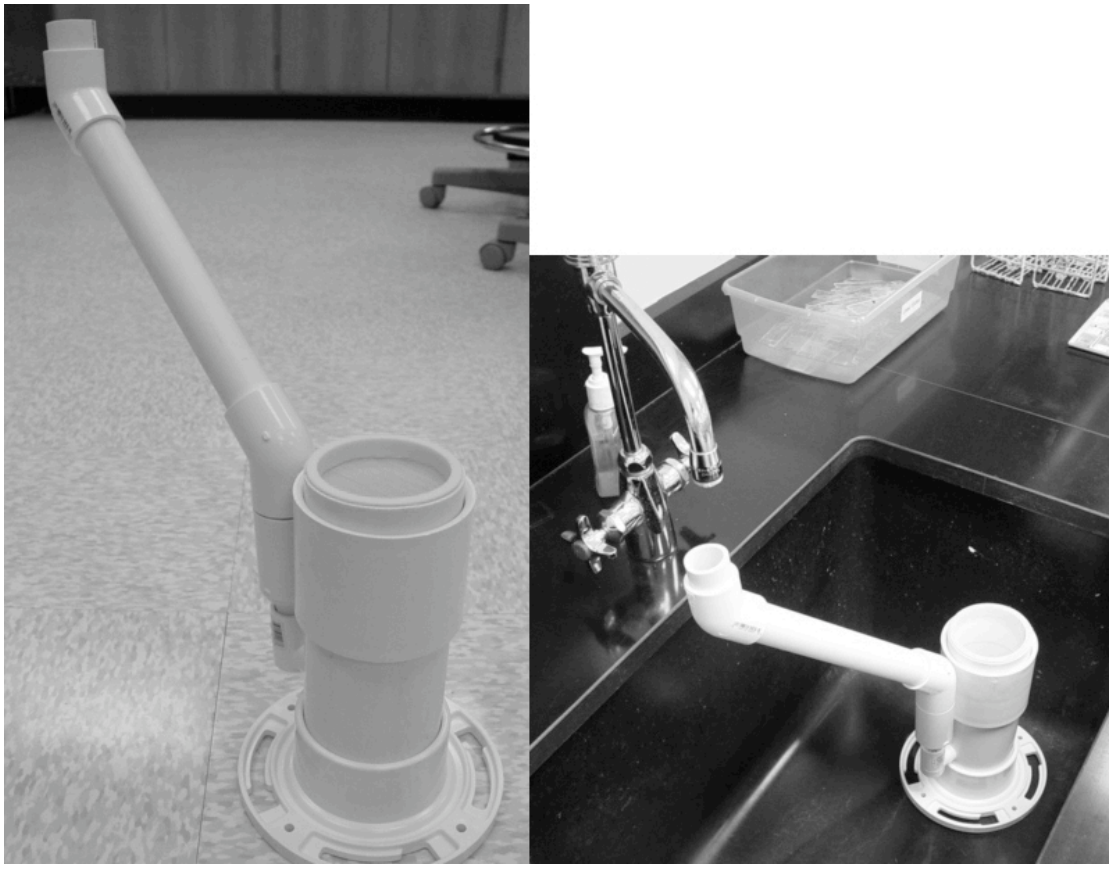

Figure 9. Completely assembled stem washer (L) and stem washer in sink ready for use (R).

Transfer boxes

The transfer boxes used in the protocol are made from 2 mm acrylic and glued together with an acrylic solvent (Weldon #3: McMaster-Carr Supply Co. Cat.# 7528A13). Acrylic from Home depot or any plastic supply company will work. Thicker acrylic is acceptable, but the dimensions would have to be adjusted for the thicker stock. Cutting acrylic requires a table saw with a fine tooth blade or a router with a flush trimming bit. The cut edges must be flush and smooth for the solvent to bond the acrylic properly. If you are inexperienced with cutting acrylic, it is suggested that you have an plastic fabrication shop do the cutting for you. Other options for transfer boxes include a 10 gallon glass or acrylic aquarium placed on its side with plastic wrap over the upper half of the opening or a clear plastic sweater box with holes cut in the side to allow access.

