A Practical Guide to Woody Plant Micropropagation

John W. Einset

The spinoff from basic research on the physiology of plants, plant micropropagation is a simple, straightforward—and commercially profitable—technique

Although its commercial use as a method for multiplying plants is still fairly new, tissue-culture propagation (micropropagation) has already had a significant impact on the way people think about and handle plants. The example of pyrethrum (*Chrysanthemum cinerariaefolium*) is especially striking. During the last 15 years this plant has been exploited extensively as a source of chemicals, known as pyrethrins, that are used as "natural" insecticides. In fact, it is estimated that over 150 million pyrethrum flowers are harvested every day of the year in East Africa or Ecuador for the production of pyrethrin insecticides. Given the size of the industry (50 billion flowers per year), it is obvious why micropropagation is being used for pyrethrum. After all, the technology enables growers to obtain rapid, clonal mulitiplication of plants that produce exceptionally high concentrations of pyrethrins. With this capability, yearly increases in superior plants equivalent to one million-fold multiplication are obtained, and the total level of pyrethrin production is increased significantly. As a matter of fact, the pyrethrum example probably represents the single most important use of plant-tissue-culture technology in the world today.

Micropropagation is also becoming very important for woody plants, although the scale of this enterprise is minor compared to the pyrethrum industry. At the present time micropropagation is utilized commonly for species in two families, the Rosaceae (roses, apples, raspberries, and strawberries) and the Ericaceae (rhododendrons, azaleas, and mountain laurels). While it is not yet clear that the technology will be feasible with all woody species, the prospects are very promising for several of them. Because of this, the most spectacular applications for micropropagation are undoubtedly still in the future.

As a technique, micropropagation represents a direct, practical extension of scientific methodology devised over 30 years ago to study fundamental aspects of plant physiology, especially the role of phytohormones in growth and development. Essentially, micropropagation takes advantage of the control of plant development that can be exerted by phytohormone treatments. Thus, although tissue-culture media contain over 20 different chemical constituents, and in

spite of the fact that environmental factors such as light intensity and temperature need to be carefully monitored, the crucial variable in micropropagation is the phytohormone content of the medium. (An article on "Chemicals That Regulate Plants," which appeared in the Spring 1985 issue of *Arnoldia*, discusses other practical uses of phytohormones.)

The Three Methods of Micropropagation

Depending on the plant, micropropagation involves one of three possible strategies: (1) regeneration from callus, (2) somatic embryogenesis (embryo formation from vegetative cells), or (3) shoot multiplication.

☐ **Regeneration from callus** was demonstrated first in the early 1950s by Professors F. K. Skoog and C. O. Miller, codiscoverers of the cytokinin class of phytohormones, both of whom were working at that time at the University of Wisconsin. These investigators showed that stem segments taken from tobacco plants will proliferate an unorganized mass of tissue, known as callus, when placed on a nutrient medium containing *cytokinin and auxin*. If the callus is then subdivided into smaller pieces and these are placed on fresh media, growth will continue. Significantly, the type of growth depends on the kinds and quantities of phytohormones added to the medium, especially the relative levels of cytokinin and auxin. Thus, high cytokinin-to-auxin concentrations result in shoot formation from callus, low ratios result in root formation, while intermediate ratios result in continued callus proliferation. Spectacular as this classic demonstration of plant developmental control is, there are surprisingly few plant species that respond in tissue cultures as tobacco does. Even though callus can be produced from practically any plant, the ability of these calluses to form shoots and roots in response to phytohormone treatments is rare.

