INBAR Working Paper

INBAR
INTERNATIONAL BAMBOO AND RATTAN ORGANIZATION

Technical Paper

Technical Manual on Tissue Culture Protocol for Bamboo

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2024

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Ministry of Foreign Affairs of the Netherlands

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About the International Bamboo and Rattan Organization

The International Bamboo and Rattan Organization (INBAR) is an intergovernmental organization dedicated to the promotion of bamboo and rattan for sustainable development. For more information, please visit www.inbar.int.

About this Working Paper

This work is an INBAR publication produced as part of the Dutch–Sino East Africa Bamboo Development Programme—Phase II, funded by the Kingdom of the Netherlands and the Chinese government. This program is a triangular South-South cooperation to support livelihood development, food security, and better environmental management by developing robust bamboo value chains and a sustainable bamboo industry in Ethiopia, Kenya, and Uganda.
https://www.inbar.int/project/sino-east-africa-bamboo-development-programme-phase-ii/

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Foreword

INBAR has encouraged initiatives worldwide to utilize bamboo's potential to transform member states' economies. Bamboo is a fast-growing, renewable, and sustainable alternative to conventional woody biomass, with applications ranging from construction to biofuel. Since 2017, the Dutch-Sino East Africa Bamboo Development Programme has been working to develop value chains and support livelihoods in Ethiopia, Kenya, and Uganda. The program aims to apply the experience gained in other parts of the world to promote the role of bamboo plantations in forest landscape restoration, soil erosion control, water conservation, and climate change mitigation and adaptation.

The technical manual provides information on the tissue culture propagation protocols for commercially utilized bamboo species. The Kenya Forestry Research Institute (KEFRI) has undertaken the commendable endeavor of creating a protocol as part of the Dutch–Sino East Africa Bamboo Development Programme. As part of its mandate to enhance forest biodiversity and environment management, KEFRI generates and disseminates technologies for bamboo propagation and product development.

The rapid growth of bamboo species makes them suitable candidates for the restoration of degraded lands, carbon sequestration, and mitigation of climate change. Some bamboo species can sequester between 100 and 400 tons of carbon per hectare. In addition, many bamboo species are used for construction and building materials, as a food source, and as a versatile raw material for fuel, kitchenware, fabric making, and various other purposes.

In Kenya, bamboo has long been an integral part of indigenous Afromontane forests. Bamboo forests used to cover over 300,000 ha of the country but have now been reduced to about 150,000 ha. *Oldeania alpina* (syn. *Yushania alpina*), commonly known as highland bamboo, is the only indigenous bamboo species that grows naturally between the altitudes of 2,200 m and 3,400 m above sea level.

The International Bamboo and Rattan Organization (INBAR) has partnered with KEFRI since 1986 to introduce and validate suitable bamboo species in various agroecological zones in Kenya. However, the lack of seeds and other appropriate propagation technologies has hampered mass production of bamboo seedlings. Recently, INBAR partnered with KEFRI to enhance bamboo germplasm production through tissue culture to accelerate the mass production of seedlings. By taking advantage of tissue culture over traditional culm cuttings and branch divisions, KEFRI aims to promote the mass production of bamboo seedlings of various species from a limited stock source. This protocol is the culmination of research that has resulted in successful tissue culture of the bamboo species *Dendrocalamus asper, D. yunnanensis, D. latiflorus, D. membranaceus*, and *Bambusa longinternode* and their subsequent establishment in the field.

This manual will be a valuable resource for producing high-quality bamboo planting materials in African settings if it is used carefully and thoughtfully.

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Acronyms/Abbreviations

BAP	6-benzylamino purine
HCl	Hydrochloric acid
IAA	Indole acetic acid
IBA	Indole-3-butyric acid
INBAR	International Bamboo and Rattan Organization
KEFRI	Kenya Forestry Research Institute
MG	Milligram
ML	Milliliter
MS	Murashige and Skoog medium
NaOH	Sodium hydroxide
pH	Acidity or alkalinity
psi	Pounds per square inch
RH	Relative humidity
RIM	Root induction medium
SIMM	Shoot induction and multiplication medium
WPM	McCown woody plant medium
°C	Celsius

Executive Summary

In Kenya, bamboo has long been an integral part of indigenous forests, represented by the species *Oldeania alpina* (syn. *Yushania alpina*), commonly known as highland bamboo. This species is the only indigenous bamboo species that grows naturally between the altitudes of 2,200 m and 3,400 m above sea level. The country is endowed with 22 bamboo species introduced from Asia in 1986 and validated for their growth performance in Kenya's various agroecological conditions. Among the best performing of these species are *Bambusa brandisii*, *Bambusa vulgaris* var. *striata*, *Bambusa bambos*, *Bambusa tulda*, *Dendrocalamus membranaceus*, *Dendrocalamus strictus*, *Dendrocalamus asper*, *Gigantochloa aspera*, *Oxytenanthera abyssinica*, and *Thyrsostachys siamensis.*

Bamboo is a valuable natural resource for both the economy and the environment. Over the years, it has continued to gain recognition in Kenya as a multipurpose plant with many uses, including as a timber substitute; a bioenergy source; a sustainable raw material source for micro-, small-, and medium-scale enterprises; and a provider of ecosystem services by protecting the water towers of Kenya. In view of all these benefits, intensifying bamboo cultivation is a vital objective, and efforts have been invested in promoting its growth and commercialization. Such efforts aim to ensure a sustainable supply of bamboo for industrial enterprises and domestic consumption while also contributing to the achievement of national forest and landscape restoration targets. Recently, the plant was declared a cash crop in Kenya, thus increasing its uptake for planting and, consequently, the demand for planting materials.

However, the large-scale propagation of bamboo has been difficult due to species' peculiar flowering behavior, which may occur at very long intervals, and sterility in some species. Moreover, a lack of seeds and other appropriate propagation technologies has hampered the mass production of bamboo planting materials. The most common propagation techniques have been asexual methods conducted through vegetative propagation and macro-proliferation.

Recently, the International Bamboo and Rattan Organization (INBAR), through its Dutch-Sino Bamboo Development Programme—Phase II, partnered with the Kenya Forestry Research Institute (KEFRI) to enhance bamboo germplasm production through tissue culture. The initiative is helping to accelerate the mass production of seedlings following the successful development of a tissue culture protocol for selected bamboo species. The advantages of tissue culture over traditional methods of propagation include greater numbers of propagules generated from a limited stock source in vitro and the compact size of the propagules, making them easier to transport and handle in large numbers. Tissue culture also permits the use of different plant parts (such as leaves, roots, and small nodal segments) that are not traditionally utilized in other propagation techniques, rendering the production of propagules independent of seasons, weather, and climatic conditions. Furthermore, plants propagated through tissue culture are clean and free of disease pathogens.

