How-To-Do-It

Plant Tissue Culture

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Tissue culture is the propagation of plants through "cloning," an asexual method. The process consists of growing a portion of the original plant *in vitro* (an artificial environment), promoting its multiplication, and then transferring it to potting medium in a greenhouse environment. Tissue culture is based on the concept of totipotency, which is the ability of a cell, given the correct environment, to develop into a new organism due to the genetic information contained within the cell. Because the new organism has exactly the same genes as the original, it becomes an identical twin to the original.

Plants have been propagated asexually for thousands of years by means of cuttings, divisions, and grafting. Tissue culture adds a new dimension to propagation by providing a means of rapid multiplication in a short time span while utilizing a small quantity of plant tissue. Starting with 50 Boston fern explants (runners), approximately 6,250 Boston fern plants may be produced in 12-18 weeks.

Tissue culture is currently being utilized to commercially propagate many plants including Boston ferns, hyacinths, lilies, caladiums, orchids, and gerbera daisies. It is used not only to mass-propagate plants economically, but also to produce pathogen-free or disease-free plants, plant mutants for crop improvement, plant hybrids, and plant products such as steroids, titerpenes (ginseng), and antitumor alkaloids.

The concept of tissue culture and totipotency has been included in introductory plant sciences for many years. Now as instructors, we have the information, materials, and usually the space to take tissue culture out of the textbook and into the laboratory. Teaching the theory behind tissue culture is good, but providing our students with the actual experience is even better.

Tissue culture can be used as a vehicle to present other concepts related to the growth of plants in general, such as sterile technique, hormone balance, anatomy, and environmental factors.

Practicing tissue culture teaches students more than just how to rapidly multiply plants. Tissue culture utilizes basic sterile techniques; poor technique is usually apparent in 4-6 days. This visible contamination is an indicator to the student of the lack of good procedure, and is usually visible soon enough after the beginning of the procedure to allow the student to start again.

The relationship between phytohormones, auxin and cytokinin, in the promotion of shoot versus root growth becomes apparent as the plant tissue is transferred from one growing medium to the next. The various stages of culture require different media. The media contain inorganic nutrients, sucrose for cellular respiration, vitamins, and growth regulators in varying combinations.

Through learning the process of tissue culture, the student will also become aware of which part of a particular species is most amenable to *in vitro* growth. Tissue culture can utilize almost any portion of a plant as the explant: flower parts, buds, runners, stem sections, bulb sections, leaves, or roots.

Tissue culture can also make the student aware of the need for specific environmental conditions for growing plants. The medium provides the essential nutrients for growth inside the test tube. The enclosed test tube environment maintains the proper humidity. Light and temperature are factors that must be regulated to ensure optimal multiplication and growth of the plants.

During the various stages of growth and multiplication, the cultured plants require different light intensities. The final stage is the growth of the new plants under controlled greenhouse conditions including humidity and light intensity until the root systems are fully developed. Temperatures for the early stages are usually kept constant.

Terms and General Procedures

The Explant—The explant, a piece of the parent plant, is chosen carefully. Leaf portions, stem sections,

apical meristems, root tips, flower parts, and embryos are all utilized as explants, some plant parts producing better growth than others for a particular plant species. The explant is surface sterilized before it is placed in the selected media. A 10% clorox solution with a wetting agent is commonly utilized as the disinfesting agent. The aseptic explant is implanted into the nutrient media.

The Media—The liquid or solid media is designed to provide all the nutrients required for the tissue to become established and start new growth. When the original explant is subcultured (divided into sections), the sections are placed in media containing phytohormones that promote either shoot or root growth.

The Stages—Each stage in the tissue culture process requires different environmental conditions and serves a definite purpose. Stage I is designed to isolate the explant and insure its sterility. During Stage I new plant growth occurs. The media usually contains organic and inorganic nutrients, and temperatures around 27°C or those close to the plants' natural environmental temperatures are maintained. Light intensities around 100 foot candles coupled with a 16-hour day have been found to be beneficial to most plants during this phase of growth.

