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Handbook for Commercial-Scale Taro *(Colocasia esculenta)* Tissue Culture in Hawai‘i

With notes on sample preparation for disease testing

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INTRODUCTION

This handbook is the result of research on the commercial–scale tissue culture of four Hawai‘i taro (*Colocasia esculenta* var. *esculenta*) varieties: Bun Long (the Chinese table and chip taro), Lehua Maoli (a purple poi taro), Niue (a pink Samoan table taro), and Mana Lauloa (a white table taro). This book covers each stage of the tissue culture process, from field selection of the mother plant, preparation for disease testing in a diagnostic laboratory, surface sterilization, initial explanting, *in vitro* propagation, cost of culturing, and acclimatization for field planting (Figure 1).

The main benefit of tissue culturing taro, previously screened for disease organisms, is the production of large numbers of uniform, disease–free plants. This provides the grower with the ability to plant new farms, fertilize efficiently, and increase production. Tissue culture also allows for better farm management and planning.

The *Handbook of Commercial–Scale Taro (Colocasia esculenta) Tissue Culture in Hawai‘i*, provides the necessary information to propagate taro in a commercial laboratory setting to anyone who has basic training and experience in plant tissue culture. As this is not a primer in the art and science of tissue culture, the following books are suggested for review:

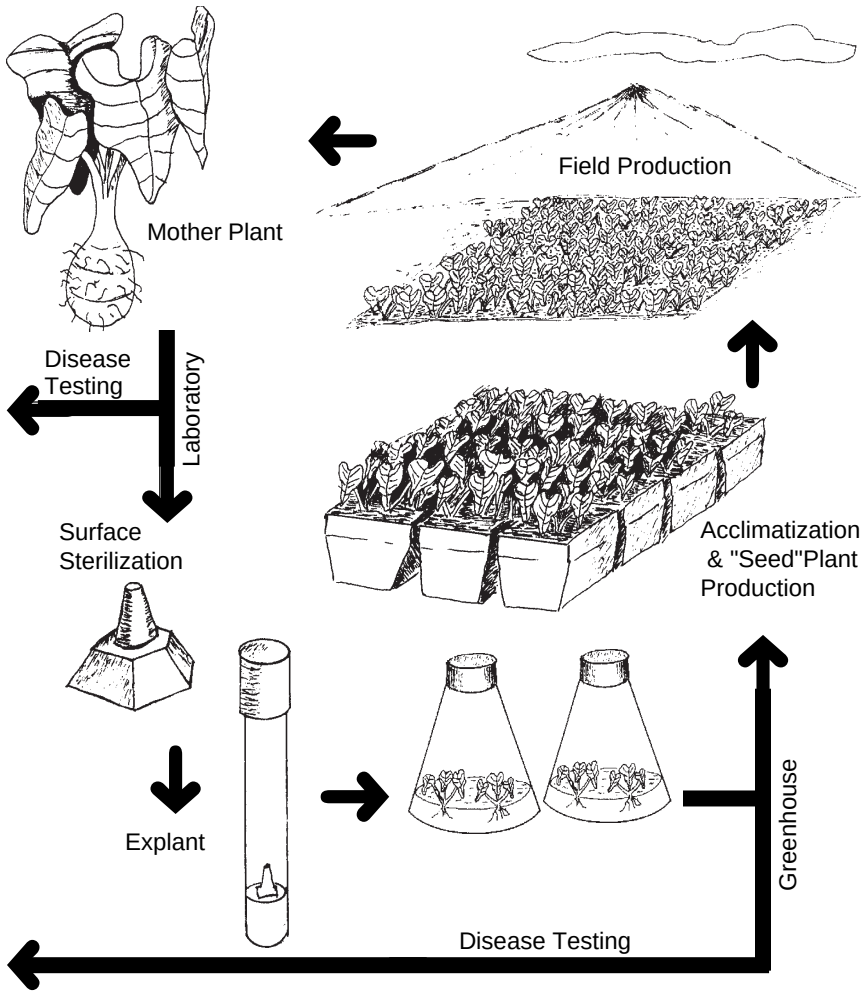
Plant Propagation–Principles and Practices, by Hartmann, Kester, and Davies. 1990. Regents/Prentice Hall Press. 647 pages.

