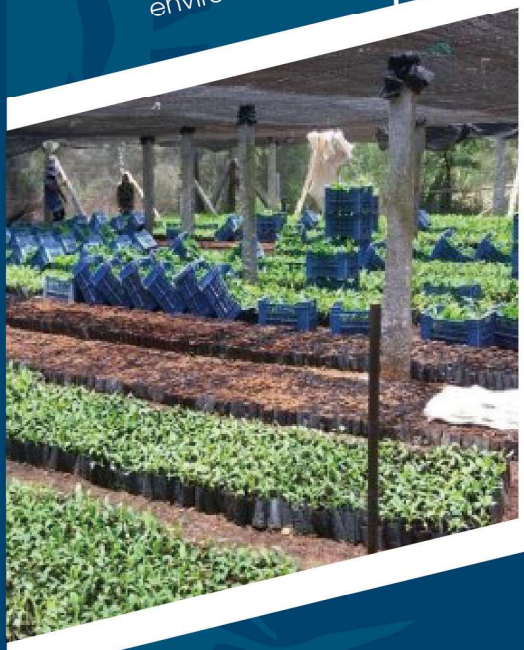
Transfer of Technology Model

Bamboo Micro-propagation (Tissue Culture) Planting Material Production

(This transfer of technology model is an outcome of the IFAD-EU-funded South-South knowledge transfer strategies for scaling up pro-poor bamboo livelihoods, income generation and employment creation, and environmental management in Africa (Phase II) Project in Tanzania, Madagascar and Ethiopia.)



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International Bamboo and Rattan Organisation

INBAR, the International Bamboo and Rattan Organisation, is an intergovernmental organisation bringing together some 44 countries for the promotion of the ecosystem benefits and values of bamboo and rattan.

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Foreword

There are continuous challenges to obtaining quality planting materials to scale-up bamboo plantations. Large-scale bamboo plantations developed for land restoration and commercial utilization mostly depend on the availability of quality planting materials. The conventional as well as the traditional methods of bamboo vegetative propagation (seeds, seedlings, natural regeneration) has limitations and reaches a bottleneck when producing bulk quantities of planting materials. The requirements of small or medium-scale cultivation have been fulfilled by farm boundaries, homesteads, riverbank, and shelterbelts. Promoting a small-scale micro-propagation (tissue culture) production unit could be an alternative approach to mitigate the demand and supply for the sector. This microscale production unit could be established with limited investment in the periphery of the bamboo cluster and address the local value chains. Further, it will encourage the community to set-up tissue culture production enterprises.

The INBAR South-south Phase-II Project has taken initiative in community-led micro-propagation (Tissue Culture) production unit as a business enterprise approach in partnership with PROSPERER (Support Micro-Enterprise Programme Poles Rural and Regional Economies) in Madagascar.

The technical manual, 'Handbook on Bamboo Micro-propagation (Tissue Culture) Planting Material Production', will be used as a ready reckoner. It will help to establish, operate and manage microscale bamboo tissue culture production unit for bare-footed community lab technicians in Africa and elsewhere.

Dr. Hans Friederich

Director General

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Acknowledgements

Commercial tissue culture is essentially the rapid vegetative propagation of bamboo plants and can be done year round. It encourages rapid multiplication within a few weeks and miniaturization of the process such that tens of thousands or hundreds of thousands of plants can be produced in a small space and a short period of time. The concept of tissue culture is based on the fact that a plant can be dissected into small or tiny parts called explants, and since each plant cell has the same genetic information and cellular machinery, it has the ability to regenerate the entire organism. The walls of individual cells can be removed, and two such wall-less cells or 'protoplasts' can be fused or hybridised *in vitro* in tissue culture.

In bamboo micro-propagation, one multiplication cycle takes 3 weeks; in a year, 17 such multiplication cycles are possible. Further, the multiplication achieved in one cycle is 2.5–3.0 times. Thus, it is helpful to produce numerous planting materials for large-scale plantations.

The present technical, 'Handbook on Bamboo Micro-propagation (Tissue Culture) Planting Material Production' is a ready reckoner to help manage commercial micro-propagation labs to produce quality bamboo planting materials. The manual has been prepared for the bare-footed community-led bamboo micro-propagation (tissue culture) sector in Africa and elsewhere. The handbook will identify the priority needs in the bamboo sector.

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The Authors

1. Bamboo Micro-propagation (Tissue culture)

Bamboo Micro Propagation (tissue culture) is essentially the rapid vegetative propagation of plants done in nurseries using sterile tissue culture methods to encourage rapid multiplication within a few weeks and miniaturization of the process such that tens of thousands or hundreds of thousands of plants can be produced in a small space within a short period of time. Unlike in nature, this can be done year round.

The concept of tissue culture is based on the fact that a plant can be dissected into small or tiny parts called explants; since each plant cell has the same genetic information and cellular machinery, it has the ability to regenerate the whole organism. This property of cells is called *totipotency*. Somatic embryos can be artificially produced that behave just like the original embryos and germinate to produce a full plant. The walls of individual cells can be removed, and two such wall-less cells or 'protoplasts' can be fused or hybridised *in vitro* in tissue culture.

