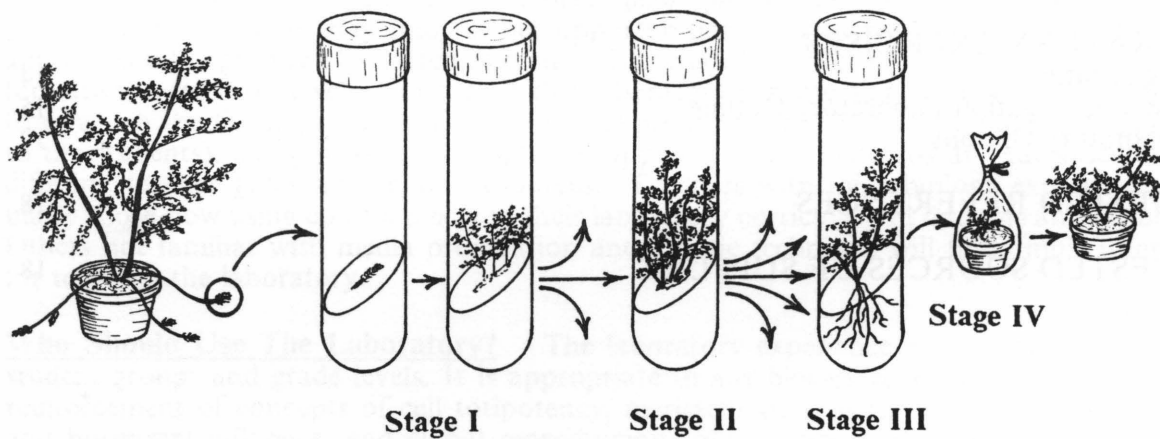


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PLANT TISSUE CULTURE



An Introductory Laboratory Teacher's Guide

Virginia Tech and Virginia State
Virginia's Land Grant Universities

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PLANT TISSUE CULTURE

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INTRODUCTION

Purpose - Biotechnology is an advanced science that draws its substance from many of the natural and physical sciences. Courses in tissue culture, a foundation technology, are commonly reserved for advanced collegiate students. Pre-college students may be provided some textbook references to the subject, but few secondary-school curricula have incorporated any direct experiences. This is unfortunate because:

- 1) biotechnology will have a major future impact that affects everyone;
- 2) students in the life sciences will have significant professional opportunities in this field;
- 3) there are applications that may be introduced to students who have only basic knowledge of biology, or that may be expanded in content for advanced students; and
- 4) any application of basic scientific study will enhance interest in and appreciation for the science involved.

The purpose of this publication is to assist basic or applied biological science teachers in providing a laboratory experience in biotechnology. For simplicity, safety, and student interest in the final product, this laboratory involves **plant propagation by tissue culture**. Advanced programs and students may find further opportunities in other plant and animal studies. An annotated bibliography of selected references is included to offer ideas and guidance. Most information needed to develop and teach this laboratory is presented (including an in-depth text from which the teacher may extract lecture and pre-laboratory information deemed appropriate to the students), along with several optional approaches for materials and facilities to match differing teacher goals and school resources. Teachers with microbiology experience, particularly those now using culture media in their laboratory curricula, will find this an easy addition. Others not familiar with media preparation and aseptic technique will find ample information for teaching the laboratory.

Who Should Use The Laboratory? - The laboratory experience may be adapted to several student groups and grade levels. It is appropriate to **any biology course** as a demonstration or reinforcement of concepts of cell totipotency, meristematic growth, organogenesis, nutritional and hormonal influence, and clonal reproduction (plus microorganism effects in the case of contaminated cultures). In advanced biology courses, there are many opportunities for student experience in media manipulation or in meristem, cell, and/or anther culture. The basic exercise is in use now as a rapid cloning technique in commercial plant propagation. As such, it should be included in **all vocational horticulture** curricula to expose those students to a major source of plant materials they will produce or handle in the future. The background science may be expanded or compressed according to individual student needs while studying the advantages and disadvantages of this technique compared to others.

The teacher may develop whatever laboratory form suits the program needs. The only limitation is the time required for completion of the full culture process. This should not be used as an end-of-the-year laboratory unless the intent is for students to take cultures home for observation and establishment of finished plants. (The Boston fern cultures require two to four months from tube to pot, depending on culture conditions and whether a plant-multiplication step is included.)

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The primary emphasis is to introduce biotechnology and tissue culture to secondary-school students, but lower grade levels may benefit also. The process is interesting and fun to watch, and a demonstration or class exercise at the junior-high level can aid orientation and provide guidance to the life sciences.

REVIEW OF BIOTECHNOLOGY AND TISSUE CULTURE

Introduction - Biotechnology involves a vast array of techniques that can provide a better understanding of basic life processes and the potential to manipulate those processes for the betterment of mankind. It is hailed in the scientific community as a key to future advances in medicine and agriculture. Many of the techniques have been in use for years, but the general public has had little information about the benefits. Unfortunately, most news media attention has been focused on the possible horror that might result from accidental release of a highly virulent pathogenic "monster" organism created through gene splicing and recombinant DNA procedures. While ethical and safety issues in biotechnology must not be minimized, it is important to recognize the contributions biotechnology has made and will continue to make.

Agricultural crop production provides an example of biotechnology's impact. Many improvements in crop productivity over the past half century have come from a better growing environment through managed soil condition, fertility, water supply, and pest control. Each of these has a high cost today in consumption of energy and other resources, and there are serious concerns over environmental changes (soil, water, air, and organisms) due to fertilizer and pesticide use. However, other advances during this time came from the breeding of crops for greater productivity and efficiency.

Conventional crop breeding uses controlled pollination for hybrid seed production. Unfortunately, there are biological limitations to maximum progress in this area. The wide array of random gene combinations (even with inbred lines) results in only a small percentage of progeny carrying the desired trait. Much time is required (many plant generations) to verify presence and stability of the trait and to build sufficient seed supplies for commercial release. In some cases, this may be 20 years or more. Sometimes a chance mutation will result in an improved trait, but it can be observed and utilized for new crop development only if the mutated cell is near the plant surface and grows into view. Most occur deep inside the tissue and never become apparent. Plants also are sexually compatible only with closely related species, limiting the use of pollination to transfer desirable traits between distantly related plants.