This technical manual has been prepared in order to illustrate the bamboo tissue culture environment and its applications based on the bamboo protocol developed for the mass production of seedlings of various bamboo species from a limited stock source. The protocol is a culmination of research that has resulted in the successful micropropagation of several bamboo species, namely *Dendrocalamus asper*, *Dendrocalamus bigasper*, *Dendrocalamus yunnanensis*, *Dendrocalamus latiflorus*, *Dendrocalamus membranaceus*, and *Bambusa longinternode*, and their subsequent establishment in the field.

The manual highlights the following key points:

- **How to set up a tissue culture laboratory**: Tissue culture should be conducted in a clean and sterile laboratory environment where all surfaces and benches are thoroughly cleaned and disinfected. This includes a **washing area** supplied with adequate quantities of clean water, a **media preparation area** fitted with the required number of suitable workbenches; an **inoculation room** for use in aseptic culture initiation and the subsequent subculture of plant materials in strictly sterile conditions; an **incubation room** where the cultures are incubated in tissue culture growth incubators and chambers with a specific set temperature, humidity, and lighting per the requirements of the cultured plant species and materials; an **examination area** where constant screening and examination for possible contamination, culture quality, and assessment of growth performance are undertaken; and a **record-keeping area** designated for data capture equipped with computer terminals where records concerning the lab, including records and results of experiments, subculture schedules, personnel records, and other important information are kept.
- **Major reagents required for bamboo tissue culture:** In vitro propagation requires several macro, micro, and minor chemicals, vitamins, hormones, (primarily) Murashige and Skoog medium (MS), McCown woody plant medium, Gelrite or agar, sucrose, 6-benzylaminopurine (BAP), indole-3-butyric acid (IBA), 1M sodium hydroxide (NaOH), 1M hydrochloric acid (HCl), vitamins, fungicide, soilless growth media (e.g., coco peat), and distilled or RO water
- **Steps for protocol development**: Methods for the establishment of explant banks, harvesting and surface sterilization of explants, basal media formulations and preparation, formulation, and preparation of hormone stock solutions.
- **Media preparation:** How to prepare MS medium for shoot initiation and multiplication, as well as WPM medium for root induction.
- **Explant inoculation methods**
- **Shoot multiplication methods**
- **Root induction and development methods (rooting)**
- **Primary hardening and acclimatization** (hardening plantlets with enhanced humidity/with reduced humidity)
- **Nursery phase or secondary hardening:** The plantlets are transferred from the soilless media to a mixture of red soil/garden soil, farmyard manure, and sand in a ratio of 1:1:1 and maintained in a nursery under 50–70% shade for 90 days.
- **Field planting:** This involves transplanting nursery-hardened plantlets into the field while observing species site-matching protocols for your area.

In a nutshell, this manual outlines standard protocols of tissue culture for the production of plantlets from six bamboo species, namely *Dendrocalamus asper, Dendrocalamus bigasper*, *Dendrocalamus yunnanensis*, *Dendrocalamus latiflorus*, *Dendrocalamus membranaceus*, and *Bambusa longinternode*, which have been developed through experimentation in the KEFRI biotechnology laboratory at Muguga. It is intended for dissemination to other tissue culture laboratories that share an interest in bamboo propagation, particularly those in KEFRI's regional centers, to enhance their capacity and standardization of procedures for the generation of bamboo plantlets through tissue culture.

1. Introduction

Bamboos are perennial, evergreen flowering plants constituting the subfamily Bambusoideae of the grass family Poaceae; they comprise 121 known genera and 1,662 species (Canavan *et al.*, 2016). Most bamboo species are native to warm and moist tropical and warm temperate climates. However, many species are found in diverse climates, ranging from hot tropical regions to cool mountainous regions and highland cloud forests, growing in altitudes ranging from sea level up to 4,300 meters (Clark, Londono, and Ruiz-Sanchez, 2015). The heaviest concentrations and largest numbers of bamboo species are naturally found in East and Southeast Asia and on islands of the Indian and Pacific Oceans (Song *et al.*, 2011; Clark, Londono, and Ruiz-Sanchez, 2015; Yeasmin *et al*., 2015; Mustafa *et al*., 2021).

Bamboos include some of the fastest-growing plants in the world due to their unique rhizome-dependent system (Clark, Londono, and Ruiz-Sanchez, 2015; Mustafa *et al.*, 2021). This rapid growth and tolerance for marginal land make bamboo a profitable candidate for the afforestation of degraded lands, carbon sequestration, and climate change mitigation, with bamboo plantations reported to sequester and fix more carbon per hectare than other tropical trees (Song *et al.*, 2011). Many bamboo species are of notable economic, social, and cultural significance and are used for construction and building materials, as a food source, and as a versatile raw material for fuel (charcoal and biofuel), kitchenware, fabric making (textiles), etc. (Song *et al.*, 2011). Bamboo's strength-to-weight ratio is similar to timber, and its strength is generally similar to that of strong softwood or hardwood timber (Anokye *et al.*, 2016; Awalluddin *et al.*, 2017; Wang *et al*., 2019).

Bamboo culms are ready for harvest and suitable for use in construction within about three to five years (Anokye *et al.*, 2016; Kaminski, Laurence, and Trujillo, 2016). Historically a dominant raw material in South and Southeast Asia, bamboo is the foundation of a global industry that has seen significant growth in recent decades, in part because bamboo is highly sustainable compared to other biomass cultivation strategies, such as traditional timber forestry.

In Kenya, bamboo is an integral part of indigenous forests. *Oldeania alpina* (syn. *Yushania alpina*), commonly known as highland bamboo, is the only indigenous bamboo species that grows naturally between the altitudes of 2,200 m and 3,400 m above sea level (Grimshaw, 1999; Muchiri & Muga, 2013; Ndirangu *et al*., 2017). Bamboo forests, which once covered over 300,000 hectares of Kenya, have been reduced to about 150,000 ha ((Ndirangu *et al*., 2017; Zhao Y. *et al*., 2018). Kenya's natural bamboo growing area is mostly concentrated in the high altitude mountain forests, namely, the Aberdare Range, the Mau Forest, Mount Kenya, Mount Elgon, and the Cherangany Hills Forest, and forest areas managed by the national government. Research to introduce and validate the suitability of various types of exotic bamboo to Kenya's agroecological conditions started in 1986. Twenty-two Bamboo species were introduced from Asia. Those found to be suitable for various agroecological zones include *Bambusa brandisii*, *Bambusa vulgaris* var. *striata*, *Bambusa bambos*, *Bambusa tulda*, *Dendrocalamus membranaceus*, *Dendrocalamus strictus*, *Dendrocalamus asper*, *Gigantochloa aspera*, *Oxytenanthera abyssinica*, and *Thyrsostachys siamensis* (Kigomo, 1994).