Plants from Test Tubes–An Introduction to Propagation, by Kyte. 1987. Timber Press. 160 pages.

Tissue Culture of Selected Tropical Fruit Plants: A Handbook on the Application of Tissue Culture to Plant Propagation, by Jona. 1987. United Nations Publications. 125 pages.

Taro Tissue Culture Manual, by Arditti and Strauss. March 1979. South Pacific Commission Information Document No. 44, 1979.

Figure 1. Flow Diagram of the Tissue Culture Process



In addition to basic experience in plant tissue culture, commercial–scale operation requires the following:

- ❏ Medium preparation room
- ❏ Transfer hood
- ❏ Sterilizing equipment
- ❏ All medium ingredients
- ❏ Cool white light bulbs
- ❏ Forceps
- ❏ Cutting board
- ❏ Dish soap
- ❏ Plastic sample bags
- ❏ Distilled water
- ❏ Small buckets
- ❏ Shade house
- ❏ Transfer room
- ❏ Culture storage room
- ❏ Air conditioners
- ❏ Mechanized stir plate
- ❏ Sterilized potting soil
- ❏ Scalpels
- ❏ Knives
- ❏ Scrub brushes
- ❏ Disposable gloves
- ❏ Bleach
- ❏ 2–quart pitcher
- ❏ 3–inch pots

Despite a general proliferation of literature and experience at the research level on taro tissue culture world–wide and in Hawai‘i, this current work establishes commercial–scale laboratory procedures for taro tissue culture and its associated disease screening in Hawai‘i. The basic difference between a research laboratory and a commercial laboratory is that a commercial laboratory must produce large numbers of uniform plants at a reasonable cost in order to be profitable. A research laboratory, on the other hand, often focuses on a limited number of plants to determine the ideal methods and medium components.

Success in the micropropagation of large numbers of plantlets can be very specific to plant type, i.e., various explants of the same species may respond differently from each other. Other factors can also affect *in vitro* proliferation such as field cultivation methods of the mother plant material (stock material), growth stage of the mother plant, season, etc.

There is a need for disease indexing of taro plants before explanting and at initial stages of shoot proliferation. The methodology is presented here for surface sterilization of stock material, nutrient medium requirements, induction of shoots, induction of rooting, and acclimatization to prepare plantlets for field conditions.

Finally, while all four varieties of taro were handled similarly throughout propagation, this guidebook is based on the Chinese cultivar, Bun Long, with notes made when necessary for a specific cultivar. Stock materials were collected from the Big Island and Moloka‘i. All tissue culture research was performed in the Hilo area.

BEFORE EXPLANTING

Mother plant material is relatively easy to clean if it is cultivated for laboratory use. Optimally, hydroponics or sterile potting medium should be used. Plants should be kept under greenhouse cover, as well as watered with a dribble system rather than overhead irrigation. All of these practices aid successful culture initiation because they lower the chance of contamination by microorganisms.

MATERIAL SELECTION

Select mother plant material that fits the following criteria:

- ❏ plants grow rapidly;
- ❏ leaves are free from irregular patterns of yellowing or lesions with surrounding water–soaked, yellow areas;
- ❏ plants are free from burrowing pests in the tubers and from weeds;
- ❏ plants are mature–not too small nor too close to harvest. Plants grown 3 to 6 months in the ground are the best.

It is most important to institute a numbering system at the beginning of the process for each corm sample and its associated leaf and tissue culture samples. Along with the numbers, you should note the following observations about each sample in your log book:

- ❏ the presence any brown spots or lesions for each corm number;
- ❏ corm size;
- ❏ the field location where the corms were selected to allow for disease identification if corms test positive;
- ❏ growing conditions in field, e.g., weedy, poor nutrition, etc.

Example of a numbering system: Corm #1 recorded in log with date. All subcultures from this corm will also be #1. Each container should show corm #, date, and passage number (how many times subcultured) or a code to indicate this information.