Table 3. Parts list for acrylic transfer boxes

Part	Dimensions	Comments
Bottom	32 cm x 45.8 cm	
Back	29.8 cm x 45.8 cm	
Top	10.2 cm x 45.8 cm	45° bevel on front edge
Front	28.4 cm x 45.8 cm	45° bevel on front and back edge
Sides (2)	30 cm x 29.8 cm	45° cut along one corner

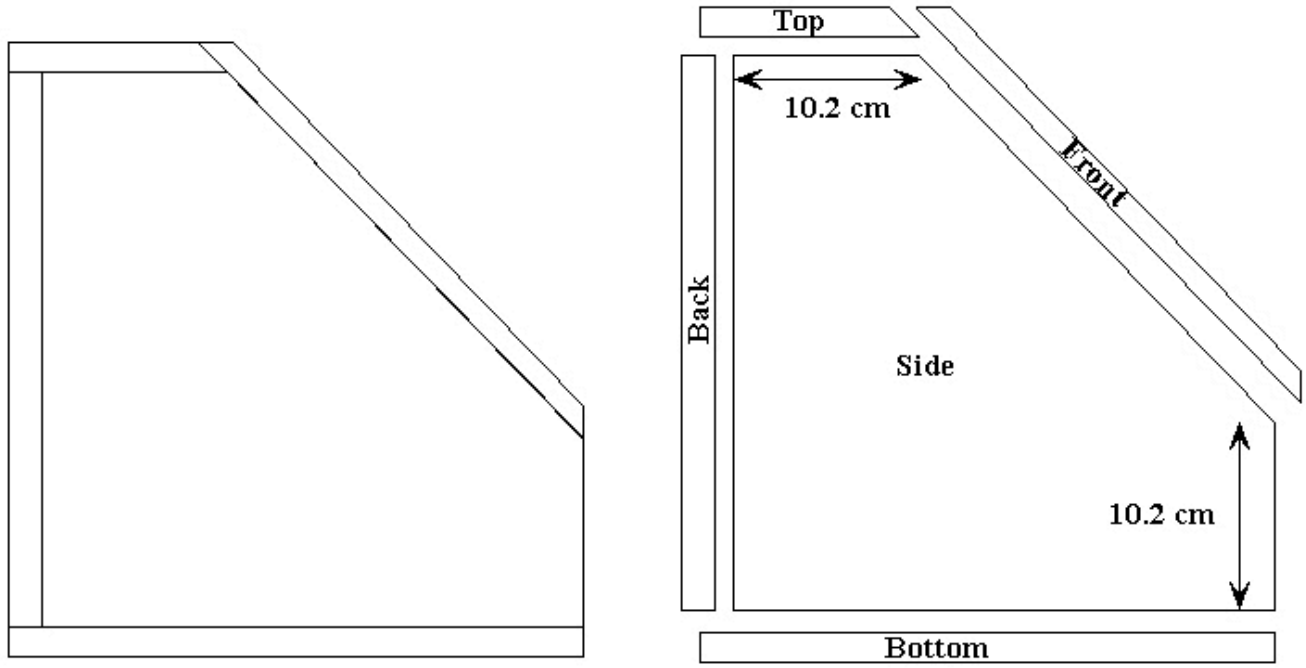

Figure 10. Side view of transfer box. The side pieces fit inside the top, back, and front. The sides and back sit on top of the bottom. Thickness of pieces is not to proper scale. Note the location of the 45° bevels on the top and front pieces.

Assembly of transfer boxes

1. Cut all pieces to the proper dimensions.
2. Set the bottom piece on a flat surface.
3. Place four pieces of masking tape along the back edge and three pieces along each side edge. The tape should go under the bottom piece and stick out approximately 5 cm with the sticky side up.
4. Place the back piece along the back edge of the bottom piece. The back piece should be laying on one of its long edges. It should be flush with the back edge. Bring the four pieces of tape up and tape the back into place.
5. Place one side piece along the side of the bottom piece. The side piece should be resting on its longest edge with the angled cut facing forward.
6. Use the three pieces of tape attached to the bottom to secure the side and bottom pieces. Use three more pieces to secure the side and back pieces together. Repeat with the other side piece.
7. Place the top onto the box. Move the side and back pieces until they are flush with the top piece. Tape into place using four pieces along the back and one piece along each side.
8. Place the slanted front onto the box and tape into place using four pieces for the top edge and three pieces for each of the sides.
9. The box is now ready to be glued together with solvent. The solvent has the consistency of water and must be dispensed with a glass syringe. Capillary action will pull the solvent into the joints.
10. Dispense a small amount of solvent to each joint between the pieces of tape. Do not allow the solvent to get to the tape as it will follow the tape up onto the faces of the acrylic pieces.