Thus, it is a very versatile technique. To initiate a culture, the explants are placed on a semi-solid medium or dispersed in a liquid medium, and the flask, tube or plate containing the cultured part and the medium is then incubated, usually at a temperature close to that of the tissue's normal environment. Sterile conditions are maintained to prevent contamination with microorganisms, since the medium constituents can equally support the growth of contaminants, such as bacteria and fungi. When the plant cells and tissues are cultured *in vitro* ('in-glass'), most generally exhibit a very high degree of plasticity, which allows one type of organ or tissue to be initiated from another type. In this way, the whole plant can be subsequently regenerated.

In bamboo, one multiplication cycle takes 3 weeks. Thus, in a year, 17 such multiplication cycles are possible. Further, the multiplication achieved in one cycle is 2.5–3.0 times. In theory, the following is the number of plants that can be produced in a year starting with one plant with continuous successive multiplication, assuming there is no loss of cultures due to contamination or other reasons. Tissue culture plants are transplanted in potting media. These are allowed to be acclimatised under humid conditions and controlled temperatures and with the controlled entry of sunlight. The potted plants are then transferred to a greenhouse.

The multiplication power of tissue culture

Weeks	Cycles	2.5x	3.0x
3	1	2.5	3
6	2	6	8
9	3	16	19
12	4	39	47
15	5	98	117
18	6	244	293
21	7	610	732
24	8	1526	1831
27	9	3815	4578
30	10	9537	11444
33	11	23842	28610
36	12	59605	71526
39	13	149012	178814
42	14	372529	447035
45	15	931323	1117587
48	16	2328306	2793968
51	17	5820766	6984919

Bamboo tissue culture can increase new avenues for large-scale bamboo planting and generate rural employment and livelihood generators.

Bamboo Micro-propagation (Tissue Culture) Planting Material Production

1.1. Micro-propagation: Benefits

- Rapid multiplication of the desired plant species.
- A limited number of explants is required.
- Small pieces of explants can be used to produce large numbers of plants in a relatively small space.
- Uniform or true-to-type plants can be produced.
- Micro-propagation provides a high degree of phenotypic/physical uniformity. Since the production cycle takes place under controlled conditions, proper planning and scheduling is possible based on the market demand. The resulting product has a very high degree of uniformity compared to traditionally propagated plants.
- Plants can be stored *in vitro* in a small space, and less labour is required for the maintenance of stock plants.
- Plantlets produced by tissue culture are usually disease-free. With a proper diagnosis and treatments, the elimination of any fungus, bacteria or viruses prior to large-scale propagation is possible.

1.2. Culture Conditions

Culture conditions vary widely for each plant, but the artificial environment in which the plant tissue is cultured invariably consists of a suitable vessel containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O_2, CO_2), and regulates the physicochemical environment (pH, osmotic pressure, temperature). Most explants are anchorage-dependent and must be cultured while attached to a semi-solid substrate, while others can be grown floating in a liquid culture medium. Plant tissue culture technology enables the mass propagation of elite, high yielding and disease-free plants throughout the year.

2. The Laboratory: Critical Infrastructure

A commercial tissue culture unit must be designed for a pre-determined level of production. The laboratory design is critical to ensure the productivity and financial viability of the unit.

Plant tissue culture techniques require the following conditions in a laboratory: (a) Aseptic conditions, (b) Temperature control, (c) Light control, (d) Proper culture media, and (e) Trained and experienced personnel.

A tissue culture laboratory should, but not necessarily, be located in an area with moderate climate conditions. An uninterrupted supply of water and power is essential. Since tissue culture has to be carried out under controlled conditions of temperature, extreme exterior climatic conditions add to the cost of maintenance of a conducive internal environment, which then adds to the cost of the plant produced. The design should facilitate maintenance of the required temperature, humidity, illumination and ventilation, and the laboratory should be free from dust particles. The laboratory may be divided into separate rooms, and its size can be decided according to the production capacity of the laboratory.

A steady water supply and drains are essential, since almost every activity in tissue culture operations uses water – washing glass vessels and other equipment, and distillation or reverse osmosis along with demineralization, with such water being used for media preparation. The lab needs 24 hours of constant and stable electricity to run many of its tests.

The cost of power is therefore an important factor in the cost of the plant produced. A power back-up in the form of a universal power supply(UPS) is required since some production cycles may be lost in case of an outage, growth may be affected, and dividing the plant clumps in a sterile transfer facility would not be possible. Reduction in the cost of energy is essential to lower the production cost in a tissue culture unit. Most of the electrical energy is used for autoclaving, lighting of the growth room, air filtration in laminar-flow cabinets and air conditioning.

The profitability of a unit can be increased by using natural lighting and/or renewable energy. The laboratory should have facilities for transport and be near the mother nursery. It is necessary to train two people to operate and manage the lab. Two people are essential so that one can look out for the other person in the interest of safety. A single person is permitted only to inspect cultures.

The lab should have washing facility well-equipped with large sinks with running hot and cold water. It must also have large buckets or tubs to soak the glassware for washing along with detergents, washing brushes of various sizes, draining racks, hot air ovens and a dust proof cabinet to store the washed labware. A good alternative is a dishwasher, in which cleaning can be done at 70°C. One bucket with a lid is required for disposing used or infected media before cleaning; this bucket should be kept outside the room and emptied each day into the toilet.