DISEASE TESTING

Because much of the taro in Hawai‘i has been infected by some type of disease, it is important to first test potential stock material for those diseases. A sampling collection kit should include:

- ❑ Record book
- ❑ Scissors
- ❑ Plastic sample bags
- ❑ Potable water
- ❑ Masking tape
- ❑ Bleach
- ❑ Scrub brushes
- ❑ Disposable gloves
- ❑ Marking pens

Samples for DMV (Dasheen mosaic virus) and Xcd (*Xanthomonas campestris* pathovar *dieffenbachiae*) tests should be prepared outside (e.g., outdoors) the lab by first spraying the newly dug up tuber with water at high pressure (Figures 2a and 2b).

DMV–Symptoms

Among other problems, taro plants are highly susceptible to DMV, a systemic disease that significantly reduces yield. Visual symptoms of DMV are yellowing of leaves in a feather–like mosaic pattern. This should not be confused with natural yellowing of older leaves as they die.

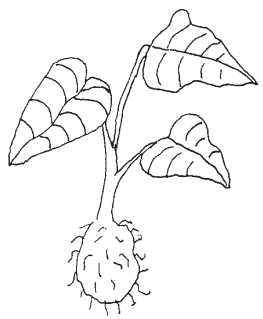
DMV–Sampling Procedure

Using sterile scissors, cut a piece of leaf roughly 2 inches square from an area of the leaf that shows DMV symptoms. If no DMV is readily evident, a piece should be sampled randomly (Figure 2c). Place the leaf sample in a plastic bag and give it the same number that the corm was given. Disinfect the scissors between samples by submerging in 20 percent bleach or dip scissors in ethyl alcohol and flame. (No research has been done to verify that these procedures kill DMV). Refrigerate the DMV test samples until they are ready to be sent for testing. Testing takes about 5 days.

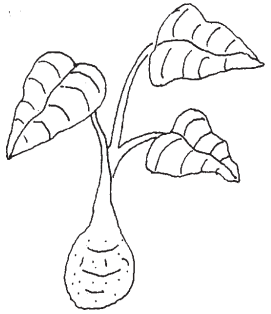
Plants were tested using the ELISA (Enzyme–Linked Immunoabsorbant Assay) technique. The test is relatively inexpensive ($5 per sample) and is commercially available from:

Agricultural Diagnostic Service Center
University of Hawai‘i
1910 East–West Road
Sherman Lab, Room 134
Honolulu, Hawai‘i 96822 (808) 956–8053

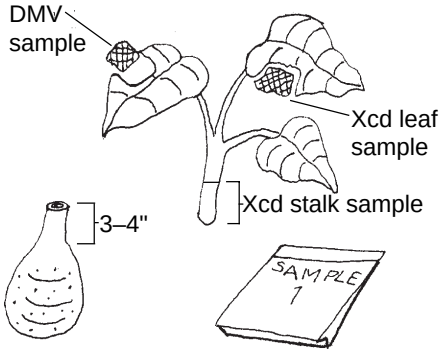
Figure 2. Initial Clean Up & Disease Sampling Method



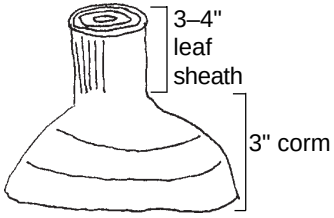
a. Newly harvested mother corm.



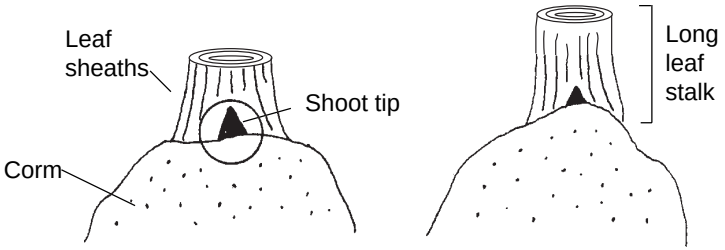
b. Corm after water rinse & removal of roots.



c. Sample method for DMV & Xcd assays.



d. Trimmed corm, ready for bleaching.



e. Left: longitudinal cross–section of shoot tip & portion of corm showing leaf primordia area to be explanted (circled). Right: typical Samoan taro; note how area to be explanted is on a pointed dome, requiring long leaf stalk be kept on corm to avoid cutting off area to be explanted.

