Training Manual

"The INBAR-UPLB-ERDB International Training on Bamboo Propagation: Techniques, Utilization and Program Development"

Micro-Propagation of Bamboo

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TABLE OF CONTENTS

	PAGE
Foreword	
Acknowledgments	
1. Micro-propagation Protocols	01
2. What Do I Need To Do Micro-propagation?	09
3. Making the Most of Seeds and Seedlings	15
4. I Wanna Be Just Like You	43
5. Getting Ready for the Real World	66
6. Try Mini-clump Division	74
7. Making Up For Lost Forests	80
8. Help Me Out	89
Appendix . Preparation of Stock Solution	101
References	112

SECTION 1. MICROPROPAGATION PROTOCOLS

In this section, we would like you to learn about in vitro technologies and their application to plant propagation. "*In vitro*" connotes artificiality and the first mental picture that you might have will be a test tube. This is essentially what micropropagation is. Plants are grown under an artificial environment within confines of a culture vessel typified by a test tube. How can a plant grow in such conditions? Will you have to use seeds?

What is micropropagation?

Micropropagation is the development of new plants in an artificial medium under aseptic conditions. You do not have to start with seeds but you can use different parts of a plant as a starting materials to establish an *in vitro* culture. These will include embryos, pollen grains and parts such as stems, shoot tips, nodes, root tips, callus and single cells.

You may consider using seeds when asexual methods using traditional horticultural techniques are not possible, for example in coconut. Seeds are not useful to initiate tissue cultures when the candidate plant are apomictic. In such plants, the embryos are not

regular embryos that form from fusion of male and female gametes. Instead, it develop from the cells in the nucellus or integuments of the seed. Seeds are also useful for initiating *in vitro* cultures of plants when it is difficult to use other plant parts and when it is not necessary that the plants are copies of a selection. Such is the present situation in bamboo.

When other parts of the plant are used for *in vitro* culture, you can anticipate the outcome. Almost exact copies of the source plant are typical.

High tech stuff?

The beginnings of this method can be traced to the earliest (although unsuccessful) attempt to grow plant tissues in aseptic culture by Haberlandt in 1902. Initially, the tissue culture methodologies were research tools. However, with a greater understanding of how cells grow and how shoots and roots can form *in vitro* in many economic plants, the methodologies were applied to propagation.

Why propagate plant through *in vitro* technologies? The primary applications of these techniques to plant propagation are: (a) to enable rapid multiplication under conditions that maintain freedom from disease, (b) to establish pathogen-free plants, and (c) to isolate genetically unique cells or cell lines that can become new plant variants (Hartmann and Kester, 1975).

Can we micropropagate bamboo?

The bamboo is among the many plants that have been successfully propagated by *in vitro* technologies, being the subject of many researches undertaken by several laboratories for many years (Zamora, 1994). The earliest report on bamboo tissue culture was in 1968 by Alexander and Rao. The increase in published papers in the last 10 – 15 years signify that it will simply be a matter of time (and research and interested scientists) before each and every economic bamboo would be cultured *in vitro*. Table 1.1 summarizes the genera and species for which reports are available on micropropagation.

What can you use for establishing cultures?

A scan of literature shows that the most amenable initial material for micropropagation is the seed. The seed, specifically the embryo, is most adapted to any propagation system, be this *in vitro* or *ex vitro*. It has several characteristics that make it ideal as planting material for tissue culture. First, it is small and as such several seeds can be disinfected in a small container at the same time. Second, the embryo is dormant and as such more amenable to harsher disinfection procedures, Third, seeds are easier to transport than other plant parts, Fourth, you may store them and initiate cultures from them throughout the year.

Other juvenile materials, e.g. seedlings, are also very amenable to culture. Seeds can be aseptically germinated and the seedlings *in vitro* can be used to initiate new cultures.

Aside from juvenile plant materials, the initial plant materials for culture may be obtained from well-grown bamboo mats. The nodes and internodes from secondary branches and tissues from the ground corm are among plant parts in mature clumps which are used for culture. Some have also used flowers of bamboo to initiate cultures.

Table 1.2 compares three explants or explant sources (i.e. the seed, seedlings and adult clump) for micropropagatiing bamboos. Some points were taken from Saxena and Dhawan (1994).

How can you approach micropropagation?

You may consider several approaches to micropropagation in bamboo (Fig. 1.1 and 1.2). You may germinate seeds or embryos directly *in vitro*. You may induce the seeds to form multiple shoots or callus depending on the culture medium and environment. You may use the seedlings as sources of nodes for multiple shoot formation in media with cytokinins and auxins. Subsequently, you may root these shoots *in vitro* with auxin-supplemented medium.

You may induce seedling tissues to form calli. Then, you may multiply the calli by subculturing them regularly in an appropriate culture medium (usually with 2,4-D or a combination of auxins) and induce them to regenerate new shoots. If you were able to induce somatic embryoids, you may germinate them *in vitro.*

These different approaches using seed, embryo, seed/seedling tissues will give you more plants. If you label the individual sources, you will be able to tell later on on that some seeds and seed sources propagate better than others. We observed this in *Gigantochloa levis*. With this observation, several questions came to mind. First, if there is no selection, will I be propagating more of the potentially good bamboos? Does growth rate in vitro match growth rate ex vitro. Will the more persistent genotypes *in vitro* also have longer life spans *ex vitro*? We still have many questions regarding the possible performance of these propagations.

Aside from seed, you may use non-seed tissues that are meristematic (or with a capacity to cell division). You may induce callus with

auxins (usually 2,4-D) and micropropagate the calli. Plants may also be obtained through the intermediary callus stage or through the germination of somatic embryoids formed directly from seed tissues or from embryogenic calli. You may use the nodes of lateral branches or culm buds for multiple shoot formatiom.

What is significant in the micropropagation protocols is that you use plant parts from mature clumps. Thus, this approach would be most useful in propagating superior bamboo clones. Succeeding sections shall describe various methodologies commonly applied to bamboo micropropagation. These will include the use of seed and seedling tissues as well as tissues and nodes from mature clumps.

Thought For the Section:

> *And God said, Let the earth bring forth grass, the herb yielding seed, and the fruit yielding fruit after his kind, whose seed is in itself, upon the earth: and it was so.*
>
> *And the earth brought forth grass, and herb yielding seed after his kind, and the tree yielding fruit, whose seed was in itself, after his kind: and God saw that it was good. Taken from the book of* ***Genesis 1:11-12 (Holy Bible).***

The striking part of this page from the Holy bible is that the first group of plants God singled out was grass. When Mt. Pinatubo erupted in the Philippines, lahar and ashfall covered many towns in the Philippines. People thought these areas would take many years before anything could grow. The first vegetation that grew without man's intervention was grass. Grass is the first among plants, in the succession of growths possible on land.

Bamboo belongs to the grass family and biblically can be used as one of the choices for man to reforest and rehabilitate degraded lands. Have you ever thought that we are in line with bible when we choose bamboo for replanting degraded lands?

SECTION 2. "WHAT DO I NEED TO DO MICROPROPAGATION?"

Now that you are interested in propagating bamboo by micropropagation, let us consider what you will need. Basic to all micropropagation technologies is a tissue culture facility. This does not have to fancy, just functional.

There are two main activities in a laboratory. These are preparative activities such as preparation of stock solutions and culture media, preparation of culture vessels when making media, preparation of plant materials for introduction to culture and when you want to pot them out. The other activities are all asepsis-related, such as disinfection and introduction to clean culture and maintenance for propagation and rooting. You should allot space in your laboratory for these activities.

A typical research laboratory would define space for distinct activities. So, separate rooms may be allotted to the storage of chemicals and equipment, culture media preparation, sterilization, inoculation, culture storage, observation and acclimatization.

On the other hand, a production laboratory needs not to be fancy. For example, orchid cultures have been stored in a nursery, in a garage, ordinary bedroom or in just a spare room in the house. What can we recommend for bamboo? It is up to you whether it is one room or more. However, you should consider the following points in your decision-making (a) mandate of the laboratory, and the (b) resources available for the laboratory. The mandate is important and covers questions like what crops will be cultured and whether it will be production only or research-production. The resources to be allocated are important. Whether you will consider an available space for renovation or construct a facility, when you will hire or whether you have to generate income to sustain your activities are all related to available resources.

With these considerations in mind, we can best help you to other references on establishing a facility and our perspective on establishing a facility. Among these references is "Mageau, O.C. 1991. Laboratory Design. Pp. 15-30. *In:* Micropropagation technology and application. Edited by P.C. Deberg and R.H. Zimmerman and published by Kluwer Academic Publishers, Netherlands." Other references for laboratory designs are included in this article. The floor layouts of a few laboratories that adapted available space are included in this section (Fig. 2.1).

Activities and suggested equipment v*is a vis* space

Enumerated are activities that you need to consider in the planning of your laboratory and the suggested equipment for these activities:

1. Stock solution preparation and storage

   This area will house (a) weighing balances, (b) a refrigerator with freezer, (c) a distilling apparatus, (d) a stirrer-hot plate and (e) a work area with drawers and cabinets.

   In places where power failure is frequent or unannounced, have pre-weighed amounts of chemicals for stock solution/media preparation. Stock solutions and crystals of growth regulators are often stored under reduced temperatures. Stock solutions are

also best stored under refrigerated temperature to delay algae growth. Growth complexes such as coconut water can be stored in plastic bags and frozen in measured volumes. This permits you to retrieve frozen coconut water in volumes sufficient for your media preparation.

Distilled water is used in small quantities in the laboratory for your stock solution preparation and in large quantities for your media preparation. You may want to fabricate a unit for single distilled water (Fig. 2.2) or buy distilled water. These are available as bottled water (check labels).

Note: If you have a good rainwater collector, rainwater is an option to distilled water. However, you must be sure you do not have a problem of factories around your laboratory location, lest you collect polluted water.

A stirrer-hot plate can make media preparation and disinfection easier.

2. Preparation of culture media

   For this activity, you will need (a) balances for macro and micro weighing, (b) a distilling apparatus, (c) stirrer-hot plate, (d) a pH meter and (e) a work table with access to sink and running water.

   Note: (a) You can melt the agar using a stirrer-hot plate, cook it on a stove and stir it to prevent burning, put it in a microwave oven for a few minutes or you can put it in a pressure cooker and let the steam cook the agar. (b) a fancy pH meter is not necessary. A small, cheaper pH pen (electronic pH paper pen) will accomplish this job well.

3. Storage of culture medium before inoculation

   You will need storage shelves where you can put the sterile culture media.

4. Inoculation area

   You will need: (a) a sterile working bench, (b) an air conditioner unit, (c) a dissecting microscope, (d) a storage shelf for small equipment, alcohol and sterile water and (e) a cart to facilitate transfer/movement of culture media from storage to inoculation area and back to storage or wash area.

   The list of small equipment includes: forceps (different lengths which depend on the type of culture vessel used), scalpel handles and blades, curved scissors, sprayer or atomizer for alcohol, heavy bottles or couplin jar for alcohol and sterile water rinses, and a sterilizer for forceps, and other small equipment (may range from alcohol lamp, Bunsen burner or more fancy sterilizers). You will need a source of gas if you have a Bunsen burner for heat sterilization of small equipment.

