COMMERCIAL MICROPROPAGATION OF BANANA (*Musa* spp.) USING A TEMPORARY IMMERSION BIOREACTOR SYSTEM

By
WAGHMARE VAIBHAV GAUTAM
(2018-11-008)

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE,
VELLANIKKARA, THRISSUR – 680656
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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **'commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system'** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara
Date: 22-10-2020

Waghmare Vaibhav Gautam
(2018-11-008)

CERTIFICATE

Certified that this thesis entitled **'commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system'** is a record of research work done independently by **Mr. Waghmare Vaibhav Gautam** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Shylaja M R 22/10/2020

Vellanikkara
Date: 22-10-2020

Dr. M. R. Shylaja
(Major Advisor Advisory Committee)
Director of Education KAU &
Professor and Head (Retd.)
CPBMB College of Horticulture
Vellanikkara, Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Waghmare Vaibhav Gautam (2018-11-008),** a candidate for the degree of **Master of Science in Agriculture**, with major field in Plant Biotechnology, agree that the theses entitled **"commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system"** may be submitted by **Mr. Waghmare Vaibhav Gautam,** in partial fulfilment of the requirement for the degree.

4/12/2020

Dr. M. R. Shylaja
(Major Advisor Advisory Committee)
Director of Education KAU &
Professor and Head (Retd.)
CPBMB College of Horticulture
Vellanikkara, Thrissur.

4/12/2020

Dr. Abida P S
(Member Advisory Committee)
Professor and Head CPBMB
College of Horticulture
Vellanikkara, Thrissur

4/12/20

Dr. Deepu Mathew
(Member Advisory Committee)
Assistant Professor CPBMB
College of Horticulture
Vellanikkara, Thrissur

4/12/2020

Dr. Jyothi Bhaskar
(Member Advisory Committee)
Professor and Head
Dept of Fruit Science
College of Horticulture
Vellanikkara, Thrissur

4/12/2020

Dr. Minimol J S
(Member Advisory Committee)
Associate Professor
Cocoa Research Centre
College of Horticulture
Vellanikkara, Thrissur

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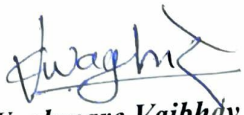

Waghmare Vaibhav Gautam

Vellanikkara

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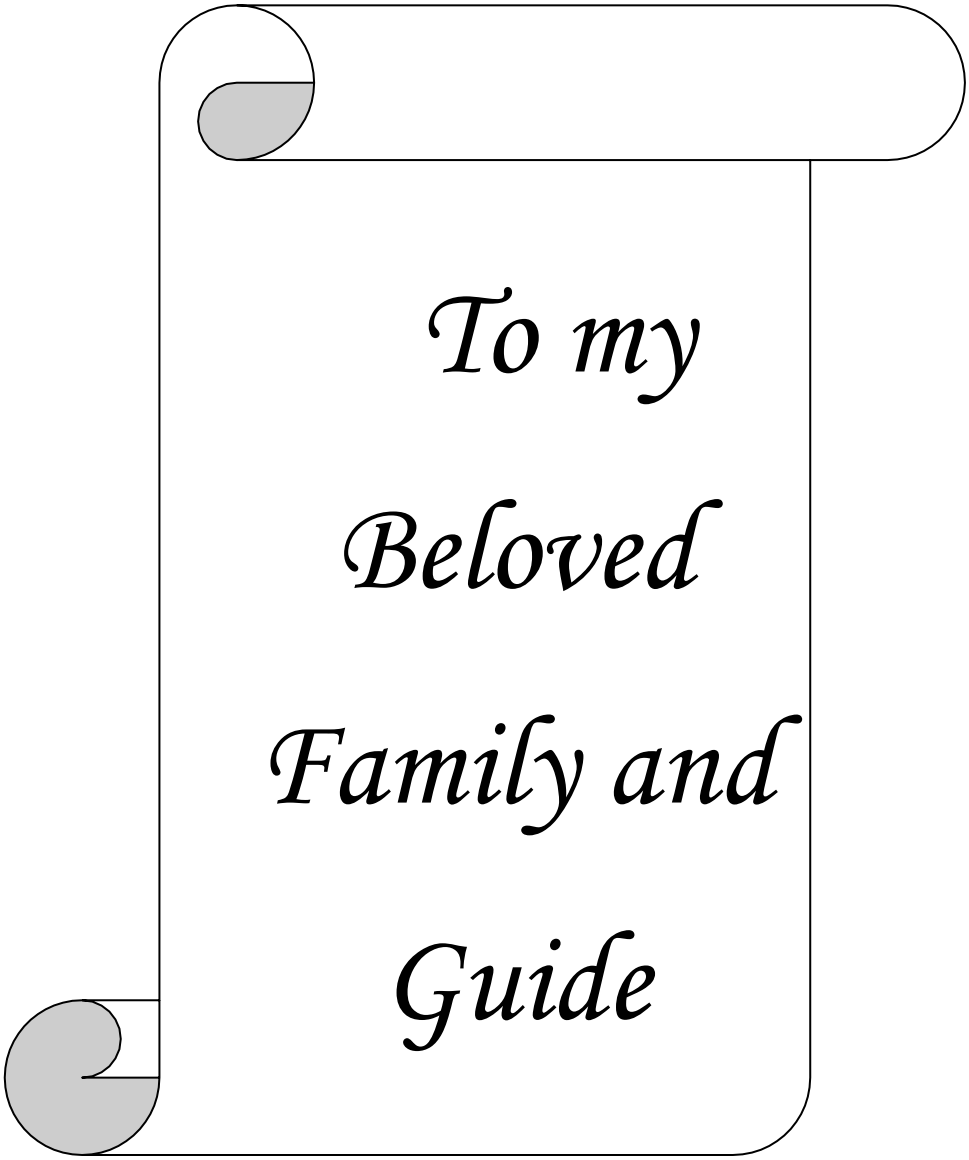
To my
Beloved
Family and
Guide

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ABBREVIATIONS

%	Percentage
β	Beta
µg	Microgram
µl	Microlitre
µM	Micro Molar
AFLP	Amplified Fragment Length Polymorphism
BA	Benzyladenine
BAP	6-Benzylaminopurine
BIB®	Immersion by Bubble
bp	Base pair
CD	Critical Difference
cm	Centimetre
CO_2	Carbon Dioxide
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
cv	Cultivar
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed Sequence Tag
FYM	Farm Yard Manure
g	Gram
GM	Gelled Medium
GoK	Government of Kerala
h	Hour
ha	Hectare
ISSR	Inter Simple Sequence Repeats
IBA	Indole butyric acid

KAU	Kerala Agricultural University
Kb	Kilo base pairs
L	Litre
M	Molar
MATIS®	Monobloc Advance Temporary Immersion System
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mM	Milli mole
mm	Milimetre
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
NaOH	Sodium Hydroxide
NCS-TCP	National Certification System for Tissue Culture Plants
ng	Nanogram
nm	Nanometre
NS	Nonsignificant
ºC	Degree Celsius
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PVP	Poly vinyl pyrolidone
Pvt. Ltd.	Private Limited
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RITA®	Recipient for Automated Temporary Immersion
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
Rs	Rupees
SE(m)	Standard Error of Mean
SSR	Simple Sequence Repeats

STR	Short Tandem Repeats
STS	Sequence Tagged Sites
t	tonnes
TAE	Tris Acetate EDTA
TC	Tissue culture
TIB	Temporary Immersion Bioreactor
TIS	Temporary Immersion System
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts

Introduction

1. INTRODUCTION

Banana (*Musa* spp.), is an important fruit crop cultivated throughout the world, mainly in tropical and subtropical regions. It belongs to the family Musaceae and is also known as the 'apple of paradise'. Among the fruit crops, banana stands second in production next to mango in India.