Xcd–Symptoms

Since taro is an aroid, it can carry the anthurium blight bacteria, Xcd. If other aroids, such as anthuriums, are propagated in the same lab as taro, it is a good idea to have stock material tested for Xcd. Symptoms of Xcd are yellow and water–soaked lesions on leaves. In general, the best protection against propagation of disease is to use only the shoot tip of the mother plant. This means that only the bud from under the main leaf stalk of large, central corms be placed into culture; the side, smaller cormels, and dormant buds are preferably not used. However, it is still necessary to have all stock material tested for this disease before the propagation process begins.

Xcd–Sampling Procedure

Xcd is detected by plating stock plant material onto four different semi–selective media. They are then observed for Xcd characteristic growth on each medium. To sample for Xcd, the leaf stalk should be cut approximately 3 to 4 inches above the tuber. (Note that the Samoan variety has a slightly different growth morphology than the other cultivars (Figure 2e, right). The area to be explanted, the shoot tip, originates from a domed area located under the leaf sheath. Due to this dome, 5 to 6 inches of leaf stalk should be left on the corm to avoid cutting off the area to be explanted.) From the leaf stalk cut a section of about 3 inches above the first cut (Figure 2c). Place this piece in a *separate* plastic bag and number it according to the corm number. Take a 2–inch–square leaf section from the suspected Xcd infected portion of any leaf and place this sample in the same bag as the stalk section. Disinfect scissors as in DMV procedure on page 5. Submitting two samples in this manner allows for systemic and foliar testing for Xcd. Testing takes about 5 days.

The test for Xcd, at $10 per sample, is commercially available from:

Agricultural Diagnostic Service Center
875 Komohana Street
Hilo, Hawai'i 96720 (808) 959–9528

When Test Results Are In

It is important to keep the numbering system intact throughout all phases of shoot proliferation in case test results come back positive. All materials with positive DMV or Xcd results should be carefully discarded immediately. *Despite the technology and hard work that goes into*

disease testing, it was found that stock material that originally tested negative for DMV in this research could test positive after initiation and shoot proliferation in vitro. Since the assay is quite sensitive to the presence of DMV, this occurrence suggests that either the sampling method was not optimum for detection of the virus or that the virus can exist at an undetectably low level. Therefore, it is advisable that all material be sampled a second and third time before large numbers of plants are proliferated (see Chapter 3).

After sampling and numbering all stock material to be initiated into culture, further clean up of the plant material is necessary.

Cleaning Up The Plants

Each corm needs to be handled separately during the bleaching and air–drying procedures. The lower portion of the corm should be cut off, leaving about 3 inches of the starchy root. Scrap away the remaining roots with a knife. Scrub the tuber with a brush and dish soap under tap water (Figure 2d). Bleach stock material (each corm individually) in a 10 percent household bleach (unscented) solution for 20 minutes in small buckets. Retain labeling system. Without rinsing, air dry material indoors in a relatively clean, well–ventilated area until disease test results are obtained. No special lighting is required during the air–drying phase. A clean room with normal household temperatures and humidity will work.

MEDIUM PREPARATION

Based on medium formulations found in the literature on other taro varieties or dasheen (*Xanthosoma*), eight different medium/plant growth regulator combinations were tried. However, none of these performed as well as Murashige and Skoog's (1962) medium consisting of major and minor salts and vitamins (modified with 1.1 mg/l thiamine), with 3 percent sucrose, 2.5 mg/l 6–benzylaminopurine (BA), and 2 g/l gellan gum (Phytagel or Gelrite) at pH 5.7. The composition of this standard medium is found in Table 1.

Prepare the medium in advance so that it can cool and solidify. The amount depends on how many corms will be put into culture. Medium can be stored in a clean room (the transfer room) for 1 month or longer. If medium is stored, it must be inspected carefully before use to ensure that no contamination has entered the container.