Genetic improvement by conventional breeding methods requires mature flowering plants, but the newer approach in biotechnology concentrates on individual cells and tissues in cultures. Considerable economy of time and resources may be achieved by selection among cultured cells; a much larger number of cultured cells may be grown and tested for desirable traits in the laboratory than can be grown and tested in the field or greenhouse. The plant growing environment may be controlled more easily in the laboratory as well. Tissue cultures also allow much more rapid multiplication of new plants. When tissues are separated into dispersed cells, chance mutations of internal cells may be observed and isolated for expanded genetic potential in the crop. Response to environmentally harmful materials may be tested in minute quantities on tissues in a culture vessel rather than on plants in a field, and with better experimental control. Through protoplast fusion, gene splicing, and other techniques, the genetic combination of unrelated plants is possible.

The preceding discussion has been based on new plant breeding potentials of biotechnology, but parallel opportunities exist in livestock production, forestry, medicine, and environmental improvement. While some of the techniques are not new, the whole field is still very much in its infancy and has a very open future. Examples of developments considered within reach are: 1) livestock with greater reproductive efficiency, better meat conversion from feed, higher nutritional quality, and ability to use feed sources other than grain diverted from

human consumption; 2) forest trees that are genetically resistant to injury from pests and fire and that produce larger yields of pulp and quality lumber in less growing time; 3) crop plants that have the ability to fix nitrogen, resist diseases, repel insects, and compete with weeds, all of which can reduce the need for applied fertilizers and pesticides; 4) cancer-specific antibiotics that can destroy cancerous cells without damaging healthy cells; and 5) organisms that require particular environmental pollutants as food sources, thereby eliminating the pollutants (and themselves after the food source is consumed). These are futuristic views, but many applications exist today both in research (e.g., in studying cancer development and testing therapeutic methods) and in commerce (e.g., in the manufacture of antibiotics and vaccines or the propagation of certain ornamental plants).

Biological Concepts in Tissue Culture and Micropropagation - The 19th-century observation that single, detached cells of lower plant forms could continue growth gave rise to the theory of *cell totipotency*, that any cell contains all required information and the ability to generate a whole plant. In reality, the theory applies only to cells capable of *mitotic division*; i.e., cells that are *meristematic* (e.g., embryos, stem/root tips, cambium) or otherwise relatively *undifferentiated* (e.g., parenchyma). In animals, at least at this time, only the zygote and initial embryo cells may be totipotent. Because animal cells differentiate very early, their cultures only continue producing cells of the original tissue type.

Among higher plants and animals, the zygote exemplifies cell totipotency occurring naturally in the whole organism (*in vivo*). Nourishment, growth factors, and a suitable environment are provided by the parent while the cell multiplies, develops tissues and organs, and prepares for an independent existence. This process is paralleled in culture (*in vitro* - separate from the whole; or literally "in glass" from the Latin). In effect, an artificial plant ovary or animal uterus is created by the culture vessel and medium. However, plants possess other regenerative capabilities in addition to the reproductive structures. For example, given a proper inducing environment, cells in the margin notches of *Kalanchoe* (kal-an-ko'-ee) leaves will become meristematic and produce rooted plantlets that detach and establish independent plants. This is totipotency within a *vegetative* or *somatic* cell.

Plant reproduction in nature is commonly sexual (by seed), a mixing of genes from both parents that results in genetically diverse progeny, which aids survival of the species. However, many plants reproduce by asexual means. A plant stem in contact with the soil may form roots, establishing an independent plant. These *adventitious* roots (arising from an unusual place) result from growth of meristematic cells in the stem. Modified stems (tubers, rhizomes, stolons, corms, and bulbs) also develop adventitious roots. On the other hand, roots (e.g., sweet potato) may produce adventitious shoots, and leaves (e.g., African violet or the *Kalanchoe* mentioned previously) may develop adventitious roots and shoots. An important feature of these asexually reproduced plants is that they are genetically identical to their original parent, and these identical progeny constitute a *clone* of that parent.

Natural reproduction is too slow and random to meet human needs for food and ornamental plants. Thus, most economic plants are *propagated*, or have their reproduction controlled. Seeds are an efficient source of new plants when the genetic diversity of sexually propagated progeny is acceptable. The desired traits of many plants are not carried by all seeds; consequently, these plants must be asexually propagated. *Cuttings* are probably the best known method. Starting with a single plant (or a single mutated branch on a "normal" plant), sections of stem are removed and stimulated to produce adventitious roots. New stems growing from lateral buds may be removed for rooting to continue the multiplication process. From some plants, leaf or root cuttings might be used instead. Given food stored in the cutting and a suitable environment of light, water, and temperature, very satisfactory results are obtained. However, the process is expensive in terms of labor and growing space and in maintaining sufficient stock plants.

Of the many techniques developed to increase efficiency in asexual propagation, tissue culture offers the greatest promise. Standard propagation methods might use a stem cutting 10-15 cm long that is placed in a greenhouse bed under mist to develop roots over a few weeks or months. With *micropropagation*, only a small piece of stem (0.1 to 1 cm) is placed on nutrient media in a culture tube, whereupon meristematic cells in the tissue begin multiplying. That growth may be subdivided in a few weeks and several new cultures established. Potentially, this growth and reculturing process may continue indefinitely, geometrically increasing plant numbers each time and yielding an infinite number of identical new plants from the single original tissue piece. Under the correct conditions, the cultures develop into rooted plantlets ready for further growth using standard methods. All of this would occur in test tubes in a small room; expensive greenhouse space would not be needed until the small plants with functioning root systems were fully established.

Developing and operating a micropropagation laboratory has a high cost, but standard greenhouse propagation is also expensive. The economic advantage for micropropagation is in the significantly larger number of new plants possible in much less time and space. Another significant benefit of tissue culture is the production of virus-free plants. Viruses are dispersed throughout plant tissues; therefore, conventional cuttings from an infected plant will retain the virus in the new plants. However, actively growing stem tips (about 0.5 mm of the apical dome) may be growing in advance of the virus infection and, consequently, *meristem culture* can effectively rid the plant of the virus. Many valuable plants that have become universally virus-infected are being reclaimed by *meristemming*.

Tissue Culture Requirements and Stages - A piece of plant tissue (an *explant*) in culture has the same requirements it had within the intact plant. Therefore, the culture must provide to the explant all things normally supplied by the plant and must be in an environment suitable for growth (i.e., *in vitro* needs to duplicate *in vivo*).

Much of the developmental work in tissue culture has involved determining specific cultural and environmental requirements for each plant type and explant source. As with intact plants, it is a question of both "what" and "how much" is needed. For example, water and mineral nutrients can be supplied in the culture media. Too little water will kill the explant from osmotic injury that results from excessive mineral salt concentrations. If a required mineral is deficient or excessive, explant growth will be restricted or abnormal. If the explant is submerged in the media without proper aeration, it will drown as do roots in a saturated soil. Fortunately, several very effective media formulas have been developed. One that is adaptable to a wide variety of plants is the MS formula, referring to the developers Murashige (mur-a-she'-gee) and Skoog.