Bamboo has, over the years, continued to gain recognition in Kenya as a multipurpose plant with many uses, including as a timber substitute; a bioenergy source; a sustainable raw material source for micro-, small-, and medium-scale enterprises and ecosystem services; and protecting the water towers of Kenya. In view of all its benefits, intensifying bamboo cultivation is therefore important, and efforts have been made to promote its cultivation and commercialization. This will ensure a sustainable supply of bamboo for industrial enterprises

and domestic consumption while also contributing to the achievement of national forest and landscape restoration targets. Recently, the plant has been declared a cash crop in Kenya, thus increasing its uptake for planting and, consequently, the demand for planting materials.

The most common propagation techniques for bamboo in Kenya have been asexual methods through culm cuttings and branch divisions, since bamboo seeds are difficult to obtain (Kigomo, 2007). Germplasm production can, however, be greatly accelerated through tissue culture techniques. The advantages of tissue culture over traditional methods of propagation include greater numbers of propagules generated from a limited stock source in vitro and the compact size of the propagules, making them easier to transport and handle in large numbers. Tissue culture also permits the use of different plant parts that are not traditionally utilized in propagation (e.g., leaves, roots, and small nodal segments). Hence, the production of propagules is not dependent on season, weather, or climatic conditions. In addition, plants propagated through tissue culture are clean and free of disease pathogens (Hussain *et al*., 2012).

This publication outlines standard protocols for the tissue culture of six bamboo species, namely *Dendrocalamus asper, D. bigasper*, *D. yunnanensis*, *D. latiflorus*, *D. membranaceus*, and *Bambusa longinternode*, which have been developed through experimentation in the KEFRI biotechnology laboratory at Muguga. It is intended for dissemination to other tissue culture laboratories that share an interest in bamboo propagation, particularly those in KEFRI's regional centers, to enhance their capacity and to standardize procedures for the generation of tissue culture bamboo plantlets.

Plant tissue culture

Plant tissue culture exploits the unique capacity of isolated plant cells and tissues to give rise to whole plants, a phenomenon known as *totipotency*. Thus, small plant parts, known as explants, which could be leaves, roots, stem cuttings, or axillary buds, are rapidly multiplied in a formulated plant growth medium to regenerate whole plants (Bhojwani & Razdan, 1983). Different plant species and, indeed, different explant sources have unique nutritional requirements and hence require different formulations of plant growth media. Generally, plant tissue culture growth media contain a mixture of inorganic mineral salts, organic nutrients, a carbon source, plant growth regulators (PGRs), and media-gelling agents in the case of semi-solid media with water (Bhojwani & Razdan, 1983; Dodds & Roberts, 1985; Duchefa Biochemie, 1996).

Plant tissue culture media components

Inorganic mineral salts

Mineral elements are important for plant growth and development in nature, as well as in tissue culture. Essential mineral elements required by the plant in relatively large quantities (concentrations > 0.5 $mmol^{-1}$) are referred to as *macro elements*, while those required in lesser quantities (< 0.5 $mmol^{-1}$) are known as *microelements*. When mineral salts are added to water, they dissociate and ionize, thereby providing ions, which are the active factors in media (Bhojwani & Razdan, 1983).

There are six essential macro elements: nitrogen, phosphorous, sulfur, calcium, potassium, and magnesium (Bhojwani & Razdan, 1983; Dodds & Roberts, 1985; Duchefa Biochemie, 1996).

Nitrogen (*N*) is a major component of almost all tissue culture media. It is provided in the form of nitrate (NO_3^-) or ammonium (NH_4^+) ions. The salts mostly used are potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), and calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$).

Phosphorous (*P*) is taken up by plant roots in the form of $H_2PO_4^{2-}$. It is important in the synthesis of ATP (adenosine triphosphate), which provides the energy required for cell metabolism. Phosphorous is also an essential component of DNA, RNA, and phospholipids, which give biomembranes their unique structure and regulate enzymatic activity as inorganic phosphate (Pi).

Sulfur (*S*) is taken up in the form of SO_4^{2-} in the roots and reduced before being used in the synthesis of reduced sulfur-containing compounds, such as amino acids, proteins, and enzymes. In its nonreduced form, sulfur is incorporated into sulfolipids and polysaccharides.

Calcium (*Ca*) in plants is in the form of Ca^{2+}, which is largely bound to the cell wall and cell membrane, where it gives strength and regulates the cell membrane structure. The presence of Ca^{2+} in the cell wall helps confer resistance against fungal infections. Calcium also stimulates the activity of some membrane-bound enzymes and has a function in cell osmoregulation. It is also important in cell and root multiplication and pollen tube development.

Potassium (*K*), of all the elements, is found in the highest concentration in plant cells in the form of the potassium ion K^+, which has functions in the regulation of osmotic pressure, enzyme activation, protein synthesis, photosynthesis, ATP synthesis, respiration, cellular extension, and maintenance of ion balance.

Magnesium (*Mg*) is essential for many enzymatic reactions; in photosynthesis; in RNA, protein, and ATP synthesis; for the regulation of intracellular pH; and in the cation-anion balance.

The **micronutrients** in plant tissue include iron, manganese, copper, zinc, boron, molybdenum, chlorine, and cobalt.

Iron (Fe) in plants is mainly bound to chelators and complex compounds. Most plants absorb iron in the form of Fe^{2+} (as opposed to Fe^{3+}) at the root surface. Iron's major function in plants is to form chelates of iron-binding proteins and reversible oxidation-reduction (redox) systems and to stabilize enzyme-substrate complexes. Iron is also important in the biosynthesis of chlorophyll.

Manganese (*Mn*) is taken up by plants as unbound Mn^{2+} ions. It is strongly bound to several metalloproteins, either as a structural part of the binding site of the enzyme or as part of MnII/MnIII redox reactions. Manganese is essential for photosynthesis and enzymatic reactions.

Copper (*Cu*) is taken up as Cu^{2+} or as a copper chelate complex, with the free Cu^{2+} ion being seemingly preferred by the plants. Copper within cells appears mostly as part of enzyme complexes, where it facilitates [$(Cu^{2+})/Cu^{+}$] redox reactions. The element also plays an important function in photosynthesis and is part of the enzyme superoxide dismutase, which neutralizes the highly reactive superoxide anion radical O_2^- formed during photorespiration.