Initiate explants in 15x125 mm culture tubes using about 15 ml of medium per tube. For shoot proliferation, use the same medium formulation in either Magenta GA–7 vessels or 500 ml glass flasks with cotton–filled, rubber stopper lids, with 40 or 50 ml of medium per vessel, respectively.

Table 1. Murashige and Skoog Medium (mg/l)

- ❏ Ammonium nitrate, NH_4NO_3 ..... 1650.0
- ❏ Potassium nitrate, KNO_3 ..... 1900.0
- ❏ Calcium chloride, $CaCl_2 \cdot 2H_2O$ 440.0
- ❏ Magnesium sulfate, $MgSO_4 \cdot 2H_2O$ 370.0
- ❏ Potassium phosphate, KH_2PO_4 ..... 170.0
- ❏ Boric acid, H_3BO_3 ..... 6.2
- ❏ Manganese sulfate, $MnSO_4 \cdot H_2O$ 22.3
- ❏ Zinc sulfate, $ZnSO_4 \cdot 7H_2O$ 8.6
- ❏ Potassium iodide, KI 0.83
- ❏ Molybdic acid (sodium salt), $Na_2MoO_4 \cdot 2H_2O$ 0.25
- ❏ Cupric sulfate, $CuSO_4 \cdot 5H_2O$ 0.025
- ❏ Cobalt chloride, $CoCl_2 \cdot 6H_2O$ 0.025
- ❏ Sodium–EDTA, Na_2EDTA 37.3
- ❏ Ferric sulfate, $FeSO_4 \cdot 7H_2O$ 27.8
- ❏ Glycine 2.0
- ❏ myo–Inositol 100.0
- ❏ Nicotinic acid 0.5
- ❏ Pyridoxine • HCL 0.5
- ❏ Thiamine • HCL 1.1
- ❏ 6–benzylaminopurine 2.5
- ❏ Sucrose ..... 30.0 (g/l)
- ❏ Gellan gum ..... 2.0 (g/l)

2

SURFACE STERILIZATION AND EXPLANTING (Stage I)

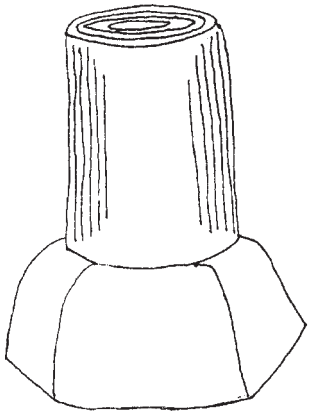
Bring the air–dried plant material into the medium preparation room. To surface sterilize an explant, use a sharp knife and cutting board to trim the leaf stalk to about 1 to 2 inches and cut away all outer tissue from tuber to create a 1– to 1H–inch cube (Figure 3a). Rinse both tools in a 20 percent household bleach solution between corms. (No research has been done to verify that these procedures will kill DMV or Xcd). Using clean hands or gloves, gently peel away 1 to 2 leaf sheaths (Figure 3b), then trim base to a 1– to 1H–inch square (Figure 3c). Use a gentle soap and a toothbrush to gently scrub the explant under running water. No dirt or dried material should remain on the explants. To further clean the explants, place them in a large beaker, set the beaker in a colander, and run tap water into the beaker, causing the explants to be gently agitated. Run tap water for 1 hour. If any explants tumble into the colander, use clean forceps to replace them in the beaker–*do not use your hands* to avoid further contamination.

Drain the explants and place them in a clean beaker containing a solution of 10 percent bleach made with sterile distilled water and 2 drops of Tween–20 surfactant/100 ml. Put clean aluminum foil over the beaker and place the beaker on a magnetic stir bar plate and stir briskly for 45 minutes.