☐ By contrast, **somatic embryogenesis** has already been utilized for species in over 25 different families. Like regeneration from callus, somatic embryogenesis involves an initial stage of callus formation, in this case using a medium containing *auxin* as the only phytohormone. The callus is then recultured on a medium lacking phytohormone or on medium with cytokinin. Often, several suc-

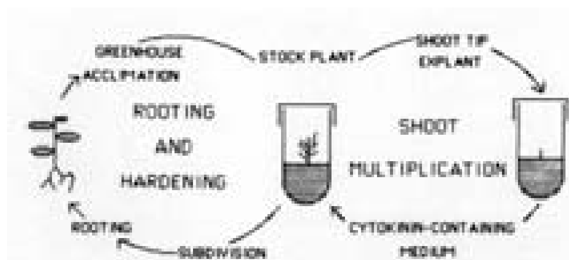

Figure 1 Steps in the Micropropagation of Woody Plants Woody-plant micropropagation involves a shoot-multiplication cycle using controlled cytokinin treatments and a series of treatments to cause the rooting of cuttings and the hardening of plantlets

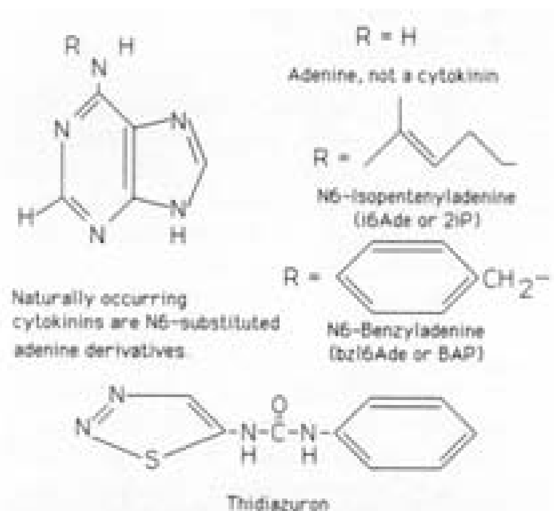

Figure 2. The Chemical Structures of Some Cytokinins. Chemically, naturally occurring cytokinins are considered to be derivatives of adenine, a basic building block of several important plant constituents Thidiazuron, a synthetic cytokinin that has been shown to be effective in micropropagation, is a phenylurea cytokinin.

cessive passages are required before true embryos are formed. The technique, therefore, depends in large part on the finesse of the tissue culturist, a skill demonstrated first by Professor F. C. Steward of Cornell University, who was able to obtain somatic embryos from carrot tissue cultures during the late 1950s.

☐ The third technique, **shoot multiplication**, can almost be considered as the "standard methodology" as far as woody plant micropropagation is concerned. Exploited especially by Professor T. Murashige of the University of California at Riverside, who was involved in the early development of this technology for propagation, the method starts with a growing shoot tip and uses media with high *cytokinin* concentrations to promote growth and to overcome apical dominance. The result of this treatment is the production of a branched shoot system, which is subdivided. Individual shoots are then used for further shoot multiplication, or they are rooted.

The Stages of Micropropagation

According to Professor Murashige, all methods of plant micropropagation involve three basic types of manipulations, designated as Stage I, Stage II, and Stage III.

☐ During **Stage I**, establishment of the aseptic culture, an "explant" (part of a stock plant) is cleaned, disinfected, and placed on a tissue-culture medium. The objective of Stage I is to obtain a living and growing plant tissue free from microbial contamination. Surprising as it may seem, this goal is usually the most difficult thing to achieve in micropropagation.

☐ **Stage II**, also known as the stage of propagule multiplication, sometimes coincides with Stage I, especially when shoot multiplication is used. The aim of Stage II is the rapid increase in shoots or other structures that ultimately can give rise to plants. Often in shoot multiplication, explants respond almost immediately to the high cytokinin concentration of the medium by proliferating new shoots. In these cases, Stage II shoot multiplication has the potential of producing one million shoots in a year, starting from a single growing tip.

☐ **Stage III** involves all the manipulations required for establishment of tissue-culture-derived plants in soil. If, for instance, shoot multiplication is used for Stage II, then Stage III technology consists of a rooting treatment that produces plantlets and then a gradual process of acclimation (hardening) of these plantlets to the lower humidity and increased light intensity of the greenhouse or outdoor environment. Depending on the tenderness of the plantlets obtained from tissue culture, hardening may last two to eight weeks.