   Note: (a) If you are serious with your micropropagation, we recommend that you get an air conditioner if your laboratory is in the tropics. This increases protection for laminar flow cabinet and cultures against dust and dust-borne contaminants and increases efficiency of working staff. (b) Dissection of embryos becomes easier with a dissecting microscope. You can mount a magnifying glass and use this instead of a dissecting microscope to help you during dissection (Fig 2.3).

5. Culture storage area with storage shelves with light fixtures and timers

   Note: (a) A room measuring 4x3 sq.m. and a ceiling height of 2.4 m when used as culture storage area can accommodate 4 shelves, each 3 m long and 40.5 cm wide, with 7 tiers. The total shelf space can accommodate more than 14,700 cultures or roughly 73,500 plants at a time. (b) Separate the ballasts of the light fixtures to minimize heating of shelves. (c) You may reduce number of light fixtures if the room has adequate natural light.

6. Kitchen/Washing area *cum* sterilization area

   You will need (a) sterilizers (pressure cookers or autoclave), (b) stove or heavy duty burners and gas or electrical power, (c) draining and drying shelves, (d) storage shelves for clean glassware, (e) sink and (f) exhaust fan.

   Washing can be segregated so that very dirty glassware or plant materials can be washed in this "dirty kitchen" area.

7. Acclimatization area

   This area should have (a) work table or working carts, (b) a storage space for cultures being acclimatized and (c) a sink.

8. Nursery

   This area should have (a) a potting area, (b) a shaded area for newly potted plants, (c) storage bins for potting mix, (d) a sterilizer for potting mixes, (e) a source of water and (f) water storage containers.

9. Staff room and lavatory

The equipment which may be needed include (a) a simple typewriter or computers, (b) filing cabinet and (c) a small library for references.

Human resources are critical. Your propagation strategies may be implemented well with well-trained personnel. Changes in technicians also affect the productivity of a laboratory. So, help your staff remain with your laboratory. A room for the staff and a lavatory area should be included in the design of the laboratory. Provide a table where they can meet and discuss problems regarding their work or just to relax and eat. A lot of the work becomes routine and boring. You should invest in your staff to encourage them to remain with you.

Other considerations

Aside from function, you must plan for safety and efficiency in the layout of the available space you want to adapt for the facility. You should consider the number of access doors, ease of monitoring potential accidents and the ease of movement in case you need to vacate a room or the facility. Monitoring for potential accidents can be made easier if your work areas have glass panels (Fig. 2.4).

Safety also includes the availability of fire extinguishers, a manual on safety precautions regarding equipment use and first aid instructions particularly with materials used in the laboratory.

Efficiency considers the flow of activities and materials from the start to the finish of the culture systems and the maximum use of available resources.

You may also want to invest in a generator so that you have stand-by power in case of power failures.

Chemicals and other supplies

Aside from work areas and equipment, chemicals, laboratory and nursery supplies are necessary for the laboratory. The chemicals for the culture media are dependent on the formulation to be used. You will also need (a) ethyl alcohol, (b) buffer solutions, potassium hydroxide and hydrochloric acid to adjust pH of the media and solutions, (c) agar and (d) sucrose (or white high grade refined sugar).

The list of supplies include: (a) glassware such as measuring cylinders, culture vessels, amber glass bottles, etc., (b) detergent, (c) bleach, (d) matches, (e) liquefied petroleum gas for cooking, (f) rubber slippers which you can wash and disinfect, (g) cleaning implements (scotch brite, brush for bottles, sink, etc.) and (h) other supplies (fluorescent bulbs, cleaning materials) to maintain the laboratory.

Thought For the Section:

> *"Yea, if thou criest after knowledge, and liftest up thy voice for understanding; If thou seekest her as silver, and searchest for her as for hid treasures; Then shalt thou understand the fear of the Lord, and find knowledge of God. For the Lord giveth wisdom; out of His mouth cometh knowledge and understanding."*
> ***Proverbs 2:3-6.***

SECTION 3. MAKING THE MOST OF SEEDS AND SEEDLINGS

In the heart of a seed, very deep so deep,

A dear little plant lay fast asleep,

"Wake up!" said the sunshine, "and creep to the light"

"Wake up!" said the voice of the raindrops bright,

The little plant heard and he rose to see

What a wonderful world God made for you and me!

-V. B. S. Manual (1992)-

Seeds just might be available for the bamboo species of interest to you. Literature on bamboo flowering in your area would be most useful. Get in contact with the Forestry Department and you may be able to contact people who have many observations on times (or months) of flowering of particular species of bamboo.

Bamboo species have different types of inflorescences. *Gigantochloa levis* have long inflorescences, which looks like a "curtain" around the culm (Fig. 3.1). *Schizostachyum lumampao* has short inflorescences that are near terminally located. When we saw gregarious flowering of *Bambusa vulgaris* and *G. levis* even the old stumps had flowers. You will have to adjust on how to proceed with each collecting situation.

A. Collecting seeds and seedlings

First, let us go through some dos and don'ts.

1. Plan the collection trip. You must have an idea where flowering clumps of bamboo have been sighted. It is helpful to have a survey trip.

2. What should you bring? The following are the things (Fig. 3.2) we found convenient to have on a collection trip:

   a. Binoculars

   b. Large net which we can lay on the ground to catch inflorescences

   c. Pruning shears

   d. "Bolo" (similar to machete) or scythe

   e. Rope, preferably nylon (long enough to lasso bamboo branches or support a collector, about 20-25 m long)

   Note: At times, the flowering clump may be located along the river or creekside. Under these conditions, it will be difficult to position the net for the collection. Once, our driver tied a rope to the bridge. Then he used the rope to swing down to the flowering culm so that he could reach the inflorescences to get us some samples.

   f. Fine nylon net bags (for the seeds and florets and for sheaths and leaves)

   Note: You may use paper, cloth or plastic bags to hold unprocessed flowers and seeds. Protect the seeds from increased humidity in these bags by rolling down their sides during the collection trip and travel back to the laboratory. Protect from heat by putting bags in shade. Do not put them in a parked vehicle because temperatures may rise above 40°C. Excessive prolonged heat could drastically affect seed viability.

   g. g. Stick broom for sweeping fallen inflorescences/ florets

   h. Permanent markers and shipping tags

   i. Polystyrene box

   j. Jacket or long-sleeved shirt which you can button up and a cap or hat

   Note: This protective gear can save you from scratches and itching.

   k. Medicine kit (insect creams, Band-Aids, etc.)

3. When you locate a flowering clump, seek the owner of the land or its caretaker.

   a. Record his name, address, date and interview notes to include when flowering was first observed and tentative identification of the species.

   b. Make a location map showing the flowering clump in relation to other structures, trees or landmarks.

   c. Collect culm sheaths. These will help you identify the bamboo. Culm sheaths of different bamboos have distinct features (Fig. 3.3). Put all the materials from one clump together.

Seeds

You may collect seeds following one or more techniques or their combinations (Pukittayacamee, 1996). These methods include (a) collecting from seeds that had fallen to the ground naturally, (b) collecting by shaking the culms and (c) collecting from the felled culms. Aside from these techniques and depending on fertility of the bamboo species, you may also cut inflorescence from unfelled culms

Of the different methods, the least preferred is to cut down the flowering culm. However, some situations warrant the use of this method. For example, some communities have an aversion to flowering bamboo because this signals bad luck or hard times to them (Fig. 3.4). The flowering bamboo will soon be cut down anyway.

Of the various methods of collection, culm-shaking yielded seeds with the highest percent germination, followed by culm branch cutting in *Bambusa bambos* while germination was closely similar for ground collection and culm-shaking for *Thyrsostachys siamensis* (Pukittayacamee, 1996). We also recommend this method for species with low fertility such as *Gigantochloa levis.*

Collecting from seeds which have fallen on the ground naturally

1. You may opt for this technique when the flowering clump is near a community and you can arrange with a possible collector. The technique is most useful if the clump is accessible and you can return to collect seeds every 3-5 days. However, daily collection would minimize the exposure of seeds to soil-borne pests.

2. Clear the area beneath the flowering clump.

3. Lay a large fine net underneath the flowering clump. A plastic sheet or canvas can be spread late in the afternoon each day to avoid morning dew and condensation that would moisten the seeds and make them more susceptible to fungal infection (Seethalakshmi and Soman, 1992 and Pukittayacamee, 1996).

   Note: If you do not have a net, canvas or sheets of plastic or woven mats, particularly for unplanned collections, sweep the area underneath the clump thoroughly with a stick broom. Collect fallen inflorescences and seeds.

4. Protect the collection area from rats, birds and other animals that eat the seed.

5. Label the bags. Include tentative identification of clump source, area of location, owner and date collected.

Collecting by shaking the culms

1. Clear the area of tall grass or small shrubs (if permitted) with your "bolo"/scythe.

2. Spread the net underneath the flowering clump. Consider the direction of the wind when positioning the net (Fig. 3.5).

3. Tie the rope on the culm so that you can tug on the rope to shake the culm. You may climb up the culm, jump on it or shake other stems and branches (Fig. 3.6).

4. Collect the florets that fall into the net. Transfer the florets into net bags.

5. Label the bags. Include tentative identification of clump source, area and, whenever possible, the owner of the land. If more than one mat is flowering within the same area, label each mat as individuals.

Collecting seed-bearing inflorescence from felled and unfelled culms

1. Inspect the culm for relative fertility. Highly fertile bamboos, e.g. *S. lima*, *S. lumampao*, have many seeds in clumped inflorescence.

2. Collect inflorescence that has turned brown (due to maturity). Pick mature seeds or cut inflorescence from low-lying unfelled culms with a pruning shears (Fig. 3.7).

   Note: If bamboo clumps are easily accessible, collect mature seeds only. Leave immature seeds to develop further and inspect after a week or so for another collection.

3. Return to collect more inflorescence as they mature.

4. Transport inflorescence in a paper or wrapped in newspapers, within net bags.

5. Put all net bags in a polystyrene box to ensure safety during transport.

Handling after harvest

1. Remember that drying and cleaning affect the quality of your seed lots since seed moisture at harvest may vary with species, e.g. 16% for *T. siamensis* to 21% for *B. tulda*. You will have to reduce moisture content.

2. If you collect seeds during the rainy season and if the seeds remain wet for a sufficient time, they may germinate before you even use them. So, dry them. You may need to lay the seeds on lots of newspapers or other absorbent material to absorb excess moisture (Fig 3.8).

3. Air dry inflorescence or seeds as soon as possible. You may hasten air drying with a fan or hair dryer during the trip.

Seedlings

It is also worthwhile to investigate if seeds had fallen earlier around the flowering clump. These seedlings can be gently lifted and potted in the nursery. You may want to use them to improve methodologies using juvenile tissues, including nodes and shoot tips.

1. Locate seedlings beneath the canopy of flowering bamboo, particularly near sources of water.

2. Use a stick or other alternative to lift the roots and soil of the seedling.

3. Put some moist soil from the vicinity of the seedling in a plastic bag or alternative container, which will help keep the humidity around the plant during the trip. Moisten soil if it needs a bit of water.