The Nendran (AAB group) also called a 'French' plantain, is one of the leading banana cultivars in Kerala. The Nendran type has special socioeconomic importance during the festive occasions especially Onam, the regional festival of Kerala. In 2018-19, the area under banana cultivation in Kerala was 52,898.61 ha with a production of 4.24 lakh t (GoK, 2019).

Propagation of banana is generally done by using suckers and there is high demand for suckers during planting season. The required number of planting material to cultivate an area of 52,898.61 ha will come to around 13.22 crores with a planting density of 2500 plants/ha. If a 10 per cent substitution of required planting material with tissue culture plants is aimed, the requirement of a tissue culture plant is around 1.322 crores per year.

The conventional micropropagation techniques are hampered by the high unit cost of production, poor multiplication, and low survival rates during acclimatization and it is highly labour intensive (Kozai *et al*., 1988; Mehrotra *et al*., 2007). With the development of a bioreactor system using liquid culture, the problems of conventional micropropagation could be rectified. The micropropagation using a bioreactor system is speedy with more number of propagules within a short period of time. Also, the cost of production can be reduced as the costliest component in the tissue culture medium, agar is not required for liquid medium and the multiplication rate achieved in a bioreactor is high.

The liquid medium in the bioreactor allows close contact of medium with plant tissues which facilitates the uptake of nutrients and phytohormones

leading to better shoot and root growth. Bioreactor also avoids intensive manual handling of cultures (Mehrotra *et al*., 2007).

Temporary immersion system has tremendous scope in commercial plant tissue culture. The temporary immersion bioreactor (TIB) system for *in vitro* regeneration of banana was first used by Alvard *et al*. (1993) who compared liquid medium and different culture methods including cellulose culture support, aeration by bubbling, permanent immersion, partial immersion, and temporary immersion with gelled medium and observed higher multiplication in cultures of a liquid medium with temporary immersion. They also reported that cultures permanently immersed in liquid medium with continuous aeration by bubbling showed hyperhydricity which was not observed in cultures with temporary immersion in the liquid medium.

Semi-automation of micropropagation in bioreactors was reported for improving multiplication and reducing the cost of production. Successful micropropagation with a high multiplication rate using TIB was reported in several species like chrysanthemum, garlic, ginseng, grape, lily and potato (Paek *et al*., 2005), pineapple (Silva *et al*., 2007), sugarcane (Distabanjong *et al*., 2018), and banana (Bello-Bello *et al*., 2019).

Commercial micropropagation is highly effective for the production of quality, disease-free and uniform planting materials (Azman *et al*., 2014). However, genetic instability has been noticed in tissue culture plants. The occurrence of genetic instability due to somaclonal variation is a problem during commercial clonal micropropagation (Lazaro-Castellanos *et al*., 2018). Hence Assessment of genetic stability of plants produced through any tissue culture protocol is important for the distribution of true to type plants.

Polymerase chain reaction techniques such as Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) are widely used marker systems in the assessment of clonal fidelity of *in vitro* developed plantlets in several crop species (Bhowmik *et al*., 2016, Fayas *et al*., 2018). The confirmation of genetic stability of bioreactor derived plantain

(AAB) regenerants using ISSR markers was described by Korneva *et al*. (2013).

The use of a TIB system for commercial plant tissue culture is popular in several species of plants within a short time of its invention. In South Africa, the plants cultured using RITA® TIB have been used by the forestry industry to distribute mother plants of Eucalyptus for hedges and to supply disease free seed cane to growers of the sugar industry (Meyer *et al*, 2009; Snyman *et al*., 2011). The propagation using of RITA® TIB has been integrated into the SmartSett® sugarcane programme in Australia (Mordocco *et al*., 2009). Commercial micropropagation of Spanish red cedar (*Cedrela odorata* L.) using TIB has been employed successfully in Mexico by Pena-Ramires *et al*. (2010). But TIB for commercial micropropagation of any of the plant species is not yet attempted in India.

The commercial micropropagation unit at CPBMB and other centers of Kerala Agricultural University (KAU) involved in banana micropropagation utilise the conventional micropropagation system. In KAU, micropropagation using bioreactor is not yet attempted. If the bioreactor micropropagation system is standardized in banana, it can be directly adopted in commercial micropropagation units and it helps in the large scale distribution of quality planting materials to farmers at reduced costs.

In this context, "Commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system" was taken up at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University to develop an efficient commercial micropropagation protocol in banana using a temporary immersion bioreactor system.

Review of Literature

2. REVIEW OF LITERATURE

The research programme entitled "Commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system" was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2018- 2020. Relevant literature on various aspects of the research is reviewed in this chapter.

2.1 General description

Banana (*Musa* spp.) is a perennial monocotyledonous herbaceous plant. Banana is a popular fruit crop available to the common man throughout the year and grown in a wide range of climatic conditions. It is delicious with seedless fruit. Banana is also called wonder berry, used as a staple food across the globe and provides a balanced diet for millions of people.

Banana comes under the order Zingiberales and family Musaceae. It is cultivated in tropical and subtropical regions. The edible banana is originated from *M. accuminata* and *M. balbisiana*. The genus *Musa* consists of 70 species, most of them were originated in Southeast Asia and the South Pacific region (Simmonds, 1962).

2.2 Morphology of banana

Bananas and plantains are giant herbaceous monocotyledonous plants belonging to the genus *Musa*. They are grown commonly up to 3 m in height, with no lignification or secondary thickening of stems (Tomlinson, 1969). The stem of a banana is called the 'rhizome' and it remains underground. The rhizome is a modified stem with buds (eyes) on the upper and middle regions of the rhizome. The pseudostem is the plant part above the ground. Leaf sheaths are slightly swollen at the base (Shanmugavelu *et al*., 1992). The top of the sheath is contracted into the petiole. Lamina is an extension of the margin of the midrib. About 30 leaves are formed till flowering. A short lived, bract like leaf emerged just before flowering protects the bunch. The inflorescence initiates from the heart of the pseudostem. Female flowers are larger, with well

developed ovary, long style and reduced stamens. Male flowers contain well developed anthers, abortive ovary with slender style and stigma. Pollen is rare in edible banana. The fruit is seedless, developed without pollination. Auxin stimulation is assumed to take part in the development of banana fruit (vegetative parthenocarpy) (Robinson, 1996).