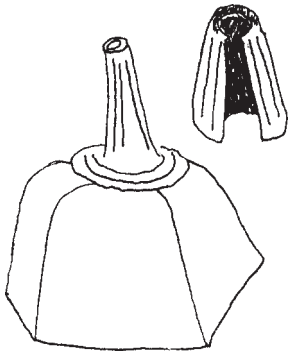
Take the beaker of explants into the transfer hood. From here, use the "sterile technique." Move explants into sterile distilled water (assume all water used from here on is distilled or deionized and then sterilized). Place explants in a second sterile water rinse. Working on one explant at a time, using a sterile paper plate as a cutting surface, remove one leaf sheath using a scalpel and forceps. Put the explant in an empty (sterile) test tube, rinse with 70 percent ethanol for 30 seconds, then rinse with water. Put the explant in an empty test tube, soak in a 5 percent bleach solution for 5 minutes. Remove a second and perhaps a third leaf sheaf, resulting in a final explant size of approximately H inch x H inch. *Using a microscope is not necessary.* Place explant directly onto the medium. It is not necessary to rinse off the 5 percent bleach solution. After

explanting is completed, place containers in a growth or culture storage room on shelves under cool white fluorescent lights for 15 hours per day at 80°F. After 5 or 6 days, carefully inspect each culture for any contamination and remove any contaminated cultures. Discard any contaminated cultures outside the lab and soak containers in a 10 percent bleach solution before reusing. When discarding contaminated cultures, be sure to note their removal in your numbering system record.

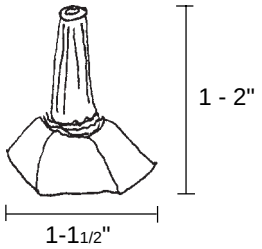
Figure 3. Steps to Surface Sterilization of Explants



a. First, trim the explant (actual size).



b. Peel way leaf sheaths (actual size).



c. Trimmed base (actual size) ready for 10% bleach solution.

3

PLANT PRODUCTION

SHOOT PROLIFERATION (Stage II)

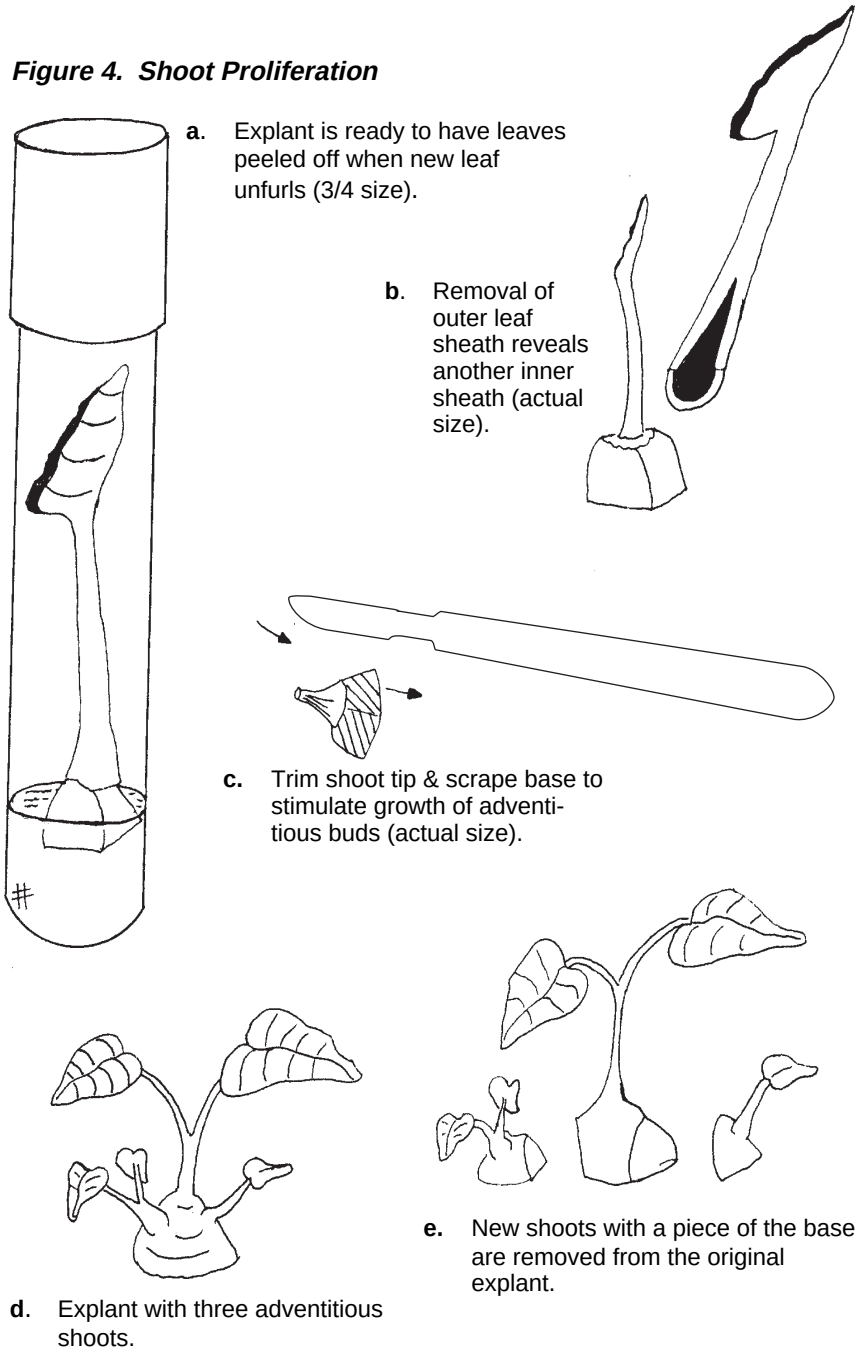
Explants are ready to be stimulated to produce adventitious shoots when the first new leaf has unfurled (Figure 4a). The new leaf is removed by carefully peeling it away with forceps at the base, rather than by cutting (Figure 4b). More than one leaf sheath may be removed, depending on the size. Trim the underlying shoot tip to about G inch above the base. The base may have darkened and the outer tissues toughened. Gently scrape the surface of the base to freshen the exposed surface and to stimulate shoot proliferation (Figure 4c). Depending on variety, an explant can produce adventitious shoots in 3 to 8 weeks (Figure 4d).