Zinc (*Zn*) is taken up by the roots as Zn^{2+} and transported in the xylem as free Zn^{2+} or as zinc salt of an organic acid. It is a common metal component of a number of enzymes through the formation of tetrahedral complexes, where it acts as the structural and/or regulatory cofactor of the enzyme complex. Zinc is also very important in protein synthesis through the activity of RNA polymerase as well as in the synthesis of indole acetic acid (IAA) by the plant.

Boron (*B*) is taken up by the roots and transported to other parts of the plant through the xylem. It is mostly found as a borate ester on the cell membrane, performing mainly extracellular functions. Boron's function in the plant is not very well understood, but it is believed to be

involved in cell wall lignification and xylem differentiation through the formation of sugar–borate esters, which are part of the hemicellulose fractions of cell walls.

Molybdenum (*Mo*) is mainly present in the form MoO_4^{2-}. Molybdenum is part of nitrogenase and nitrate enzymes; hence, it is involved in nitrogen assimilation in plant cells and in nitrogen fixation in the root tubers of leguminous plants.

Chlorine (*Cl*) is taken up as Cl^- and is very mobile in plants. Its main functions are osmoregulation and the compensation of charges. It plays a role in photosynthesis' photosystem II during the splitting of H_2O to O_2 and $2H^+$ and regulates the opening and closing of stomata and the osmotic potential of vacuoles. Chloride is also important in nitrogen metabolism through the activation of asparagine synthetase, which converts glutamine to asparagine and glutamic acid. Asparagine is the main carrier of nitrogen over longer distances in some plants.

Cobalt (*Co*) is important in nitrogen fixation in the root tubers of leguminous plants through the activity of the cobalamin enzyme, which comprises the metal component of the enzyme. In this case, Co is required for bacterial methionine synthesis, ribonucleotide synthesis, and synthesis of methylmalonyl-coenzyme A mutase, which is involved in the synthesis of leghemoglobin. However, the functions of cobalt in higher plants are unknown.

Organic nutrients

Organic nutrients in tissue culture media comprise nitrogenous substances and carbon sources.

These *nitrogenous substances* include vitamins and amino acids, which are added to the media to supplement the low quantities produced by tissues in vitro. Vitamins play a catalytic function in enzymatic reactions and are required only in trace amounts. Thiamine (vitamin B1) is considered an essential nutrient, while pyridoxine (vitamin B6) and nicotinic acid (vitamin B3) promote plant growth (Dodds & Roberts, 1985). Other vitamins that have been used in tissue culture media include calcium pantothenate (vitamin B5), ascorbic acid (vitamin C), biotin, cyanocobalamin (vitamin B12), folic acid, riboflavin (vitamin B2), p-aminobenzoic acid (PABA), choline chloride, and inositol. There is wide variance in the requirements for these substances by different cultured plant materials; hence, their quantities vary in different media. Although other nutritive materials, such as casein hydrolysate, tender coconut water, corn milk, malt extract, tomato juice, and yeast extract, have traditionally been used to promote the growth of tissue cultured material, the composition of their growth-promoting constituents is undefined and hence may affect the reproducibility of results. In addition, the quantity and quality of these substances may vary with the age, variety, or environment of the donor organism; therefore, their use in tissue culture media should generally be avoided (Bhojwani & Razdan, 1983). Amino acids are not usually added to tissue culture media, with the exception of glycine, which is a constituent of several types of media. However, a few authors also include L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, and L-arginine in their media. Very few have included L-methionine or L-serine (Dodds & Roberts, 1985; Duchefa Biochemie, 1996).

Carbon source. Tissues in culture depend on an external source of carbon, mainly provided in the form of sucrose at concentrations of 2–5% (Bhojwani & Razdan, 1983). Glucose and fructose have both been reported to support the growth of tissues. Maltose, galactose, mannose, and lactose are also known to be utilized by plants, although their use in tissue culture is limited (Bhojwani & Razdan, 1983).

Plant growth regulators (PGRs)

PGRs are naturally occurring or synthetic substances which, at low concentrations, influence physiological processes in the plant, such as growth, differentiation, and development. Plant growth regulators are classified into three main types: auxins, cytokinins, and a group of miscellaneous compounds that affect plants' physiological processes in one way or another. Auxins are a class of compounds that are naturally involved in cell growth, cell division, vascular tissue differentiation, tropism, leaf senescence, leaf and fruit abscission, fruit setting, fruit growth, and ripening (Bhojwani & Razdan, 1983; Dodds & Roberts, 1985; Duchefa Biochemie, 1996). In tissue culture, auxins are used to stimulate the growth of shoot apices, initiate and elongate roots, initiate callus, and induce somatic embryogenesis. The activity of an exogenous source of auxin on cultured plant tissue is believed to be influenced by endogenous levels. IAA is the main naturally occurring auxin in many plants. However, it is readily degraded by light and enzymatic oxidation, limiting its use in tissue culture. Instead, synthetic auxins are more widely used in tissue culture due to their relative stability. The most widely used auxins in plant tissue culture are 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), and IBA. Other compounds used as auxins in some cases include p-clorophenoxyacetic acid (4-CPA), dicamba, picloram, paclobutrazol, and 2,4,5-trichlorophenoxy acetic acid.

Cytokinins are substances which, in combination with auxins, stimulate cell division and also determine the route which cell differentiation takes. They are involved in cell division, morphogenesis, shoot initiation, modification of apical dominance, leaf expansion, delay of senescence, and chloroplast development (Duchefa Biochemie, 1996). In tissue culture, cytokinins are mainly used to induce cell division, morphogenesis, and auxiliary and adventitious shoot proliferation from callus and organs. The most commonly used adenine-related cytokinins in plant tissue culture are zeatin (6-[4-hydroxy-3-methylbut-2-enylamino]purine), kinetin (6-furfurylaminopurine), BAP, and 2-iP (isopentyladenine). Some phenylurea derivatives (e.g., thidiazuron [1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea]) also possess strong cytokinin activity and are used in tissue culture for shoot proliferation (Duchefa Biochemie, 1996).

Solidifying/gelling agents

A gelling agent is usually added to tissue culture media to provide a stable surface on which to plant the tissue. The most commonly used gelling agent is agar, a polysaccharide obtained from seaweeds, at a concentration of 0.8–1.0%. Using a very high concentration of agar can make the media so hard that nutrient diffusion is hampered. A semi-solid medium is preferred. At present, agar is widely replaced by Gelrite, a clear and cost-effective gelling agent.

