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STRENGTHENING OF TISSUE CULTURE LAB

INVESTIGATORS

Suma Arun Dev
Genetics & Tree Breeding Department
Forest Genetics and Biotechnology Division

EM Muralidharan
Biotechnology Department
Forest Genetics and Biotechnology Division

Kerala Forest Research Institute
(An Institution of Kerala State Council for Science, Techno logy and Environment)
Peechi, Thrissur, 680653, Kerala

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PROJECT DETAILS

1.	Project No.	:	KFRI RP 787/2019
2.	Title	:	Strengthening of Tissue Culture Lab
3.	Principal investigator	:	Dr. Suma Arun Dev, Senior Scientist, Genetics & Tree Breeding Department, Forest Genetics & Biotechnology Division
4.	Associate investigators	:	Dr. EM. Muralidharan, Senior Principal Scientist, Biotechnology Department, Forest Genetics & Biotechnology Division
5.	Objectives	:	To strengthen the existing infrastructure of the Plant Tissue Culture Facility
6.	Duration	:	2 Years
7.	Funding Agency	:	National Bamboo Mission (NBM), Govt. of India

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ABSTRACT

The plant tissue culture laboratory of the Institute which was established two decades back, essentially required an upgradation of the infrastructure. With the financial support received from National Bamboo Mission (NBM), Govt. of India, the facility has been strengthened to meet the current research targets in the field of Plant Tissue Culture. The following equipments such as laminar flow, autoclave, electronic balance, refrigerator, vertical deep freezer, centrifuge, microbial hot mixer, PCR Thermal Cycler and nano spectrophotometer were purchased as part of the project. The laboratory is well equipped to undertake and standardise micropropagation protocols in various bamboo species and other forest tree species with reproductive constraints, which requires a large number of propagules in a limited time span. With this, the facility can perform the genetic fidelity testing which is essentially required in mass micropropagation programs to assess the true-to-type nature of the tissue culture raised plantlets.

BACKGROUND

Clonal forestry is an option that addresses the limitation of conventional tree improvement so as to make available planting material of proven performance in large numbers (Libby and Ahuja, 1993). Micropropagation has been increasingly used for large scale cloning of forestry species. While *in vitro* culture of woody perennial species is difficult compared to herbaceous species, protocols have been developed over the years to circumvent the major constraints such as higher incidence of microbial contamination and phenolic exudation, poor multiplication and rooting frequencies. Several species have now been commercially micropropagated all over the world. Clonal forestry with the best performing clones derived from selections has been adopted as an alternative strategy. In the case of forest trees, the long lifecycles make conventional breeding difficult since several generations of scientists are required to contribute to the programme.

Tissue culture of woody plants has a history of several decades and a wide range of trees species have been successfully micropropagated and commercialization is yet to be achieved for many of the reported successful tree species (Mascerenhas and Muralidharan 1989). The situation remains more or less the same even today with a handful of species, where the method of choice for commercial propagation is micropropagation. Simlarly, commercial species of bamboos has also been successfully micropropagated by several workers using two major plant regeneration pathways viz. axillary bud proliferation (Sandhu *et al*., 2017, Gielis and Oprins, 2002) and somatic embryogenesis (Gillis *et al*., 2007). While the potential for commercialization has been shown for both the methods, the axillary bud proliferation is preferred in general due to the lower risk of genetic variation. National Bamboo Mission (NBM) has prioritized *Dendrocalamus stocksii* for large scale cultivation in Peninsular India as a substitute in furniture industry owing to its solid nature of culms and good culm wall thickness (Viswanath et al., 2013; Chandramouli et al. 2014). The efficiency to regenerate very profusely in *in vitro* conditions would enable larger scale production of plantlets of the species even in the absence of seed set. A few species of *Bambusa* and other genera also routinely micropropagated in India and other countries.

Commercial utilization of tissue culture plantlets warrants genetic stability among the *in vitro* raised plantlets so as to ensure the intended productivity in line with the selected superior mother genotypes. Somaclonal variation is a major constraint which usually occurs during the de-differentiation and re-differentiation processes in *in vitro* culture when a plant tissue is

maintained through *in vitro* culture process, generally due to the stresses in the culture environments (Venakatachalam *et al.* 2007). These are manifested due to DNA methylations, chromosome rearrangements and point mutations (Phillips *et al.* 1994). Especially in ling lived woody perennials, these variations will be manifested only very late in their lifecycle and the assured productivity cannot be guaranteed in those instances.