When newly proliferated shoots are about 1H inches tall, cut them away from the explant, removing a portion of the explant base with each shoot separated (Figure 4e). If new shoots are very small (H inch or less), divide so that two shoots remain together. Trim the shoot tip off all resulting pieces (propagules) and scrape the bases as described above (Figure 4c). Cut off all roots to encourage shoot development. Label all culture vessels resulting from one corm with that corm's appropriate number. *At this time it is necessary to resample and test propagules for DMV*. After trimming the shoot tips, remove one leaf from each of the original clumps of proliferating shoots and submit it for disease testing (individually labeled with the appropriate corm number). Proliferation progresses in this fashion every 3 to 8 weeks depending on the variety. However, if results from the disease testing come back positive, carefully discard cultures and record immediately. The testing process should be repeated until a total of three tests have been done on each culture (including the first test on the corm prior to initial culture).

ROOT INITIATION (Stage III)

Roots may form to some extent during shoot proliferation, especially on the Chinese variety. However, to enhance rooting, select large plantlets for placement on root promoting medium. Rooting medium consists of the same medium previously used with the growth regulator (BA) omitted.

Figure 4. Shoot Proliferation



a. Explant is ready to have leaves peeled off when new leaf unfurls (3/4 size).

b. Removal of outer leaf sheath reveals another inner sheath (actual size).

c. Trim shoot tip & scrape base to stimulate growth of adventitious buds (actual size).

d. Explant with three adventitious shoots.

e. New shoots with a piece of the base are removed from the original explant.

ACCLIMATIZATION (Stage IV)

After proliferation for three to four passages or whenever the number of propagules is large enough for your needs, plantlets can be acclimatized to the outside environment directly from Stage II or Stage III. Ideally, the plantlets would be 2 to 3 inches tall with some roots. Rinse each plantlet with lukewarm water to remove the medium. Plant in sterile potting soil in 3–inch pots. (The commercial potting soil mix Sunshine #3 works well). Prior to field planting, place potted plantlets in 50 percent shade for 3 weeks, followed by 1 week in full sun. Keep plants watered while in the shadehouse. A liquid fertilizer such as Miracle–Gro can be applied 2 weeks after potting, following manufacturer's guide.