Several organic components are required in the media. Primary is a carbohydrate source (carbon and energy), usually supplied as sucrose, but other sugars may be used. This is needed because the explant is not autotrophic, even if from a leaf. Some plant cultures need certain vitamins (mainly B complex) added because they are unable to produce sufficient amounts. Either purified vitamins or yeast extract are used. Plant hormones, which are compounds produced in other plant parts that affect development of the explant tissue, also must be present in the media. Generally, these are *auxins* and *cytokinins*, supplied either in pure form or from such things as coconut milk (a liquid endosperm quite appropriate to embryonic growth in an explant).

The growing environment must not be ignored for culture growth to be successful. As growth rate varies with temperature, a warm area (25°C) is commonly used, but higher or lower temperatures may be better depending on the plant. Moderate lighting is provided, under time clock control, to provide the plants a diurnal cycle and to stimulate photosynthesis when functional leaves develop. Pest control is also required. The explant has lost its epidermal barrier to bacteria, fungi, and yeasts, which also grow well on the culture medium and would quickly kill the explant. Thus, preparation of the explant includes a disinfecting step. In addition, all

media and equipment must be carefully sterilized before use, and *aseptic technique* is used to avoid microbial contamination whenever the cultures are handled.

For micropropagation, the changing requirements during explant-to-plantlet development may be considered as a series of four steps or stages. (The following descriptions are for a stem-tip explant as used in the laboratory exercise.) **Stage I: Establishment** - The explant is prepared and established in sterile culture. During this period (about four weeks), freedom from contamination is determined, and the explant begins to elongate and form small leafy structures.² **Stage II: Multiplication** - The explant is cut apart for culturing on a new medium. Each new culture may cycle through this stage over two to six weeks, providing up to a 50-fold increase in explants each time. Once a satisfactory number of stock cultures is available to continue the multiplication, the extras may be transferred to the next stage. **Stage III: Pretransplant** - This is the rooting stage for the explant cutting before it is moved out of the artificial culture environment. The new medium contains a different hormone balance.³ With some plants, rooting may be achieved with the multiplication medium, or the Stage II plants may be grown to sufficient size for direct rooting (at significant cost savings when this is possible). **Stage IV: Transplant** - The plantlet is moved from the closed and aseptic culture environment. Because the plantlet has a limited root system and minimal cuticle development, a period of *acclimitization* to the harsher conditions is needed. A soilless potting medium is used in a shaded and plastic-enclosed growing area. This stimulates rapid root growth and minimizes desiccation. The plastic cover is progressively opened over two to three weeks, encouraging morphological and physiological adjustments to normal greenhouse conditions. (A transparency master is provided on page 19 to illustrate the sequence of culture stages.)

LABORATORY MATERIALS AND PREPARATIONS

Teachers may elect from several options on how this laboratory can be incorporated into their curricula. Each is presented for adoption according to individual program goals and resources.

General Laboratory Options - If the teacher needs to minimize investment in supplies and equipment, and only minimal scientific refinement is acceptable, there is a "home tissue culture" method that uses readily available materials, measurements, etc. It will give satisfactory results with only a few limitations. Another approach is to use standard science laboratory equipment, reagents, and procedures; this enables better demonstrations of research and commercial methods but requires more specialized materials. However, a combination of these approaches is possible and will be discussed in more detail. The third option is to use a "kit" available from educational supply companies. These offer a wide variety of plant studies and are complete with pre-mixed medium samples, containers, tools, and supplies. Kits can save teacher time and expand options for additional studies, but they have a cost commensurate with the convenience. Before deciding to use a kit, the teacher should study the remaining discussion. The process of developing this laboratory from scratch is not difficult or expensive. It can be quite enjoyable and stimulate ideas for further student involvement and educational development.

Program Coordination - In schools offering both horticulture and biology, an excellent opportunity exists for a coordinated effort that offers mutual benefits of economy, materials, and expanded educational experience. Horticulture teachers can provide plants, expertise on propagation, fertilizer for culture media, and Stage IV culture materials and technique. Biology

² This growth may be from the immature axillary buds in the shoot tip, adventitious shoots along the stem, or embryoids arising from *callus* (undifferentiated cell mass) formed at the explant base. Low levels of both auxin and cytokinin favor axillary shoot growth, high levels of both are needed for adventitious shoot formation, and high auxin/low cytokinin is commonly used to stimulate callus production.

³ Auxin levels are increased in Stage III to promote rooting, similar to its use with conventional cuttings. Cytokinins, which favor shoot formation, are reduced or eliminated.

teachers can provide laboratory equipment and technique, reagents, and cell and microbiology expertise. While educational objectives may differ between the programs, there are mutual benefits for both student groups from the combination of basic and applied science in these techniques. This does not require a joint laboratory of horticulture and biology students, only recognition that resources may be combined to achieve full educational value of the laboratory experience. (Additional note: Coordination with chemistry is also advised. Many reagents needed in very small quantities for media are stock items in chemistry laboratories, and some equipment -- e.g., a magnetic stirrer/hot plate -- may be available there.)

Laboratory materials required - An overview of supplies and procedures for a generalized laboratory is presented in figure 1. These are described below in more detail, including options the teacher may consider according to available resources.

Explant type and source - The choice of explant and the corresponding medium tends to be specific to particular plants, even to particular varieties and cultivars of the same species. However, the exercises in this laboratory have been adopted in successful commercial propagation. Rhizomes of Boston fern are readily available, the explants grow easily and relatively quickly, and the final product is of interest to the students. (Carrot callus can be interesting to observe but not to take home.) For African violet, leaf tissue (blade or petiole) is the usual explant source. Stem tips of coleus or chrysanthemum also may be easily micropropagated. Advanced students seeking a science-fair project may wish to try stem tip, meristem, or axillary bud cultures of cattleya orchids (see reference #4 or #5).

Culture medium - The simplest medium formulation,⁴ presented in figure 2, is designed for home preparation using grocery and garden store items. As such, the measurements are only approximate (kitchen equipment), and some variability is to be expected among medium batches, but the results should be satisfactory.

The second option is to use reagent grade chemicals. Although the plant growth regulators and some other materials might not be common school laboratory items, they are readily available in small quantities (only trace amounts are needed each time) at a relatively low cost. Media used in this laboratory are modified MS formulas, and the formulas are presented in figure 3.