4. Put the seedlings with some leaf mold on the soil and ensure that these are protected from desiccation.

5. Place these seedlings in the shade until all collection activities have been completed. You may put them in the vehicle when it is time to go.

B. Processing of the inflorescences for seeds

You need to process the collected inflorescence and florets for seed. The technique for processing will depend largely on the characteristics of the flowers. The flowers may vary, depending on the bamboo species. Glumes may be absent, loosely enveloping the seed or closely clinging to the seed. Seed-bearing florets and seeds, before and after processing, are shown for *G. levis*, *S. lumampao* and *D. strictus* (Fig 3.9). Processing would mean selection of florets with seed and cleaning seeds.

1. Winnow the florets to separate chaff from the filled florets. Most of the empty florets will separate from those with seed during winnowing.

2. Press each floret. Florets with seed are hard to the touch. If the seed is immature, the floret will be more malleable to the touch.

Note: We have tried to improve on the processing for G. levis using seed processing equipment but to no avail. For other species without glumes, postharvest equipment may make work simpler.

3. Extract the seed from the glumes manually.

1. Sort the seeds to mature (hard dough stage), less mature (soft dough stage) or immature (milk stage).

Note: The seeds can also be differentiated by the flotation method. Put some seeds in water. Those that float are most likely immature and at the milk stage. Those that sink are mature seeds. Inspect the seeds and differentiate each batch based on color and "feel". Use these observations in subsequent batches to sort seeds. For G. levis, the immature seeds are green while the mature seeds are brown. For S. lumampao, immature seeds are purplish-green, mature seeds are brown.

2. Discard seeds that are at the milk stage.

3. Store mature seeds.
4. Germinate embryos from less mature seeds.

C. **Storage of seeds**

1. You may need to store seeds since their availability is very limited. Bamboo seeds are orthodox. They can withstand desiccation and storage.

2. Test each seed batch for viability and percentage moisture content if you want to store the seeds for several months.

Note: If your seeds are too few, you may not want to waste any of the seeds by using them for a viability test. You will have to rely on visual appraisal of the embryos. Healthy embryos are white and plump. With prolonged storage, the embryo may look shriveled and on the creamy side. Not all of these will germinate, but some will.

3. You may use a desiccant to dry seeds to 5-6% MC. You may also sun dry or kiln dry the seeds at 40-50°C for 3-6 hrs (Pukittayacamee, 1996).

3. Line a plastic bag or plastic/glass bottle with an absorbent material such as cotton or paper.

4. Put in the seeds and seal the container.

5. Label properly. Include species, collection data, percentage viability, and date of storage.

6. Store in a refrigerator (about 15°C) for 18 months. You can prolong storage life with storage at even lower temperatures (2°C).

Testing for Seed Viability *(Taken from Pukittayacamee, 1996)*

a. *Deglume seed to get the embryo.*
b. *Soak seeds in 1% H_2O_2 solution (100 ml/50 seeds) and incubate overnight in the dark at room temperature. Change the solutions daily for 3 days.*
c. *Observe for radicle protrusion. Renew the solution for another 3 days if the radicle has not yet protruded.*

d. Evaluate radicle protrusion. Viable seed would have radicles longer than 5 mm.

D. Aseptic germination

In vitro germinated seedlings represent opportunities for a micropropagator. They serve as sources of nodes, shoots and rhizomes for other micropropagation techniques.

1. Disinfection

Disinfection is successful if aseptic materials are established from the tissues of interest. Seeds of different bamboo species vary in shape and characteristics (Fig. 3.9). Although seeds are generally amenable to disinfection, the harshness and duration of treatment are influenced by the characteristics of the seed. Some seeds have very smooth and tough surfaces; these are easily disinfected. Others have fine hairs and a thin skin. These characteristics make them more difficult to disinfect. Contaminants may not be easily disinfected since air bubbles may be trapped in the tufts and the thin skin makes the seed less resistant to the disinfecting solutions.

With variations among species, it is best to look for literature on the species of interest to you or to choose a procedure for a species with seed characteristics like your species. Table 3.1 summarizes some disinfection procedures for seeds of some bamboo species. Whenever data is available, the characteristics of the seeds are also indicated.

The following disinfection protocol works for D. *strictus* and G. *levis.* Seed batches of *G. levis* stored for 7-8 weeks and used for embryo culture showed 79-84% germination.

a. Obtain an appropriate quantity of seeds from storage.

b. Wrap the seeds loosely in a piece of gauze, fine net, nylon stocking or similar material.

c. Tie the "seed bag" to the water tap (faucet) and leave the water running for two hours.

Note: If your water source warms up with time, you need to modify the system. Collect water in a container (e.g. plastic jug, clay water container) with a tap. Allow the water to cool. Wash your seeds.

d. Transfer the seed bag to a flask or appropriate vessel, add pure commercial bleach and cover with a piece of foil. Shake the solution or stir the seeds with a magnetic bar on a magnetic stirrer for fifteen minutes.

e. Transfer the flask to a clean air bench or a sterile inoculation box.

f. Wash the seeds thoroughly with sterilized (distilled or tap) water to remove the bleach.

Table 3.1. Disinfection procedures for seeds of bamboo species.

SPECIES	DISINFECTION PROTOCOL	AUTHOR, YEAR
Dendrocalamus strictus	Keep the dehusked seeds in running water for 1 hr, then dip in 5% Teepol for 5 min.; wash in running tap water and distilled water; wash seeds with rectified spirit for few seconds; sterilize with 0.1% $HgCl_2$ for 15 min.	Niraula and Bhandary, 1987
D. strictus	Wash seeds with 2% Teepol solution on a magnetic stirrer for 5 min.; wash in running tap water for 15-20 min.; rinse in distilled water; immerse in chlorine water (saturated chlorine water diluted 5 times with distilled water); wash thoroughly in sterile distilled water.	Rao et al., 1987
D. strictus	Wash the dehusked seeds with 2% Teepol solution on a magnetic stirrer; wash in running tap water for 15-20 min.; rinse in distilled water; immerse in chlorine water for 5 min. (saturated chlorine water diluted 5 times with distilled water); wash thoroughly in sterile distilled water.	Rao et al., 1985
D. membranaceus	Surface-sterilize with 75% ethanol for 1 min.; dip into 10% sodium hypochlorite for 15 min. and, then rinse thoroughly in sterile distilled water.	Vongvijitra, 1988
Otatea acuminata O. aztecorum	Wash for 10 min. in water containing a few drops of detergent; rinse in distilled water; dip in 70% ethanol for 1 min.; immerse for 15 min. in 30% commercial bleach (1.6% sodium hypochlorite final concentration) and rinse 3 times in sterile water. Final rinse with sterile water at a pH of 3.5 (achieved by addition of HCl).	Woods et al., 1992
S. latiflorus	Sterilize with 75% ethanol for 1 min.; dip into 2% sodium hypochlorite for 10 min.; rinse thoroughly in autoclaved distilled water.	Yeh and Chang, 1987
54 species from 15 genera*	Spray with 70% ethanol; surface-sterilize for 30 min. in 1% sodium hypochlorite; wash 3 times in sterile water.	Prutpongse and Gavinlertvatana, 1992

*Arundinarea pusila, A. superecta, Atatea aztecorum, Bambusa srundinacea, B. flexuosa, B. glaucescens, B. gracilis, B. humilis, B. longispiculata, B.multiplex (variegata), B. nigra , B. multiplex, B. oldhamii, B. polymorpha, B. ventricosa, B. ventricosa (variegata), B. vulgaris, B. cv. Dam Khan, B. cv. Bong ban, B. Bong Naew, Cephalostachyum pergracile, Dendrocalamus asper, D. giganteus, D. hamiltonii, D. Bong Kaiy, D. latiflorus, D. membranaceus, D. nutans, D. strictus, Dinochloa scandens, Gigantochloa albociliata, G. apus, G. auricurata, G. compresa, G. densa, G. hasskarliana, G. hossuesii, Hibanobambusa triangullans, Helocatamus compactiflorus, Oxytenanthera albociliata, Phyllostachys aurea, P. bambusoides, P. gramineus, P. nigra f. megurochiku, P. pubescens, P. sulphurea, P. viridis, P. iaponica, Sasa fortunei, Sasaella sumekoana, Schizostachyum aciculare, Sch. brachycladum, Sch. zollingeri, Semiarundinaria fastuosa, and Thyrsostachys oliveri

2. Excision and inoculation of embryos

a. Place the sterile stainless steel dishes lined with sterile paper, scalpels, forceps, alcohol lamp, a couplin jar with 95% ethyl alcohol, bottles with sterile water and soapy solution (Fig. 3.10), in the sterile working area. A dissecting microscope is also helpful.

Note: Kitchenware steel plates may be replaced with petri dishes.

b. Open the flask with caution to maintain sterility of seed.

c. Transfer some seeds onto the sterile paper on petri dishes or steel plate with a pair of sterile forceps.

d. Hold the seed aseptically with the forceps and make a slit on the seed coat over the embryo with the tip of the scalpel blade.

e. Excise the embryo from the rest of the seed tissues. Use a dissecting microscope to make your work easier.

f. Inspect the embryo.

f. Inoculate the embryo onto the culture medium.

Note: (a) In literature, others use the intact seed. We have also used the embryonal end, without the endosperm. However, the bigger the explant, the higher the risk of contamination. With many grooves in the bamboo seed, it is best to reduce the size of the explant to the essentials. For callus and somatic embryogenesis, clean the embryo of other tissues. (b) A culture medium recommended for seed germination consists of half-strength MS macronutrients and micronutrients, full strength MS vitamins and iron and 20 g/l sucrose, pH 5.7.

h. Position of the embryo on the culture medium embryo-side up and away from the culture medium (Fig. 3.11).

Note: The components of the culture medium vary depending on the route for multiplication. See Table 3.2. Routes of micropropagation and culture media for seed-based tissue cultures of B. arundinacea and D. strictus.

i. Maintain the cultures under 16-hour photoperiod, 42 uE m^{-1} sec^{-1} in an air-conditioned room at 22-27 ^{o}C.

j. Inspect for bacterial and fungal contamination. Discard contaminated cultures.

k. Observe for germination 4 to 8 days after inoculation.

Table 3.2. Routes of micropropagation and culture media for seed-based tissue cultures of *B. arundinacea* and *D. strictus.*

MICROPROPAGATION/ BAMBOO SPECIES	CULTURE MEDIA	AUTHOR, YEAR
In vitro germination a. *B. arundinacea* b. *D. strictus*	WP/MS Whites + 2% sucrose	Vasana, 1985 Nadgir et al, 1984
Multiple shoot formation a. *B. arundinacea* *b. D. strictus*	Medium + 1 mg/L NAA + 4 mg/L 6-BAP -	Vasana et al, 1985
Callus/somatic embryogenesis a. *B. arundinacea* *b. D. strictus*	N6 + 7 mg/L 2,4-D B5 (0.5x) + vitamins + 30 uM 2,4-D + 2% sucrose + 0.8% agar, pH5.8	Metha et al, 1982 Rao et al, 1987

Note: You will first observe swelling of the embryonal end, a change in color to more opaque to creamy, radicle protrusion and lastly shoot growth.

h. You are successful! Your seedlings are slowly putting on more leaves and nodes! (Fig. 3.12). You are now in business.