2.1. Sterilization Room

This room is the section of the laboratory where most activities take place. The media are prepared here and sterilised along with any glassware needed for culture. This room should have sufficient space for a working bench of suitable length for working while standing and be well-equipped with critical tools and equipment, such as a refrigerator/deep freezer to store growth regulators, vitamins, stock solutions for preparing media, vacuum desiccators, double distillation or reverse osmosis systems, analytical balances, digital thermometers, cotton plugs, polycarbonate caps, dispensing pumps, washed bottles, glass rods, etc.

Dos	Don'ts
√ It is very important that cables/wires in the media room are kept away from any source of heat such as heaters, hotplates, magnetic stirrers with heating, and the like. If the insulation were to melt, a short circuit might happen. √ Cotton or similar insulating gloves should be worn while handling hot water and hot media. √ Keep the sterilised media for at least 2–3 days to observe for contamination. Once none has been noted, only then use the media or inoculation. √ Bottles can be wrapped with cling wrap after autoclaving.	√ Do not fully tighten the lids of glass bottles during autoclaving. Keep them slightly loose to allow the steam inside to escape. Tighten the lids after sterilization and, even then, only lightly tighten them. √ As the steam inside the bottle condenses, negative pressure is created which can cause the thin part of the cap to cave in and make it very difficult to open the bottle. For other bottles where the cap does not cave in, there is a chance that the ingress of air from outside that takes place possibly draws in contamination, if the air is not clean. Ideally, the cooling of the medium should be in as sterile a place as possible, though this might not be an issue. The presence of contamination will demonstrate whether it is an issue or not.

2.2. Autoclave Room

Autoclaving relies on the sterilization effect of superheated steam under pressure (a domestic pressure cooker can be used). The size of the equipment used can be as small as 10 litres or as large as several hundred litres depending on the size of the lab.

Most instruments/nutrient media are sterilised with an autoclave, which has a temperature range of 115–1350C. The standard temperature condition for autoclaving is 121ºC at a pressure of15 pounds per square inch (psi) for 15–30 minutes to achieve sterility. This figure is based on the conditions necessary to kill thermophilic microorganisms. The time taken for liquids to reach this temperature depends on their volume in the container and may also depend on the thickness of the vessel. A temperature of 1210C can only be achieved at 15 psi. The efficiency of the autoclave can be checked in several ways. The most efficient way is to use an autoclave tape.

When the autoclave tape is autoclaved, dark diagonal stripes appear on the tape that indicate that it has been autoclaved. The autoclave room must have a fire extinguisher and a slab to put the glassware on both before and after autoclaving.

Dos	Don'ts
√ Check the water level at the bottom of the autoclave and also the pressure setting before switching on the autoclave. √ Ensure that the lid of the autoclave is properly closed. √ Ensure that the air-exhaust is functioning normally. √ Wear heat-insulating gloves while unloading the autoclave.	√ Do not agitate containers of super-heated liquids or remove caps before unloading. √ Do not increase the reduction of pressure after the required time of autoclaving. If the temperature is not reduced slowly, the media will start to boil again. √ Do not screw the bottle tops tightly when they are being autoclaved.

√ The release of steam after switching off the autoclave must be SLOW, or the media in the containers might burst out from their closures because of the fast and forced release of pressure. √ Allow sterilised material to stand for 10 minutes in the chamber after opening the autoclave. This allows the steam to clear and the trapped air to escape from hot liquids, reducing the risk to the operator. √ The bottle tops should be slightly loose. After autoclaving, these bottles are kept in the laminar air-flow. Their tops are tightened after cooling.	√ Inoculation room √ The first and foremost activity of the inoculation room is to maintain aseptic conditions under all circumstances. The room should not have any windows or ventilators. It should be air-conditioned and dust free (even shoes must be left outside). The room should have double doors with an automatic door closer. The floor inside should have a fitted rubber mat to facilitate cleaning. The inoculation room should preferably not have shelves to avoid the accumulation of dust.

2.3. Culture Room

This room has specially designed shelves with slides in several tiers; the slides help in easily placing and accessing culture bottle trays that may be inside.

It is best to use stainless steel for shelf construction, which enhances cleanliness, although other materials could be used as well. The culture room is where the cultures in bottles/tubes are incubated in an environment with controlled temperature, humidity, air circulation, light quality and duration.

The culture room is fitted with a door to make it dust free and to maintain a constant room temperature. Typically, a culture room for the growth of plant tissue cultures should have a temperature between 20–30ºC, with a temperature fluctuation of less than ±1ºC.

The room should have an alarm system to indicate when the temperature has reached a pre-set high or low temperature or a remote read out. The temperature should be constant throughout the entire culture room (no hot or cold spots).

Fluorescent lighting in the culture room should be able to reach 10,000 luces, and both the quantity and photoperiod duration of light should be adjustable. Light and temperature are to be programmable for a 24-hour period. The culture room should have uniform forced-air ventilation; a relative humidity (RH) of 60 per cent is required to maintain healthy growth. In addition, fungus contamination can be an issue. Hence, 40 per cent RH is recommended since this makes it difficult for fungi to thrive and cause contamination. Insects can also be an issue and should be exterminated. A timer device is usually installed for controlling the illuminating system of the room. Cultures can be grown in light or in dark conditions.