During Stage II, the new growth from Stage I is divided into sections that can be grown into separate plants or subcultured to produce more plants. The process of dividing and obtaining new growth may go on indefinitely until there are thousands of plants genetically like the original. The Stage II media usually contains phytohormones to induce shoot growth. The temperature and light conditions are similar to those appropriate for Stage I. Multiplication of shoot material occurs during Stage II. The resulting plant growth can then be divided and transferred to Stage III conditions or it can be divided and each division transferred to new Stage II media. The subcultured divisions will again multiply and produce more shoot growth.

Stage III involves the transfer of Stage II divisions to media that will favor root growth. For some plants, this media is the same as Stage I media, the new shoot growth providing the stimulus for the initiation of roots. Sometimes an auxin supplement is necessary to stimulate root formation during this phase. Light intensity is increased during Stage III and the 16-hour day is maintained. Growth during Stage III is enhanced by light intensities of 300-1,000 foot candles. Temperatures similar to the other stages are maintained and plants that require chilling are exposed to cold.

The final stage, Stage IV, takes the newly rooted divisions from the test-tube environment into the greenhouse. This change is a critical step for the young plant. The new plants must be kept humid until their root systems become well established. A mist system helps maintain humidity during the change from the test tube to pot. Shading also helps acclimate the new plants gradually to greenhouse conditions. Photoperiod is taken into consideration at this time.

Fertilizer may be applied to the leaves (root systems are poorly developed). Sterile soil will help prevent infestation.

The acclimated plants are then ready for actual greenhouse growing conditions.

FIGURE 1. A runner is removed from the Boston fern. The removed section of the runner is the explant. (Photographs by Steve Smith and True Kelly)

Procedure for Boston Ferns

We have been successfully culturing Boston ferns for two years. The process is rather straightforward. Equipment and materials needed are listed in table 1.

TABLE 1. Equipment and Materials Needed

Equipment
Autoclave or pressure cooker
Laminar flow hood or transfer chamber
Balance
Growth (environmental) chamber with light and temperature controls
Greenhouse facilities or table with lights
Materials
Scalpels
Forceps (10" and fine tip)
Test tubes
Test tube rack
95% alcohol
Petri dishes
Mason jars (optional)
Kaputs (test tube covers)
Media

Boston fern runners are cut at approximately 10 cm lengths and kept moist until needed (fig. 1). One runner is necessary for each test tube.

Surface sterilization of the explants (runners) is accomplished by putting them in a 10% clorox solution with a drop of wetting agent and agitating for 5 minutes. The explants are transferred to a second 10% clorox solution for 5 minutes, again with agitation to insure complete coverage by the clorox solution. While in the second 10% clorox solution, the cut ends of the explants are trimmed so that 7-8 cm remain. The explants are then rinsed with *sterile* distilled water three times, thoroughly agitating each time.

The disinfested explants are now ready to be implanted in the media. The media needs to be prepared ahead of time and cooled before use. We use prepackaged Boston fern media from Gibco (Grand Island Biological Company, 3175 Staley Road, Grand Island, NY 14072). The Stage I Boston fern media is prepared according to package in-

FIGURE 2. Transfer of the explant into the test tube containing the nutrient media is accomplished in aseptic conditions.

FIGURE 3. The cut end of the explant is pushed into the media and the remaining portion is placed in contact with the surface of the media.

FIGURE 4. Test tubes containing the implanted explants are maintained at 27°C in a lighted environmental chamber.

structions which include autoclaving for 15 minutes at 15 pounds of pressure. About 25 cc of the prepared agar is poured into 15 cm by 2.5 cm sterile test tubes and allowed to cool at a 45° angle. The cooled media is kept refrigerated until needed.

The media necessary for Stages II and III are also prepared ahead of time and kept refrigerated until needed.