E. Multiple shoot formation from nodes of aseptic seedlings

Using aseptically germinated seedlings as sources of node cuttings can amplify your propagation scheme. Seedlings would have the advantage of the full utilization of its biological life span.

1. Inspect cultures of germinated seedlings. In a month or so after seed/embryo establishment, sort the seedlings according to vigor.

   Note: If you had opted to use the culture medium for multiple shoot formation right at germination, shoot growth will be slower.

2. Establish your coding system so that you can sort out the sister lines from one embryo and from one clump source. This will help us release plants from several genotypes to supply requests of communities responsibly.

3. Prepare appropriate culture media for multiple shoot formation.

   Important! The culture medium for multiple shoot regeneration in juvenile sources of nodes consists of MS at full strength, 30 g/l sucrose, 1 mg/L NAA,and 4 mg/L BAP, pH 5.7.

4. Using aseptic techniques and working in a clean air cabinet cut the stems near the base of the plant with a pair of curved scissors (Fig. 3.13). Leave some nodes at the base to keep the remainder of the seedling productive.

5. Draw out the cut stems (upper part) and place them on a stainless steel dish.

6. Prepare node cuttings. Cut shorter stub on the upper part of the node cutting to differentiate polarity and favor early bud break.

7. Inspect node cuttings for lateral buds by pulling on the leaf sheath encasing the node area.

   Note: Some cuttings may have blind nodes (i.e. lateral buds are not observed). Discard these nodes.

8. Inoculate cuttings unto a culture medium for multiple shoot formation.

9. Maintain the cultures under 16 hours photoperiod, 42-uE m^{-1} sec^{-1} in an air-conditioned room at 22 - 27 ^{o}C.

10. New stems developed within 2 to 4 weeks.

11. You may repeat the whole cycle of multiplication.

Note: If your species does not form multiple shoots on the suggested culture medium, try these published media. Table 3.3 summarizes literature on multiple shoot formation using seed and seedlings of various bamboo species. If none of these works, experiment!

F. Micro-division

With the stems cut short to the base, it is unnecessary to throw away the remaining basal tissues. The rhizome developed during the germination is best used as planting material. You may extend its usefulness by adding fresh medium and waiting for new shoots. You may also trim the roots near the rhizome and transfer the whole shoot to a new culture medium. Alternatively, you can propagate them by micro-division.

1. Trim roots with close to the rhizome with a pair of curved scissors

2. Take out the shoot from the culture vessel and transfer to a sterile paper.

3. Cut it along the rhizome to get small shoot clusters.

4. Inoculate to fresh medium.

5. Repeat the whole cycle of multiplication.

G. Calli and somatic embryogenesis from embryo tissues

Embryos are easier to induce to callus and to form somatic embryoids. This approach is potentially more productive within a shorter period of culture than multiple shoot formation. However, the risk of getting somatic variations is potentially higher among plants that were regenerated from calli/somatic embryoids than from multiple shoot formation.

Use procedures for aseptic germination. Follow disinfection procedures. You may use intact seeds (Rao and Rao, 1985) or excised embryos following our procedures adapted from institute procedures for wheat tissue culture. The procedures described below worked for *G. levis*, *D.strictus*, and *B. bambos* and are based on Zamora and Gruezo (1989).

1. Inoculation

a. Disinfect the seeds to be used for embryo culture.

b. Use aseptic techniques.

c. Transfer to sterile petri plates lined with paper to absorb excess moisture.

d. Hold the seed aseptically with the forceps and make a slit on the seed coat over the embryo with the tip of the scalpel blade.

e. Excise the embryo from the rest of the seed tissues. Use a dissecting microscope to make your work easier.

f. Inspect which is the scutellum-side of the embryo.

g. Inoculate the embryo with scutellum-side up onto a solid culture medium for callus induction. The embryo-side should face the culture medium.

   Important! The culture medium we found useful for inducing and propagating calli/somatic embryos consists of full-strength MS, 20 g/L sucrose, 2 mg/L 2,4-D, 5.0 g/l agar, pH 5.7.

   Note: Table 3.4 summarizes the culture media for callusing and somatic embryogenesis in various bamboo species.

h. Maintain the cultures in eight hr-diffused lights in an air-conditioned room at 22-27 ^{O}C.

   Note: Cooler temperatures are very favorable for callus induction and propagation.

i. Inspect for bacterial and fungal contamination. Discard contaminated cultures.

2. Propagation of Calli/Somatic Embryoids

a. Inspect callus growth after 4 weeks inoculation.

b. Sort callus cultures into compact, loose-type or combination (Fig. 3.14). Discard the cultures showing only loose-type calli.

Note: (a) If callus is still small, subculture the whole callus to fresh culture medium. (b) The base medium and the growth regulators are important for the type and extent of callus growth (Fig. 3.15).

c. If compact callus is big enough, segregate it from the loose-type. Subculture onto freshly prepared callus medium.

Note: (a) It generally takes two subcultures (a month apart) before the callus sufficiently enlarges for you to decipher the presence of compact calli.

(b) Embryogenic compact calli give rise to somatic embryoids.

d. Propagate the compact-type calli/somatic embryoids by subculturing every 2 weeks to fresh culture medium. Break up the proliferations along weak points.

Note: (a) Do not break up calli forcibly with a scalpel. Cutting up will cause the calli to turn brown die. (b) Compact calli the size of an eraser head of a Mongol pencil will yield callus more than four times its size in 4 weeks (Fig. 3.16).

e. Inspect for any signs of bacterial contamination. Discard contaminated cultures.

Note: Any clouding of the culture medium at the immediate vicinity of the calli makes the culture suspect.

f. Strategize the propagation of calli/somatic embryoids and regeneration of plants.

Note: When you observe albino callous or plants transfer all calli stock belonging to this batch to the regeneration medium.

g. Initiate a fresh batch of seeds every six months to ensure a continuous stock of embryogenic calli capable of regenerating green plants.

3. Plant Regeneration

a. When you have built up the stock of e-calli/somatic embryoid stock, subculture some stock to the culture medium for plant regeneration and to fresh 2,4-D culture medium to propagate the e-calli/somatic embryoid stock.

Important! The culture medium we found useful for plant regeneration consists of full-strength MS macronutrients, vitamins and iron, half-strength MS miconutrients, 20 g/l sucrose, 150 ml/l coconut water (from young green nuts), 0.1 g/l myo-inositol, 5.0 g/l agar, pH 5.7.

b. Maintain the cultures under 16 hours photoperiod, 42 $uEm^{-1}sec^{-1}$ in an air-conditioned room at 22 - 27 ^{o}C.

c. You will observe the greening up of the compact calli and soon you will see shoot points that develop which will form plantlets within 4 to 8 weeks (Fig. 3.17).

a. You may use the plants for multiple shoot formation and micro-division if you still need more plants.

H. Wildings or Nursery-Grown Seedlings

Remember the wildlings you collected? These can be cultured. However, the major obstacle is decontamination. We get about 55% clean cultures from greenhouse-grown materials. This could have been improved by improving on nursery culture.

Briefly, our procedures are as follows:

1. Harvest shoots with at least seven leaves.

   Note: Each leaf corresponds to a node so that by counting the leaves, you can approximate the number of nodes.

2. Cut off the top 3 leaves, including the extended spindle leaf. Discard.
3. Scrub the remaining shoots under running tap water. Use cotton wad dipped in soapy water. Rinse thoroughly.
4. Cut the stem into cuttings with one to two nodes, depending on closeness of internodes.
5. Put the segments in a flask.
6. Soak the segments in soapy water for 10 min. Decant solution.
7. Add bleach to flask, soak segments for 15 min. Decant the disinfectant, and rinse with sterile tap water.

8. Transfer segments to 70% ethyl alcohol. Decant solution after 1-2 seconds.
9. Add 0.12% calcium hypochlorite ($CaOCl_2$) and soak segments for 10 minutes. Decant bleach.
10. Add streptomycin (1 g/L) solution and soak segments for 10 min.
11. Put the segments onto a sterile petri dish lined with sterile paper.
12. Cut along the base of the sheath along the node. Remove the sheath.
13. Trim the internodes below and above the bud. Make a shorter stub above the node.
14. Inoculate onto the culture medium.

    Note: The culture medium varies with the propagation methodology. Prepare appropriate medium.

15. Observe new shoot growth after three to four weeks (Fig. 3.18).

I. *Invitro* Rooting

1. Prepare appropriate culture media for *in vitro* rooting.
2. Using aseptic techniques and working in a clean air cabinet cut the stems near the base of the plant with a pair of curved scissors. Leave some nodes at the base to keep the remainder of the seedling productive.
3. Draw out the cut stems (upper part) and place them on a stainless steel dish.
4. Inoculate shoots or cuttings unto a culture medium for rooting.
5. Maintain the cultures under 16 hours photoperiod, 42 uE m^{-1} sec^{-1} in an air-conditioned room at 22 - 27 ^{o}C.
6. Observe roots after 2 to 4 weeks.
7. Acclimatize them for potting out.

Thought for the Section:

Hearken; behold, there went out a sower to sow;

And it came to pass, as he sowed, some fell by the way side, and the fowls of the air came and devoured it up.

And some fell on stony ground, where it had not much earth; and immediately it sprang up, because it had no depth of earth;

But when the sun was up, it was scorched; and because it had no root, it withered away,

And some fell among thorns, and the thorns grew up, and choked it, and it yielded no fruit.

And the other fell on good ground, and did yield fruit that sprang up and increased; and brought forth, some thirty, and some sixty, and some a hundred. ***Mark 4:3-8.***

We hope the seed you collected will all fall under the last category. For bamboo, seed is precious. "Limited quantity" is not necessarily a hindrance to propagation with tissue culture because we can multiply whatever seed is available by many techniques *in vitro* instead of waiting for just one plant to arise from one seed. As the word in Mark 4:3-8 says that if seed falls on good ground, and is taken care of, it will increase.

SECTION 4. I WANNA BE JUST LIKE YOU!

"When I grow up, I will be just like you".

If you grow bamboo, you may observe that their performance in terms of growth rate, yield, culm diameter, fiber strength or other characteristics vary. If you have a better plant, you may prefer propagating this selection to the others. You can increase this clone by

macropropagation and by micropropagation. We expect the plants propagated by micropropagation techniques to be identical to the source of the initial plant material. They are somaclones or sister lines from somatic tissues.

There are several candidate sources of initial tissues for micropropagation. You may culture small bits of tissues from the soft internode tissues in extended and unextended branches. If you look at the bamboo during the onset of the rainy season, there will be many new branches. However, from each branch, the number of sections you can get is rather limited compared to the fast growing ground corm. What is a ground corm? Are you familiar with the less technical name "bamboo shoot"? When the new shoot is developing and fast elongating, you can get many tissues from the soft internode and node areas for tissue culture work. Common to these tissues in the branches and ground corms are the highly active and dividing groups of cells that can be induced to further divide in culture to form calli.