2.4. Hardening Centre

The process of acclimatization of *in vitro* grown plants after the rooting step to the normal environment is called hardening. It refers to the nursing of plantlets for the final transfer to field beds. The transition is achieved by gradually exposing the plantlets from conditions of high humidity and diffused light to decreasing humidity and increasing light intensity.

Carbohydrate concentration has an influence on the acclimatization process as the plantlets shift from heterotrophic to autotrophic mode. Carbohydrate treatment increases the photosynthetic capacity and improves plant establishment. The plants are then transferred to a mist chamber or greenhouse, from where they are moved to partial shade and gradually to open areas.

Plantlets can suffer high mortality rates when transferred from *in vitro* to *ex vitro* conditions since they do not have functional leaves and roots. Proper care must be taken during hardening to minimise mortality. It is necessary to aid plantlets in initiating the development of cuticles and stomata/root functions before transferring them to the field growth complex environment.

Transferring plantlets from their culture vessels to polybags or plastic trays (with 20–25 cavities 4–6 inches deep or even 96 cavities 2 inches in depth) should be carried out with a steam sterilised rooting substratum prior to hardening. Misting is done, and repeated treatment with fungicide and nutrient sprays is essential, given the high humidity. The hardening potting mixtures include vermiculite**,** perlite**,** coco peat (coir pith)**,** peat soil, compressed peat pellets**,** soil and sand. They impart protection from transplantation shock, diseases and pests and promote the growth of the plants.

Box: Hardening mixture ratios are plant specific; For example,

Soilrite mix = 75% peat moss: 25%perlite, pH: 5–6.5

Peat moss: perlite: vermiculite (1:1:1)

Red soil: coir pith: sand (2:1:1)

Peat moss: sand: vermiculite (1:1:1)

Peat moss: perlite (2:1)

2.5. Greenhouse or poly-tunnels

Plants are hardened in the greenhouse or poly-tunnels after rooting in the *in vitro* medium or for rooting and acclimatization. This structure consists of transparent walls and a roof that separate the inside environment from the outside. Cooling pads and exhaust fans are present at opposite ends to create a gradient of relative humidity and temperature inside.

Protection from sunlight is provided by placing a shade-net for gradual hardening of the plants. Normally, no trees are allowed to grow over the greenhouse because they may damage the structure if any branches fall. Concrete flooring is not recommended because it may stay wet and slippery from soil mixed with media. The floor should be covered by several inches of gravel for good drainage and to maintain the proper humidity. Good drainage facilities should be provided because various experiments may invite pests and diseases during hardening. Plants are irrigated with inorganic salts for about two weeks.

2.6. The Mother Nursery

The mother nursery is an open stretch of land with facilities for irrigation where the plants are retained until they are dispatched. The mother nursery should be well protected from animals grazing. The mother bed should be equipped with plant-specific nameplates along with an updated register. In addition, water, fencing, electricity and tools are all necessary for a well-established nursery.

Photo: Hardening centre and mother nursery

3. Micro-propagation (Tissue Culture) Techniques

The micro-propagation (tissue culture) should follow certain techniques used to run and manage the production lab or unit. A professionally managed unit will generate revenue to operate successfully and meet any recurring expenses. Customised techniques will guarantee the sustainability of the commercial production unit of micro-propagation (tissue culture).

3.1. Selection of the Mother Plants

Mother plants serve as a source of tissue (explants), and their successful propagation depends solely on the health and vigour of mother plants. Healthy mother plants provide vigorous explants. Proper care and maintenance of these mother plants ensures vigorous and healthy explants. Plants should be well-watered and not experience water stress. Mother plants not only need timely irrigation but also the input of manure and fertiliser at the proper stages. Fungicides and insecticides should also be applied to control diseases and pests.

3.2. Explant Selection

Explants are parts of plants – the shoot tips (or only meristem) or nodal buds– which are taken from the appropriate plant part. The part to be selected often depends on the species to be propagated and the propagation method. Tips of apical or lateral shoots contain meristems, which are most commonly used. The process consists of actively dividing the cells in an organised manner. The top 0.1 mm of the meristem in diameter and 0.25–0.30 mm in length are excised under sterile conditions and implanted on the medium.

Though explants should always be chosen from typical, healthy, disease-free, well-tested mother plants cultivated under controlled conditions, which reduces contamination and promotes the growth of tissues to be cultured, explants may also be subjected to virus testing and elimination.

Box: Criteria for the selection of mother plants:

- √ Mother plants should be vigorous, healthy and high yielding with the desired characteristics or quality parameters.
- √ Mother plants should be pest-, disease- and virus-free.
- √ Mother plants must necessarily be genetically pure and superior in quality and certified from agriculture universities or government nurseries.
- √ Purchase receipts for mother plants should be preserved as they authenticate their origin and quality.

Photo: Quality mother clump

3.3. Preparation of the Media

The methodology for media preparation involves the preparation of stock solutions (in the range of 10x to 100x concentrations) of highly purified chemicals using de-mineralised water. Stock solutions must be stored in glass or plastic containers and either used within a week or frozen until they are needed.

A plant tissue culture medium is determined by the type of plant and tissue to be cultured.