2.3 Genetics

Most of the domesticated bananas are triploid with genome constitutions of AAA, AAB, or ABB. There are also seedless cultivated AA and AB diploids, and tetraploids with genome constitutions of AAAA, AAAB, AABB, and ABBB (Simmonds and Shepherd, 1955; Ortiz, 1997). Simmonds (1962) reported five plant characteristics used by farmers for picking plants for cultivation: plant vigour, seedlessness, hardiness, fruit quality, and yield, the first four of which are related to polyploidy (triploidy).

There is a wide range of variations available in this crop. Changes in chromosome number have influenced the development of phenotype. Because of the sterility, breeding based on segregating populations has not been possible in the triploid bananas and efforts for mapping diploid *Musa* species have not been successful.

2.4 Breeding

Conventional crossbreeding programmes using elite cultivars are not practical in banana because cultivated bananas are sterile, the diversity in cultivars is derived from wild collections that are vegetatively propagated. The deliberate creation and selection of synthetic hybrids by breeders have been carried out since the 1920s (Ortiz and Vuylsteke, 1995). By the early 21st century, the use of radiation mutants or cultivation of synthetic cultivars was reported (Jain and Swennen, 2004). Rowe (1984) has summarized sexual methods for the breeding of bananas. Vuylsteke *et al*. (1997) and Ortiz (1997) reviewed a wide array of breeding schemes that use conventional and innovative approaches for producing new improved cultivars from primary tetraploids, secondary triploids, and other populations.

Roux (2004) developed two commercially released banana lines by using gamma-ray-induced mutations. Both lines have useful agronomic characteristics, 'Novaria', as compared to the original parental clone, flowers about 10 weeks earlier ('Grande Naine' in the 'Cavendish' group), while the Thai variety KU1 has a larger bunch size with a cylindrical shape and larger banana fingers. Several other traits that are derived from mutation treatments or by using a somaclonal variation include resistance to dwarfism, Fusarium wilt, and Black Sigatoka *etc,* (Hwang and Ko, 2004).

Some alternative ways to generate transgenic bananas have been developed. Transformations with selectable markers and alien genes have low efficiency or it completely depends on the genotype and state of the material, although procedures are improving. Both particle bombardment (Sagi *et al*., 1995) and Agrobacterium-mediated (May *et al*., 1995; Khanna *et al*., 2004; Rodriguez-Zapata *et al*., 2005) strategies have been applied. Along with marker genes, anti-fungal, anti-nematode (Atkinson *et al*., 2004), virus-resistance genes and some for control of ripening have been the earliest targets for transformation, and clearly, both the precision and speed of this technology (e.g. using cell suspensions and BIBAC vectors, Ortiz-Vazquez *et al*., 2005) are increasing as plants have experimented in the field. The transformation of banana using promoters for fruit with selectable protein genes has also been considered to deliver and produce edible vaccines (Mason *et al*., 2002).

2.5 Nendran

According to Shanmugavelu *et al*. (1992), Nendran which is also known as Rajeli (French Plantain) is an important group of banana speculiar to the West Coast of Kerala. This group comprises of a few varieties which differ from one another in some respects only. The pulp is firm and is suitable for the preparation of chips, banana figs, and a varied number of other preparations.

2.6 Propagation

2.6.1 Seed propagation

The propagation of banana is achieved by sexual as well as asexual methods. Sexual propagation of banana is done by using seeds. Propagation through seed is not adopted in all cultivated banana species, but it is possible in some of the diploid wild species which have abilities to carry out normal meiosis, fertilization, and seed set. The extent of seed set, germinability, and dormancy depends on the species. Seed propagation is important in breeding programmes.

The propagation of banana is generally done by the vegetative method through suckers, (Baneerjee and De-Langhe, 1985). Tissue culture based micropropagation systems are well developed for bananas and, consequently, can be exploited to multiply elite genotypes. As regard to yield performance in banana, the plants which are developed by tissue culture produce 39 per cent higher yield than sword suckers (Pardeep*et al*., 1992; Farahani *et al*., 2008).

2.6.2 Vegetative propagation

Many of the cultivated commercial bananas are triploid and sterile, except a few parthenocarpic bananas AA and AB diploids. Sucker propagation is the only natural means of their propagation; artificial methods of propagation include macropropagation and micropropagation.

2.6.3 Sucker propagation

Quality planting material is the key to the successful cultivation of all crops. Sucker is the primary and major source of propagation material in banana. The suckers are the major source of planting material and normally remain true-to-type. Continuity of the production process can be maintained only by balancing good bunch yield and the optimum number of quality suckers (Bhende and Kurien, 2015).

The natural regeneration of cultivated bananas through suckers is very slow due to the apical dominance of the mother plant. A plant produces only 5-10 suckers during its lifetime of 10-12 months. The selection of appropriate mother plants for raising new propagules either through *in vivo* or *in vitro*

methods is important.

The advantages of propagation through suckers are mainly the low planting material cost, easy availability, lesser input requirements, scope for immediate planting in the field, less physical damages and easiness in conserving native and rare varieties (Swennen *et al*., 1984).

2.6.4 Macropropagation

Macropropagation is an excellent option for producing good quality planting material at a low cost. This is a simple method because of the ease of multiplication, saves the cost of producing planting material, and has the potential of producing 50-60 propagules per sucker in 4-5 months. Macropropagation is achieved by two methods and could be adopted either in the field conditions (*in situ)* or in the nursery (*ex situ*). It involves decapitation, decortication, shoot production and hardening of micro suckers.

Baiyeri and Ndubizu (1994) compared the suckers produced by six cultural methods (including decapitation techniques and mulching) that revealed that 98 per cent of the suckers survived in the first six months after planting.

2.6.5 Micropropagation

The goal of micropropagation is to obtain large numbers of genetically identical, developmentally normal plants with high yield potential. Micropropagation aims at producing a replica of the original plant selected for its desirable characters in many plant species (Bhojwani, 1980; Wang and Hu, 1982).

Propagation by *in vitro* tissue culture system is very efficient in *Musa* spp. The method can produce a large number of uniform high-quality plantlets that are free from disease and nematodes. Shoot tip cultures have been most widely used (Strosse *et al*., 2004), but suspension cultures are also being developed. In some of the tissue culture systems, high levels of chimerism are found, where the number of chromosomes and genotypes are varied (Roux *et al*., 2001) in the resulting plants. The valued South Indian ‘Red’ sweet banana

shows regular reversion of the colour character to green, particularly in tissue-culture propagated plants (Stover and Simmonds, 1987), although the basis of this has not yet confirmed. A programme for checking the varietal characteristics of material grown after a decade of storage *in vitro* has shown very few morphological or ploidy variants (Van den Houwe *et al*., 1995). Although true-to-type plantlets are the desired goal of micropropagation, many times somaclonal variants are obtained due to tissue culture induced variability. Applications of molecular markers do show some DNA changes (Ray et *al*., 2006) arising following tissue culture. Notably, Oh *et al*. (2007) demonstrated that some genomic regions of *Musa* show higher rearrangements and mutation rates than others under the stresses imposed during tissue culture.

