How to Grow a Ridiculously Large Number of Sundews

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Introduction

Let's face it: carnivorous plant people are a little nutty about their plants, and I am an excellent example. And if you are nutty about one single pygmy sundew or lowland *Nepenthes* in your collection, wouldn't it be even better to have fifty? Of course it would! So how do you propagate and maintain that many? Seeds and cuttings are an excellent way to multiply plants, but keeping many copies, of many species, happy in a greenhouse or terrarium is beyond the reach of most enthusiasts' growing space. Add to that the difficulties from some species of carnivorous plants that just do not reproduce easily—for example *Drosophyllum* or *Roridula*.

Tissue culture can solve many of these problems. Although the technique probably scares away many amateur growers who imagine that a prohibitive amounts of money, space, and expertise are required to do it, this is not a fair prejudice! Tissue culture is a relatively straightforward way to multiply plants, and some carnivorous plants will even maintain their normal morphology and annual cycle, to the point of flowering, *in vitro*. That term, *in vitro*, means "in glass," and originated when the early practitioners used glass containers. Many still do, although plastic dishes are also common.

Tissue culture is also a tool for conservation! By rapidly multiplying rare species, tissue culture helps remove pressure from natural populations—widely available, inexpensive plants propagated from tissue culture offset the incentive to poachers. Also, plants from tissue culture are often more vigorous than seed-raised plants of the same species, perhaps because of the optimal conditions given to them when young, especially with regard to mineral nutrition.

A Brief History of Plant Tissue Culture

Tissue culture began sometime in the late 19th or early 20th century, depending on how strictly "tissue culture" is defined, as a means for testing the growth requirements of plants—if a plant could be isolated from the soil the importance of inorganic compounds such as iron, and organic compounds such as vitamins, could be studied with scientific precision. For example, by withholding a particular substance, any developmental defects in the plants could be observed.

In vitro cultivation of plants has also played a large role in the study of plant development. These studies expanded knowledge of what plants parts can be used for cloning. White and Knudsen were two early tissue culture practitioners (ca. 1930-1960) who furthered our knowledge of growth requirements and developmental biology; some of their recipes for plant tissue culture media are still used even today.

Today tissue culture is used for a wide variety of applications. Many agricultural laboratories use a variety of tissue culture techniques. For example, all transgenic crops originated in tissue culture. Furthermore, tissue culture is still used in its traditional role in studies of growth requirements and of development.

A number of national and international societies are dedicated to the science of botanical tissue culture, including the International Association for Plant Tissue Culture and Biotechnology and the Society for In Vitro Biology, both of which are easily found on the internet.

Basics of Tissue Culture

"Aseptic technique" refers to the removal of all the microorganisms, bacteria, fungi, and protists from the surface of material to be grown in tissue culture. Aseptic techniques are necessary in tissue culture because of the rich growing media used. Plant tissue culture media are very rich because they are intended to last for relatively long periods, and any microbes entering the cultures quickly overwhelm the plants being cultured. A special challenge in plant tissue culture, as opposed to laboratory culture of

bacterial or other eukaryotic cell or tissue cultures, comes from the inability to add antibiotics to plant tissue cultures. The antibiotics used for killing most microbes in culture harm plant cells. They are also quite expensive. A new compound called Plant Preservative Mixture (PPM)[1] suppresses the growth of microbes, although some reports in the scientific literature say that PPM can cause abnormalities in plants. Since PPM only suppresses the growth of microorganisms (and does not kill them) a culture grown using PPM must always use the compound, or else the microorganisms will resume growth and overwhelm the plant cells.

All of the water that comes into contact with specimens used in tissue culture work needs to be sterilized to kill any microbes in it. Sterile water can be purchased from some lab suppliers but is very expensive. Use an autoclave or a pressure cooker made for cooking to sterilize water. An autoclave is essentially a larger lab-grade pressure cooker. Some smaller autoclaves may be in the price range of home users and can be plugged into a home outlet.