The Medium

A surprisingly large number of nutrients are needed by tissue cultures, at least in comparison to the requirements of whole plants. Thus, in addition to the expected inorganic (mineral) nutrients, media for tissue cultures need to contain sugar (*e.g.*, sucrose, or cane sugar), at least two vitamins, and one or more phytohormones. Presumably, whole plants generate all of these additional nutrients internally, although their production must be restricted to specific tissues. In fact, it is likely that localized vitamin and phytohormone synthesis is an important mechanism coordinating growth and function within plants.

Usually, inorganic nutrients are added to media as a standard mixture of salts. The so-called "Murashige and Skoog salts" ("MS salts"), for example, contain about 15 different salts, carefully formulated into a mixture that furnishes all of the inorganic requirements of tissue cultures, *e.g.*, nitrogen (N),

phosphorus (P), potassium (K), and sulfur (S). Even though the MS salts mixture was originally devised for tobacco tissue cultures, experience has shown that it is adequate for most other plants, at least during initial attempts at micropropagation.

Sucrose and vitamins (thiamine, *i*-inositol, pyridoxine, and nicotinic acid) can be added separately or, alternatively, in preformulated mixes. At the Arnold Arboretum, we use a formulation called "Murashige's Minimal Organics Medium" (actually a misnomer), which contains sucrose, the vitamins thiamine and *i*-inositol, and MS salts, all in the proper proportions. After dissolving this mixture in the appropriate volume of deionized water, we add pyridoxine and nicotinic acid to complete the basal medium. Most commercial nurseries, on the other hand, prefer to add every component, including each of the MS salts, separately. As so often happens, the scale at which one is working determines the most economical method of operation.

Of course, the key component of the medium is the phytohormone; specifically, when micropropagation involves shoot multiplication, the cytokinin. Although over 200 different cytokinins are available, they all seem to have similar effects on plants, so it is usually only necessary to test a few compounds to find an effective cytokinin. Almost

Figure 3 The Tissue Culture Rooms at Nourse Farm in Whateley, Massachusetts. Nearly 500,000 strawberry and raspberry plants are produced annually by micropropagation at Nourse Farm

all plants, for example, respond well to a medium containing the basal components plus 1 milligram per liter (mg/l) to 5 mg/l of the cytokinin N6-benzyladenine (abbreviated BAP, BA, or, preferably, bzl^{6}Ade). (One mg/l is equal to one part per million [ppm].) Likewise, N6-isopentenyladenine (2iP or i^{6}Ade), kinetin, and thidiazuron are usually also effective as cytokinins, though bzl^{6}Ade is generally the best choice. A curious exception to this rule involves ericaceous species such as rhododendrons, azaleas, and kiwifruits, which respond poorly, if at all, to bzl^{6}Ade but exhibit extensive shoot proliferation with i^{6}Ade. Obviously, there is something unique about the biochemistry of cytokinin in these plants.

Once all nutrients have been incorporated into the medium, the mixture is supplemented with 1 percent agar and then heated to dissolve the agar, and then the medium is dispensed into the culturing container. Practically any type of container can be used, the only requirements being that it permit light to enter, provide for ventilation, and not be destroyed by the heat involved in sterilization. At the Arnold Arboretum, we use glass test tubes with plastic caps, but have used baby-food jars, canning jars, and even kitchen cooking pans.

The agar provides an inert, jelly-like support that prevents the plant tissues from

Figure 4. Subdividing Clusters of Shoots and Planting Individual Shoots on Fresh Cytokinin-Containing Medium under Sterile Conditions. This procedure is carried out after each cycle of shoot multiplication

sinking to the bottom of the culture vessel and suffocating from lack of oxygen. To most tissue culturists, however, agar (several different brands are available) is one of the most troublesome aspects of micropropagation. As a way of illustrating some of the problems involved, consider that, first, it is necessary to dissolve the agar by heating so that it will be uniformly distributed throughout the medium. Next, this very hot, agar-containing medium needs to be dispensed into the culture vessels both accurately and quickly, before the agar solidifies. If all has gone well to this point, the culture vessels can now be sterilized (for 15 minutes at 120 C in an autoclave, or for 30 minutes in a pressure cooker) and, after cooling, used for tissue culture. Unfortunately, this usually is not the end of problems with agar because, after growth is completed and the plant tissues have been removed, it becomes necessary to redissolve the agar so that the used medium can be discarded—not by pouring it down the sink, however, as the agar will gel and plug the drain!