These formulas are provided so that the teacher will understand different media requirements among the explants and culture stages. While preparing reagent media is not at all difficult (and may be appropriate for advanced studies), the more practical approach is *pre-mixed media* available from tissue-culture supply companies. For these exercises the teacher may obtain crystalline salts in single-liter quantities of **Fern Multiplication Medium** (Stages I and II) and **Minimal Organic Medium** (Stage III ferns). The African violet leaf discs are grown on **Violet/Gloxinia Multiplication Medium**. (Although differing in BA content and auxin source, this medium should be effective for the petiole explants. Also, some companies offer a separate violet/gloxinia pretransplant medium.) These premixed salts will cost about \$.75-\$1.75 per liter of medium, and their use is recommended. (Pre-mixed liquid media are available for the ultimate in convenience, but the cost may be \$20-25/liter.) Later, advanced laboratories may use only a minimal organic medium (to manipulate the vitamin and hormone components) or another formulation according to the plant of interest.

Crystalline pre-mixed media usually do not include sucrose or agar, which are listed as supplements in figure 3. The sucrose is easily supplied by table sugar. Agar may be added to provide a diffusive, semi-solid support for the explant. This allows the explant to absorb nutri-

⁴ Bridgen, M. *et al.* 1983. Plant tissue culture for the homeowner. VCES Publ. 426-385. 2 pp. Teachers may obtain up to six classroom reference copies at no charge from their local Virginia Cooperative Extension Service (VCES) offices.

Figure 1. Generalized Laboratory Outline

Supplies - for each work station

- Boston fern and/or African violet
- Sterilized culture vessels with medium and closures
- Disinfectant cleaner (e.g., Lysol) and sponge
- Small container of 1:10 bleach solution
- Small container of sterile water
- Sterile scalpel or razor blade
- Sterile forceps
- Sterile paper towel or petri plate

General Procedure

Stage I

- Remove explant and place in bleach solution. Soak 10 minutes (submerge frequently) to disinfect surface.
- Wash hands and disinfect work surface. Soak ends of forceps and scalpel in bleach solution.
- Immerse explant several times in sterile water to rinse.
- Unfold sterile paper towel and transfer explant onto it. Remove any bleach-damaged tissue with scalpel.
- Aseptically transfer explant to culture medium.
- Place culture in growing area.

Stage II

- When sufficient growth has developed, remove culture to sterile towel. Cut apart explant growth, discarding any dead or inactive parts.
- Aseptically transfer explants to fresh medium (Stage II or III) and return to growing area.

Stage III

- When sufficient roots have formed, remove plant from culture (need not be aseptic) and carefully wash agar from roots.
- Transplant to growing medium (Stage IV).

ents from the medium while avoiding problems of poor aeration due to submergence. Liquid media may be used if a support or bridge is provided; this may be a cotton ball or a folded or rolled piece of paper towel to hold the explant just above the medium's surface and provide a wicking action. Therefore, agar *may* be omitted for in-class cultures if they are carefully handled, but agar addition is recommended if the cultures are to be taken home (in a shirt pocket or purse) or if petri plates are used.

Some pre-mixed media now contain pH buffers, but most will require adjustment during mixing. This and the other mixing procedures are outlined in figure 4.

Culture vessels - Any glass or plastic container that will tolerate steam sterilization might be used for tissue cultures. The simplest choice, at no cost, for the home project is used baby food jars with lids. The other option is to select from the wide array of laboratory culture vessels available.

The standard container for most laboratories is a 25x150 mm glass tube, as this size provides both good access into the tube and an adequate volume of medium to allow efficient intervals between the culture transfers. A smaller tube (e.g., 16x100 mm) might be more economical. Standard tubes are made of heavy borosilicate glass that will take multiple washings and uses, and their cost (about \$.35-.45/tube) reflects this. Disposable tubes are now

Figure 2. Home Tissue Culture Medium Preparation

Mix the following in a one-quart canning jar:

- **1/8 cup table sugar.**
- **1 tsp all-purpose soluble fertilizer** (e.g., Peters General Purpose or Peat-Lite Special). Read the label carefully. If fertilizer does not contain *both* ammonium and nitrate forms of nitrogen, add 1/3 tsp of ammonium nitrate (35-0-0) fertilizer. If the available fertilizer lacks micronutrients, add 1/8 tsp of a soluble trace element mix (e.g., Peters S.T.E.M.).
- **1 tablet (100 mg) of inositol** (from health-food store).
- **1/4 of a pulverized multi-vitamin tablet.** This is needed with African violet leaves but not the petioles or fern rhizomes. However, its inclusion should not interfere with the fern cultures.
- **4 tbs coconut milk** (cytokinin source). Drain a fresh coconut, using the milk as needed and freeze the remainder for later. Omit when procedure specifies a Stage III culture.
- **3-4 grains (1/400 tsp) of commercial rooting powder** (an auxin source) containing 0.1% active IBA (indole-3-butyric acid; e.g., Hormodin #1).
- **Water to fill jar.** Distilled or deionized water is normally used, but tap water boiled for several minutes is satisfactory. Heating drives off chlorine and precipitates excess minerals in the water.

Shake or stir to dissolve all ingredients, dispense medium into culture vessels, and sterilize.

available as an alternative, made of either borosilicate or less-expensive flint glass and in differing glass weights. The true disposable (#1 weight) is appropriate for classes where the students will take Stage I cultures home. Appropriate sizes for these might be 16x100 or 20x150 mm (25 mm not currently available), at about \$.03 and \$.08/tube, respectively. If tubes are to be washed and reused for in-class projects, the heavier (#2 weight) glass is better as a compromise between economy and durability, considering the normal breakage expected in school laboratories. Costs for these range from \$.04 (16x100), \$.10 (20x150), to \$.15 (25x150) per tube. As a general recommendation, the #1 weight 16x100 mm disposable is best for take-home, and the #2 weight 25x150 mm disposable is best for in-class.

Closures for culture tubes function as barriers to air-borne microbes while allowing for gas exchange. Specially designed plastic caps (e.g., Kaput or Kim Kap) are commonly used. These are purchased according to the tube diameter at a unit cost of \$.04-.09 depending on size and quantity. These are very convenient and reusable. Other tube closures include heat-resistant foam plugs or rolled cotton plugs (about 4-6 cm long with 2 cm placed in the tube). These may be used if desired or available, but there is greater risk of contamination due to wetting, and the cotton plugs require considerable preparation. Aluminum foil crimped tightly over the tube top is the simplest closure but is difficult to handle during transfers. However, it is very appropriate in several other applications discussed later.