Ideally, a specimen of plant material to be used in tissue culture work is no more than a cm or two in all dimensions (and is often much smaller). Before it can be placed on sterile media, it is first surface sterilized (made aseptic on its surface) usually by using a dilute mixture of bleach (NaOCl, sodium hypochlorite), or a close chemical relative such as CaOCl (calcium hypochlorite)[2]. To make this sterilizing solution, household bleach is diluted with sterile water to 5-40% concentration. (Since the bleach concentration was originally only about 10% at the time of purchase, the additional dilution reduces the concentration to about 0.5-4%.) Tween 20 (polyoxyethylene sorbitan monolaurate, in syrup form) is added to the sterilizing solution to make a detergent concentration of about 0.1-1% by volume. The detergent acts as a wetting agent and allows the bleach to better access nooks and crannies in the plant material where bacteria and fungi may be hiding. The plant material is then rinsed with sterile water to remove the bleach solution, which would otherwise eventually kill the sample.

With luck, the plant specimen is now aseptic, and can be placed on tissue culture media. There are many formulations of media, but they all include a number of nutrients needed by plants: inorganic salts of macronutrients and micronutrients, and organic compounds such as vitamins and *myo*-inositiol. Sugar (usually sucrose) is included at the rate of about 3% w/v (weight/volume; 1ml=1g; so 30 g of sucrose is dissolved into 1000 ml of medium). Most media are designed to have a pH of 5.8, and are autoclaved to destroy any microbes or spores. The components of the medium survive the pressures and temperatures applied in the autoclave, although some media are filter sterilized instead, to better preserve the organic components of the media. These media will not be discussed further since they are expensive and impractical for home use.

Many media formulations have been devised over the years, and they are often specialized for particular types of plants. The three best-known formulations are called "Murashige and Skoog," "Linsmaier and Skoog," and "B5" (which is also called "Gamborg's Medium"). Combinations of the mixtures can be purchased from many supply houses. For example, MS/B5 is a dry ingredient formulation which has the macro and micro salts from the MS medium, and the B vitamins from the Gamborg *et al.* medium).

All these media should all be suitable for culturing carnivorous plants, although since carnivorous plants prefer lower rates of nutrients the media should be diluted to 5-20% normal concentration.

Instead of being fluid, media used in aseptic culture are solid. This is because most plants, including most carnivorous plants, prefer to grow upon a solid substrate (obviously, this is not necessary for *Aldrovanda vesiculosa* and aquatic *Utricularia*). Since the mixture of nutrients used in tissue culture is in a liquid form at room temperatures, it must be gelled into a solid form. Agar, often sold as Bactoagar is the compound most commonly used to effect this change[3]. The agar is usually used at 0.5-1% (w/v), and must be dissolved by heating the media to near boiling temperatures either during or before autoclaving. Conventional cotton balls are sometimes used as an alternative to agar. While it is cheaper and more easily available than agar, plants may be harder to disentangle from this solid support.

The medium (including the agar) is placed in a glass jar. Many plastic containers are rated for autoclaving, such as medical containers used to collect urine samples (hey, you have got to be creative). The containers are sealed using either a waxy tape-like material called parafilm or a breathable dressing,

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[1]This is available for purchase on the internet.

[2]Sometimes this step is preceded by briefly rinsing the plant tissue in 70% ethanol.

[3]While agar is by far the easiest gelling agent for the home user to obtain, others are also available but are not discussed here. Systems based upon using porous solid supports and liquid media are sometimes used, and the interested reader may wish to investigate these more.

available from most pharmacists in the surgical supplies or first aid sections (Figure 1). These reduce the loss of water from the cultures. This is important because it extends the lifespan of a culture, thus minimizing how frequently you must transfer the plants to fresh media.

Table 1: A Sample Schedule for Tissue Culture of a carnivorous plant	
Event	Time
Sterilize seed or stem, place in culture.	0
Culture begins to germinate/grow.	1-3 weeks
Complete plants develop.	2-4 weeks
Plants begin to crowd container.	4-12 weeks
Subculture required or culture lost.	4-20 weeks

All the necessary media ingredients can be obtained from Sigma Chemical or a companies which provide supplies to schools for science classes—*e.g.* in the USA, Carolina Biologicals or Ward's Natural Science. A simple search of the web will turn up many such sources with moderately priced components (see Table 2). One savings involves the use of table sugar in place of scientific-grade sucrose, a perfectly acceptable choice for home use. Some media can be bought already autoclaved and ready to use, which can be a big time and trouble saver.