Aside from these tissues, nodes from unextended and extended branches as well as unenlarged buds are candidate materials. There are differentiated buds, which can serve as the source of new growths by multiple shoot formation. However, the requirements for culturing nodes from mature clumps, whether non-flowering of flowering, are different from nodes from seedlings. Researches using tissues and nodes from mature clumps are limited compared to seed and seedling tissues (Saxena and Dhawan, 1994; Zamora, 1994).

The following protocols were developed in the Institute of Plant Breeding of the College of Agriculture, University of the Philippines Los Baños, Philippines.

I. GROUND CORMS

A. Collecting ground corms

Let us start with some dos and don’ts.

1. Plan the collection trip. Bring what you need because you might end up not being able to excavate the ground corm. What should you bring? The following is a list of things we found convenient to have to collect ground corms:

   a. Sharp pruning shears

   b. Desuckering bar, "bolo" or machete

   c. Net bags (heavy duty) and newspapers for wrapping

   d. Clean water to wet newspapers

   e. Markers and shipping tags

   f. Polystyrene box, if collection area is far from the laboratory

   g. Jacket or long sleeved shirt which you can button up

   h. Cap or hat, boots and gloves (or plastic bag)

2. Visit the clumps two weeks well in the rainy season and inspect if new shoots have come out. Cover with some decomposing leaves or litter fall to protect your discovery!

   Note: Guard your mats when two to three weeks of rain has fallen! By this time, the

   bamboo would have new shoots and, boy, are they tasty at this time!

3. Return after a few days to about a week. Harvest when the shoots are about 1.5 to 2 feet high from ground level.

   Note: Growth rates of shoots vary with species so familiarize yourself with the species of interest! If you missed the preferred height of the bamboo shoot, make do with your harvest. Your cultures will be restricted to the younger nodes.

4. When you return, dress for the occasion, with long sleeves, a cap and boots. Be prepared in case it rains.

   Note: Some bamboos have many trichomes on them that can be a nuisance to you.

5. Remove the litter cover to expose the shoot and excavate around the base of the new shoot.

6. Extract a new shoot with a "bolo" or desuckering bar (Fig. 4.1).

   Note: Exercise great care during extraction because the new shoot is tender. Avoid shaking movements that tend to crack the base and open wounds. Make clean cuts. Avoid bruising the sides of the shoots.

7. Once separated from the rhizome, gently lift the shoot to flat ground and clean the base of adhering soil.

8. Dampen the newspapers and lay the shoot on them. Wrap the shoot with damp newspaper.

9. Label. Include species, date/area of collection, and if taken from someone's backyard, the name of the owner.
10. Transport in a polystyrene box to prevent cooking or dehydrating the corm.

B. Handling of corms for culture (Fig. 4.2)

1. In the laboratory, wash the shoots thoroughly in running water.
2. Trim off the ligules (small leafy structure at the tip) from the leaf sheaths with the pruning shears.
3. Chop off the very hard tissues in the ground corm.
4. Remove the outermost leaf sheaths and all loosely fitted sheaths.
5. Scrub the remaining surfaces with a scrubbing pad or soft brush with soapy water to remove dirt and possibly pests such as insect eggs, aphids and mites.
6. Rinse with clean water. Air dry.

C. **Storage of corms**

If you come in from your trip on a weekend and dead-tired from the trip, store the corms. Anyway, who wants to work on a Sunday! They will have to be stored until the next workday!

1. Remove the damp newspapers.

2. Wash the shoots thoroughly in soapy water dry with newspapers and when air-dried, re-wrap in a dry newspaper.

3. Store in the refrigerator.

4. Remember to use them for culture within a week.

Note: The longer you keep them in the refrigerator, the poorer will be your results. In other words, don't procrastinate! It is best to use them freshly collected.

D. Disinfection of corms (Fig 4.3)

1. Wrap the shoots in a thin layer of cotton.
2. Transfer to a big plastic bag.
3. Wet the entire surface with pure bleach and let this soak for 60 minutes.
4. Transfer the bag containing the ground corm to the clean air bench.
5. Remove shoots from the bleach by peeling away the cotton layer.
6. Transfer to a small tray that will hold the shoot and the water to be used for rinsing.
7. Hold the shoot by the tip, position the shoot perpendicular to the tray and rinse with sterile water.
8. Transfer the shoot to a dry tray.

E. Preparation and inoculation of explants (Fig. 4.4)

1. Remove the bottommost sheath by making an incisive slit at the base of the sheath and pull the sheath away from the shoot to expose the internode and node.
2. Cut the internode tissues just above the nodal ring. This will be about 3 - 4 mm thick.
3. Put the layer in petri dish lined with paper and wrap the dish with a piece of paper to minimize exposure to light. From our experiences, these tissues are very susceptible to browning when left exposed to light. The browning is irreversible.
4. Continue processing the shoot and extracting internode layers until you have finished nearly all layers.
5. Discard sections less than 2 cm wide.
6. From each layer, make a strip 3-4 mm wide that includes the epidermis and inner tissues.

7. From the strip, cut perpendicular to the epidermis so that you will have 3-4 mm cubes. Each cube should have a piece of the epidermis. These tissues are more responsive for callusing.
8. Inoculate each explant cube onto the solid culture media consisting of MS, 40 g/L sugar, 1 ppm 2,4-D and 1 ppm BAP, 5 g/L agar (HiMedia), pH 5.7.

Important! If there are protocols reported for your bamboo species, first try these protocols. If your cultures do not form callus, try other media summarized in Table 4.1. It will be a good idea to try several culture media at the onset of experimentation.

Note: See appendices for the preparation of culture media and aseptic techniques. Depending on the width of the vessel used, the number of explants may be more than one. In vessels 18-20 mm diameter (like test tubes), inoculate one explant. In screw-capped bottles (4.5 cm outer diameter), you may inoculate up to 5 pieces.

9. To minimize browning, you may opt to extract the internode layer and immediately process this to get the explant cubes for inoculation. However, you are stuck with a shoot in the laminar flow cabinet, which is not very convenient.

10. Place the cultures in the dark immediately after inoculate to control browning.

Note: You may put cultures in a cabinet, in paper bags or wrap your vessels in carbon paper. Cultures are very susceptible to heat and require cool temperatures from 20 - 25 ^{o}C. The lower setting was attained using a growth chamber. However, in the absence of a growth chamber, cooling was achieved with the use of an airconditioner. Temperature ranged from 23-25 ^{o}C. Expect some growth (either enlarging, callusing or both) within a month.

F. Subculture and selection of calli (Fig. 4.5)

1. Inspect the explants two weeks after culture.

2. Transfer all explants to fresh media every two weeks.

3. At the end of the second month (after three transfers), sort out cultures that have formed callus.
4. Discard brown and unresponsive cultures.
5. Transfer the remaining cultures (those with callus growth) to fresh media and incubate in darkness under recommended temperatures.
6. Inspect after a month and select cultures with compact type-calli.

7. Discard the rest of the cultures, including the loose-type calli.

G. Propagation of compact-type calli

1. Transfer the compact calli to fresh medium containing BA and 2,4-D monthly.
2. From the compact-type calli, you will recover somatic embryos, which may be propagated by regular transfers to fresh 2,4-D medium.

Note: Avoid making cuts or injuries on the cultures that may induce browning.

H. Shoot and shoot-like structures and plant regeneration (Fig. 4.6)

1. Observe shoots and shoot-like structures that will form from the compact-type calli even in the presence of 2,4-D or BAP in the medium.
2. Transfer the cultures bearing shoots and shoot-like structures to semi-solid medium (one-fourth strength MS macronutrients and micronutrients) with 5 ppm NAA to recover plants. Check Table 4.1 for other culture media for germination of somatic embryoids/rooting of shoots.

3. At this point, you may micropropagate by multiple shoot formation to maximize the plantlets.

4. Each plant can be used as the initial plant source of nodes for multiple shoot formation.

II. UNEXTENDED AND EXTENDED BRANCHES

A. **Collecting branches** (Fig. 4.7)

1. Prepare for a collection at the onset of the rainy season

2. Prepare the necessary equipment and paraphernalia for collection, including the culture medium to be used when you return to the laboratory.

   a. Sharp pruning shears

   b. Plastic bags/pail, net bags (heavy duty) and newspapers for wrapping

   c. Clean water to wet newspapers

   d. Markers and shipping tags

   e. Polystyrene box, if collection area is far from the laboratory

   f. Jacket or long sleeved shirt which you can button up

   g. Cap or hat, gloves and boots

3. Inspect the clumps for extended and unextended branches.

   Note: Avoid collecting branches with mealy bugs or aphids. They make decontamination difficult.

4. Cut these branches with a pruning shears and put them in plastic bags or pail with water.

   Note: The branches dry up easily so keep the environment most favorable for them. You may wrap them in moist newspapers and transport them within a plastic bag or net bag, depending on travel time.

5. Bring the branches to the laboratory.

B1. Preparation of nodes and disinfection (Fig. 4.8)

There are other protocols for preparation and disinfection based on literatures that are summarized in Table 4.2. The following are the procedures presently used by the authors.

1. Cut branches into sections. Each section should contain a node. Discard the upper stem bearing the spindle and two-three expanded leaves.
2. Trim the section 0.5-cm below the node and 2 cm from the node to the tip.
3. Select sections with tight enclosing sheaths.
4. With a pad of cotton dipped in 95 % alcohol, wipe each nodal section clean to remove the trichomes.

   Note: You can use 70% ethyl alcohol but it is easier to wipe of trichomes with 95% alcohol.

5. Soak in soapy sterile tap water for 30 minutes, change the soapy water and soak the nodal sections again for 30 minutes. Use a magnetic stirrer. Decant the soapy solution.
6. Soak in antibiotic (1 g/l streptomycin) for 60 minutes. Use a magnetic stirrer. Decant the antibiotic solution.
7. Soak in commercial bleach (5.25% a.i.) for 60 minutes. Use a magnetic stirrer. Decant the bleach.
8. Rinse thrice with sterile water.
9. Transfer the sections onto a sterile steel plate lined with sterile paper.
10. Make a cut along the base of the leaf sheath and remove the enclosing sheath.
11. Inspect for buds. If the section is blind, discard it.
12. Trim stem 1-2 mm above the bud and 3-5 mm below the node.

    Note: Use the bud section for multiple shoot formation and the soft internode tissues above the bud section for establishing callus cultures.

13. Section the soft internode tissues above the bud section into 2-2.5 mm thick slices.

B2. Preparation of unextended branches and disinfection (Fig. 4.9)

1. In the laboratory, wash the unextended branches thoroughly in running water.

2. Trim off the ligules from the leaf sheaths and the hard basal portion with the pruning shears.

3. Wipe the remaining surfaces with a cotton pad wet with 95% ethyl alcohol.

4. Wrap the shoots in a thin layer of cotton.

5. Wet the entire surface with pure bleach. Cover with aluminum foil and let this soak for 60 minutes.

6. Transfer the disinfected unextended branches to the clean air bench.

7. Remove shoots from the bleach by removing the aluminum foil and peeling away the cotton layer.

8. Put each branch in a flask and rinse thoroughly with sterile water (tap or distilled).

9. Take out unextended branches and peel off all sheaths until all soft tissues are exposed.

10. Section the soft tissues (about 3-4 mm thick slices) and inoculate onto a culture medium for callus induction consisting of MS, 40 g/L sugar, 1 ppm 2,4-D and 1 ppm BAP, 5 g/L agar (HiMedia), pH 5.7.