It is preferable to grow the plantlets in an area where temperatures and humidity are not excessively high. The taro leaf blight, *Phytophthora colocasiae*, can show up suddenly on taro under these circumstances.

FIELD INTRODUCTION

The field–ready plants should be 6 to 12 inches tall. Plant by hand or with a mechanical transplanter at the density desired. By planting "clean" plants in an infected area or mixing some "clean" with other, perhaps infected planting materials from your current fields, you may realize some increase in yield for a few generations, but eventually, the "clean" plants will become infected and the growth slowed. It is recommended that "clean" plants go in areas where taro or dasheen has not been grown. Any time yield is down, check the taro plants for disease and, if positive, begin anew with "clean" plants in a "clean" field.

4

COST OF PRODUCTION

The production costs for tissue cultured plants will vary from lab to lab. One way to assess these costs is to use a computer spreadsheet template (Figure 5). This template is available via modem from the "AgNet–Hawai'i" computer bulletin board at (808) 969–3025. It is located under the "Production Economics" file area and the program is *Tissue$.WK1*. It is also available by sending a DOS–formatted 3.5" diskette to:

Tissue Culture Cost of Production Template
c/o Dr. Ruth Iwata
Hawai'i Branch Station
461 W. Lanikaula Street
Hilo, Hawai'i 96720
USA

Figure 5. Example of the Tissue Culture Cost of Production Spreadsheet

5

POTENTIAL PROBLEMS AND SOLUTIONS

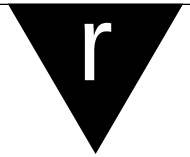
There are several problems associated with *in vitro* propagation of Hawai‘i’s taro varieties. One important concern is the existence and possible proliferation of DMV in cultures. Although sensitive techniques are available to detect the virus, current sampling methods may not be able to detect low levels of DMV. More thorough understanding of the virus and how it spreads throughout the plant is needed. It is troublesome that stock material that first tests negative can yield shoots later found to be DMV positive.

Another concern during commercial production of taro plantlets is the relatively slow proliferation rate of certain varieties. The Chinese variety was the easiest of the four varieties to initiate *in vitro* on most every medium tested. This variety also had the greatest proliferation rate; 3 to 5 new shoots were typically produced in about 3 to 6 weeks. The Mana Lauloa responded best to the standard medium described, but had a slower proliferation rate than the Chinese type, about 3 to 4 new shoots every 3 to 8 weeks. The Lehua and Samoan varieties were the hardest to initiate and the slowest to proliferate. A few explants of each type never produced new shoots. They either lost vigor or merely lacked any signs of growth. Both varieties could produce only 2, sometimes 3 new shoots in 8 weeks.

The period of research was too short to answer questions such as: would proliferation rates vary with number of passages; how many passages are possible without obtaining mutations; could growth regulator level be decreased without sacrificing productivity; and conversely, is there an optimum level not yet tested? It appears that each plant within a variety has differing growth rates *in vitro*. One explant may reproduce prolifically, while another explant of the same variety reproduces slowly or produces very few adventitious shoots. A mutation that appeared *in vitro* was flattened petioles (leaf stems). This occurred most often when growth regulators BA and IAA (indole–3 acetic acid) were used in

combination. Such an offtype reverted back to normal when propagules were placed onto the standard medium. Other offtypes were not seen, but time has not allowed for field evaluation of the plants. Tissue cultured plants should produce marketable corms in 8 or 9 months.

Finally, due to the time and expense involved in producing clean, disease–free taro plants in the laboratory, it is highly recommended that such plants be used as “seed” to produce many more clean plants. That is, tissue cultured plants should be cultivated in a disease–free area, preferably a greenhouse, and cormels that are produced should be separated using disinfected tools and then further propagated to make seed plants for field planting.



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