One of the earliest applications of Biotechnology was in the tissue culture or micropropagation of commercial or RET tree species through *in vitro* regeneration of plantlets from selected genotypes. KFRI has standardized efficient tissue culture protocols for various forest trees such as teak, rosewood, sandal, bamboos, cane, etc. (Muralidharan, 1994, 1997; Valsala and Muralidharan, 1998; Renuka *et al.* 1998; Muralidharan and Pandalai, 2017) as well as many endangered, endemic and threatened tree taxa (*Dipterocarpus bourdillonii, Humboldtia bourdillonii, Syzygium travancoricum, Cinnamomum veerum, Gluta travancorica, Vateria macrocarpa,* etc.) (Swarupanandan *et al.* 2013) and medicinal plants (*Malaxis rheedii, Wrightia tinctoria, Embelia* spp.) (Muralidharan, 1995; Raghu *et al*. 2017). Most of these protocols have been successful and can be recommended for large scale commercial propagation of forest trees. Further, decades of research could also bring out cost-effective innovations and technological advancements which have great potential to transform a capital intensive micropropagation technique to an entrepreneur-friendly practice through simple modifications of conventional procedures and equipments (Muralidharan, 1995).

The plant tissue culture laboratory of the Institute established two decades before, essentially requires an upgradation of the equipments involved. With the financial support received from National Bamboo Mission (NBM), Govt. of India, the facility has been strengthened to meet the current research targets in the field of Plant Tissue Culture (Table 1).

TABLE 1. LIST OF PURCHASED EQUIPMENTS

No	Equipments	Cost (Rs.)	Sanction Order	Page no.
1	Laminar Flow (2nos.)	1,49,100	No. KFRI/PO 132/787/19-20	1
2	Autoclave	4,17,900	No. KFRI/PO 154/787/19-20	2
3	Electronic balance	60,900	No. KFRI/PO 147/787/19-20	3
4	Refrigerator	55,400	No. KFRI/188/20-21-P-981/20-21	4
5	Vertical Deep Freezer	65,898	No. KFRI/PO 131/787/19-20	5
6	Miniplate spinner centrifuge	56,175	No. KFRI/PO 146/787/19-20	6
7	Microbial hotmixer	2,50,000	No. KFRI/198/20-21/P869/20-21	7
7	PCR Thermal Cycler	3,84,300	No. KFRI/PO 170/198/NBM/2020-21	8
9	Nano Spectrophotmeter	6,93,000	No. KFRI/PO 155/787/19-20	9

1. LAMINAR AIR FLOW (LABLINE, INDIA)

A laminar flow cabinet or tissue culture hood is an enclosed bench which consists of a filter pad, fan and a HEPA (High-Efficiency Particulate Air) filter. The fan sucks the air through the filter pad where the dust is trapped. Then the pre-filtered air passes through the HEPA filter which will remove at least 99.97 per cent of particles whose diameter is equal to 0.3 µm, with the filtration efficiency increasing for particle diameters both less than and greater than 0.3 µm. HEPA filters capture pollen, dirt, dust, moisture, bacteria (0.2-2.0 µm), virus (0.02-0.3 µm), and submicron liquid aerosol (0.02-0.5 µm). Unidirectional air moving at a fixed velocity along parallel lines (laminar flow) could reduce turbulence and aid in the capture and removal of air borne contaminants from the air stream.

Application in Tissue Culture

- Prevent contamination of semi-conductor wafers, biological samples, or any particle sensitive materials
- It has a UV-C germicidal lamp to sterilize the interior and contents before usage to prevent contamination of the experiment

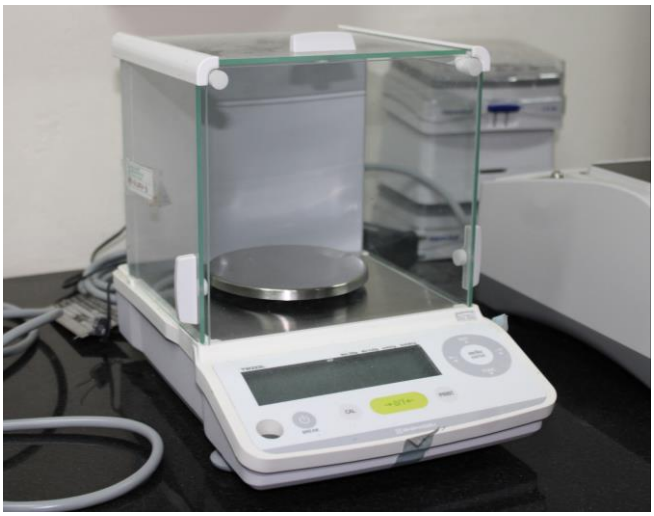
2. AUTOCLAVE (MEDIPHARM, INDIA)