2.6.5.1 Studies on banana micropropagation

There are many reports on banana micropropagation. The first report on *in vitro* adventitious bud formation from the shoot apex of banana after decapitation was reported by Ma and Shii (1972) in Taiwan. After two years from the first report, Berg and Bustamante (1974) used meristem culture for virus free banana plant production.

Wong (1986) established a successful *in vitro* culture of 22 banana cultivars on Murashige and Skoog's medium supplemented with 6-benzylaminopurine (BA) and indole butyric acid (IBA).

A report on the callus development in banana on a nutrient medium containing picloram and solidified with gelrite was described by Huang and Chi (1988).

Ganapathi *et al.* (1999) studied the embryogenic response of young male flowers of banana from different genotypes. Rasthali (AAB) has a good embryogenic response as compared to Lokhandi (AAA), Trikoni (AAA), Shreemanti (AAA) and Basrai (AAA).

Assani *et al*. (2001) developed the regeneration protocol for Grande Naine (AAA) via somatic embryogenesis from cell suspension culture.

A commercial micropropagation protocol for *in vitro* mass propagation of *Musa acuminate* cv. Dwarf Cavendish was developed by Rout *et al*. (2001).

Kosky *et al*. (2002) established cell suspensions of FHIA 18 (AAAB) from embryogenic tissue. Embryogenic suspension cultures of *Musa acuminata* from male inflorescence was established by Jalil *et al*. (2003).

Multiplication of banana (*Musa* spp.) cv. Basrai was studied by Muhammad *et al*. (2004) and observed differences in the rate of multiplication among the explants from the same genotype and also shoot tips from different rhizomes behaved differently.

Resmi and Nair (2007) reported changes in hormonal combinations in MS medium gave different *in vitro* responses of diploid and triploid banana cultivars.

The effectiveness of BAP than the other cytokinins to induce growth of cultures in different cultivars of bananas was described by several authors such as Venkatachalam *et al*., 2007, Farahani *et al*., 2008, Buah *et al*., 2010, Jafari *et al*., 2011, Robert *et al*., 2013, and Deo *et al*., 2020.

Mohamed (2018) established a successful commercial banana micropropagation protocol using low cost alternatives.

Hasan *et al*. (2020) successfully developed the *in vitro* regeneration protocol in some selected banana cultivars such as Grand Naine, Monthan, and Red Banana.

2.7 Bioreactors for plant micropropagation

Bioreactors are the vessels used for the culture of a large-scale cell, tissue, or organ in the liquid medium. The vessel is designed in such a way that it regulates various nutritional or physical factors, for the production of large scale cultures (Afreen, 2006). The bioreactor system consists mainly of two parts, a culture vessel and an automated control system. The design of the culture vessel provides an aseptic environment, maintains microenvironmental conditions, nutrients, and gases and ensures the maximal growth of cultivated

cells. The automated control system is either computerized with automation or a semi-automated system, that monitor and control the culture conditions in the vessel, such as the temperature, agitation speed, dissolved oxygen and carbon dioxide (CO_2) concentrations, illumination regime, pH, and the level of the liquid medium.

Different designs of bioreactors have been developed specifically for crops, culture types, and stages. According to functions, bioreactors for plant culture can be divided into two broad types: continuous and temporary immersion of medium in the culture.

2.7.1 Continuous immersion system

In a continuous immersion system, the plant cultures are immersed completely in a liquid medium. Based on the mode of agitation there are different liquid-phase bioreactors which are mainly mechanically, pneumatically and hydraulically agitated and membrane bioreactors. A few authors noted that the permanent immersion bioreactors to be the most suitable for multiplication of *Anoectochilus formosanus* (Wu *et al*., 2007) and *Vaccinium crymbosum* (Ross and Castillo, 2009). Continuous immersion of plant cultures in the liquid nutrient medium is the reason for asphyxia and hyperhydricity (Debnath, 2011).

2.7.2 Temporary immersion system

The development of a Temporary Immersion System (TIS) was an important step for the commercialization of plant micropropagation. Temporary Immersion System is either semi-automated or automated, where the plant cell, tissue, or organ cultures are immersed into the liquid nutrient medium followed by draining and exposing the plant tissue to a gaseous environment. The initial attempt to design the TIS was in 1983 when Harris and Mason developed an apparatus called “auxophyton” which was capable of merging aeration and liquid medium.

A temporary immersion system (TIS) is a simple plant culture system with automation that maintains microenvironment, optimal nutrients, and gas

transfers in the *in vitro* culture. The system provides the most natural environment to the cultures during *in vitro* growth. In the TIS, the cultures are immersed periodically into a liquid phase and then exposed to a gaseous phase. Different types of TIS have been developed and are widely used in the commercial micropropagation of economically important plant species. Moreover, because of their flexibility in design and simple operation system, TIS has been widely adopted in plant metabolite production, molecular farming and even in phytoremediation of toxic compounds (Steingroewer *et al*., 2013).

The TIS is usually made with glass or plastic vessels that would be transparent, that allow external light in the vessel required for the growth of cultivated cells. Construction of vessels is done by using interchangeable plastic materials that make this system simple to design, easy to handle, compact to store, autoclavable, or ready for multiple uses (Georgiev and Weber, 2014).

A temporary immersion design improves the physiology and mass growth of plants *in vitro*. Several different TIS have been applied successfully for the micropropagation of a variety of plant species of agricultural, medicinal, and conservation value. Today, different types of designs are available in TIS, some of them which are widely studied by a number of researchers, which include Ebb-and-Flow bioreactor (Tisserat and Vandercook, 1985), Recipient for Automated Temporary Immersion (RITA®) (Alvard *et al*., 1993), Temporary Immersion Bioreactor (TIB) (Escalona *et al*., 1999), Monobloc Advance Temporary Immersion System (MATIS®) (Etienne *et al*., 2013), and SETIS™ Bioreactor (Vervit, 2018).

According to the available literature, the studies were mainly focused on investigating the validity of the single design of TIS in improving mass culture, as compared with semisolid medium or continuous immersion (Jova *et al*., 2011). Few reports were available in which different TIS designs were tested for *in vitro* culture of plants (Sankar-Thomas *et al*., 2008; Scheidt *et al*., 2011).

Most of the reports showed an improved rate of multiplication in TIS, although some reported no differences (Hanhineva *et al*., 2005; Jo *et al*., 2008;

Stanly *et al*., 2010).

2.7.3 Micropropagation of plants using bioreactors

The first study on the use of a bioreactor for micropropagation was reported by Takayama and Misawa (1981) who used shake culture in *Begonia*. Different systems available in temporary immersion with a variety of characteristics make this highly efficient for use in semi-automated micropropagation. In the continuous immersion, the plant cultures are immersed constantly in a liquid phase, which often affects the negative impact on plant growth and morphogenesis. Temporary immersion systems provide adequate nutrient and oxygen supply, relatively infrequent subculturing, ease of medium changes, and limited shear damage that reduces the risk of contamination. For these reasons, Ziv (2005) and Arencibia *et al.* (2008) have described TIS as the "most natural tissue culture approach".