Implantation of the sterile explants in the media should be accomplished in an aseptic environment (fig. 2). A transfer chamber that has been surface sterilized or a laminar flow hood work best. If a transfer chamber or hood is not available, the process can be accomplished in a room where there is relatively little air movement. The working surface that is being used should be wiped with a germicidal agent. Instruments are sterilized by dipping them into 95% alcohol and blotting them dry with sterile gauze. Using standard sterile technique, prepared explants are transferred to Stage I media by inserting about half of the explant into the media (fig. 3). The test tubes are placed in racks at a 45° angle in a growth chamber (fig. 4). The growth chamber is set to provide a 16-hour day at 27°C and 100-300 foot candles of light.

Budding will occur in 4-6 weeks (fig. 5). Contamination will be visible in 4-6 days. Contaminated tubes should be removed and autoclaved before washing.

The Stage II media contains phytohormones to induce shoot growth. This is the multiplication stage of tissue culture (fig. 6). Each of the Stage I test tubes usually provides enough plant material (buds) to be divided into five Stage II test tubes. This division occurs in the same aseptic environment as the initial implantation. The growth is removed from a test tube and transferred to a sterile petri dish. All instruments are sterilized and dried as before. The plant

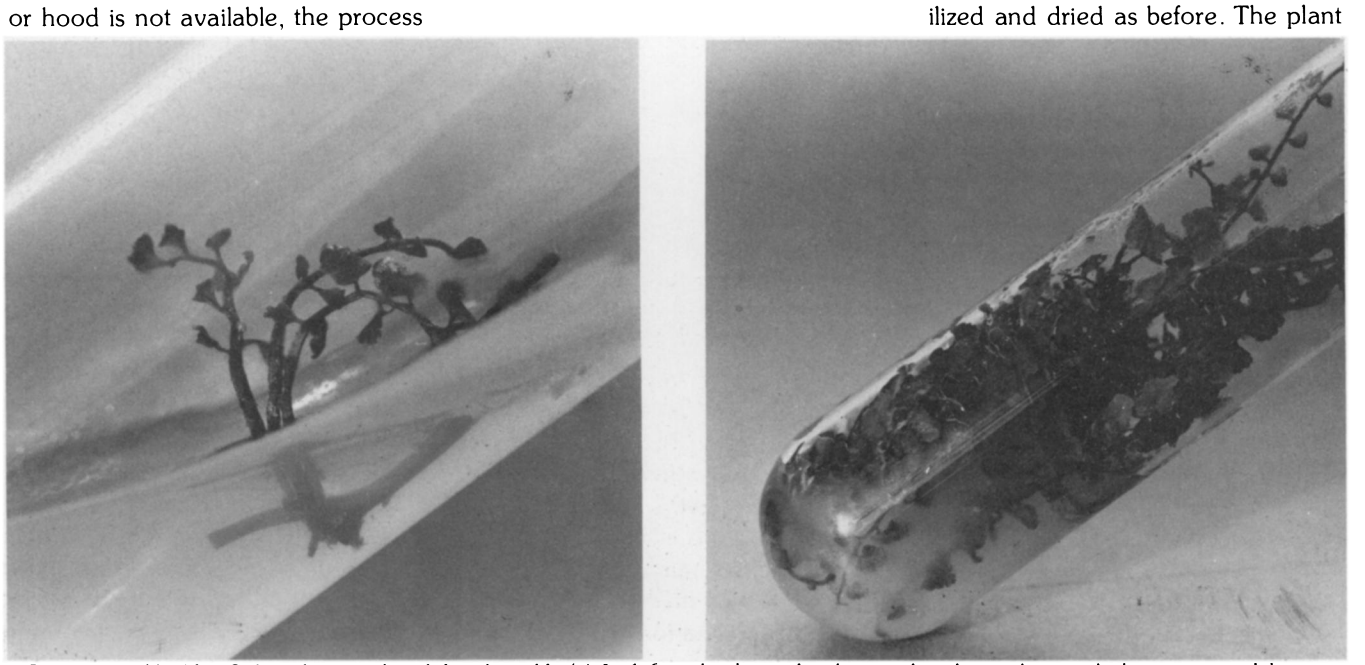

FIGURE 5a and b. After 3-4 weeks, growth is definitely visible (a). In 4-6 weeks, the explant has produced many leaves which start to crowd the test tube environment (b).