11. Allow the solvent to set for 10 minutes, then carefully remove the tape.
12. Dispense solvent to the entire length of all joints. Allow to sit undisturbed for 24 hours before use. If any of the joints crack during usage, hold the pieces together and dispense a small amount of solvent into the broken joint.

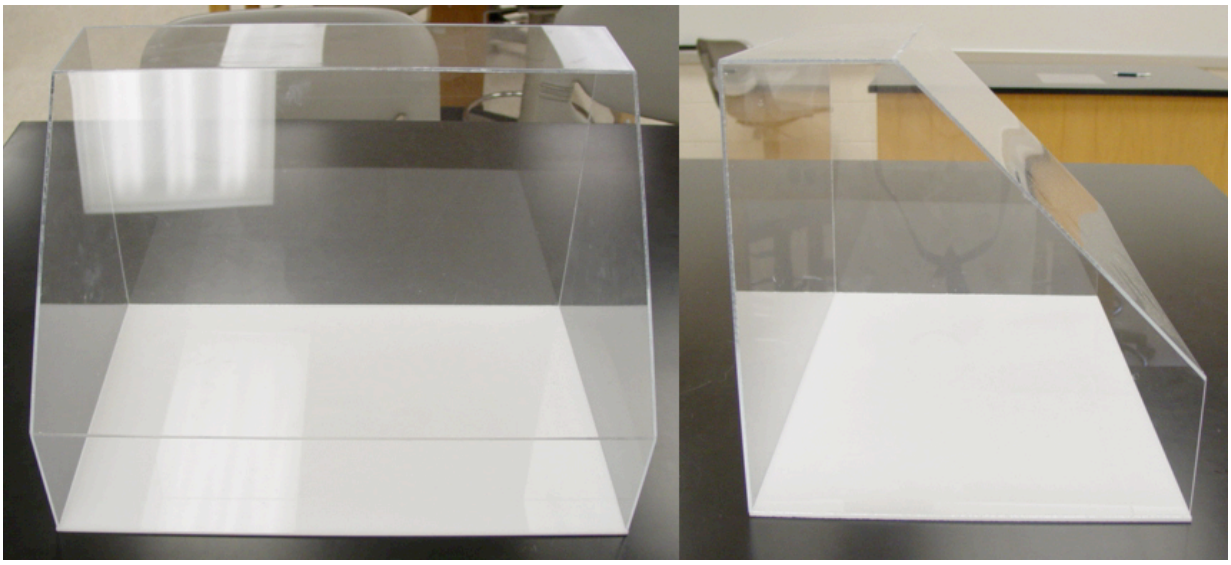

Figure 11. Front and side view of completed transfer boxes

Lighting unit

The lighting unit consists of three "shoplight" type fluorescent fixtures bolted together and suspended from a 3/4-inch black iron pipe framework. The fluorescent fixtures should hold two 48-inch 40-watt cool white or daylight fluorescent bulbs. Thirty-two watt "energy-saver" bulbs are also acceptable. The three fixtures are held together by three pieces of rigid angle iron mounted to the back of each fixture. The angle iron should be spaced evenly with one piece at each end and one piece in the middle. Each fixture can be powered separately or all three fixtures may be wired to a single electrical cord. Observe proper grounding and orientation of hot and neutral wires when working with fluorescent ballasts. The ballasts in cheap fluorescent fixtures tend to produce a lot of heat. Initially we had problems with the shoplights shutting off after 7-8 hours of use due to overheating and activation of the built in thermal shutdown feature. Two 3-inch computer fans mounted so that air is blown across the ballast solved the problem and the lights now run 24 hours a day with no problem.

The lights are suspended from a simple frame constructed of 3/4-inch black iron pipe which is commonly used to run natural gas service. The pipe sections are threaded on each end and then screwed into the appropriate fittings to form the frame. Home improvement stores usually carry this pipe and will cut and thread the pipe for a minimal charge. The fluorescent fixtures are suspended from the frame using chain that is attached to the fixtures then to two inch threaded eyes that are attached to the horizontal part of the frame. Carolina Biological sells a similar unit (cat.# HT-15-8998) constructed using plastic PVC pipe for approximately $140.