C1. Callus cultures

1. Inoculate the soft internode tissues from lateral branches on the culture medium.

2. Follow procedures for tissues extracted from ground corms for inspection, sorting for

compact and embryogenic calli, e-calli propagation, and plant regeneration.

1. Maximize plants by multiple shoot formation.

C2. Multiple shoot formation from nodes (Fig. 4.10)

1. Inoculate the node sections onto the culture medium for bud break, bud germination and shoot growth.

Note: Culture media for node culture in other species are listed in Table 4.2.

2. Observe bud break within two weeks.

3. Observe shoot growth within four weeks.

Note: You may not be successful the first time you try your hand on multiple shoot formation. Remember that Prutponge and Gavinlertvatana (1992) had success in 54 species but not in 13 species. So, let us hope that your species shall respond to any one of the reported protocols. Otherwise, you guessed it. Back to the drawing board!

4. You may repeat the cycles of cutting up and reculturing to get more multiple shoots.

Note: Saxena and Dhawan (1994) demonstrated that multiplication could be induced for 15 cycles. Beyond this limit, the material did not form any more shoots.

D. *In vitro* rooting

1. Prepare appropriate culture media for *in vitro* rooting.

Important! First, check Table 4.3. Is your bamboo species in the list? Try the medium for rooting others have been used for your species.

2. Using aseptic techniques and working in a clean air cabinet, break up the multiple shoots.

3. Inoculate the shoots onto a culture medium for rooting.

4. Maintain the cultures under daylength and incubation temperature appropriate for your species.

5. When roots have formed and developed, you may acclimatize them for potting out.

Thought For the Section:

> *Either make the tree good, and his fruit good; or else make the tree corrupt, and his fruit corrupt; for the tree is known by his fruit.* ***Matthew 12:33***

A good bamboo? Micropropagators are generally not the ones tasked to determine whether a bamboo is good or not. Technically trained breeders are not a common people for bamboo because of technical limitations including the infrequency of flowering, non-synchrony in flowering, sterility and long juvenile growth stage. However, there is also a perception that bamboos are unlimited natural resources and that bamboos can readily adapt to changes in the environment.

We rely on field men who have observed clumps in field trials to determine a better bamboo clone. As micropropagators, we hope that we can become partners with field men in propagating better bamboo.

Section 5. Getting Ready for the Real World

An excellent micropropagation protocol needs to address potting out requirements of the tissue cultured plants. Within the confines of the culture vessel, plantlets are pampered with a continuous supply of nutrients. Humidity is high as evidenced by water droplets on the sides of the vessels. These pampered plants are fragile and require hardening to prepare them for the conditions in the nursery.

Acclimatization is the process whereby these plants become adjusted to the real world! The possible shift from the environment provided within the culture vessel to the rigors of a nursery is to be addressed. *In vitro* plants are prepared for these changes by one of several techniques or by their combinations:

1. Change the culture medium.

   Some opt to increase the sugar concentration in the last stage of culture, while others reduce sugar.

2. Reduce the relative humidity within the vessel.

   The relative humidity can be reduced by removing the vessel closures. The stomates of the plantlets need to get used to some stress to do work!

3. Transfer the cultures to room temperature.

   Laboratories in the tropics need to address the possible negative effects of the shift from air conditioned culture rooms to elevated temperatures in the nurseries. Generally, the cultures are grown under ambient temperatures before moving them to the nursery area.

4. Increase light intensity in the culture room during the rooting stage.

The typical laboratory combines several techniques so that the plants are better acclimatized.

After acclimatization, the next step is to get the plantlets out of the sterile confines of the culture vessel and into a potting mix which will support their subsequent growth and development. The readiness of the plant for potting out, the potting mixture and the environment (light intensity, temperature and wind factor) and the hardening techniques prior to potting out all combine and contribute towards the successful transfer of tissue cultured plants to the real world.

In this section, are the techniques we found workable in Los Baños, Philippines. Some published methodologies are also included.

A. Acclimatization of plantlets for potting out

1. Inspect cultures if the plants have formed sufficient roots (Fig 5.1). Plants should have at least two roots because unrooted plants have poor chances of survival.

2. Transfer the cultures to room temperature in the laboratory or nearby nursery.

Note: Los Baños, Laguna (Philippines) has rainy and dry seasons. Temperatures range from 22.7 – 31.5 ^{o}C. A specialized chamber for acclimatization from culture vessels has not been necessary to increase potting out successes.

3. Select a brightly lit area of the laboratory or nursery (49 – 61 watts/m^2). A window sill that is exposed to morning light is a good spot.

Note: Take care that the cultures are not exposed to direct sunlight by midday. They may cook!

If humidity is a problem:

The Institute of Forest Genetics and Tree Breeding (Tamil Nadu) recommends the use of poly-globules for 2 weeks to harden the tissue cultured bamboo plantlets. The poly-globules are cylindrical frames enclosed with clear polyethylene sheets. The poly-globules are opened and the plantlets are transferred to a shade house for a month (ICRFE Technical Bulletin, 1992).

If you have a growth chamber:

There are many types of growth chambers which can be used for acclimatization, but these generally are expensive. Rao, et al. (1990) reported the fabrication of a simpler and less expensive growth chamber for establishing tissue cultured plantlets from culture vessels.

The growth chamber provided light to plantlets by 4 40-W tubelights (cool-white daylight type) fixed at a distance of 30 cm from the plantlets. The fluorescent tube ballasts were located outside the chamber to reduce heat load.

Temperature was addressed through air-conditioning which also took care of dehumidification. Aerosol humidifiers are included in their design to increase humidity and reduce the heat load through evaporative cooling. Two fans which were provided permitted adequate air circulation. There was a provision also for the intake of fresh air. Temperature and humidity were monitored and controlled by digital controllers. A thermostat and an alarm were also installed to ensure safety of the plants in the facility.

The facility could accommodate 12,000 plants for acclimatization at any one time.

If you have imported micropropagated plantlets from another laboratory, you may want to consider the following before potting them out:

1. When you receive the cultures, take them out from the box used during transport. Inspect for contamination. If with fungal infection, transfer them to the nursery area for acclimatization and potting out.
2. Transfer the cultures to a bright location in the laboratory for a few days. Keep them separated from your own cultures. Observe for possible mite infection. This is generally associated with fungal contamination.

   Consider using plantlets as source of some nodes to initiate your own cultures of the species.

B. **The nursery**

1. Consider the area you want to designate as nursery.

   Note: You may want to take the following questions into account:

   Is there enough protection from excessive light and wind at the initial stage of potlet establishment? Is there a way for you to increase the light once the plants are established? Is there sufficient space for transportation, e.g. truck, to maneuver to pick up plants? Will stray animals be a problem? Will you able to recoup your investment?

2. In the tropics, we have several options as to the type of nursery area you may develop (Fig. 5.2). Basically, lathhouses are more than sufficient to meet the requirements.

Potting out and nursery care

Several potting mixes have been evaluated for tissue cultured plantlets of bamboo during the cool (November to January) and warm (February to April run) months under greenhouse conditions (Fig. 5.3). Survival and growth were better during the warm months.

A good potting mix should always retain moisture, have good drainage, be "light" and not easily compacted and provide sufficient nutrients to the plantlets.

If a two-step potting strategy is employed, one can select for a medium which allows for very high survival and repot to mixture which allows for very good plant growth. Or alternatively, one can use a potting mixture that allows high survival and good growth at the same time or a one-step potting strategy.

During the warm months, survival of potted plants was very high (80 - 100%) in soil:sand:compost (2:2:1) while growth was very good on soil:compost and soil:sand and sand:compost. Survival was high (60 - 80%) and growth was good to very good on soil:sand and sand:compost and soil.

During the cool months, survival was very high in sand:coirdust and soil:sand:coirdust and growth was good to very good on soil from creekside and soil:compost. For a one-step potting strategy during the cool months, survival was high and growth was good on soil from the creekside.

Procedures

1. Prepare suitable potting mixture. The mixture of 1:1 (v:v) soil:compost gives consistent results. When possible, sterilize mixture or apply fungicide to the mixture after potting.
2. Moisten potting mixture before use. Put enough potting material to fill pots or plastic bags or plastic cups (or equivalent material) to two cm below the brim of the pot.
3. With a pair of forceps, grasp plantlets at the base of the shoots. Pull gently from the culture bottle. A small amount of water added to the culture bottle can make the harvest of plants easier.
4. Wash the agar medium from the roots thoroughly with plain tap water. Be careful with the root system.
5. Make a hole in the moist potting mixture large enough to plant the tissue culture bamboo. Place bamboo in the hole and gently press soil around the root system. Label plants if several species are to be potted out.
6. Transfer the plants to shaded conditions. Water when needed. Keep surroundings shaded and moist. Avoid overwatering. The clump of young leaves show yellowing and leaf drop when plants are overwatered.
7. Maintain plantlets in the nursery for three to four months (Fig. 5.4). The plants are now ready for further division by miniclump division, repotting to a bigger plastic bag or transport for field planting.

Thought For the Section:

> *"For when the time ye ought to be teachers, ye have need that one teach you again which be the first principles of the oracles of God; and are become such as have need of milk, and not of strong meat. For everyone that useth milk is unskillful in the word of righteousness: for he is a babe. But strong meat belongeth to them that are of full age, even those who by reason of use have their senses exercised to discern both good and evil.* ***Hebrews 5:12-14 (Holy Bible, King James Version).***

In this section, we addressed the need to prepare the tissue cultured plants for the new environments. Tissue cultured plants have been called test-tube babies. They have been pampered in the artificial world of a culture vessel where they were fed daily with nutrients from the culture medium and bathed in moist air. They need to be prepared for the real world so the majority, if not all, may survive and be of use as planting materials.

SECTION 6. TRY MINI-CLUMP DIVISION

A nursery filled with bamboo plants looks like an overgrown lawn. Once you have potted out tissue culture-derived plants in the nursery, it is easy to build up planting materials for bamboo. However, one of the issues raised against mass propagation through tissue culture methodologies is the lack of control of the common propagator over the technology. He has to rely on laboratory-based technicians to produce planting stocks for him. Costs will be high because of the nature of the propagation techniques.

The idea of using tissue cultured plants as mother stocks came about after trying to divide wildlings of bamboo. Wildings were collected from Abra and Zambales, grown in the nursery, and used for an experiment on macroproliferation (Banik, 1991). Macroproliferation makes use of one to two-year-old seedling stocks for propagation. The seedlings are divided to yield several clumps with one or more stems. From our own experiments, we noted that even one-stemmed propagations from the seedlings recovered and developed new stem growths. However, seedling stocks take more than a year to form new stems. It is about a year for *S. lumampao* and up to 2 years for *B. bambos*.