Table 1. Chemical Constituents of a Standard Tissue-Culture Medium Used at the Arnold Arboretum for the Micropropagation of Several Woody Species by Shoot Multiplication

Based largely on research conducted at the University of Wisconsin in the early 1960s by F. Skoog, T. Murashige, and E. M. Linsmaier–Bednar.

Component	Concentration, milligrams per liter
Inorganic Components	
NH_4NO_3	1,650.0
KNO_3	1,900.0
$CaCl_2 \cdot 2H_2O$	440.0
$MgSO_4 \cdot 7H_2O$	370.0
KH_2PO_4	170.0
$Na_2 \cdot EDTA$	37.3
$FeSO_4 \cdot 7H_2O$	27.8
H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83
$NaMoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Organic Components	
Sucrose	30,000
Agar	10,000
i-Inositol	100
Pyridoxine·HCl	5.0
Nicotinic acid	5.0
N6-Isopentenyladenine (i^6Ade)	5.0
Thiamine·HCl	0.4

Explants for Shoot Multiplication

By far the best starting material for micropropagation is a growing stem tip from a vigorous, healthy plant. Although seedlings are usually better sources for tips than mature specimens, the disadvantage of using seedlings is that the characteristics of the resulting adult plants are unpredictable. On the other hand, when shoot explants are taken from mature plants, one can be fairly certain that the individuals produced by micropropagation will be identical (that is, "clonal") to the stock plant.

Optimally, shoot tips are collected during the early flush of vegetative growth in spring rather than during summer, when growth has ceased, or during fall, when buds have entered their dormant period. The size of the explant depends on the objective of the micropropagation procedure. If the goal is to use micropropagation to obtain virus-free plants, for example, it is usually necessary to excise only the terminal millimeter of the growing point, to clean it, and then to plant this tissue onto the nutrient medium. Unfortunately, these manipulations require con-

siderable manual skill and, therefore, the probability of success when small explants are used is quite low. Because of this, if the goal is solely clonal multiplication, it is easier to begin with shoot tips that are 0.5 to 1 centimeter long.

After they have been collected from the stock plant, the tips need to be disinfected thoroughly, to remove all traces of microbial contamination. At the Arnold Arboretum, we normally wash explants in detergent and then rinse them under tap water. Since these steps effectively clean the shoot tips of nearly all bacteria and fungal spores, the few remaining microorganisms can be killed simply by a soak in 1/10-strength household bleach (the active ingredient being sodium hypochlorite) for 2 to 15 minutes. Tips from most plants can withstand a 10-minute bleach treatment, although some tissues are very tender and will brown and die under these conditions. For this reason, it is best to experiment with different times for the hypochlorite treatment when a new plant is being used for micropropagation.

After they have been treated with hypochlorite, tips are transferred to sterile petri plates and a fresh cut is made at the base of each explant. The tips are then planted in nutrient medium with sterile forceps, and the cultures are incubated under light and temperature conditions that promote the optimal multiplication of shoots. We use artificial lighting recommended for houseplants and normal room temperature (75 F, or 24 C).

Growth normally becomes apparent after one to two weeks. Within about six weeks, it is usually necessary to subdivide the resulting shoot cluster and to use individual branches for further shoot multiplication on fresh medium or for plantlet production following a rooting treatment. Of course, the frequency of subculturing varies from species to species, as does the rate of multiplication. On the average, one can expect approximately a fivefold increase in shoot number every six weeks, a rate that theoretically would produce more than a million shoots, starting from a single tip, within 12 months.