Alvard *et al*. (1993) used RITA® TIS successfully in micropropagation of banana (cv. 'Grande Naine'). High multiplication rate, shoot length, and leaf number were observed in TIB as compared to semi-solid medium in plantain cv. 'CEMSA ¾' (AAB) (Roels *et al*., 2006).

Ziv *et al*. (1998) reported the stimulation of bud cluster formation in banana cv. 'Williams' on 19.5 µM/L ancymidol by using inexpensive disposable plastic film bioreactor. Similar results were reported in plantain cultivar 'FHIA-18' (AAAB) using paclobutrazol in TIB (Daquinta *et al*., 2001).

Automation of micropropagation in bioreactors has been reported by several authors as a possible way of reducing the cost of micropropagation. The successful mass propagation of pineapple plants using a temporary immersion system has been reported by Escalona *et al.* (1999, 2003). The hybrid banana cultivars 'FHIA-18' (AAAB) was micro propagated in a bioreactor through somatic embryogenesis Kosky *et al*. (2002). Later, the evaluation of these plants at the field level does not show any genotypic difference (Kosky *et al*., 2006).

A simple protocol for *in vitro* mass propagation of apple rootstock M.9

EMLA using an automated low cost bioreactor system has been developed by Chakrabarty *et al.* (2003). Propagation of apple, chrysanthemum, garlic, ginseng, grape, lily and potato by using temporary immersion bioreactor was described by Peak *et al*. (2005).

Optimisation of micropropagation protocol using a temporary immersion bioreactor (TIB) system in plantain (*Musa* AAB) was reported by Roels *et al*. (2005).

A rapid multiplication protocol for banana cv. Basrai from meristematic stem tips, by using a medium with optimised concentration of the auxins/cytokinins through temporary immersion system and permanent immersion system (medium solidification) bioreactor was done by Haq and Dahot (2007).

Micropropagation of the banana cv. 'Dwarf Cavendish' (AAA) in TIS was compared with stationary liquid or solid medium and observed better quality in plant growth in culture from TIS (Farah and Freshteh, 2008).

Niemenak *et al*. (2008) developed a protocol for somatic embryo formation in *Theobroma cacao* using a twin flask bioreactor and reported, 13-fold more yield in TIS compared to the semi-solid medium after three months of culture.

Mordocco *et al*. (2009) studied SmartSett® and RITA® temporary immersion bioreactor and observed a high number of shoot proliferation in the SmartSett® system.

Scheidt *et al*. (2009) compared immersion by bubbles (BIB®) culture system with the RITA® system for pineapple micropropagation and observed higher number of shoots in BIB® when compared with the RITA® system.

Temporary immersion bioreactor has been used successfully in some species which had not been investigated previously, includes some endangered and medicinal plants (Debnath, 2009; Yan *et al*., 2011; Malosso *et al*., 2012).

Farahani and Majd (2012) studied the growth, development and multiplication of banana (*Musa*, cv. Dwarf Cavendish) in the TIS system compared with liquid or solid medium and found the highest shoot multiplication in the TIS system.

Aragon *et al.* (2014) compared the physiological characteristics of plantain (*Musa* AAB) plantlets micropropagated in temporary immersion bioreactors (TIB) and on a gelled medium (GM). Temporary immersion bioreactors (TIB) improved rooting and gave rise to longer shoots and higher dry mass. Respiration rate was the highest at the beginning of shoot elongation in both the TIB and GM plantlets. The photosynthetic rate in TIB was significantly higher than in GM.

In *Vanilla planifolia,* the highest multiplication rate of 14.27 shoots per explant in temporary immersion when compared to the partial immersion system (8.64 shoots per explant), and solid medium (5.80 shoots per explant) was reported (Ramos-Castella *et al*., 2014).

Ribeiro *et al*. (2016) studied different culture systems on *in vitro* culture of *Bambusa vulgaris* and found that a greater number of established explants in the bioreactor system.

Godoy *et al*. (2017) developed a micropropagation protocol using temporary immersion bioreactor in different cultivars of Sweet cherry (*Prinus avium* L.) and observed the culture of Maxma-14, Colt, and 'Van' from TIS had better performance compared to the solid system, whereas 'Rainier' showed no differences.

Osbel *et al*. (2017) tested immersion frequency (6, 8, and 12 h), additional ventilation (1 min every 2 h) and length of duration of shoot culture (14, 21, 28, and 35 days) in gerbera. They noted that immersion every 6 hrs has higher fresh and dry weight but shoots displayed hyperhydricity. According to the author, this was the first study on gerbera using TIB.

Large scale micropropagation of blueberries, raspberries and poplars

using TIB with improved plant quality, greater efficiency during acclimatization phase and higher luminosity in reduced sucrose concentration were reported by Arencibia *et al*. (2018).

Successful micropropagation of sugarcane using temporary immersion bioreactor, with high multiplication rate of 29.4-31.2 microshoots per starting microshoot within 4 weeks in MS liquid medium supplemented with 0.5 μM paclobutrazol, 0.2-0.4 μM benzyladenine (BA) and three per cent sucrose was reported by Distabanjong *et al*. (2018).

In vitro regeneration of *Capparis spinosa* L. using the traditional solid medium with comparison to Plantform temporary immersion bioreactor (TIB) was evaluated by Gianguzzi *et al*. (2019) and reported significant differences in plant growth in the Plantform liquid culture with those grown in solid medium regarding the number and length of shoots.

Luciana-Cardoso *et al*. (2019) evaluated the performance of cactus using bioreactors and the conventional method and reported a good response of bioreactor cultures in cladode length and fresh matter mass.

Martinez-Rivero *et al*. (2019) studied the effect of Vitrofural® on sugarcane micropropagation using a temporary immersion system and found a higher number of shoots per explant, length of the shoots and number of leaves in comparison with the shoots obtained in semi solid medium.

Date palm micropropagation using Plantform temporary immersion system (TIS) was examined by Nayyef *et al*. (2019) and reported more number of shoots, fresh weight of biomass, and sugar content in TIB as compared with solid media.

Abahmane (2020) compared the temporary immersion system and semisolid system in date palm and reported higher shoot and root length with a higher fresh and dry weight of shoots in TIS.

Debnath and Arigundam (2020) reviewed the *in vitro* propagation

strategies of medicinally important berry crop, Lingonberry (*Vaccinium vitis-idaea* L.) and noted that micropropagation using a bioreactor system has significantly increased the multiplication rates of *in vitro* produced shoots.

Kim *et al*. (2020) compared the different *in vitro* culture systems, including a temporary immersion bioreactor (TIB), a continuous immersion bioreactor, and conventional solid and liquid culture systems (controls) for the production of virus free apple plantlets. The highest fresh weight, shoot length, higher root number, root length, with the largest total stem area was reported in TIB plants.

2.7.4 Immersion time and immersion frequency

The frequency and time of immersion are the critical factors of TIS which determine the absorption of nutrients and the control of hyperhydricity (Etienne and Berthouly, 2002). Although these parameters vary considerably due to the diversity in existing species, different micropropagation processes use different frequency and time of immersion. The studies conducted reported high growth of fresh mass and number of shoots when smaller immersion intervals were used, although hyperhydricity was sometimes more severe (Carvalho *et al*., 2019).