Petri plates are an alternative to tubes or baby food jars for Stage I and II cultures. One advantage is that they allow more explants to be established in a single culture. The plates may be reusable glass or disposable plastic. The latter are common in most kits because they are low in cost and are presterilized. This can save preparation steps and allow students to have direct involvement in culture preparation. (The medium is mixed, measured into separate closed containers, and sterilized by the teacher. Students are issued medium bottles, which they heat in boiling water to soften the agar and aseptically pour into their plates. After cooling, the medium is ready for transfer of explants.) Disadvantages of plates are the restricted growing height and the increased potential for explant losses from culture contamination. Plate (and tube) prepa-

Figure 3. Sample Media Formulas

Component	Boston Fern Medium Rhizome Tip		African Violet Medium Stage I, II, and III	
	Stage I,II (mg/liter)	III	Leaf (mg/liter)	Petiole
MS Major Salts				
KNO ₃	1900	=	=	=
NH ₄ NO ₃	1650	=	=	=
CaCl ₂ •2H ₂ O	440	=	=	=
MgSO ₄ •2H ₂ O	370	=	=	=
KH ₂ PO ₄	170	=	=	=
NaH ₂ PO ₄ •H ₂ O *	0	0	170	0
* Supplement to MS formula for violet leaf discs only.				
MS Minor Salts				
MnSO ₄ •H ₂ O	22.3	=	=	=
ZnSO ₄ •7H ₂ O	10.6	=	=	=
H ₃ BO ₃	6.2	=	=	=
KI	.83	=	=	=
Na ₂ MoO ₄ •2H ₂ O	.25	=	=	=
CoCl ₂ •6H ₂ O	.025	=	=	=
CuSO ₄ •5H ₂ O	.025	=	=	=
Iron				
Na ₂ EDTA **	37.3	=	=	=
FeSO ₄ •7H ₂ O	27.8	=	=	=
** EDTA = ethylenediaminetetraacetic acid (a chelating agent).				
Vitamins				
Inositol	100	100	170	100
Pyridoxine	0	0	0.4	0
Niacin	0	0	0.4	0
Thiamine	0	0	0.4	0
Growth Regulators ***				
BA	1.1	0	0.08	0.01
Kinetin	0.04	0	0	0
IAA	2.0	2.0	2.0	0
NAA	0	0	0	0.1
*** BA = benzyladenine, IAA = indole-3-acetic acid, NAA = 1-naphthaleneacetic acid.				
Supplements				
Sucrose	30,000	=	=	=
Agar	8,000	=	=	=
pH	5.7	5.7	5.5	5.5

ration is included in the laboratory outline for teachers interested in providing this experience. Plate unit cost is about \$2.50 (glass) and \$.15-.25 (plastic) depending on size.

Stage III cultures are provided a container of greater volume to allow for the increased size of these plantlets. These may be "community" vessels with several plantlets sharing a common

Figure 4. Preparing Pre-mixed Medium

- Select a beaker or flask of 1.5-2 liter volume. Mark *exact* liter by measuring in 1000 ml of water. Empty and dry.
- Place about 700 ml of distilled water in container.
- Add medium salts and dissolve (hand-stir or use magnetic stirrer/hot plate, if available).
- Add sucrose (30 gm granulated table sugar) and dissolve.
- Fill to volume with distilled water.
- Adjust pH, using meter or test paper, adding *drops* of 1M HCl (if too high) or NaOH (if too low) with stirring.
- Add agar (8 gm) if desired. Heat to boiling with constant stirring (changes from cloudy suspension to clear tan solution). Need larger container to avoid boilover.
- Dispense medium (hot if with agar) into culture vessels.
- Insert paper bridge for explant support if not using agar.
- Attach closures and sterilize.

volume of medium. Pint or quart canning jars (upright or laid on their sides) are very effective. These may be closed with aluminum foil secured with a rubber band or with standard lids and bands. (Note: Canning lids will seal during the sterilization process. Breaking the vacuum seal draws contaminated air into the jar. Therefore, inverting the lid to prevent sealing is advised.) Heat-tolerant-plastic vessels for Stage III cultures are also available at a unit cost of about \$1.70.

The medium is dispensed into tubes or jars by simply pouring the desired volume from a spouted container or by placing the medium in a large buret for more accurate transfer via tubing and pinch clamp. As a general guide, the volume of medium is typically 15-20% of the tube volume. Standard 25x150 mm tubes (about 74 ml) are filled with 12-15 ml of medium (thus, 60-80 tubes/liter), but a 16x100-mm tube would only need 8-12 ml. A 100x15 mm petri plate would need 20-25 ml for a 0.5 to 0.75-cm medium layer. Canning jars for Stage III cultures should have a 2 to 4-cm (upright) or 1.5 to 2-cm (on side) layer of medium. Test-measure this volume with water for estimating the medium requirement.

Other materials and equipment - Tube racks or baskets will simplify handling culture tubes. Small jars or beakers are satisfactory for the sterile water and bleach solution. A standard biology dissecting kit will provide the scalpel/razor blade and forceps needed. Note that 25-cm-long forceps are the preferred size, but smaller ones will suffice; dissecting needles or similar instruments also may substitute. A common but optional item is a Bunsen or alcohol burner, which is used to flame-sterilize tube mouths and metal tools during the transfer process. While this step helps reduce culture contamination, there are problems of safety for students and for the cultures (picking up tissue before forceps cool sufficiently). Another option is Parafilm or a parafin tape stretched around the plastic tube caps or petri plate tops to reduce evaporation from the medium and provide an additional barrier to contaminants. Finally, be sure to provide an appropriate means for labelling all cultures.

Aseptic procedures and preparations - A wide variety of fungi, bacteria, and yeasts can be supported by the explant and the culture medium. These microorganisms must be destroyed for successful culture establishment. Alcohol (75-90%) may be used to surface-disinfect explants and tools, but bleach (sodium hypochlorite) is safer and cheaper. (Prepare this as a 10% sol-

ution using one part common household bleach with nine parts water. Prepare a fresh batch daily, dispensing only the amount needed in each period from a sealed container.) Once the explant is removed from the bleach solution, it should contact only sterile items.

Initial sterilization of all equipment and materials is provided by heat. Dry heat may be used (320°F oven for 4 hours), but most plastics will be damaged by this treatment. More common is pressurized steam (30 minutes at 15 psi), either with an autoclave or a home pressure canner. (Important: Timing begins when full steam pressure is reached, and slow cooling to atmospheric pressure is required to prevent liquid boilover.)