Photo: Lab equipment, chemical and preparation of media

Typically, a nutrient medium consists of the following components:

- Inorganic nutrients (micronutrients – Fe, macronutrients – N, P, K, Ca, Mg and S).
- A carbon source and energy source: sucrose is the most preferred.
- Organic supplements to achieve good growth of cells; vitamins, amino acids and organic acids. Sometimes antibiotics are also added to the media to prevent the growth of microorganisms.
- Plant growth regulators(PGRs), also called as growth or plant hormones, include:
    - Auxins (for root formation): the most commonly added auxins are NAA and IBA.
    - Cytokinins (for shoot formation): the most commonly added cytokinins are 6-Benzyl-Aminopurine (BAP) and Kinetin.
    - Gibberellins: Gibberellin Acid$_3$ (GA_3) is most commonly used for tissue culture. GA_3 enhances linear growth and induces dwarf plantlets to elongate.
- The ratio of auxins and cytokinins play an important role in the morphogenesis of culture systems:

Low auxin to cytokinin ratio = shoot formation

High auxin to cytokinin ratio = root formation

Agar-agar (or agar) is a solidifying agent added to the liquid medium; without agar, the tissue would be submerged in the liquid medium and die due to a lack of oxygen. Gelrite is now increasingly being used because of its transparency. Plant tissues may be

grown in a liquid medium with neither agar nor gelrite, in which case these cultures are aerated regularly either by bubbling sterile air, gentle agitation or the inclusion of a plant part with an internode that reaches the bottom of the tube while the bud is at the meniscus (water level) or just above. There are compounds such as casein hydrolysate, and complex additives, including coconut milk, malt extract, yeast extract and tomato juice, which may be added as prescribed in the protocol. The optimum pH is considered to be 5.7, and this is also very important because the plant cells stop growing in cultures where the pH is higher than 7.0 and lower than 4.5.

Box: MS medium: formulated by Murashige and Skoog (MS) in 1962, it is the most widely used for many types of culture systems; B5 medium: developed by Gamborg for cell suspension and callus cultures and for protoplast culture; and White's medium: one of the first plant tissue culture media.

3.4. Propagation of Cultured Clones

Plant tissue culture techniques can only be used in aseptic conditions created from highly filtered air in an enclosed, sterile environment. Since the surfaces of living plant materials will be naturally contaminated by the environment, which contains microorganisms, the surface sterilization of the starting materials (explants) in chemical solutions is a critical preparatory step. Explants are placed on the surface of a solid culture medium or in liquid medium such that the internode is in the liquid but the bud is at or above the surface. The cultures develop; as they grow, the brown areas are peeled off or removed otherwise. These are then sub-cultured (transferred to a new media) to allow continued growth.

3.5. Shoots/propagules

Shoots are produced from excised shoot tips or nodes. Hormones (cytokinins) are used to induce multiple branching. This is the most common method followed in commercial units. The rate of multiplication must be kept low at 2.5–3.0x to avoid somaclonal variations creeping in. Shoots are likely to be genetically stable.

Normally, a multiplication cycle (the period from incubation of the plant parts in a medium to the visible formation of shoots varies from 3–6 weeks. However, this process is repeated many times by sub-culturing to obtain the required multiplication rates. In addition, the culture must be refreshed with a new mother culture after the specified numbers of cycles are completed.

3.6. Micro-cuttings

Young axillary or adventitious shoots are finally separated from clusters for the initiation and development of their roots. After separation, they are transferred individually to a medium containing rooting hormone (auxin) and maintained in the growth rooms until the roots are formed. It may also be possible to transfer the micro cuttings directly to soil or compost in a humid green house or poly-tunnel for root formation.

4. Lab Management

The most critical stage of propagation by tissue culture is the establishment of the plantlets into the soil. During their growth in a laboratory, plantlets do not photosynthesise, and their control of water balance is very weak. They use sugar contained in the medium as a source of energy.

They exist like bacteria (heterotrophy) and instead need to be converted to a more plant-like existence (autotrophy) and to utilise carbon dioxide from the air and solar energy for producing sugars for their growth. New roots that can function also need to be produced. This acclimatisation to the harsh real environment outside the artificial laboratory milieu takes place gradually.

4.1. Acclimatisation

High mortality rates are noticed when tissue cultured plantlets are transferred from the laboratory to the outside environment. Therefore, plantlets need slow acclimatization to *ex vitro* conditions and are provided with a high intensity of light in low humidity conditions.

Hardening is a treatment that allows firming or hardening of plant tissue. It reduces the growth rate, thickens the cuticle and waxy layers and reduces the percentage of freezable water in the plant. Such plants often have smaller and darker green leaves than non-hardened plants. Hardening results in an increased level of carbohydrates in the plant, permitting more rapid root development than in non-hardened plants.

4.2. The Hardening Process

A starter solution or liquid fertiliser may be applied to the hardened transplants one or two days prior to transplanting or at the same time of transplantation. The plantlets are gradually exposed to greater amounts of sunlight, which results in the development of a thicker cuticle layer, thereby reducing water loss.