It works on the principle of moist heat sterilisation, where saturated steam is generated under pressure in order to kill microorganisms such as bacteria, viruses, and even heat-resistant endospores from various types of instruments. It is done by heating the instruments within the device to temperatures surpassing the boiling point of water. In the process where pressure and temperature are directly proportional to each other, a high temperature of about 121 °C kills the microorganisms by coagulating the proteins that microbes thrive on, and higher pressure of 15 psi or 775 mm of Hg increases the boiling point of water, which thus increases the temperature of sterilisation. High pressure also helps heat to rapidly

Application in tissue culture

- Used for the sterilization of cuture media, autoclavable containers, plastic tubes, and pipette tips.
- Decontaminate specific biological waste and sterilize media, instruments, and lab wares.
- Regulated wastes including used media that might contain bacteria that are resistant to boiling water and other powerful detergents, viruses, and other biological materials can be inactivated by autoclaving before disposal.

3. ELECTRONIC WEIGHING BALANCE (SHIMADZU, JAPAN)



Weighing balance is used to calculate the weight or mass of an object/chemicals used for plant tissue culture. It has higher readability, measuring to the nearest 0.0001 g, drought-proof camber and anti-vibration tables which enable high accurate measuring. Precision balance has a resolution near 0.1-0.01 g. Both analytical and precision balance don't directly measure the mass but measure the weight (force) acting downward on the measuring pan using an electromagnet. An electromagnetic servomotor is used to generate a force to counter the weight of the mass being measured

Application in tissue culture

- Measurement of biochemicals and media components

4. REFRIGERATOR (HAIER HRF 2984BS-E, CHINA)

Refrigerators work by causing the refrigerant circulating inside them to change from a liquid into a gas. This process, called evaporation, cools the surrounding area and produces the desired effect. To start the evaporation process and change the refrigerant from liquid to gas, the pressure on the refrigerant needs to be reduced through an outlet called the capillary tube. Refrigerators were used to keep samples for short term duration in a low temperature (4 °C–10 °C) which are reactive or degradable at room temperature.

Application in tissue culture

- Used for storing various thermos labile chemicals like vitamins, hormones, amino acids, casein-hydrolysate, yeast extract etc.
- Storage of stock solutions of salts to prevent contamination.

5. DEEP FREEZER (ELANPRO, INDIA)

The Deep Freezer works based on the principle that coils absorb heat from the surrounding air and it is designed and fitted with cooling compressors and CFC free refrigerants. These freezers are installed to create highly effective cooling consistently inside the cabinet. The air cooling compressors of the freezer are designed with aerodynamic fans and washable condense filters which keep the internal environment free from dirt and dust. A deep freezer can reach temperatures of -50 °C to -60 °C within an hour to a few minutes and this allows for rapid freezing of products and is especially useful in industries. Apart from that a freezer which is equipped with two compressors can used to create the temperature below the mentioned limit.

To utilize the efficiency of the compressors and to extend the life of the compressors, the small compressors automatically get turn off whenever the set temperature is reached whereas the big compressor should remain ON continuously for maintain the consistency in temperature.

Application in Tissue Culture

- Used to preserve and store samples at low temperatures for a longer period Deep freezer can be used for the preservation of stocks and reagents
- Unstable media, serum, and reagents can be also stored in a Deep Freezer
- It can also be used for the freeze-preservation of Plant Cell culture

6. SPINNER CENTRIFUGE (LABNET, USA)

Spinner Centrifuge is a small robot-accessible automated centrifuge that provides both vibration and noise control in a small, low-maintenance package. It offers a stable high-speed centrifugation for 3-second cycle times. Ideal for high- or medium-throughput applications such as PCR purification, cell harvesting, and air bubble removal in high-density micro plates, the spinner centrifuge is capable of rapid customizable acceleration and deceleration, minimizing the required cycle time.