All media and supplies may be sterilized at the same time, and covering or packaging will protect them until used. For example, jars or beakers of water may be covered with heavy gauge aluminum foil. Scalpels, razor blades, forceps, etc., may be wrapped in either two layers of paper towel (secure with masking tape) or one layer of foil. Paper towels may be sterilized by placing a handful in a large beaker covered with foil to prevent wetting by condensation. Petri plates also may be stacked and wrapped in paper towels for sterilizing.

Any sterile item exposed to air is potentially contaminated. Aseptic transfer procedures are designed to minimize the risk. A laminar-flow transfer chamber provides the ultimate protection by moving ultra-filtered air over and away from the transfer zone. This item, at \$1500+, is only for the serious laboratory. Another way to minimize contamination is to use a still-air chamber that minimizes air currents around the transfer zone. Information on both of these is available in the references. However, neither is a requirement for a successful exercise. If the laboratory is reasonably free of air drafts, work tables are cleaned (using the diluted bleach, Lysol, 80% ethanol, or another disinfectant), and the students follow aseptic procedures, there should be few problems with excessive contamination.

Stepwise instructions on aseptic technique are provided for students in the exercises. Teachers may help the students obtain better results by: 1) using only a few work stations for easier student observation and guidance, 2) providing a nonsterile practice culture so the students may experience the transfer process beforehand, 3) ensuring that all tools are returned to the bleach between transfers, and 4) reminding students about contamination from talking and breathing toward or reaching across the sterile work surface.

Culture growing facilities - Environmental requirements for incubating plant tissue cultures can be easily provided in school laboratories. Both fern and violet cultures need only 300 ft-c of fluorescent lighting (16 hours light/8 hours dark). In lieu of using the violet pretransplant medium, rooting may be achieved on multiplication medium under 50-100 ft-c. (Rooting occurs along etiolated stems.) A lighted plant table with time clock is very satisfactory, if available, with some of the lights turned off. A north window (no direct sunlight) also may be used for the higher light level while an interior location may be used for the lower light level, using natural photoperiods only. Growth will be faster with warmer temperatures (25-27°C, 77-80°F), but room temperature can be satisfactory. Placing the cultures on top of a refrigerator may provide additional warmth.

Transplant growing facilities - A commercial soilless rooting medium (or 1:1 mix by volume of shredded peat and vermiculite) is very good for growing plantlets. A soilless medium is preferred as the components are essentially sterile (i.e., free of weed seeds and pathogenic organisms), and no special preparation is required. However, the medium must be clean and unused to insure its sterility. Flats, pots, or cell-packs, all with drainage holes, are satisfactory containers. Used containers must be thoroughly washed and rinsed in 10% bleach solution before use for Stage IV cultures. Use care when transferring the plantlets; handle them by the leaves only. Any agar remaining on the roots may lead to problems with fungal growth and should be carefully rinsed off with tap water (need not be sterile).

Place the plants in a warm area out of direct sunlight and under a plastic cover to provide high humidity. Large flats may have wires bent over them to support a sheet of plastic. After a few days, cut a single hole (one to two inches long) in the plastic. Enlarge the hole a little each day until the plants are fully exposed and acclimatized in two to three weeks. An alternative for small flats and pots is a plastic bag, opened for five minutes the first time and progressively longer periods each day thereafter.

LABORATORY EXERCISES

Organization - Rather than duplicate similar information among different exercises, a single basic exercise is presented with several optional components following. The teacher may then photocopy those sections to be used, revise them as necessary, and create a cut-and-paste student handout as appropriate. The exercise may be shifted from in-class to at-home at any point the teacher desires.

The basic laboratory exercise is centered on propagation of Boston fern by removing explants of rhizome tips. This duplicates commercial production procedures. For economy of stock plants and fewer cultures per student, the teacher may pre-cut the rhizomes into 4 sections of 2.5-cm length for storage on wet toweling. These sections may be distributed to the students who begin with the bleach disinfection and then proceed with the aseptic steps (Part 2).

Although the laboratory outline is presented as a single exercise, please recognize that time is required for growth after each culture transfer. Parts 1 and 2 constitute one laboratory period, and Parts 3 and 4 are separate laboratories two or more weeks after the preceding part. Note that optional Part 0 can be included in the same period with each of these, or it may be part of the pre-lab activities.

Required materials are listed within the laboratory parts as a checklist for each work station, based on one student working at a time. The actual number or volume of the items required will vary with the number of students and the item's usage and sterility. For example, the disinfectant for the initial laboratory may be prepared in bulk and transferred into non-sterile containers at each station. A larger volume is provided for use by each student in cleaning the work table, and two smaller volumes are provided for soaking the tools and for treating the explants. Multiple usage of the disinfectant solutions (and the tools) is appropriate. This is not possible with the sterile water used for rinsing the explants. Covered containers of water should be sterilized for each student, and these containers must remain closed until needed. The paper towels used to provide a sterile cutting surface are also sterilized in a closed container. In this case, there should be one container for each work station, containing one or more towels per student assigned to that station. If students carefully remove their towels with clean hands and replace the cover immediately, the risk of contaminating the other towels is minimal. The used towels are then discarded. Petri plates, the alternative cutting surface, will be used by only one student each. These do not need to be sterilized within another container (since the plates include a cover), but they will be easier to handle if several plates are wrapped together in paper towels for sterilization and distribution to the work stations.

MICROPROPAGATION LABORATORY OUTLINE

Part 1: Preparation of Explants (Non-Sterile)

Materials required Stock plant
 Scalpel or razor blade (non-sterile)
 10% bleach solution (50 ml) + 1 drop liquid detergent

Procedure

- 1.1 Cut a 10-cm tip from an actively growing fern rhizome, and cut this piece again into 4 equal lengths.
- 1.2 Place each 2.5-cm piece in the bleach solution.