I use a mixture of 20% MS/B5, 3% sucrose, and 0.8% Bactoagar (which has a pH=5.8 before autoclaving). This medium has been successful with *Drosera* of all types, *Sarracenia*, *Utricularia*, *Cephalotus*, *Genlisea*, *Heliamphora*, *Pinguicula*, and *Stylidium*. Most other carnivorous genera should do well on this same medium.

Setting up Tissue Culture at Home

To set up a home laboratory to do tissue culture, you will need a place with little traffic. Even small air currents are undesirable because they can introduce microbes and their spores, which could contaminate your media. In addition, some sort of "hood" is needed to further reduce contamination. The simplest and least expensive such device is a wooden box with a viewing window and holes in the side for inserting your arms. Protective gloves can be awkward to use and difficult to decontaminate, so bare hands and arms (with all jewelry removed), and skin wiped with 70% ethanol is the best procedure. This box can be equipped with an ultraviolet lamp—usually a fluorescent bulb of at least 60 cm (2 feet) in length for greater coverage of the work area. Microbes and spores inside the box will be killed by about 30-60 minutes of irradiation. You should not work while the ultraviolet lamp is operating.

A more effective (but also more expensive) alternative to such a box is a laminar flow hood. The laminar flow hood passes a constant flow of filter-sterilized air over cultures, ensuring that microbes do not waft into containers and spoil cultures (see Figure 2).

Both a wooden box and the laminar flow hood can be purchased from the same sources that provide tissue culture media. Wooden boxes generally cost about US$150, while laminar flow hoods cost ten times as much. You can save money by building your box and hood—plans can be found on the internet or in books on tissue culture. Older books are a particularly good source of construction plans, and you can often find these in most large libraries.

Autoclaves are not hard to find and not too expensive, although a cheaper alternative you may already own is a pressure cooker used for canning—the same pressures and temperatures which kill microbes in preserved food will kill microbes in tissue culture media.

You will require tools. Scalpels and forceps like those sold on campus supply stores for college dissection courses are just fine. At least one pair of long forceps, at least 15 cm (6 inches) long, is a great help when working with some types of culture vessels.

You will require a means of sterilizing tools, and keeping them sterile while you are using them. Tools are sterilized by first dipping them in a container filled with 95-100% alcohol, and then heating them in either an alcohol lamp flame or a heated sterilizing device containing either a ceramic cylinder or a heated container of glass beads. An alcohol lamp flame will cost US$10, the heated sterilizing device will cost US$75 or more and will require use of an electric outlet.

Once cultures have been established, they will need lights. Even though the sucrose in the media will provide all the sugars the plants will need, so they do not need to photosynthesize, light will promote the formation of normal plant structures. Either standard fluorescent bulbs or grow-bulbs may be used, and they should be turned on for 12-24 hours each day. Be sure to place cultures no more than 15

Figure 1: A tissue culture (*Stylidium lineare*) in a urine specimen container, in front of tissue cultures growing in smaller, 50 ml centrifuge tubes.