5. Sterilisation and Decontamination

Sterilisation is defined as the maintenance of an aseptic environment for successful experimentation. To maintain a sterile environment, all culture vessels, media and instruments used in handling tissues as well as the explant itself must be sterilised. All operations are carried out in a laminar air flow cabinet where UV light helps in the elimination of microorganisms.

Decontamination/disinfection of culture waste, work surfaces and equipment is critical to minimizing risks. Personal protective equipment, such as gloves and eye protection, must be used when concentrated forms of disinfectants are used. The surfaces may be cleaned with 70 per cent ethanol solution and not with bleach, which oxidises and corrodes metal. Ethanol solutions are broad spectrum and suitable for most materials. Any tissue culture waste (culture medium) should be inactivated for a minimum of 2 hours in a solution of hypochlorite (10,000 ppm) before disposal by draining in water (in the toilet).

5.1. Dry Sterilisation

The sterilisation of glassware and metallic instruments can be carried out in dry heat for 3 hours at 160–180°C. Moist heat sterilisation can be done at 121°C, 15 psi for 15 minutes. Glassware and metal instruments like forceps, scalpels, surgical blades, etc., should be wrapped in aluminium foil and autoclaved followed by heating in an oven. Domestic pressure cookers are very useful in sterilising a small amount of media. House-foil is normally of a thinner gauge than needed.

5.2. Steam/Wet Sterilization

Nutrient media are sterilised by using either an autoclave or pressure cookers. For glass containers with a capacity of 20-40 ml of nutrient medium, autoclave at 121°C and 15 psi for 15–20 minutes. An autoclave has a normal temperature range of 115–130°C.

Proper sterilization relies on time, pressure, temperature and volume of the object to be sterilised. The pros of using an autoclave are its speed, easiness to handle and destruction of viruses and microbes, while the demerits are the change in pH by 0.2–0.5 units, components becoming isolated and occurrence of chemical reactions, resulting in a loss of activity of media constituents.

5.3. Filter Sterilisation

Vitamins, amino acids, growth regulators and toxins are heat labile and get destroyed during autoclaving along with other nutrient compounds. Those compounds can be sterilised by filtration through filter membranes of 0.45–0.22 µm. Other membrane filters (Sartorius, Labgene, Millipore, etc.) and related equipment are available for the sterilization of different volumes of the liquid in the range of 1–200 ml. Most filters are made of cellulose acetate, cellulose nitrate or nitrocellulose. During filter sterilisation, all the particles, microbes and viral particles, which are bigger than the pore diameter of the filter used, are eliminated.

Box: Explants taken from the main plant are thoroughly washed in tap water and then in Teepol or Tween 20 for a period specified in individual species protocols and the detergent rinsed off by washing thrice in demineralised water. The explants are then decontaminated and sterilised by one of the following chemical sterilants as per the protocol:

- √ Mercuric chloride 0.01–1% for 2–5 minutes
- √ Sodium hypochlorite 1–1.4% for 5–30 minutes; hydrogen peroxide 10–12% for 5–15 minutes
- √ Calcium hypochlorite 4–10% for 5–30 minutes; bromine water 1–2% for 2–10 minutes
- √ $AgNO_3$ 1% for 5–30 minutes; Antibiotics 4–50 mg/l for 30–60 minutes.

After treatment with sterilants, the explants must be thoroughly rinsed with several changes of sterile distilled water because any retention of such noxious chemicals will seriously affect the establishment of culture. A final rinse with 70% ethanol is often recommended.

5.4. Fumigation

Fumigation helps to minimise contamination and maintain the hygiene of the lab. Hydrogen peroxide (H_2O_2) is a safe fumigant which breaks down into H_2O and O_2, both of which are natural and safe. The virosil pharma is a patented formulation based on hydrogen peroxide stabilised with silver (in cationic forms). It is an excellent fumigant and disinfectant with a broad spectrum of activity on all kinds of viruses, bacteria, fungi, yeasts, moulds, protozoa and algae. It is non-carcinogenic and non-mutagenic. It is a very effective fumigant and disinfectant providing an environment with microbial containment and a completely safe, sterile area.

6. Conclusions

Bamboo micro-propagation (tissue culture) is a new area of value chain promotion in the bamboo sector. The success of micro-propagation (tissue culture) on bamboo depends on 'cloning' from healthy mother clumps. The seeds and seedlings are not always available, and tissue culture is an alternative approach to produce the bulk quality of planting materials within a short span of time and investment. It will meet the demand and supply for a large-scale plantation. Setting up a production unit on bamboo tissue culture also presents challenges in sourcing readily available chemicals and developing 'protocols'. There are readily available protocols for South Asian bamboos. This technique can be used through south-south knowledge transfer in Africa for bamboo sector development.

Glossary

Adventitious: The development of organs such as buds, leaves, roots, shoots and somatic embryos from shoot and root tissues and callus.

Asepsis: without infection or contaminating microorganisms.

Aseptic technique: procedures used to prevent the introduction of microorganisms such as fungi, bacteria, viruses, and phytoplasmas into cell, tissue and organ cultures, and cross contamination of cultures.

Axenic culture: A culture without foreign or undesired life forms but may include the deliberate co-culture with different types of cells, tissues or organisms.

Callus: An unorganised mass of differentiated plant cells.

Chemically defined medium: A nutritive solution or substrate for culturing cells in which each component is specified.