Part 2: Preparation of Stage I Cultures (Sterile)

Materials required Disinfectant (diluted, 500 ml) and sponge or paper towels
 Explants in bleach solution
 Sterile scalpel and forceps (tips soaking in bleach solution)
 Sterile water (50 ml) in covered jar
 Sterile paper towels in covered jar or sterile petri plate with cover
 Sterilized culture vessels with Stage I medium

Procedure (*Note: Instructions are for right-handed persons.*)

- 2.1 Soak explants in bleach solution for 10 minutes. Submerge floating explants frequently to insure full contact of disinfectant with all surfaces.
- 2.2 While explants are soaking, clean yourself and the work area. Tie back loose hair and wash hands thoroughly. Use the disinfectant to clean the transfer table surface. Arrange materials on clean surface for convenient work without having to reach over open center area.
- 2.3 Using sterile forceps, transfer explant to container of sterile water. Rinse explant for 1 minute, submerging it several times to remove bleach. Return forceps to bleach solution between uses.
- 2.4 Prepare sterile paper-towel or petri-plate cutting surface. Uncover container of sterile paper towels, grasp one at edge to remove, and spread open on table. If using sterile petri plate, simply uncover. *Do not* contact sterile area with any unsterile object.
- 2.5 Transfer rinsed explant to the paper towel or petri plate using the sterile forceps. Hold explant with forceps in left hand and pick up sterile scalpel or razor blade with right hand. Cut off any brown (bleach-damaged) tissues.
- 2.6 Cut each 2.5-cm piece into 3-5 sections (about 5-10 mm each). Each explant may be cultured, as directed by the teacher. Return tools to the bleach solution.
- 2.7 Aseptically transfer explants to medium in culture tubes, as follows:
 - Pick up culture tube in left hand. Carefully twist closure to loosen (*do not remove*).
 - Pick up forceps from bleach solution with right hand and gently pick up one explant.
 - Tilt top of closed tube at angle toward right. Wrap little finger of right hand around closure (still holding forceps and explant).
 - Remove tube cap or plug with twisting motion. Carefully insert forceps (without touching sides of tube) and plant basal end of explant into the surface of the medium. (Do not submerge entire explant.) Then remove forceps and close tube.
 - Return forceps to bleach solution. Label tube as directed by the teacher and place it in the rack.
 - Repeat preceding steps according to the number of explants and tubes.
- 2.8 Move all Stage I cultures to the growing area. Observe during next 3-5 days for signs of contamination. If any become contaminated, remove those tubes immediately and wash or discard them.

Part 3: Preparation of Stage II and III Cultures (Sterile)

Materials required Disinfectant (diluted, 500 ml) and sponge or paper towels
Stage I or Stage II cultures
Sterile scalpel and forceps (tips soaking in bleach solution)
Sterile paper towels in covered jar or sterile petri plate with cover
Sterilized culture vessels with Stage II and/or Stage III medium

Procedure (*Note: Instructions are for right-handed persons.*)

- 3.1 Wash hands, disinfect work table, and organize materials.
- 3.2 Prepare sterile paper-towel or petri-plate work surface.
- 3.3 Remove forceps from bleach solution with right hand.
- 3.4 Holding Stage I or II culture vessel tilted with left hand, remove closure with right little finger. Remove explant with forceps and place on sterile towel or petri plate. Set used culture vessel aside.
- 3.5 Holding explant with forceps in left hand, pick up scalpel from bleach solution and trim off any dead tissue. Cut the explant into separate plantlets. (These must be entire plantlets; leaves alone will not grow.) If the Stage I explant growth has been slow, trim the dead tissue only and transfer whole explant to new medium. Return scalpel to bleach solution.
- 3.6 Transfer explants to new medium in culture tubes, as follows:
 - Pick up Stage II culture tube or Stage III jar in left hand and loosen closure by twisting.
 - Gently pick up one explant, using sterile forceps.
 - Tilt top of closed tube at angle toward right and remove closure (or carefully lift lid or foil cover if using canning jar). Carefully insert forceps (without touching sides) and place explant into the surface of the medium. Remove forceps and close tube or jar.
 - Return forceps to bleach solution. Label tube as directed by the teacher and place it in the rack.
 - Repeat preceding steps according to the number of explants and tubes.
- 3.7 Move all new cultures to the growing area. Observe them during next 3-5 days for signs of contamination. If any become contaminated, remove those tubes immediately and wash or discard them.

Part 4: Preparation of Stage IV Cultures

Materials required Rooted Stage III cultures
Forceps in bleach solution
Wash bottle with water (nonsterile) and waste container
Pot or flat with sterile rooting medium
Plastic cover for container - bag (for pot) or sheet (for flat)

Procedure

- 4.1 Wash hands, clean off work table, and organize materials.
- 4.2 Prepare pot or flat with rooting medium. Use a pencil (nonsterile) to make one or more small planting holes in the medium as directed by teacher.
- 4.3 Remove forceps from bleach solution. Open Stage III culture and remove one plantlet.
- 4.4 If agar-based medium clings to plantlet roots, carefully remove the medium with water from wash bottle.
- 4.5 Transfer plantlet to rooting medium. Be careful to set plant at correct depth. Carefully water from wash bottle to settle medium around roots.
- 4.6 Repeat these steps according to number of plantlets.
- 4.7 Cover Stage IV culture container with plastic bag or sheet and move to the growing area as directed.
- 4.8 Observe plants for dessication or other problems during next 3 days. After third day, begin opening plastic cover as directed by teacher.

LABORATORY OPTIONS

Each entry below is a possible modification to the basic exercise. The step numbers correspond to those given earlier to allow cut-and-paste addition or replacement in the basic exercise.

Part 0: Preparing Culture Tubes or Plates

Materials required Sterile culture tubes or petri plates
 Premeasured containers of sterile medium with agar (solidified)
 Boiling-water bath

Procedure

- 0.1 Place container with agar medium in boiling-water bath. Loosen (*do not remove*) closure.
- 0.2 When agar has liquified, remove the container (use heat-proof glove) from heat and do the following quickly and carefully:
 - *For culture tubes* - Pick up empty tube and carefully twist closure to loosen (*do not remove*). Remove top of medium container. Now remove top of the culture tube, pour in medium, and close. Prepare agar slant by laying culture tube at angle as directed by teacher.
 - *For petri plates* - Set empty plate on table. Remove top of medium container. Raise (tilt) plate cover on one side only, pour in the medium (do not splash), and close cover.
- 0.3 Leave tubes or plates undisturbed to cool (agar solidified) and proceed with next part.

* Using petri plates rather than culture tubes *

- 2.7 Aseptically transfer explants to medium in petri plates, as follows:
 - Pick up forceps from bleach solution with right hand and gently pick up one explant.
 - With left hand, raise one side of petri plate cover. Carefully insert forceps and place explant on the medium. Then remove forceps and close plate cover.
 - Return forceps to bleach solution. Repeat above steps as directed by teacher, placing additional explants in same culture plate. Space explants no closer than 1-2 cm apart.
 - Label plate as directed by the teacher.
- 3.6 Transfer explants to new medium in petri plates, as follows:
 - Gently pick up one explant, using sterile forceps.
 - Open Stage II culture plate with left hand by tilting cover to one side, or the Stage III container by lifting lid or foil cover. Carefully insert forceps and place explant on the medium. Remove forceps and close plate or jar.
 - Return forceps to bleach solution. Repeat above steps as directed by teacher, placing additional explants in same culture plate or jar. Space explants no closer than 1-2 cm apart.
 - Label plate as directed by the teacher.