Macropropliferation has been used to describe divisions using tissue-cultured plants by Institute of Forest Genetics and Tree Breeding (ICFRE Technical bulletin, 1992). However, we decided to differentiate plant materials propagated by dividing tissue culture-derived mother stocks apart from macroproliferation by coining the term "mini-clump division". Why did it matter to have a new name? For one, established tissue cultured plants may be divided when sufficient stems and rhizomes have developed in the nursery. This can be three to four months after potting out unlike macroproliferation that can commence only after one to two years after establishment. Division can be done every 2-3 months, depending on species. Although mini-clump division and macroproliferation both involve the

division of juvenile clumps, mini-clumps permit earlier division and shorter intervals between division cycles.

The nursery propagation techniques are simple and can be translated into livelihood projects. The propagation activities can be fanned out from the laboratories to the elderly, housewives or out-of-school youths in the community, providing them income.

Advantages of tissue cultured plants and mini-clumps

Tissue cultured plants of bamboo have the following advantages as planting materials. They are light, small, and compact, easily transported and have high survival rates in the field. Tissue cultured plants potted in small plastic bags planted out in Bataan, Pampanga and Zambales when they were about four, six and eight months old in the nursery showed high survival of 95-100. Older plants had lower survival.

The advantages of tissue cultured plants as planting materials are shared by mini-clumps. They are light. About 4-5 clumps (potted in soil mix) would weigh a kilo. They are small. About 10 to 14 mini-clumps can be packed in an ordinary plastic grocery bag. They are compact. About two thousand plants can be loaded in a long-bed truck to transport them for field planting. Lastly, their survival is high when planting is timed with the onset of the rainy season.

Limitations of mini-clumps

The initial stock plants come from a laboratory and this is where the linkage of propagators to a source laboratory is important. The propagator has to rely on the tissue culture propagator for proper identification of the bamboo.

The nursery propagator has to inquire about the age of the stocks. How many cycles of propagations have these clumps undergone. How old are they? How many genotypes are available?

How is mini-clump division done?

1. Check if the tissue culture-derived plant stocks are ready for division. Inspect the potted out tissue cultured plant if there are at least 5 stems and if the new shoots are developing from the rhizome (Fig. 6.1).

2. Lift the plants to be divided and cut roots outside the plastic bags. Bring plants to the potting area.

3. Check if the other materials needed for potting are available and bring them to the potting area. All these materials should be within reach (Fig. 6.2). We will need the following:

a. Plastic bags (2.5 in x 2.5 in) or disposable cups

   a. Potting medium (1:2 compost and soil or other well-drained potting mixes high in composted plant material)

      Note: Select a potting mix which is not prone to compaction.

c. Water and watering can

d. Sprinkler

   a. Labels and marking pens

1. From among the potted out tissue cultured plants, select clumps with at least 5 stems.

2. Remove the clump from the plastic bag to expose the root system and the rhizome (Fig. 6.3). Gently shake the soil from the root system or dip the roots in water to facilitate root separation.

   Note: (a) It is best to divide the mini-clump before the rhizome strike out of the plastic bag. (b) If clumps are over-grown, trim some stems or leaves. Retain at least 40% of the existing growth or plant recovery is slow.

3. Inspect the rhizome and check for weak points.

   Note: (a) Each division should have 2 or more roots and as much as possible, a new shoot growth. (b) If the soil is compacted and does not easily fall away from the roots (particularly in over-grown plants), dip the root system in water to remove as much mix around the roots.

4. Divide at the weak points along the rhizome to get one to three stems per division (Figs. 6.4 and 6.5).

   Note: There are two options in dividing the clump: (a) Separate the clumps at the weakest points of the rhizome by gently tugging and breaking the rhizome,

   (b) Use pruning shears or scissors to cut the rhizome.

5. Keep all divisions moist, avoiding drying up of leaves.
6. Put the plants into plastic bags half-filled with potting mix (Fig. 6.6). Position the plants in the middle of the bag. Fill with the potting mix.

Note: Minimize time interval between division and repotting.

7. Water plants thoroughly (Fig. 6.7).
8. Transfer to plots and again water.
9. Ensure sufficient shade for the first two weeks.

Note: Some leaves may wilt or dry up.

10. Water sparingly for three days but keep the potting mix moist. Apply water more generously after this period.

11. New stems will grow within 4 weeks.
12. Repeat the procedures when you have sufficient new growth.

Note: (a) The interval between division cycles may be 2 to 3 months, depending on the growth rate of the species and cultural practices in the nursery. (b) Growth rate is faster in open sun with regular watering and nutrient application.

16. If you want to use plants for field planting, grow them for at least another month. Three-to-four month old plants have very high transport and field planting survival.

Micro-enterprises using mini-clump division

The mini-clump divisions can be used to start a livelihood project in the community. Table 1 is a production strategy for multiplying bamboo by mini-clump division. This permits the multiplication of stocks, maintenance of stock plants and sale/disposal of propagating materials.

The division cycle is two months, based on growth rates of mini-clumps of *D. strictus* under open culture and regular nutrient feeding. The interval need to be adjusted to the species. Growth rates vary. Among the species grown in the nursery, *D. strictus* and *B. bambos* produced 4-8 new stems in two months compared to 1 stem in 3 month for *G. levis*.

The methodology is simple and does not require sophisticated materials or structures. It requires the basic skills of a nursery propagator.

Table 6.1. Production strategy of bamboo by mini-clump division after one year following at 2-month division cycle.

MONTHS	MOTHER STOCKS	MINI-CLUMP DIVISIONS	DIVISIONS FOR SALE / DISPOSAL
1	170	500	None
2		Grow on	None
3	500	1, 500	None
4		Grow on	1,000
5	500	1, 500	
6		Grow on	1,000
7	500	1, 500	
8		Grow on	1,000
9	500	1, 500	
10		Grow on	1,000
11	500	1, 500	

12		Grow on	1,000

Based on growth and multiplication rates of *D. strictus*.

Thought For the Section:

"Give us this day our daily bread..." ***Matthew 6:11***

We ask God for food daily. The Lord Jesus instructed his disciples to pray for food daily. This is the same way with plants. In natural stands and once established in the field, bamboos are sturdy and may grow inspite of neglect. However, plants in small plastic bags in the are very prone to drying. You will have to care for them.

Section 7. Making up for lost forests

A. **Preparation of plants, transport and field planting**

1. You can bring the tissue cultured plantlets to the field when the rhizome system is well developed (Fig. 7.1). From our experience, this takes about three months after potting out.

> *Note: With good potting mixes that are loose, rich and well watered, the wait may even be shorter than three months for tissue cultured plants. You may also field plant later if you prefer bigger plants but you have to be careful to feed them well particularly if you will retain them in small plastic bags.*

Mini-clumps are ready two to four weeks after repotting and best for field planting three month after re-potting. Older clumps may also be transported and field planted but the bigger the plant and bag, the heavier the material to be transported.

2. Field-plant bamboo clumps before or during the rainy season. Otherwise, be ready to irrigate them.

3. When you are ready to transport the plants, work within a schedule. A week before transport, lift each bag . Sometimes the roots of the plants have gone beyond the bag and lifting them is part of the acclimatization process. Lifting breaks the roots. However, since plants remain in the nursery, they are recovered in time for transport the following week. Reduce the shade also. Two days before transport, do not water as frequently to further harden the plants, but do not allow them to dry out.

4. Pack the plants according to any of the following suggested techniques, depending on the volume of plants to be transported:

For small quantities:

a. Put plants in plastic bags.

b. Pack them in trays or boxes (Fig. 7.2).

For large quantities:

a. You may use several bags, boxes or trays.
b. Put second or third layers of bagged plants on top of the first layer of bag (Fig. 7.3).

5. Dig a hole sufficient to accommodate some organic fertilizer and the root ball of the plant in the selected site. Mix the organic matter with the soil. (Fig. 7.4).

6. Put the plantlet in the hole and replace the ameliorated soil. Cover the base of the plantlet with enough soil and press firmly around the root system.

7. Water the plantlet after transplanting. Put some dried grass or other mulching material around the base of the plant (Fig. 7.5).

8. Protect plantlet from animals with some twigs (fig. 7.6) of bamboo or other appropriate plants or materials from around the site. Branches of spiny bamboo (*Bambusa blumeana*) are suitable.

9. You can expect rapid growth after initial establishment phase on flat and good soils. Canopy closure was attained 2 years after planting in Bataan (Fig. 7.7a and b).

B. Degraded soils along the slopes of a mountain

Bamboos are very hardy plant materials most suitable for hard environments which you would like to reforest. Bamboos are weeds and compete well with persistent grasses such as "talahib" and cogon.

We have planted TC bamboos and mini-clumps along denuded mountainsides and where the soil was degraded and acidic. The soil was very thin. Stones, rocks and boulders were visible (Fig. 7.8).

To plant along denuded mountainsides, we suggest the following:

1. Plant along the contour. Use an A-frame and other techniques associated with Sloping Agricultural Land Technology (SALT).

2. Try to choose best spot along the contour, inspecting for better soil depth.

Note: If none, create a spot by putting rocks or stones along the contour. Put grass or similar material that will form a matting so that added soil will not be eroded by rain.

3. Dig as deep as possible and put some soil mixed with organic matter at the base of the hole.

4. Position the plant and refill the remainder of the hole with soil ameliorated with organic matter. Composted grass, fresh or composted leguminous leaves are good materials to put around the plants.

Table 7.1. Common leguminous trees.

Tree Species	Common name
Cassia siamea	Thailand shower
Delonix regia	Fire tree
Gliricidia sepium	Kakawate
Leucaena diversifolia	Acid ipil-ipil
L. leucochepala	Ipil-ipil

5. Secure plants with stones and rocks to prevent soil erosion (Fig. 7.9) and help retain moisture around the plants.

6. Cover stones with some grass. Uncovered stones absorb heat and may result in excessive heating of plants.

7. Do not remove the grass around the plants for about two years. This permits good root development. Soil erosion is controlled by the grass, water is retained for a longer period of time around the root zone and humidity around the plants is higher (7.10).

Note: You may cut the grass but leave the roots intact.

8. Whenever possible, apply organic matter to hasten growth.

Bamboo takes two years to re-establish along slopes of degraded lands.

C. Planting in Lahar

Lahar-covered areas are difficult to make profitable because organic matter is low, of its tendency to compaction, retention of heat during the day and rapid cooling at night. However, some areas have become seeded with grass. Although our experience with planting bamboo in lahar is still limited, let us share with you our experiences with this kind of "soil" in Porac, Botolan, Zambales where bamboo mini-clumps survived planting on lahar.

Follow the suggestions that may apply to the site.

1. Dig a small hole sufficient to accommodate some organic fertilizer and the root ball of the plant in the selected site. Mix the organic matter with the lahar.

2. Put the plantlet in the hole and replace the ameliorated lahar. Cover the base of the plantlet with sufficient lahar and press firmly around the root system. Composted grass or leguminous leaves are good materials to put around the plants.

3. Water the plantlet after transplanting. Protect plantlet from animals with some twigs of bamboo or other appropriate plants or materials from around the site.

4. Allow grass to grow near the bamboo plants to function as "nurse plants" (Fig 7.11). Water is retained for a longer period of time around the root zone and humidity around the plants is higher. Temperature in the microenvironment shall also be cooler with the companion grasses.