2.7.5 Contamination

Contamination due to the growth of fungi, yeast, viruses, bacteria, thrips, and mites is one of the major limiting factors for achieving truly aseptic conditions in bioreactors for successful micropropagation. Microbes grow rapidly on established cultures, once they grow it causes nutrient reduction from medium and production of toxic compounds, which affect the mortality, tissue necrosis, or variation in growth of cultured plantlets resulting in partial or total loss of culture (Kane, 2003).

McAlister *et al*. (2005) noted that the more number of clumps placed in each bioreactor is the main cause of culture losses in TIS due to contamination.

2.7.5.1 Control of Contamination

The loss of culture due to microbial contamination is one of the major problems in the temporary immersion bioreactor system for successful micropropagation. Probably it depends on the self hygiene or proper handling of the plant cultures and equipment during transfer and production. The surface sterilized starting material is initially established in small vessels and disease free indexed cultures are then used for large scale production in bioreactors.

The control of contamination can be achieved by using a single or a combination of anti-microbial compounds, acidification of the media, and micro-filtration of the medium (Schmidt *et al*., 2002). The most commonly used method is treating starting culture with antibiotics and incorporating it into the culture medium, the use of antibiotics is costly, they degrade quickly and require optimum pH (Luna *et al*., 2008).

Klayraung *et al*. (2017) noted that the contamination in the TIB system will depend on plant species, explant types and environmental conditions. Good skill and practice of the operators in the plant tissue culture laboratory are required when working with the TIB system. Furthermore, the sanitary state of the laboratory is another important factor for the success of culture in the TIB system.

Rahman *et al.* (2017) identified bacterial and fungal contaminants isolated during potato shoot growth in the TIB system. They evaluated four different antifungal agents and reported 0.15 per cent Propiconazole gave 100 per cent inhibition against fungal contaminants. They examined the effect of three antibacterial (Gentamycin, Vancomycin, and Tetracycline) and four antifungal agents (Mencozeb, Propiconazole, Bavistin, and Copper oxychloride) on the contaminants and potato shoot growth. They found that Gentamycin (50 mg/L) and Propiconazole (0.15 %) were most effective against the isolated bacteria (35 mm inhibition zone) and fungi (100 %) respectively, whereas Gentamycin in combination with Bavistin showed better performance on potato shoot and root development in a bioreactor.

2.7.5.2 Hyperhydricity

The growth of plant cultures in a liquid medium causes shoot malformation. The cell or tissues from a liquid medium accumulate more water that affects the morphology of a plant called hyperhydricity. The plant shows spongy or glassy tissues and succulent leaves (Kevers *et al*., 2004; Berthouly and Etienne, 2005).

Etienne and Berthouly (2002) observed that the volume of nutrient medium and the volume of culture container substantially influence shoot proliferation in bioreactors. Temporary immersion generally improves the quality of plant material. Hyperhydricity can be controlled by adjusting the immersion time. Plant material propagated by temporary immersion can perform better during the acclimatization phase than material obtained from the semi-solid or liquid medium.

The oxidative stress increase accumulation of ethylene and the level of humidity in the culture vessel and create an unsuitable condition for culture growth and cause physiological abnormalities such as hyperhydricity (Chakrabarty *et al*., 2006; Rojas-Martinez *et al*., 2010; Dewir *et al*., 2014). The vitrified shoots are very sensitive to liquid media and continuous contact of shoots with liquid medium causes poor survival of *in vitro* raised plants during *ex vitro* acclimatization due to the environmental stress (Robert *et al*., 2006; Shaik *et al*., 2010).

The process of immersion of explant into the liquid medium and then exposure to the air can help to reduce hyperhydricity. The duration and immersion frequency in TIS are the most critical factors for successful *in vitro* culture. Teisson and Alvard (1995) reported frequent immersion but a short duration can help to eliminate embryo hyperhydricity.

Albarran *et al*. (2005) reported changing in immersion from 15 min every 4 h to 1 min every 4 h showed a decrease of hyperhydricity and an increase in embryo formation in coffee.

Hyperhydration of shoots was found to be less in TIS as compared to semi-solid or liquid medium (Wawrosch *et al*., 2005; Yan *et al*., 2010).

The use of less nutrients and sucrose in medium, with 2 min/12 h immersion showed successful removal of hyperhydration of *Eucalyptus globulus* shoots regenerated in a twin-flask system (Gonzalez *et al*., 2011).

According to Marzieh *et al.* (2017) increasing aeration and reducing the immersion time help to control hyperhydricity. They evaluated three different numbers (2, 4, and 6) of immersion per day and found that six time immersion per day caused the highest number of new shoots (17.33), although these treatments lead to the highest rate of hyperhydricity. Four immersions per day at an interval of 6 h (14.33 new shoots) were observed as the most desirable treatment.

2.8 Hardening and acclimatization

The *in vitro* plantlets were grown under controlled conditions i.e. in high humidity and low temperature than the outside environment. When plantlets are transferred to *ex vitro* conditions, they are exposed to abiotic and biotic stresses.

Martre *et al*. (2001) reported that the success of any commercial micropropagation industry does not only depends on the high multiplication rate but also the recovery of good quality and vigorous plants in large numbers. Transfer of microshoots to *ex vitro* environment causes mortality to plantlets due to physiological and biochemical changes (Mathur *et al*., 2008). The success or failure of any tissue culture industry is depending on the rate of survival during hardening (Radheshyam and Subramani, 2008). Therefore hardening and acclimatization of *in vitro* raised plantlets are very important in tissue culture technology.

Rahman *et al*. (2005) and Ali *et al*. (2011) reported higher survival and growth of banana plants in the potting mixture containing soil: sand: FYM (2:1:1). After primary hardening for 5-6 weeks, the plants are transferred from micro pots to polybags for secondary hardening in soil and sand along with low

cost materials like coir pith, sawdust, or rice husk (Vasane and Kothari, 2006). Parkhe *et al*. (2018) studied different potting mixtures for hardening of tissue cultured banana plantlets and reported 100 per cent survival in potting mixture with garden soil and FYM (3:1) after secondary hardening.

A higher survival rate of the plant after acclimatization was reported in plants which are derived from a liquid medium in TIS (Etienne and Berthouly, 2002).

A study on *Kalopanax septembolus* plantlets obtained from the TIS system showed 100 per cent survival as reported by Kim *et al*. (2011). Plantlets of *Artocarpus altilis* from semi solid system exhibited lower (83%) survival as compared to 100 per cent survival of plant from bioreactors (Shandil and Tuia, 2015).

Based on studies on different culture systems for acclimatization, the environmental conditions in TIS prepares the plantlets for the stress of acclimatization (Yang and Yeh, 2008; Aragon *et al*., 2010; Regueira *et al*., 2018).

The plants obtained from TIS showed a high percentage of closed stomata and lower stomatal index as compared to plants from semisolid medium and exhibited a lower rate of transpiration and higher rate of photosynthesis (Chakrabarty *et al*., 2007; Aragon *et al*., 2014; Vieira *et al*., 2015; Martinez-Estrada *et al*., 2019).