Clonal propagation: Asexual multiplication of plants from a single individual or explant.

Clones: A group of plants propagated from vegetative parts, which have been derived by repeated propagation from a single individual. Clones are considered to be genetically uniform.

Explant: An excised piece or part of a plant used to initiate a tissue culture.

In vitro: Latin: 'in glass'–a culture of an organism or a portion of it in glass or plastic ware on synthetic media.

Ex vitro: Latin: 'outside glass'.

Meristem: A group of undifferentiated cells situated at the tips of shoots, buds and roots, which divide actively and give rise to tissue and organs.

Micropropagation: The multiplication of plants from vegetative parts using tissue culture.

Propagule: A portion of an organism (shoot, leaf, callus, etc.) used for propagation.

Subculture: The aseptic division and transfer of a culture or portion of that culture to a fresh nutrient medium.

Sterilization of Glassware: This may be done in a hot air oven at 160–180ºC for 2–4 hours. Sterilization can also be done in an autoclave.

Sterilization of instruments: Stainless steel instruments are dipped in 70 per cent ethanol for incineration followed by flaming and cooling.

Sterilization of nutrient media: Culture media in bottles and tubes are sterilised by autoclaving at 15 psi for 15 minutes, but this time should be extended based on the volume and experience with contamination. Autoclaving denatures the vitamins, plant extracts, amino acids and hormones; therefore, the solutions of these compounds should be ideally sterilised using filters with a pore size of 0.2 micrometre. However, sterilization is commonly done using the autoclave by compensating for losses.

Sterilization of plant materials: Disinfectants are used to sterilise the surface of the plant material. Some disinfectants are Sodium Hypochlorite, Hydrogen Peroxide, Mercuric Chloride or Ethanol. Washing with sterile demineralised water to remove the disinfectants was also done.

Sterilization of the culture room and transfer area: Exposure to UV light can be used for sterilization, and it is also used to sterilise the laminar airflow cabinet before starting work. The duration of exposure to UV light is 30 minutes, during which the laminar flow should be on. The laminar flow can be kept off if there is a front cover for the work area.

Tissue culture: *in vitro* culture of cells, tissues, organs and plants under aseptic conditions on synthetic media.

Totipotency: Capacity of plant cells to regenerate into a whole plant when cultured on appropriate media.

Table 1: Chemical Composition

S. No.	Components	Quantity (g/l)	Volume/litre of final medium
1. Macronutrients			
	NH_4NO_3	1.64	1000 ml
	KNO_3	1.94	
	$CaCl_2.2H_2O$	0.44	
	KH_2PO_4	0.17	
	$MgSO_4.7H_2O$	0.37	
	Meso inositol	0.1	
2. Micronutrients		g/100 ml	
	H_3BO_3	0.620	1 ml (from stock)
	$Na_2MoO_4.2H_2O$	0.025	
	$CoCl_2.6H_2O$	0.0025	
	$CuSO_4.5H_2O$	0.0025	
	$ZnSO_4.7H_2O$	0.860	
	$MnSO_4.4H_2O$	2.230	

S. No.	Components	Quantity (g/l)		Volume/litre of final medium
3.	KI	0.839		1 ml (from stock)
4.	Fe EDTA	0.73		5 ml (from stock)
5. Vitamins		g/50 ml		
	Thiamine-HCl	0.005		1 ml (from stock)
	Nicotinic acid	0.025		
	Pyridoxine-HCl	0.025		
6.	Glycine	0.04	(g/100 ml)	5 ml (from stock)
7.	Sucrose	30 g/L		
	pH	5.5 to 5.8		
8.	Agar	8 g/L		

(Concentrated solutions of the desired composition of media, called stock solutions, are prepared and can be diluted adequately each time according to the requirements. Simple stock solutions contain a single constituent, whereas complex stock solutions consist of several chemicals. Stock solutions of macro- and micro-nutrients, vitamins and growth regulators are prepared for distilled or demineralised water. Chemicals should be of the highest analytical grade.)

Table 2: Quick Solutions

Growth Regulators (used as mg/l)	Mg needed for 50 ml for 1 millimole	Mol. wt.
Auxins		
Indole -3 acetic acid (IAA)	8.25	**175.2**
Indole -3 butyric acid (IBA)	10.16	
1-Napthaleneacetic acid (NAA)	9.31	**186.2**
2-4 Dichlorophenoxyacetic acid (2-4D)	11.05	**221.0**
2,4,5 Trichlorophenoxyacetic acid (NOA)	12.78	
Picloram	12.06	
Dissolve the desired auxin in 2.5 ml of 95% ethanol or 1M KOH or NaOH, in the glass test tube, stir and heat gently, when dissolved make volume with water. Adjust the pH to 5.0		
Cytokinins		
Benzyl adenine (BAP)	11.25	**225.2**
Isopentenyl Adenine (2ip)	10.15	**203.3**
Kinentin (K or Kn)	10.75	215.2

Growth Regulators (used as mg/l)	Mg needed for 50 ml for 1 millimole	Mol. wt.
Zeatin (Z or Zea)	10.95	**219.2**
Dissolve the desired cytokinin in 2.5 ml of 0.5 N hydrochloric acid in the glass test tube, heat gently, when dissolved make volume with water, adjust the pH to 5.0. Alternately, the cytokinins can be dissolved in 0.5 N NaOH.		
Gibberellic acid (GA_3)	17.32	**346.4**
Abscisic acid (ABA)	13.20	
Others		
Thiodiazuron	11	
Silver Nitrate	9	
Dissolve in 0.5 ml of 95% ethanol in a glass test tube, Stir and heat gently. When dissolved, bring the solution up to volume with water. Silver nitrate must be filter sterilised.		