2. Scope

This protocol applies to the production of *Dendrocalamus asper, Dendrocalamus bigasper, Dendrocalamus yunnanensis, Dendrocalamus latiflorus, Dendrocalamus membranaceus, and Bambusa longinternode* plantlets through tissue culture.

3. Set-up of a tissue culture laboratory

3.1 Set-up

Plant tissue culture is conducted in a clean, sterile laboratory environment where all surfaces and benches are thoroughly cleaned and disinfected. A tissue culture laboratory should be spacious enough to contain all equipment and material requirements. It should be well lit with an adequate amount of natural light, and it should be organized in a logical sequence to

support the tissue culture process. This layout should ensure a seamless flow of activities and free movement of people, supplies, cultures, and final products (Dodds & Roberts, 1985; Beyl, 2011; Suttle, 2011). The lab should adhere to quality standards and ensure security and safety of personnel. Ideally, the main entrance to the lab should have a disinfecting area containing a footbath, washing area, and changing room where persons entering the lab change into clean, dedicated laboratory attire, which they remove when leaving the lab. Traffic to the aseptic sections of the lab should be kept to a minimum.

To maintain sterile working conditions and minimize cross-contamination, it is important to designate specific functional areas as “clean areas” and “semi-clean areas” and isolate the respective activities. Broadly, the main sections of a tissue culture lab include those involved with media preparation, aseptic manipulation of plant material, growth of cultures under controlled environmental conditions, examination and assessment of cultures, and a record-keeping area (Dodds & Roberts, 1985). Examples of key tissue culture activities, each of which should ideally have its own working area, are described in the following sections.

3.2 Washing area

This area is devoted to the cleaning, sterilization, and storage of assorted glassware, plasticware, and tools used in the lab (Figure 1). Common glassware and plasticware found in a tissue culture lab include graduated measuring cylinders, beakers, Erlenmeyer flasks, pipettes, test tubes, petri dishes, culture bottles, and reagent preparation bottles. The washing area requires adequate quantities of water, ideally a large washing sink in the absence of a dishwasher, as well as glassware drying equipment (Beyl, 2011). Cleaned glassware is dried in a drying oven, dry-sterilized in a hot-air oven, and then stored in a dustproof cabinet.

Item	Description of use
 Assorted glassware	Measuring cylinders, beakers, and Erlenmeyer flasks used for measuring, transferring, and dissolving solutions, as well as glass culture vessels for the culture of plant material.
 Hot air drying oven	For dry sterilization of glassware and other tools, such as forceps and scalpel blade holders.

Figure 1 Description of assorted glassware and hot air drying oven used in washing area

3.3 Media preparation area

The media preparation area is fitted with adequate workbenches suitable for working while standing, as well as media preparation equipment, including pH meters, weighing balances,

an RO water purifier, refrigerators, and autoclaves (Figure 2). Chemicals for media preparation should be safely stored in chemical cabinets and clearly labeled (Figure 3). The area should also have enough space for the storage of culture vessels and prepared media prior to utilization.

Item	Description of use
 pHeter	For the precise measurement for reading and setting the required pH of the media and other solutions
 Analytical balance	To provide precise measurement of chemicals and reagents
 Water still	To provide distilled water for the tissue culture processes
 Refrigerator	- For short term storage of stock solutions, chemicals, plant materials, hormones and media
	To wet-sterilize appropriate equipment, tools, and media.

Top-loading autoclave

For the safe storage of chemicals and reagents used in the lab.

Chemical storage cabinet

For dissolving and mixing solutions.

Hot plate with magnetic stirrer

To ensure the accurate measurement of microliter volumes.

Micropipettes

Used with micropipettes to draw accurate microliter volumes.

Micropipette tips

 Sterilizing indicator tape	Used during the autoclaving of materials. A color change on the strip indicates that the sterilization of materials has been successful.

Figure 2 Description of equipment and materials used in the media preparation area (2)

3.4 Inoculation room

This room is used for the aseptic culture initiation and subsequent subculture of plant materials. Strict sterile conditions should be maintained in this room through disinfection of the floor, walls, and benches with a surface disinfectant to minimize contamination during inoculation. If it is not possible to dedicate a separate inoculation room, laminar air flow clean benches (Figure 4) may be located in an isolated corner of a common research lab away from direct wind, drafts, and traffic (Bhojwani & Razdan, 1983; Dodds & Roberts, 1985). Ideally, there should be an easy transfer of media prepared in the media preparation area to the inoculation room through a hatch box to minimize contamination and direct movement by lab technicians. The room should also be supplied with an adequate amount of light and should be comfortable to work in, as it is generally the heart of the laboratory.

Item	Description of use
 Laminar air flow clean bench with gas burner	For the inoculation of plant materials with minimal contamination owing to laminar airflow currents. The gas burner provides a flame for the sterilization of forceps and blades after dipping in 70% ethanol. Ideally, one should avoid gas burners and use a Steripot, which is the most efficient and risk-free method.
 Scalpel blades with blade holders	For cutting and trimming plant tissues to the appropriate size for inoculation.

Figure 3 Description of equipment and materials used in the inoculation room

3.5 Incubation room/Plant growth room

After inoculation, the cultures are incubated in tissue culture growth incubators and chambers with specific set temperatures, humidity, and lighting per the requirements of the cultured plant species and material. Lighting is usually provided using regular or high-output warm white fluorescent tubes, and temperatures are maintained using heaters and air conditioners. Temperatures generally range from 18–26°C, although exceptions may occur for some plant materials. The incubation room should be well aerated with good air movement of 40 air changes per hour to equalize temperatures throughout the room (Figure 5). It should also be kept clean and contamination-free at all times.

Item	Description of use
 Growth room	For the incubation of cultured plant material under controlled temperature, humidity, and lighting conditions. This room has a larger capacity than the growth cabinet. Trays are used for the efficient handling of culture containers inside the growth room. The shelves should be made of transparent glass or preferably mesh that allows air movement.
 Growth cabinet	For the incubation of cultured plant material under controlled temperature, humidity and lighting conditions. Smaller growth cabinets can be used for the culture of just a few samples.

Figure 4 Description of equipment and materials used in the incubation room

3.6 Examination area

The incubated cultures require constant screening and examination for possible contamination and assessment of growth performance. Hence, a clean and sterile examination room provided with enough lighting should be near the incubation room. This room should also be equipped with binocular and compound microscopes to observe microscopic contaminants (Figure 6).