Rooting and Hardening

Probably because they develop in the humid environment of the culture vessel and therefore have leaves that lack a protective cuticle, shoots produced through micropropagation are particularly sensitive to desiccation. It is essential, therefore, that they be maintained under moist conditions during Stage III. Several strategies have been used to accomplish this. In our laboratory, for instance, after dipping the bases of micropropagated shoots in a rooting powder, we transfer them to a humid plastic box containing moist vermiculite and a transparent cover. On the other hand, at Weston Nurseries in Hopkinton, Massachusetts, tissue-culture shoots of mountain laurels, rhododendrons, and azaleas are planted in the greenhouse, in beds of moist peat moss covered with polyethylene tents to maintain a humid environment. At Nourse Farms in Whately, Massachusetts, strawberry shoots are planted in a moist peat moss–soil mix, in a greenhouse equipped with a fogger-type humidifier.

If the appropriate treatments are used, roots usually form on tissue-cultured cuttings within about two weeks. Once rooting has occurred, the resulting plantlets begin to grow vigorously, and the gradual process of hardening them to lower humidities and higher light intensities can take place. With lilacs, for example, we incubate our covered, plastic boxes in the culture room for two weeks while roots are being initiated, and then we remove the covers from the boxes. Two weeks later, we transfer the boxes from the culture room to the greenhouse, where

they are kept initially under shade for four weeks and then in full sun. By this time, the micropropagated plantlets are hardened enough to be handled as any other plant would be.

Often, plants produced by micropropagation are considerably more vigorous than conventionally propagated plants. This is hardly surprising in view of the optimal conditions of nutrient supply, moisture, and lighting under which they are grown. Genetically, micropropagated plants are identical to their stock plants, at least as long as shoot multiplication is used to produce them. If, however, the micropropagation method involves either regeneration from callus or somatic embryogenesis, then variant (mutant) plants are common. For some purposes this is desirable and, in fact, the variability that can be produced in tissue cultures is already being exploited commercially to obtain disease-resistant potato cultivars.

The Economics of Micropropagation

For about $250, one can purchase practically all of the supplies needed to set up a micropropagation laboratory. This price includes enough Murashige's Minimal Organics Medium, vitamins, cytokinins, and agar for 2,500 cultures ($100), 500 sterile plastic petri dishes plus covers ($50), 250 culture tubes with plastic caps ($50), flasks and beakers for media preparation, and stainless-steel for-

Figure 5 The Rapid Multiplication of Raspberry Shoots by Micropropagation

ceps, scalpels, and blades. In addition to these items, one needs to be able to heat a medium (on a hot plate, for example) so that all of its components will be dissolved before it is dispensed to the culture tubes, and to sterilize a medium (with a pressure cooker, for example). Forceps and scalpels can be sterilized simply by dipping them in 95 percent alcohol and then burning the alcohol with a flame. A balance is also needed, unless one purchases chemicals already preweighed into lots. Of course, a transfer bench is required free from drafts, as is an artificially lighted incubation room equipped with racks for cultures.

Figure 6 A Micropropagator at Nourse Farm Subdividing Shoots before Transferring Them to Soil in the Greenhouse (Shoots produced by tissue culture can either be used for further shoot multiplication, or they can be rooted)

Commercial tissue-culture laboratories estimate that they spend approximately $0.50 for each plant they produce beyond Stage III of the micropropagation process. This estimate is calculated based on a minimum level of production (about 250,000 plants per year), at which several economies of scale become significant. In addition, the estimate fails to take into account expenses involved in developing refined technology for a new plant. Because of this, a newcomer to micropropagation will probably find that the costs per plant are much higher.

Research in universities and private firms during the last few years has resulted in a rapidly expanding catalog of information about the precise conditions of Stages I, II, and III for over 200 different plant species. Unfortunately, this information is not always presented in a style that is comprehensible to the beginner. Even more serious is the tendency of some commercial laboratories to explain micropropagation in an almost mystical, surrealistic fashion when, in fact, the technology involves a principle (phytohormone control of development) that is both simple and straightforward. If you are genuinely interested in micropropagation, remember that patience, flexibility, and confidence in the scientific basis of the methodology are the most important requirements for success.

John W. Einset, associate professor of biology in Harvard University, is a member of the staff of the Arnold Arboretum.