Scherer *et al*. (2015) reported that there was more than 95 per cent survival during the acclimatization stage of pineapple plants derived from the bioreactor system. Bello-Bello *et al*. (2019) compared semi solid, partial immersion and temporary immersion culture systems for banana micropropagation and reported no significant difference in plant survival in the different systems studied. Arigundam *et al*. (2020) also reported the highest (95%) survival of bioreactor-cultured lingonberry plants under greenhouse conditions.

2.9 Clonal fidelity analysis using molecular markers

Clonal fidelity analysis is done using hybridization based and Polymerase Chain Reaction (PCR) based molecular markers. Hybridization based detection system uses Restriction Fragment Length Polymorphisms (RFLP) molecular marker (Botstein *et al*., 1980). The PCR based detection uses markers such as Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), Simple Sequence Repeats (SSR) (Hearne *et al*., 1992), Sequence-Tagged Sites (STS) (Fukuoka *et al*., 1994), Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz *et al*., 1994) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995).

2.9.1 Studies on clonal fidelity analysis in the conventional micropropagation system

2.9.1.1 Random amplified polymorphic DNA (RAPD)

The principle of RAPD markers is based on the probability that a sequence of DNA, which is homologous to a short, oligonucleotide primer occurs at different positions on the template strand of a DNA amplifiable by PCR (Waugh and Powell, 1992).

Vidal and Garcia (2000) used the RAPD marker to characterize the somaclonal variant 'CIEN BTA-03' of banana and suggested that as a suitable tool for evaluating the genetic stability of plantlets obtained *in vitro*.

A monomorphic banding pattern was observed in RAPD analysis in *Musa acuminate* cv. 'Berangan' plantlets regenerated by shoot inflorescence (Harirah and Khalid, 2006).

Ray *et al*. (2006) studied the genetic variability of micropropagated banana by using RAPD and ISSR primers (*Musa* spp.) in three different cultivars and reported minor genetic variation in cv. Robusta and Giant Governor, whereas, plantlets of cv. Martaman showed complete genetic purity.

Propagation of banana cv. 'Nanjanagudu Rasabale' *in vitro* without any

sign of somaclonal variation was reported by Venkatachalam *et al*. (2007) using 50 RAPD and 12 ISSR primers.

Viswanath *et al*. (2008) used RFLP, RAPD, and AFLP markers for somaclonal variation detection in tissue cultured banana plantlets.

Variability at the genetic level in cv. Rasthali was examined by Uma *et al*. (2009) using RAPD markers.

Prabhuling (2010) also studied the RAPD marker system for assessing stability at a genetic level in banana plantlets.

2.9.1.2 Inter Simple Sequence Repeats (ISSR)

Several DNA markers can be used to detect somaclonal variations in tissue culture raised plants. Inter Simple Sequence Repeat (ISSR) is one of the most commonly used PCR based systems. It involves the synthesis of a DNA region between two identical repeat sequences oriented in the opposite direction. Compared to other marker systems ISSR is simple to use and the cost involved is less (Ng and Tan, 2015).

Compared to RAPD primers (10-meters), ISSR is highly reproducible due to its length (16- to 25 meters) which allows the use of high temperature (45 to 60°C) during annealing which leads to higher stringency (Reddy *et al*., 2002). The designing of ISSR primers does not require any prior sequence information and the DNA sample required is very low quantity (5-50ng) (Kumar *et al*., 2009).

Use of ISSR marker assay to examine homogeneity at genetic level was reported by many authors in different plant species such as *Brassica oleracea* (Leroy *et al*., 2001), Almond (Martins *et al*., 2004), *Musa* spp. (Lakshmanan *et al*., 2007), *Oryza sativa* L. (Frederic *et al*., 2007), *Prunus mume* (Guo *et al*., 2007) and *Phoenix dactylifera* L. (Kumar *et al*., 2010).

Clonal fidelity of banana (*Musa acuminata* cv. Grand Naine) was successfully done by using ISSR and REMAP molecular markers and observed

a high degree of monomorphism in both ISSR (0.96%) and REMAP (0.95%) markers (Borse *et al*., 2011). Amar (2012) investigated morphological and molecular analysis to confirm genetic purity in banana (*Musa* spp.) var. Nendran produced *in vitro* and observed an increase of variation in the higher subculture cycles (above 12).

Awchar (2014) successfully studied the genetic stability of micropropagated gerbera plantlets using ISSR assay and reported 80 per cent uniformity of mother plant to its regenerants while, high variability (40%) was observed in plants regenerated through an indirect pathway.

Mohamed (2018) also assessed the clonal fidelity of banana plants of different cultivars at different subculture passages using specific ISSR markers and recommended multiplication up to 8^{th} subculture stage for Attunendran, Nedunendran and Grand Naine, up to 9^{th} subculture passage for Chengalikodan and up to 10^{th} subculture passages for low multiplying cultivars like Poovan and Njalipoovan.

2.9.1.3 Simple sequence repeats (SSR) or Microsatellites markers

Simple Sequence Repeats (SSR) is a 1-6 bp length repeat sequence. It is also called microsatellite markers or Short Tandem Repeats (STR) (Tautz and Renz, 1984; Morgante and Olivieri, 1993). The term microsatellite was introduced to examine the simple sequence stretches amplified by PCR (Hearne *et al*., 1992).

For monitoring genetic variations in cultures developed by *in vitro* regeneration methods, microsatellite markers are reported as a highly sensitive marker (Lopes *et al*., 2006).

Pandey *et al.* (2012) tested genetic fidelity in sugarcane microshoots based on SSR assay and observed a strong similarity in the amplification pattern of *in vitro* plants with parent genotype.

2.9.2 Studies on clonal fidelity analysis in TIS

Alteration in the genetics of plants which is produced by *in vitro* culture system is due to the establishment of culture into extremely stressful conditions. The factors which are responsible for variation in somaclones are not well understood (Bairu *et al*., 2011). There are different hypothesis which presents the causes of somaclonal variation. According to Jain (2001), the *in vitro* culture system conditions itself acts as a mutagen because cells are subjected to different conditions than *ex vitro*. One another hypothesis suggests that the variations at the genetic level like aneuploidy, polyploidy, chromosomal mosaicism, and chimerism were the reason for the somaclonal variation. Somaclonal variation may be due to various types of genetic and epigenetic changes, including chromosomal rearrangements, deletions, insertions, recombinations, and changes in DNA methylation patterns.

Assessment of the genetic stability of plantlets produced through any tissue culture system is important for the distribution of true to type plants. The use of ISSR markers for the assessment of genetic fidelity was well documented by many scientists.

Lorenzo *et al*. (2001) studied the TIS system in sugarcane micropropagation with a two year field trial and the phenotypic data showed minor differences between conventional and TIS propagation systems regarding stem diameter and length which disappeared after six months of observations.

Feuser *et al*. (2003) compared TIS with a permanent immersion system and detected less percentage of somaclonal variation in pineapple.

An increase in the occurrence of variants due to progressive subculturing in *Musa* spp. was observed by Bairu *et al*. (2006) and reported that the multiplication increases more somaclonal variation in the next culture.