Annex-I: Necessary Glassware/Plastic ware

1.5 L or 2 L plastic measuring containers to prepare the growth medium for small labs during start-up.

Tissue culture bottles of flint glass and non-soda glass (300 ml)

Caps for bottles

Wash bottle (500 ml)

Wash bottle (250 ml)

Thermometer 110°C including a turkey-thermometer

Laboleen, Tween or a similar detergent

Conical flask (250 ml)

Beaker (100 ml, 500 ml and 1 litre in a glass). 1 L, 2 L plastic beakers. Later 5 L plastic beakers should be bought when production has scaled up. For larger laboratories, the media can be prepared in several large batches with appropriate mixers to ensure media uniformity.

Pipette (0.1 ml, 1 ml, 5 ml and 10 ml)

Petri dish (150 x 20 mm, and 75 mm x 20 mm)

Test Tube (25 x 150 mm)

Measuring cylinders (250 ml, 500 ml, 1 L and 2 L)

Round bottom culture tubes with closures like cotton plugs.

Conical flasks (100 ml and 250 ml)

Beakers (100 ml and 250 ml)

Glass rods for mixing

Annex-II: Necessary Chemicals

Potassium Hydroxide	Potassium Iodide
Ammonium Nitrate (or substitute)	Thiamine Hydrochloride
Potassium Nitrate 500 gm	Nicotinic Acid
Calcium Chloride Dihydrate	Pyridoxine HCL
Zinc Sulphate Heptahydrate	Myo-Inositol
Magnesium Sulphate	Glycine
Potassium Dihydrogen Phosphate	Agar Powder
Potassium Nitrate	Gelrite
Cobalt (II) Chloride	Sulphur-free crystalline sugar
Boric Acid	6-Benzyladenine
Sodium Molybdate	Kinetin
FeEDTA salt	Alpha-naphthalene Acetic Acid
Silica gel as a desiccant	Indole-3-Acetic Acid
Ethyl Alcohol absolute 500 ml	Indole-3-Butyric Acid
Gibberellic Acid	

Annex-III: Assessment of the field performance of the plantlets

Key benchmarks	Record the following daily. It might seem tedious, but you will find it helps troubleshoot problems and to benchmark yourself and the lab.
Media produced	Number of litres of media Number of trays and bottles of media produced/day; number of initial media, number of multimedia, number of rooting media Media consumed. Media surplus
Transfer	Number of explants/bottle Trays/lab technician
Contamination (daily)	Fungi Bacteria
Temperature (daily)	Maximum temperature Minimum temperature Maximum humidity Minimum humidity

(The tissue-culture raised plants are assessed for various parameters like the height of the plant, its diameter and the overall field performance. Other data can be included depending on the end use of the plant or flowers that are produced or green leaves if it is a houseplant. The performance data werecompared with those from seed raised control plants of the same age).

Annex-IV: Dress codes

- √ Clothes: pants and a top/shirt are best for mobility. Material could be cotton-polyester mix or cotton. Clothes made of only synthetic materials are not allowed since they are a firehazard.
- √ Lab garments: a cotton-polyester lab coat with short sleeves should be used at work in the lab. This should be washed and sterilised and best changed each day. Alternatively, use disposable overalls.
- √ Gloves (disposable)- it is recommended to work in the sterile areas including the laminar flow cabinet using disposable gloves. This process reduces contamination and dryness of here hands due to frequent dipping in alcohol.
- √ Cap (disposable)- Wear a disposable cap before entering the lab. All personnel, including visitors must do so. The cap must cover all hair. Hair must be tied into a tight bun so that it can be covered.
- √ Mask (disposable)-wear a disposable mask before entering the lab and remove it only on exit. Do not reuse disposable masks.

Annex- V: Pictures depicting the process, production and plantation of Bamboo Micropropagation (Tissue culture)

Preparation of Media

Chemicals Used for the Medium

Preparing stock solutions

Common 12L pressure cooker for autoclaving media bottles

Larger 29L pressure cooker type autoclave

RO unit with demineraliser

Pouring media into culture bottles

Inoculation Process

Initial multiple shoots

Multiple shoots -subculture

Hardening Centre

Transplanting Plants

Bamboo Nursery

Cultivated Tissue Culture Plant

Farm Plantation

The International Organisation for Bamboo and Rattan (INBAR) is an intergovernmental organization established in 1997. INBAR is dedicated to improving the social, economic and environmental benefits of bamboo and rattan. INBAR plays a unique role in finding and demonstrating innovative ways of bamboo and rattan to protect environments and biodiversity, alleviate poverty and facilitate fairer pro-poor trade. INBAR connects a global network of partners from the government, private, and not-for-profit sectors in over 50 countries to define and implement a global agenda for sustainable development through bamboo and rattan.