Application in tissue culture

- To assist in genetic fidelity of propagated clones.
- Gene expression studies
- Metagenomics and barcoding studies to identify the endophytic contaminations

7. MICROBIAL HOT MIXER (EPPENDORF, GERMANY)

Thermo Mixer (Microbial hot mixer) is a device for the temperature control and mixing of liquids or samples. The device offers a wide temperature control range (1 °C–100 °C), high flexibility with the use of diverse tube and plate formats and advanced mixing performance with a mixing speed of 3000 rpm. Application includes gene expression analysis, immunological methods and protein detection. Cloning DNA fragments, culture of organism, radioactive/non-radioactive labelling of DNA and DNA purification.

Application in tissue culture

- To assist in genetic fidelity of propagated clones.
- Gene expression studies
- Metagenomics and barcoding studies to identify the endophytic contaminations

8. THERMOCYCLER (PCR MACHINE) (EPPENDORF, GERMANY)

The thermocycler is a laboratory apparatus most commonly used to amplify segments of DNA via the polymerase chain reaction (PCR). Most models have a gradient function to allow for different temperatures in different parts of the block. The PCR process is conducted via thermal cycling, a process of heating and cooling that creates the conditions necessary for DNA replication.

The desired DNA fragment is amplified using appropriately designed primers which flanks the region of interest (DNA fragment), dNTPs and DNA polymerase enzyme to catalyse the reaction along with Mg ions present in buffer.

PCR Amplification is achieved by a series of three steps:

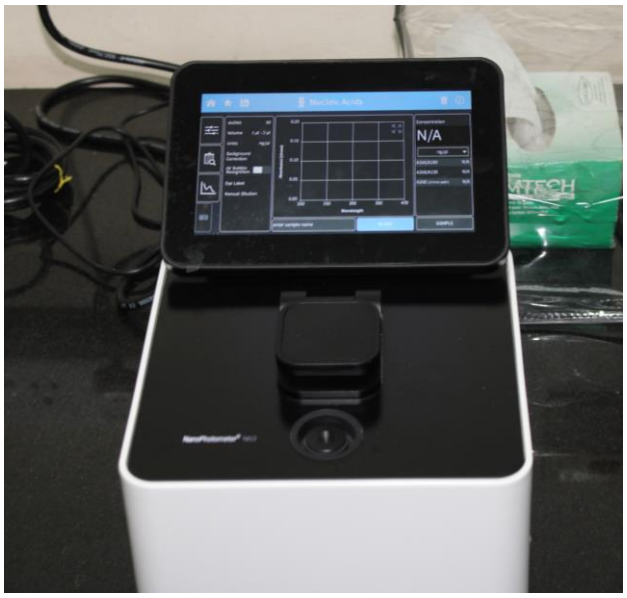
(1) Denaturation, in which double-stranded DNA templates are heated to separate the strands, (2) Annealing, in which short DNA molecules called primers bind to flanking regions of the target DNA, (3) Extension, in which DNA polymerase extends the 3′ end of each.

The thermocycler raises and lowers the temperature of the samples in a holding block in discrete, pre-programmed steps, allowing for denaturation and reannealing of samples with various reagents. PCR steps of denaturation, annealing, and extension are repeated (or "cycled") many times to amplify the target. The number of cycles is usually carried out 25–35 times but may vary upon the amount of DNA input and the desired yield of PCR product.

Application in tissue culture

- To assist in genetic fidelity of micro-propagated clones.
- Gene expression studies

9. NANODROP SPECTROPHOTOMETER (IMPLEN, USA)



The NanoDrop Spectrophotometer is a simple, easy-to-use spectrophotometer for measuring DNA, RNA, and protein concentrations in small volume samples. The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector employing Beer's law. 1 ul sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1 mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array is used to analyze the light after passing through the sample. The sensitivity range for DNA detection is between 2 and 3700 ng/ul. The spectral range of the device is 220 to 750 nm and it is possible to scan all of the wavelengths. The A260/280 ratio shows the purity of the sample analysed. Pure DNA sample gives a 260:280 ratio of ~1.8 and for pure RNA the ratio is ~2. A single measurement cycle takes only 10 sec. The instrument is driven by a PC, which allows you to archive a large number of measurements.

Application in tissue culture

- DNA quantity check for genetic fidelity test
- Quantify RNA for Gene expression studies (callus maturation and shoot regeneration)

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