* Using African violet leaves for explants *

- 1.1 Remove healthy African violet leaf blade and/or petiole from plant and place in beaker of water (nonsterile) containing 2 drops of liquid dish detergent.
- 1.2 Wash leaf blade and/or petiole and then transfer to bleach solution.
- 1.3 Cut additional leaves with attached petioles for rooting by conventional methods. (optional)
- 2.6 Prepare final explants by cutting leaf blade into several 1-cm squares or petiole into 2-mm-thick cross sections. Each explant may be cultured, as directed by teacher. Return tools to the bleach solution.

SUGGESTED REFERENCES

1. Bottino, P.J. 1981. Methods in Plant Tissue Culture. Kemtec Educational Corp., P.O. Box 57, Kensington, MD 20895. 72 pp.
Designed as college laboratory manual. Many advanced project ideas.
2. Conger, B.V. (Ed.). 1981. Cloning Agricultural Plants Via In Vitro Techniques. CRC Press, Inc. Boca Raton, FL. 273 pp.
Reviews of developments throughout agriculture field. Rather advanced and expensive but of particular value in the extensive bibliography.
3. Freshney, R.I. 1983. Culture of Animal Cells: A Manual of Basic Technique. Alan R. Liss, Inc. New York, NY. 295 pp.
For the student interested in medicine and zoological studies. Limited possibilities for advanced student projects.
4. Hartmann, H.T. and D.E. Kester. 1983. Plant Propagation: Principles and Practices. 4th ed. Prentice-Hall, Inc. Englewood Cliffs, NJ. pp. 523-594.
The reference on plant propagation, and this edition includes separate chapters on micropropagation principles and techniques. Strongly recommended reading for both teachers and students for general information, present and future applications, and project ideas.
5. Kyte, L. 1983. Plants from Test Tubes. Timber Press. Portland, OR. 132 pp.
Excellent coverage designed to introduce micropropagation to commercial nurserymen. Includes numerous media formulas and procedures for different plants. Recommended along with #4.
6. Wetherell, D.F. 1982. Introduction to In Vitro Propagation. Avery Publishing Group, Inc. Wayne, NJ. 87 pp.
Excellent introductory coverage comparable to #5, with photographs of cultures but without the guide to different plant media and procedures.

SUGGESTED SOURCES OF SUPPLIES

Most scientific supply companies offer glassware, plasticware, and reagents appropriate for plant tissue culture. A few companies that offer specialty products in tissue culture are listed below. Other equally appropriate companies offer similar product lines.

Bellco Glass, Inc., 340 Edrudo Road, P.O. Box B, Vineland, NJ 08360
Cultureware

Carolina Biological Supply Co., 2700 York Road, Burlington, NC 27215
Complete line of kits, media, reagents, supplies

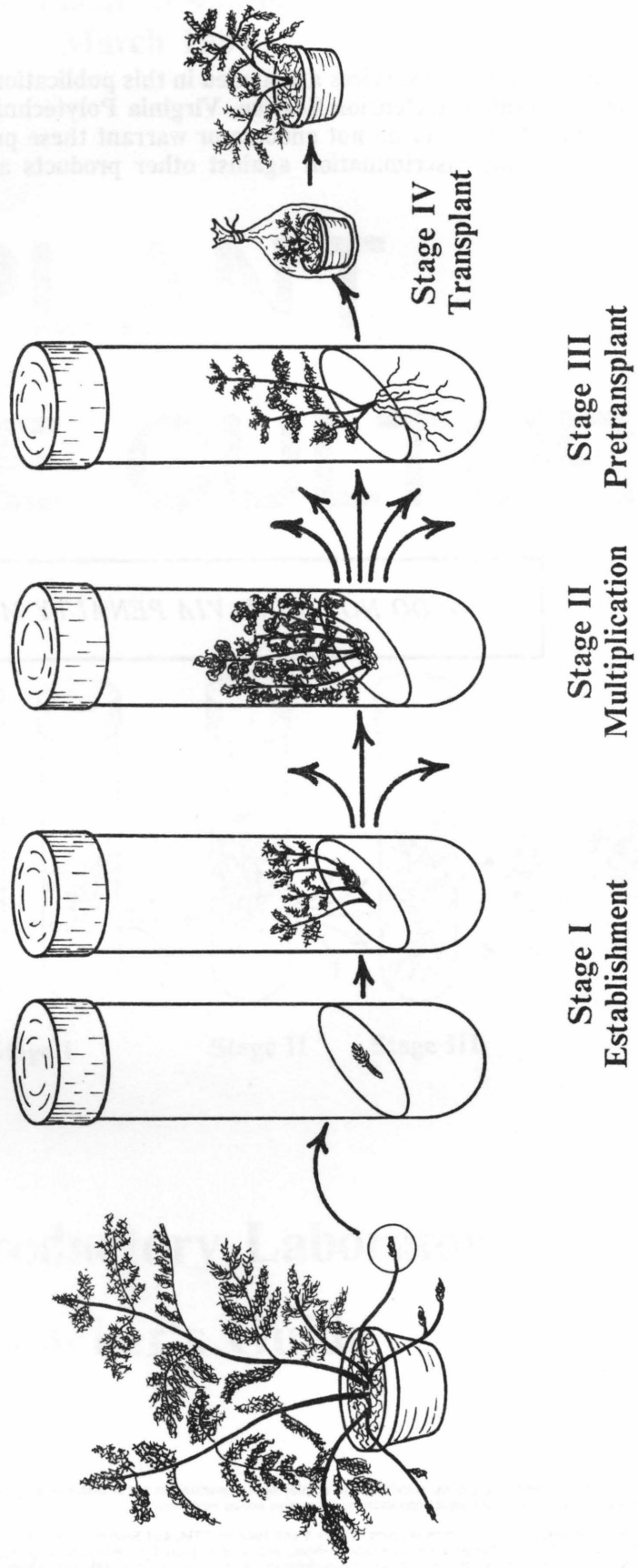
Flow Laboratories, Inc., 1710 Chapman Avenue, Rockville, MD 20852
Media

GIBCO, 3175 Staley Road, Grand Island, NY 14072
Media

KC Biological, Inc., P.O. Box 5491, Lenexa, KS 66215
Media

Kimble (Owens-Illinois), P.O. Box 1035, Toledo, OH 43666
Cultureware

Sequence of Culture Stages



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