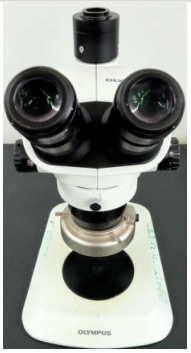
Item	Description of use
 Microscope	For the examination of plant cells, tissues, calluses, and contaminants.

Figure 5 Description of binocular and compound microscopes used in the examination area

3.7 Record-keeping area

This is the area where records concerning the lab are kept, including records and results of experiments, subculture schedules, personnel records, and other important information. It is crucial for the orderly running of activities. Nowadays, software is available for keeping records and storing data on mother plants and cultures. A general sketch of a tissue culture laboratory set-up is shown in Figure 7.

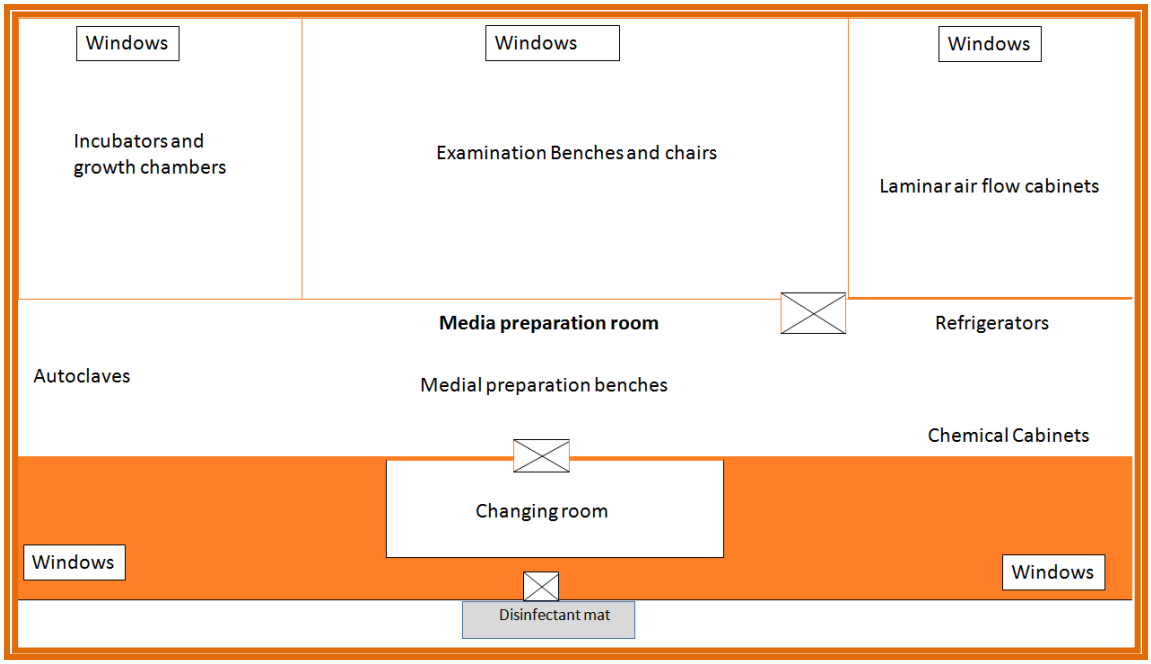


Figure 6 A general sketch of a tissue culture laboratory set-up

4. Major reagents required for bamboo tissue culture

Table 1 List of major reagents required for bamboo tissue culture lab

No.	Reagent	No.	Reagent
1	Murashige and Skoog medium (MS)	8	1 M Hydrochloric acid (HCl)
2	McCown woody plant medium	9	Vitamins
3	Gelrite or agar	10	Fungicide
4	Sucrose	11	Soil less growth media (e.g., coco peat)
5	6-benzylaminopurine (BAP)	12	Sand and natural soil
6	Indole-3-butyric acid (IBA)	13	Distilled water
7	1 M sodium hydroxide (NaOH)		

5. The protocol

5.1 Establishment of explant bank

Method

1. Establish the explant bank from selected sources. Three main commonly used explant sources for bamboo are:
    a. seedling- or wildling-based explant sources,
    b. culm-based explant sources, and
    c. tissue culture-based explant sources.
2. Maintain the explant bank in a greenhouse to minimize cross-contamination from fungal pathogens. Constant spraying with contact and systemic fungicides and insecticides will reduce fungal contaminants.

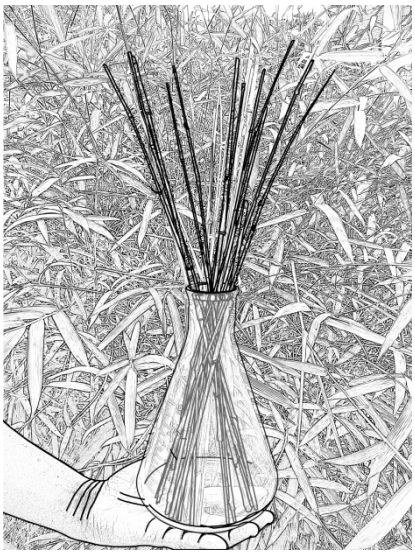
Figure 7 Seedling-based explant bank (left) and tissue culture-based explant bank (right)

5.2 Harvesting and surface sterilization of explants

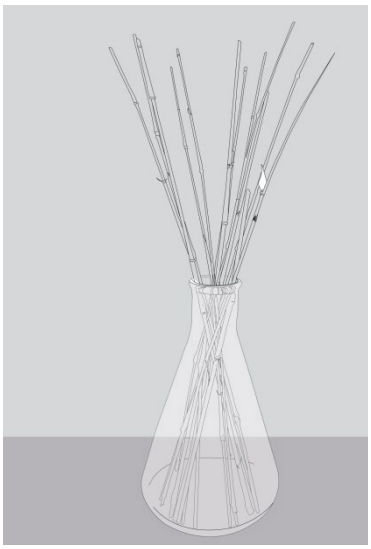
Method

1. Harvest young shoots containing at least four nodal segments from the tip using clean and sterile secateurs.
2. Place the cut shoot segments into a clean sterile flask with sterile water, and immediately transport the materials to the lab in an air-conditioned vehicle.
3. Swab the surface of the shoots with cotton wool doused with 70% ethanol.
4. Remove the covering sheath to expose the lateral buds and cut the shoots into 5–6 cm nodal segments with one to two dormant buds.
5. Surface-sterilize the segments by shaking in 70% ethanol for 30 seconds and draining off the ethanol inside a sterile hood or laminar flow chamber
6. Immediately soak the segments in 15% sodium hypochlorite solution added with a drop of detergent (Tween 20 will do) for 10–15 minutes.
7. Rinse the segments 3–4 times with sterile distilled water on a laminar air flow clean bench.