5. Whenever possible, apply organic matter to hasten growth.

Note: As a general rule, see to it that the plants are supplied with some kind of fertilizer. With problem soils, it is best to use organic fertilizers or if the budget permits, slow release pelletted fertilizers.

Thought For the Section:

"If my people, which are called by my name shall humble themselves, and pray, and seek my face, and turn from their wicked ways; then will I hear from heaven, and will forgive their sin, and will heal their land." ***2 Chronicles 7:14 (Holy Bible, King James Version).***

How we desire to see a barren land greened up, denuded mountainside reforested and riverbanks strengthened to prevent further erosion! How we desire the land to be healed.

Man’s programs address the rehabilitation of land from the natural prospective-"…replant, manage and make sure that all will profit from the venture…" When the grass, the bamboo, the trees have fully covered the mountainsides, will others after our generation conserve and enrich them? Or will the cycle of misuse and neglect arise again? Will the underlying factors to poverty, inequity in sharing fruits of labor, lack of access to appropriate knowledge remain to drive resource-poor communities to cut down the remaining trees?

SECTION 8. HELP ME OUT!

Have you been having some problems with your culture and nursery work? We have many experiences with *in vitro* propagation of bamboo and with tissue culture in general that may help you. In this section, we present possible solutions to the problems we have personally encountered or have heard others encounter.

The proposed solutions were found workable under our conditions. Since your laboratory may have different conditions, some proposed solutions may not be applicable to your situation.

A. SECURING THE INITIAL PLANTING MATERIAL

PROBLEMS	**POSSIBLE CAUSE(S)**	**PROPOSED SOLUTION**
Seed Collection and Transport		
1. Locating flowering clumps and identification of species	a. Lack of contacts b. Lack of knowledge on the geographical location of species	a. Make inquiries with closest forestry department (For Philippines, CENRO/PENRO-DENR, ERDB/ERDS-DENR offices, etc.). b. Look up for the geographical location/distribution of the species of interest. c. Learn about the descriptions/characteristics of the species of interest through available literature to be able to identify

	c. Lack of knowledge about the description or characteristics per species	the flowering clump. d. Conduct surveys and personal interviews with people within the place.
2. Strategy/methods of collecting seeds	Lack of contacts and experience in collecting seeds	Make an inquiry with the closest forestry department – how are the authorities on the bamboo species of interest. Look up the geographical location of the species. Learn about the species through available literature to be able to identify the flowering clump.

A. SECURING . . . (Continued)

PROBLEMS	**POSSIBLE CAUSE(S)**	**PROPOSED SOLUTION**
3. Germination of seeds during transport (before proper processing)	a. High moisture during collection trip b. Wet/moist seeds	a. Avoid transporting materials in plastic bags which allow moisture to build up. Use fine net bags. Store dry in polystyrene boxes that are cool. b. Reduce moisture of seed lot using dessicant, dry charcoal, similar materials that will draw moisture. c. Use a thermos to transport seeds. d. If you collected seeds during a rainy day, dry them as soon as possible by laying them on very absorbent materials, e.g. towel, newspaper,or disposable nappies. You can get the moisture-drawing gels from the baby's nappies and put the seeds on them to dry the seeds during transport.
4. Seeds difficult to collect	a. Flowering culms are tall b. Relative access to flowering clump, i.e. location of the culm.	a. Lay large net underneath the culms, shake culms to bring the seeds down (See Section) b. Use a rope to pull the culm to where the collecting net is laid.
5. Unknown species	Lack of familiarity of seed collectors with the descriptions of the flowering bamboo species	a. Collect plant parts for identification in the laboratory (See Section on bamboo species identification) b. Take a photograph and note down outstanding plant characteristics
Collecting ground corms and lateral branches		
1. Unavailability of ground corms and young lateral branches.	Seasonality of materials.	a. Time introduction to culture of explants during the rainy season. Ground corms and young lateral branches are readily available during the rainy season. b. Irrigate clumps until ground corms are induced. Continue irrigation to ensure rapid growth of corm or lateral branches.
2. Difficulty of cutting lateral branches.	Branches fully mature are hard and not suited for culture work	a. Time introduction to culture of explants during the rainy season. b. Initiate new growths by irrigation.

A. SECURING . . . (Continued)

Transporting ground corms and lateral branches		
1. Drying out of ground corms and lateral branches	Difficulty in maintaining moisture in the materials	Wrap ground corm/branches in damp newspapers, towels or cloth or other similar materials
2. Heating up and cooking of ground corms	Avoiding getting corms cooked during transport	Do not put unprotected ground corms in back of pick-up during transport. Wrap them properly and transport using polystyrene box and other materials that would ensure ground corms are kept cool and acceptable.

B. LABORATORY-BASED ACTIVITIES

PROBLEMS	POSSIBLE CAUSE(S)	PROPOSED SOLUTIONS
General		
1. Preparation of the culture media		
a. Precipitation of salts	High concentration of stock solutions	a. Reduce strength of stock solutions during mixing. b. Add water to your mixing container before adding any stock solution. After adding one stock solution, mix well before slowly adding the next solution. c. Add water to the measuring cylinder before using it for the next stock solution. d. Check sequence of addition of stock solutions.
b. Agar medium does not gel after media sterilization.	a. Gelling quality of new brand of agar b. Low pH	a. Check concentration appropriate for the desired gelling consistency b. Check pH, buffer solution and pH meter.

B. LABORATORY-BASED . . . (Continued)

PROBLEMS	POSSIBLE CAUSE(S)	PROPOSED SOLUTIONS
2. Contamination before inoculation	a. Improper sterilization b. Contaminated storage space (You might have mites in the laboratory). c. Improper storage	a. Check procedures of technicians for proper use of pressure cookers or sterilizers. Check also the pressure cookers/sterilizers gauge if it is working well. b. Check and disinfect storage space for medium.

		c. Check that culture media closures (e.g. plugs, covers) are dry when stored.
Establishment of Cultures from Seeds		
1. Contamination of cultured seeds (fungal mycellium, milkiness around seed or embryo which increases with time, Fig. 8.1)	a. High microbial load b. Inadequate disinfection procedure	a. Reduce microbial load by washing in running water and proper disinfection. b. Bamboo seeds vary in characteristics, e.g. hairy, marked, with grooves, smooth, which influence ease of disinfection
2. Death of embryos	a. Decline of viability associated with storage conditions and length of storage time. b. Disinfection too rigorous and inappropriate.	a. Improving conditions of storage. b. Use seeds as soon as possible. c. Check procedure to minimize over-disinfection or bleaching.
3. Uneven growth of seed batch.	Most probably a genotype response. Each seed is different.	Discard very slow growers. Tag individual seedlings and carry the tag to all subsequent propagations.

B. LABORATORY-BASED . . . (Continued)

PROBLEMS	POSSIBLE CAUSE(S)	PROPOSED SOLUTIONS
Establishment of Cultures from Nodes and Tissues from Lateral Branches		
1. Browning of explants and medium	a. Polyphenols; expected to some degree b. Exposures to light esp. at initial stage of culture.	a. Try activated charcoal (0.1g/l medium). Antioxidants such as citric acid (0.1 g/l, filter sterilized, soak tissues before implanting in medium) and ascorbic acid (0.1 g/l, filter sterilized, soak tissues before implanting in medium). b. Cut explants under water or anti-oxidant solutions c. Shield sections of tissues from light during handling in laminar flow. d. Frequent subcultures (i.e. every 2 weeks or until browning has subsided for *D. latiflorus*) e. Dark incubation
2. Milkiness of culture media	Bacterial contamination associated with the explant or techniques	a. Chec a. techniques for disinfection and tissue culture b. Discard cultures
3. Fungal growth on new cultures	Contamination associated with high microbial load on explant, particularly when pest eggs are on the nodes, inadequate disinfection or improper techniques of handling	a. Evaluate techniques for disinfection and tissue culture b. Pre-treat branches of clumps before nodes are taken for culture to reduce microbial load and pests.

B. LABORATORY-BASED . . . (Continued)

PROBLEMS	POSSIBLE CAUSE(S)	PROPOSED SOLUTIONS
4. Slow response to rooting	a. Inappropriate stage of growth of explant b. Improper growth medium	a. Try getting mature materials. b. Try adding different concentrations of IBA or NAA to medium (0-10 ppm).
Establishment of Cultures from Tissues of Ground Corms		
1. Browning of ground corm tissues during handling	Oxidation	Protect corm after stripping away all sheaths by covering the tissues with sterile paper.
2. Browning of tissues during culture storage (Fig. 8.2)	Oxidation	Protect explants by storage in the dark in an air-conditioned room at 20-22°C.
Subcultures		
1. Slight discoloration of the medium (Fig. 8.3)	Phenolics	a. Avoid making too many new cuts during subculture. b. Check for bacterial contamination. c. Check subculture cycles if overdue.
2. *In vitro* flowering (Fig. 8.4)	Associated with stress; mayne induced by growth regulators in medium	a. Subculture regularly. b. Check incubation temperature and adjust accordingly. c. Monitor other cultures from the same batch.
3. Slowing down in growth rates (Fig. 8.5)	Stress	a. Subculture regularly. b. Check incubation temperature and adjust accordingly.
4. Plant variegation and albinism (Fig. 8.6)	Continuous subculturing	a. Check culture medium requirements for higher subculture cycles. b. Avoid prolonged subculture interval to prevent ageing of cultures. c. Check number of subculture cycle.

C. NURSERY-BASED ACTIVITIES

PROBLEM	POSSIBLE CAUSE(S)	PROPOSED SOLUTIONS
Potting out and nursery cultivation		

1. Plants look dry and leaf are very limp.	Insufficient hardening or acclimatization. Most probably, plants were potted out directly from the airconditioned culture room storage.	Practice acclimatization. Transfer cultures to bright, natural but indirect light before potting out. Put plants that are potted out in a moist place for a week or so, then transfer to a brighter location.
2. Survival is low.	Conditions are not suitable to survival.	a. Make sure the plants are well rooted. b. Use sterile soil mixes. c. Increase shading at potting out and during the first week of cultivation in the nursery. d. Do not expose plantlets to excessive wind.
3. Leaves yellowing	a. If restricted to specific areas, may be due to a pest problem. b. If in general, may be a problem of insufficient feeding	a. Mealy bugs can be washed off with soap and water. (See section on nursery pests) b. Check if plants are regularly fertilized. With compost, plants will require extra feeding after 8 weeks.

D. FIELD ACTIVITIES

PROBLEM	**POSSIBLE CAUSE(S)**	**PROPOSED SOLUTIONS**
1. Low survival	a. Improper timing of planting. b. Brush fire c. Rhizome not developed	a. Time field planting at the onset of rainy season. Avoid planting during summer unless you have irrigation facilities. b. Ensure you have a fire line and clean grass around the plants (about 1 m). c. Check your plants for good rhizome development.
2. Plants wilted after field planting	Root systems were disturbed during nursery operation of moving plants out	a. Reduce leaves in the nursery before field planting. b. Provide shade to newly planted out bamboo.
3. Stems black, burnt look	Brush/grass fire	Water the plants to hasten regrowth from rhizomes if rainy season to far off.

4. Stems always cut back; slow height increases but good new growths	Grazing animals most probably	Put some protection around young clumps.