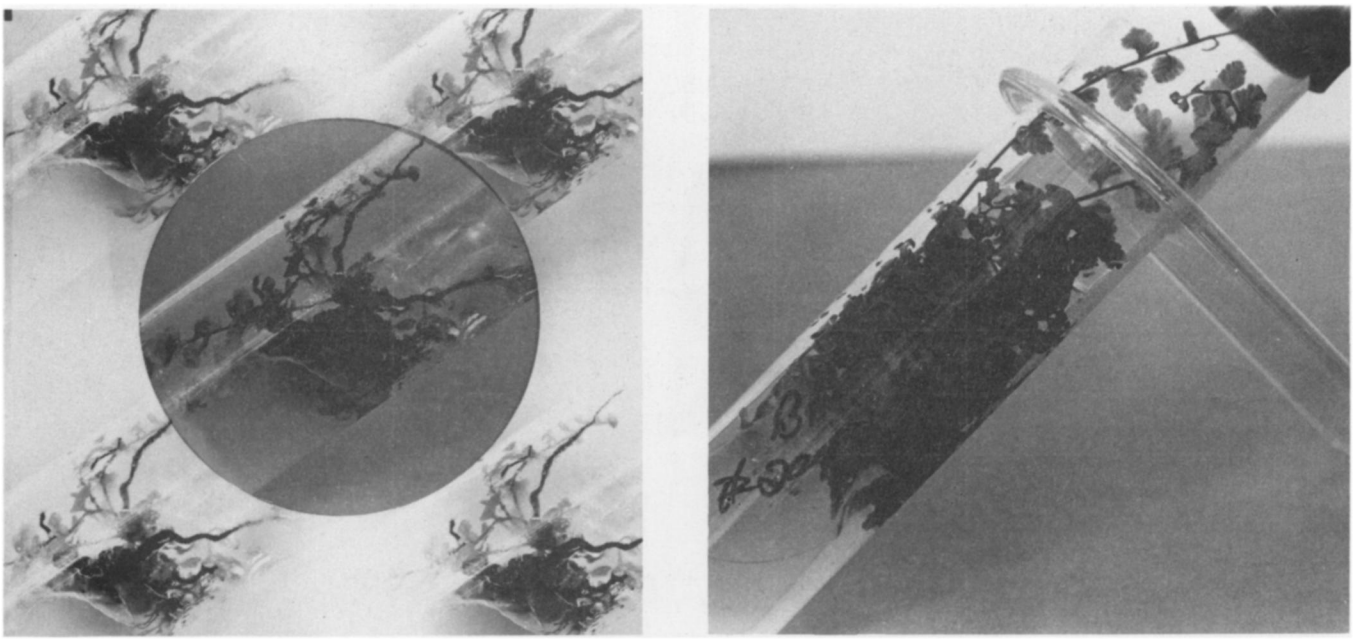

FIGURE 6a and b. Five separate cultures resulting from division of the original explant (a). Each of the five tubes will become filled with new growth in 3-5 weeks (b). The growth from each of the five tubes can then be transferred into five more tubes, giving us 25 potential plants from the original explant.