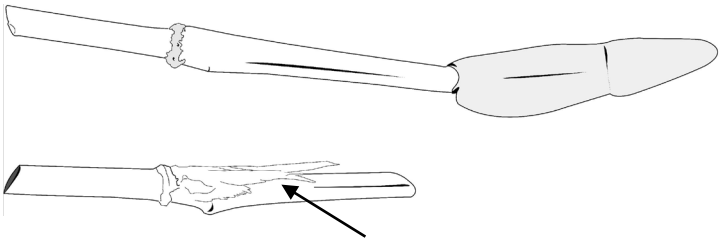
Harvesting of explants (shoot segment with at least four nodes)



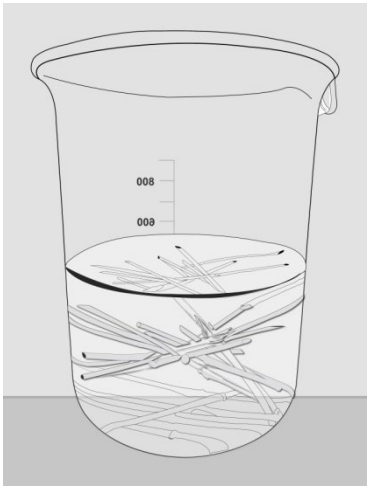
Harvested explants



Explants in the lab



Explant with sheath (top) and with exposed bud (bottom)



Explants soaked in sterilization solution

Figure 8 Illustration of harvesting and surface-sterilization of explants

5.3 Basal media formulations and preparation

The basal media formulation and preparation is to be done based on the procedures below and the stock medium solution in Tables 2 and 3.

Method

1. For basal media use:

    a. commercially available media per the manufacturer's instructions or
    b. stock solution formulations in the lab.

2. In order to prepare stock solutions, make 1 L of MS or 1 L of WPM stock solution as shown in Table 1 for MS and Table 2 for WPM media, respectively.
3. Dissolve each compound **separately** in 100 ml of distilled/RO water.
4. Mix individual solutions together in a 1 L measuring cylinder and top up to 1 L with distilled/RO water.
5. Transfer to a reagent bottle. Label and store in a refrigerator at 4–8°C for up to 1 month.

Table 2 Stock solutions for Murashige and Skoog (MS) medium

Stock I (20X)	**Amount in grams (g/l)**
Ammonium nitrate (NH_4NO_3)	33.0
Potassium nitrate (KNO_3)	38.0
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	8.8
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	7.4
Potassium dihydrogen phosphate (KH_2PO_4)	3.4
Stock II (200X)	
Potassium iodide (KI)	0.166
Boric acid (H_3BO_3)	1.24
Manganese (II) sulfate tetrahydrate ($MnSO_4 \cdot 4H_2O$)	4.46
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	1.72
Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.05
Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$)	0.005
Cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	0.005
Stock III (200X)	
Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	5.56
Disodium ethylenediaminetetraacetate dihydrate ($Na_2 \cdot EDTA \cdot 2H_2O$)	7.46
Stock IV (200X)	
Inositol	20.0
Nicotinic acid	0.1
Pyridoxine HCl	0.1
Thiamine HCl	0.1
Glycine	0.4

Table 3 Stock solutions for McCown woody plant medium

Stock I (20X)	**Amount g/l**
Calcium chloride ($CaCl_2$)	1.45
Calcium nitrate ($Ca(NO_3)_2$)	7.736
Potassium dihydrogen phosphate (KH_2PO_4)	3.4
Potassium sulfate (K_2SO_4)	19.8
Magnesium sulfate ($MgSO_4$)	3.611
Ammonium nitrate (NH_4NO_3)	8.0
Stock II (200X)	
Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$)	0.05
Iron sodium ethylenediaminetetraacetate (FeNaEDTA)	7.34
Boric acid (H_3BO_3)	1.24
Manganese (II) sulfate monohydrate ($MnSO_4 \cdot H_2O$)	4.46
Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.05
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	1.72
Stock III (200X)	
Glycine	0.4
Myo-inositol	20
Nicotinic acid	0.1
Pyridoxine HCl	0.1
Thiamine HCl	0.2

5.4 Formulation and preparation of hormone stock solutions

Method

To formulate 25 mg/l BAP and 25 mg/l IBA stock solutions:

1. Separately dissolve 25 mg of BAP and 25 mg IBA in 10 ml of 1 M NaOH in a flask.
2. Top up with distilled water to make up 1 L.
3. Transfer the stock solution into a reagent bottle. Label, including the date of preparation, and store in a refrigerator at 4–8°C for up to two weeks; preferably, prepare fresh solutions every week.

5.5 Media preparation

5.5.1 To prepare a 1 L MS medium for shoot initiation and multiplication

Method

1. Measure 50 ml of stock I, 5 ml stock II, 5 ml stock III, and 5 ml stock IV into a 1 L Erlenmeyer flask.
2. Draw 160 ml (4 mg/l) from the 25 mg/L BAP stock solution, and add to the result of Step 1 above.
3. Add 30 g of sucrose to the above solution and dissolve in 500 ml of distilled water.
4. Top up the volume to 1 L and adjust the pH to 5.8 using 1 M NaOH and or 1 M HCl as necessary.
5. Add 3 g of gelling agent (2 g of Gelrite will do) and heat to dissolve.
6. Dispense the media into culture bottles, and cap the bottles.
7. Label the bottles to include shoot induction and multiplication medium (SIMM), date of preparation, and any other key information, such as the media batch.
8. Sterilize by autoclaving the medium at 121°C at 15 psi for 15 minutes
9. Once sterilization is complete, remove the media from the autoclave and allow it to cool and solidify. **Open the autoclave only when the temperature falls below 50°C**.