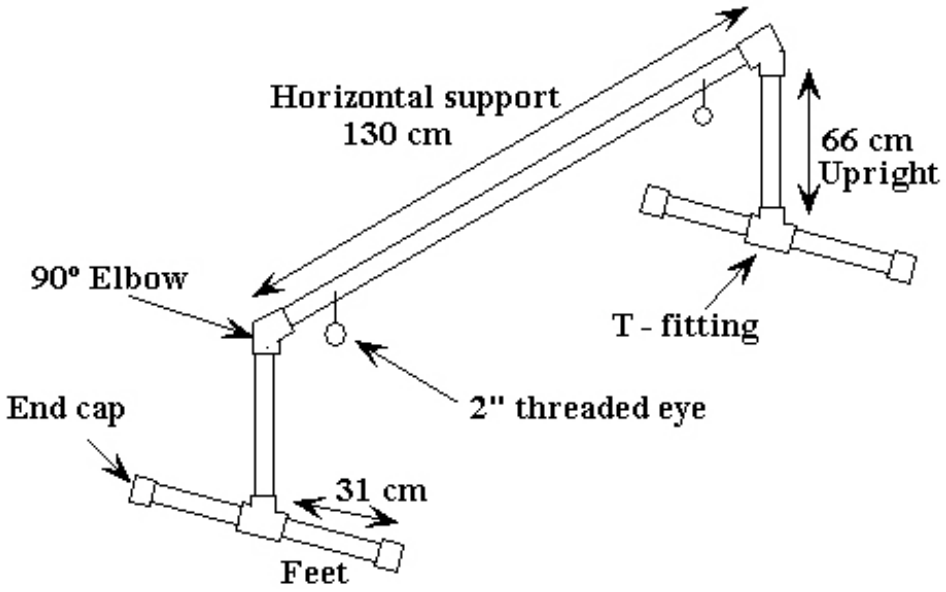

Figure 12. Diagram of lighting unit support frame.

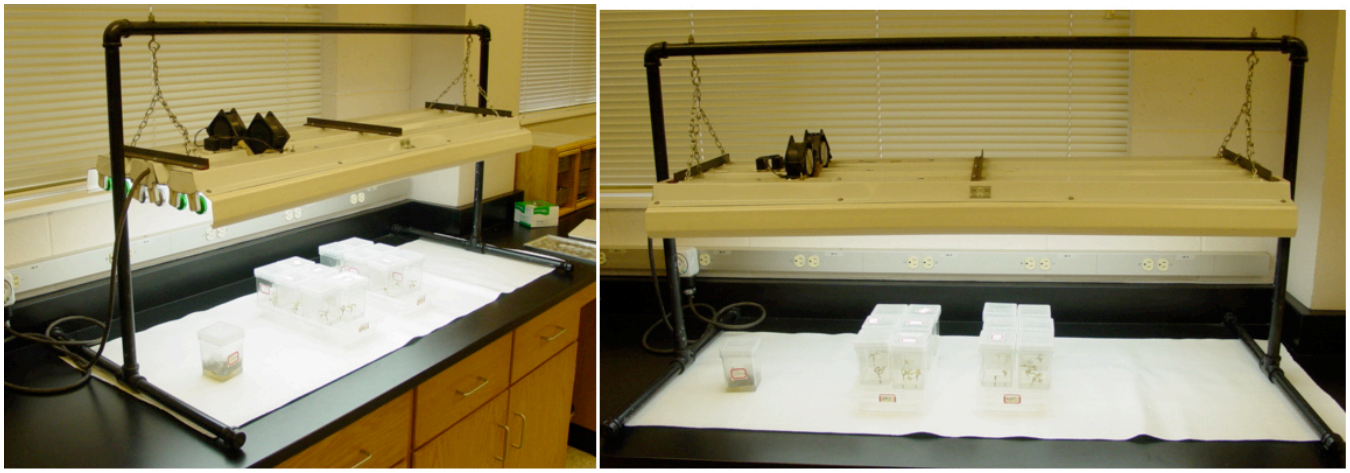

Figure 13. Completed lighting unit. Note the presence of the 3-inch computer fans on top of the light fixtures.