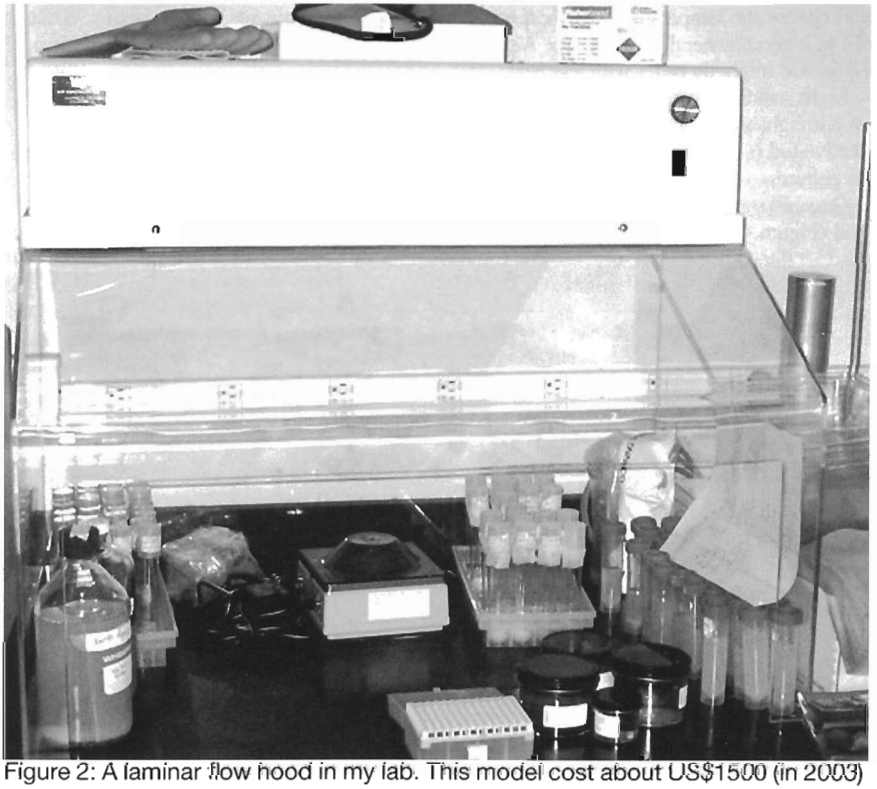

Figure 2: A laminar flow hood in my lab. This model cost about US$1500 (in 2003) and is about 1.3 m wide by 0.8 m deep by 1 m high (4 ft by 2.5 ft by 3 ft).

Figure 3: My light stand for tissue cultures. Only two shelves are in use in the picture. The stand is about 2 m high by 1.7 m wide by 0.7 m deep (6 ft by 5 ft by 2 ft).

Figure 4: A tissue culture of a pygmy sundew, with the fine roots visible against the side of the container.

cm (6 inches) from the lights for best results. The lights will generate significant amounts of heat, so temperatures in the culture vessels will be higher than ambient temperatures when the lights are lit. You should choose the temperatures that best match the growth requirements of your plants. While 23°C (75°F) is a good, general choice, some *Nepenthes* might grow better with higher temperatures while *Darlingtonia* might do better with less heat.

Light stands are constructed to support both your lights and your tissue culture media (see Figure 2). While light stands are commercially available, many carnivorous plant growers build their own. Reflective foil is used on the light stand supports, floors, walls, etc., to reflect as much light as possible to the cultures.

Once plants in culture are large enough, they can be removed from the sterile culture and grown on soil (Figure 4). This process is known as deflasking since cultures are sometimes grown in large flasks. Use standard soil mixes, but be sure to keep humidity extremely high for freshly deflasked plants. This is necessary because the humidity is very high inside culture containers, and sudden decreases in humidity may overstress and kill plants. You can keep the humidity high by enclosing the newly potted plants in a plastic bag or similar cover. The plant is hardened to lower humidity gradually, either by opening the enclosure by successive small amounts over a week or two, or by removing the plant from the cover completely for short (but increasingly long) amounts of time each day.

Using these methods, you will soon have more plants than you know what to do with, which when you think about it is a good thing.

Two excellent and recent books on tissue culture are listed in Table 2 as references. Many other older books also exist in libraries and might be better sources of plans for home users.

Table 2: Useful resources.

Web Sites
Sigma Chemical: http://www.sigmaaldrich.com/Brands/Sigma.html
Carolina Biologicals: http://www.carolina.com/
Ward's Natural Science: http://www.wardsci.com/
Kitchen Culture Kits: http://www.kitchenculturekit.com

Books
Roberta, H. 2000, Smith Plant Tissue Culture Techniques and Experiments, 2nd edition Academic Press, New York.
Trigiano, R.N., and Gray, D.J. (editors) 2000, Plant Tissue Culture Concepts and Laboratory Exercises 2nd ed. CRC Press, New York.