5.5.2 To prepare a 1 L WPM medium for root induction

Method

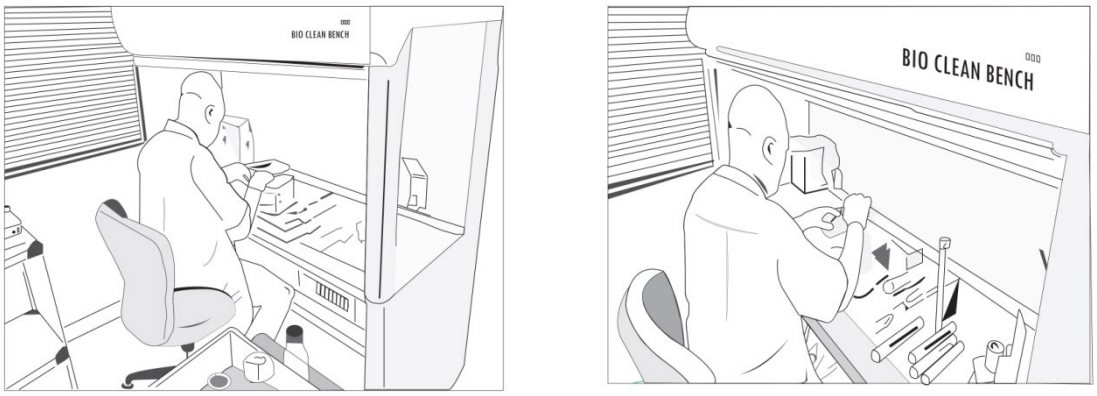
1. Measure 50 ml of stock I, 5 ml stock II, and 5 ml stock III into a 1 L Erlenmeyer flask.
2. Draw 80 ml of IBA (2 mg/l) from 25 mg/l of IBA stock solution.
3. Add 30 g of sucrose to the above solution and dissolve in 500 ml of distilled water.
4. Top up the volume to 1 L and adjust the pH to 5.8 using 1 M NaOH and or 1 M HCl as necessary.
5. Add 3 g gelling agent (Gelrite will do) and heat to dissolve.
6. Dispense the media into culture bottles, and cap the bottles.
7. Label the bottles to include root induction medium (RIM), date of preparation, and any other key information.
8. Sterilize by autoclaving the medium at 121°C at 15 psi (1 bar) for 15 minutes.
9. Once sterilization is complete, remove the media from the autoclave and allow it to cool and solidify**. Only open the autoclave when the temperature falls below 50°C**.

6. Explant inoculations

Method

1. Place the surface-sterilized shoots on a sterile laminar air flow clean bench.
2. Using a sterile scalpel blade, trim the nodal segments to 2.0–3.0 cm in length.
3. Using sterile forceps, inoculate the nodal segment into the SIMM medium in an upright position, with the bud above the medium.
4. Label the cultures with the species name, date of inoculation, and treatment.
5. Incubate the cultures in a growth chamber at 24 ± 1°C at a 16-hour photoperiod.
6. Monitor and assess the cultures regularly and discard contaminated or necrotic cultures by autoclaving as noted above.

The explant inoculation is also illustrated in Figures 10 and 11 below.



Explant trimming

Explant inoculation

Figure 9 Explant trimming and inoculation inside the laminar flow

Freshly inoculated explants

Emergence of shoots

Figure 10 Freshly inoculated explants (left) and the emergence of shoots (right)

7. Shoot multiplication

Method

1. Constantly check for bud break from 7–21 days.
2. After 21 days, aseptically transfer the initiated shoots into freshly prepared SIMM media.
3. Incubate the cultures in a growth chamber at 24 ± 1°C at a 16-hour photoperiod for 14–21 days.
4. Every 21 days, make subcultures of clusters containing 3–5 shoots until the desired multiplication ratio is achieved.
5. Use the subcultures to establish an in vitro explant bank (Figure 12).

Multiplied shoots of *D. membranaceus*

Multiplied shoots of *D. latiflorus*

Figure 11 In vitro shoot multiplication of D. membranaceus and D. latiflorus

8. Rooting

Method

1. Once an adequate number of multiplying cultures have been achieved, transfer clusters of 2–3 shoots into a freshly prepared RIM medium.

2. Incubate the cultures in a growth chamber and maintain them at 24 ± 1°C at a 16-hour photoperiod.
3. Continually check for root induction and development from 14–28 days.
4. Full root induction and development are achieved within 28 days (Figure 13).

Rooted plantlet of *D. asper* 14 days in RIM media Rooted plantlet of *D. asper* 21 days in RIM media

Figure 12 Rooted plantlet of D. asper in RIM media

9. Hardening and acclimatization

Method

1. Sterilize a soilless potting media (coco peat will do) by autoclaving at 121°C at 15 psi (1 bar) for 20 minutes.
2. Fill your preferred containers with the sterile potting media.
3. Carefully remove the rooted plantlets from the culture bottles and thoroughly wash off the media using sterile or distilled water.
4. Transfer the rooted plantlets into the sterile potting media moistened with water or antifungal/antibiotic solution.
5. Grow the plantlets for 28 days in a misted growth room or greenhouse at 26 ± 3°C and 75 ± 5% RH.
6. Constantly check for fungal contamination and manage any contamination with an appropriate fungicide.

7. Reduce the RH to 55 ± 5% for another 28 days at 27 ± 3°C or transfer the plantlets to another misted growth room/greenhouse room with similar conditions.
8. Watch out for fungal/bacterial infections and manage them with an appropriate fungicide/antibacterial.

Hardening plantlets with enhanced humidity

Hardening plantlets with reduced humidity

Figure 13 Hardening of plantlets with enhanced and reduced humidity.

10. Nursery phase

Method

1. Transfer the plantlets from the soilless media to a mixture of forest soil, farmyard manure, and sand in a ratio of 1:1:1, and maintain them in a nursery under 55% shade for 60 days.
2. Transfer the plantlets to 30% shade for another 30 days (Figure 15).
3. Transfer the plantlets to an open nursery for at least 10 days. Ensure regular watering and management of pests and diseases.

Figure 14 Plantlets in the nursery under a 55% shade net.

11. Field planting

Method

1. Transplant your nursery-hardened plantlets into the field while observing species site-matching protocols for your area.
2. For field planting in Kenya, refer to guidelines for the establishment and management of bamboo plantations in Kenya (Kigomo, 1995).

Figure 16: Four-month-old tissue cultured D. bigasper planted in the field

12. Conclusion

Bamboo is a versatile and fast-growing plant that plays a crucial role in both economic and ecological contexts. However, traditional propagation methods, such as seed or macro-proliferation, have their limitations. This manual discussed the requirements for bamboo tissue culture protocols that have been developed through experimentation in the KEFRI biotechnology laboratory at Muguga to overcome the limitations of conventional propagation methods, allowing for the potential production of large amounts of uniform and vigorous plantlets. This protocol applies to the production of *Dendrocalamus asper, Dendrocalamus*

yunnanensis, Dendrocalamus latiflorus, Dendrocalamus membranaceus, *and Bambusa longinternode* plantlets through tissue culture. The manual will be a useful resource to enhance the capacity and standardization of procedures for the generation of tissue culture bamboo plantlets aimed at producing high-quality bamboo planting materials.

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