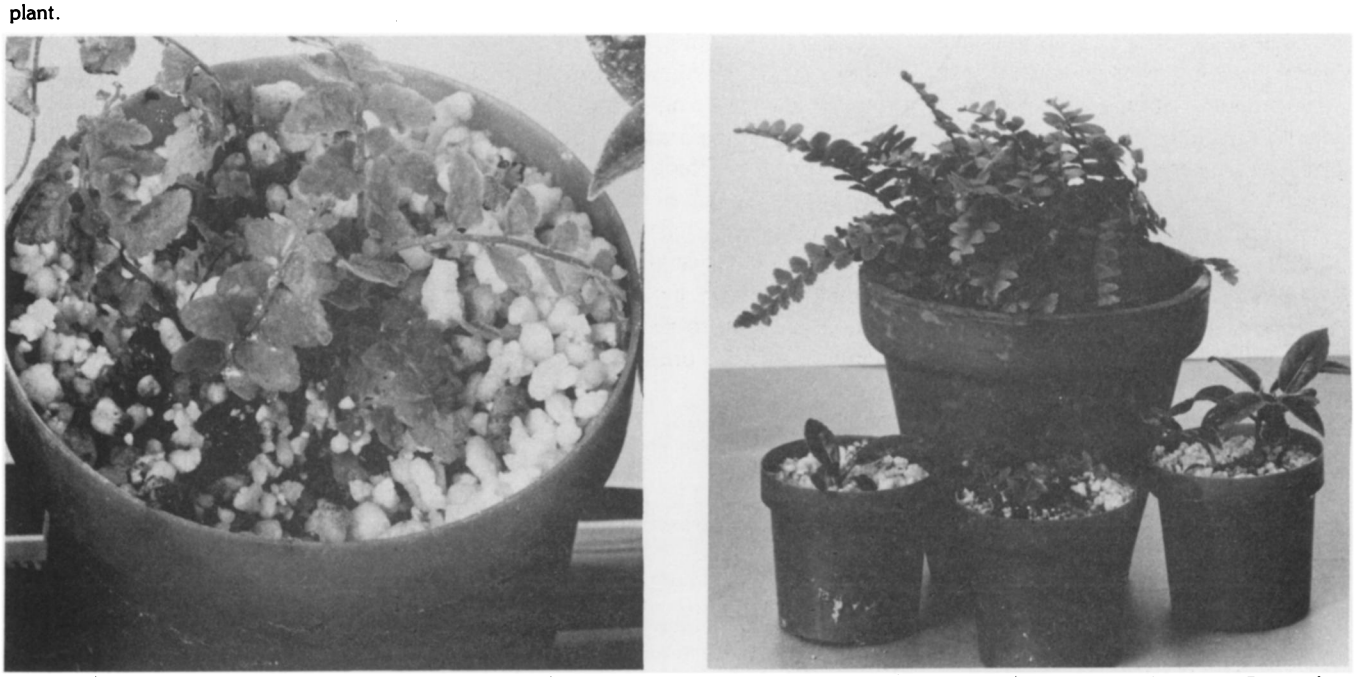

FIGURE 7a and b. The growth from each tube may also be transferred to a sterile mixture conducive to root growth (a). Since the young Boston fern cannot withstand a direct change from its test tube environment to regular greenhouse conditions, it is placed under a mist system. When the young plants have established roots and produced new leaves, they are repotted in soil and set out in the greenhouse (b).

growth is cut into five or more sections, each containing a bud (new growth). Each section is then implanted in a test tube containing Stage II media. The test tubes are returned to the growth chamber and maintained at the same environmental conditions as Stage I.

In 4-6 weeks the growth will be such that it is ready to be removed from the test tubes. The growth can be divided and implanted in fresh Stage II media or divided and implanted in Stage III media and provided with conditions that will promote root growth. In either case, the transfers are accomplished in a sterile environment with sterile instruments and petri dishes.

Stage III media for Boston ferns is the same media used for Stage I. The divisions can be implanted in test tubes or more than one can be implanted in larger vessels such as mason jars that have 2-3 cm of agar solidified against one side at a slant. Light intensity should be approximately 1,000 foot candles. General Electric Power Groove tubes have been recommended for this stage. The 16-hour day is maintained and temperatures are not changed. After three or four weeks the divisions

will be ready for transfer to modified greenhouse conditions.

Stage IV acclimates the young growth to greenhouse conditions (fig. 7). Natural light can be reduced by using shading cloth. A mist setup will help maintain high humidity for the transplants. Bottom heat will encourage root growth but is not essential. Fertilization of leaves with a dilute solution is recommended. Soil should be loose and sterile. Using deionized water will cut down on salt build-up. The acclimated plants are then ready for regular greenhouse growing conditions.

Summary

The value of propagation via tissue culture is unquestioned. Let us provide our students with practical as well as theoretical knowledge of the process.

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