Debnath (2009) compared strawberry plants derived from bioreactor-with semi solid medium and noted that monomorphic bands after ISSR assay confirmed clonal fidelity.

Goel *et al*. (2009) used a simple bioreactor for the regeneration of St

John's wort plants. They analyzed clonal fidelity using RAPD markers and observed 99 per cent similarity. Fki *et al*. (2011) produced 400 date palm plants in RITA® bioreactor and reported no phenotypic difference in plants as compared to the mother culture. A similar observation was reported in American ginseng plants derived from TIS by Uchengu *et al*. (2011).

Remirez-Mosqueda *et al*. (2016) and Liu *et al*. (2011) reported a low percentage of somaclonal variation in *Stevia rebaudiana* and *Hydrangea microphylla* respectively.

Snyman *et al*. (2011) evaluated genotypic and phenotypic stability of sugarcane plantlets developed using the RITA® system and scored only 0 to 0.9 per cent polymorphic bands.

Noceda *et al*. (2012) evaluated field performance and epigenetic profile in plantain clones produced by TIS and observed 94.2 per cent of true to type plants.

Watt (2012) reviewed the field performance of plants obtained through TIS and reported no somaclonal variation as the cultures were not regenerated via a callus stage and used low hormone concentrations and a relatively short culture period.

Successful regeneration (84.5%) of 'Barraganete' plantain (AAB) plants from somatic embryos, using a temporary immersion system with a low percentage (1.55 %) of somaclonal variation was reported by Korneva *et al.* (2013).

Debnath (2014) performed an SSR assay and confirmed clonal fidelity in red raspberry plants developed through the bioreactor.

Besides these, different researchers evaluated clonal fidelity in TIB which include *Vaccinium Angustifolium* Ait (Debnath, 2011), strawberry (Debnath, 2013), coffee (Landey *et al*., 2013), *Capsicum chinense* (Bello-Bello *et al*., 2014), *Olea europaea* (Bradai *et al*., 2016), and *Jatropha curcas* (Soares

et al., 2016).

Martinez-Estrada *et al*. (2017) evaluated somaclonal variation using ISSR markers during sugarcane micropropagation in TIB. Somaclonal variation due to *in vitro* establishment and the number of subcultures in TIB was detected and recommended up to eight subcultures cycles.

Recently Nasri *et al*. (2019) propagated myrobolan (*Prunus cerasifera*) in the RITA® system and accessed genetic confirmation by ISSR-based assay and observed monomorphic amplification pattern.

However, few authors reported a high level of somaclonal variation when the subculture cycles increased in TIS such as six to seven subcultures in *Olea europea* (Farahani *et al*., 2011), five subculture cycles in *Stevia rebaudiana* (Soliman *et al*., 2014), 6, 9 and 11 subcultures in *Pistacia lentiscus* (Kilinc *et al*., 2015).

High levels of genetic variation were observed in *Vanilla planifolia* plantlets produced *via* callus in TIS (Divakaran *et al*., 2015; Ramrez-Mosqueda and Iglesias-Andreu, 2015).

Arigundam *et al*. (2020) used EST-PCR, and ISSR markers to confirm clonal fidelity in lingonberry derived from the stationary bioreactor and TIB and observed uniform banding pattern for regenerants from both the systems.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during 2018 to 2020. The objective of the study was to develop an efficient commercial micropropagation protocol in banana using a temporary immersion bioreactor (TIB) system. The study mainly focused on the following aspects:

1) Development of a micropropagation protocol using the TIB system. 2) Clonal fidelity analysis of micro propagated plants from TIB and conventional systems using specific ISSR marker.

3.1 Materials

3.1.1 Source of culture

Studies were conducted in banana cultivar Nendran (AAB) which is commercially micropropagated at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara.

Established cultures of Nendran cultivar (Nedunendran) were received from the commercial micropropagation unit of CPBMB at the 5^{th} subculture cycle and were utilized in the study.

3.1.2 Laboratory chemicals, glassware, and plastic wares

Good quality (AR grade) chemicals obtained from various agencies like Merck India Ltd. and HIMEDIA were used. The chemicals used for the PCR reaction *viz*., *Taq* DNA polymerase, dNTPs, *Taq* buffer, $MgCl_2$, DNA ladder were supplied by Bangalore Genei Ltd. All the plastic wares used were obtained from Axygen and Tarson India Ltd. and the glasswares were procured from Borosil and Vensil. The ISSR marker was supplied by Sigma Aldrich Chemicals Pvt. Ltd.

3.1.3 Laboratory Equipment and machinery

The present study was carried out using plant tissue culture and molecular biology facilities available at CPBMB, College of Horticulture. The Plantform temporary immersion bioreactor designed by Professor Margareta Welander, Sweden and Dr. Abdullah J Sayegh, Ireland was used in the present study. All aseptic manipulations were carried out in laminar airflow (LABLINE INDUSTRIES). Media sterilization was done in the autoclave (Nat steel equipment Pvt. Ltd.). Centrifugation was done in a high-speed refrigerated centrifuge (KUBOTA 6500). The quality and quantity of DNA were estimated using the NanoDropRND-1000 spectrophotometer. DNA amplification reaction was carried out in AB thermocycler. For agarose gel electrophoresis, a horizontal gel electrophoresis system (BIO-RAD, USA) was used. For imaging and documenting the agarose gel profile, the BIO-RAD gel doc system was used. The details are given in Annexure I.

3.2 Methods

3.2.1 Culture media

3.2.1.1 Preparation of MS medium

The preparation of Murashige and Skoog (MS) plant tissue culture medium was done as per Murashige and Skoog (1962). Five nutrient stock (I, II, III, IV, and V) solutions were prepared and stored in pre-cleaned glass bottles under refrigerated conditions. Stock III was stored in an amber-colored bottle. Plant growth regulators stock solutions were also stored under refrigerated conditions.

The Beaker used for the preparation of the medium was cleaned by using distilled water. After taking the 1/3rd the volume of distilled water in the beaker, aliquots from stock (I, II, III, IV, and V) solutions were pipetted in proportionate volume. For preparing MS medium of full strength, 20 ml was pipetted from 50X stocks and 10 ml from 100X stocks. Required quantities of sucrose, inositol, and hormones were added and dissolved. The desired volume was made up by adding distilled water. The pH of the medium was adjusted to 5.7 using 0.1 N NaOH.

For preparing solid medium the required quantity of agar was added to the final volume and then the medium was heated in a microwave oven to melt the agar. After melting of agar, 50 ml of hot medium was poured into pre-sterilized glass culture bottles and autoclaved. The liquid medium was poured in pre-sterilized plastic conical flasks, plugged with non-absorbent cotton, and autoclaved directly without adding agar or any other solidifying agent. The chemical composition of MS medium is given in Annexure II.

3.2.1.2 Autoclaving

The conical flasks or culture bottles with nutrient media were autoclaved at a pressure of 15 lbs/sq. inch (121ºC) for 20 min. After autoclaving, the conical flasks and glass bottles were removed and allowed to cool at room temperature. After sterilization, the medium was kept in the media storeroom for 4-5 days to ensure that the medium was free from contamination.

3.2.2 Installation of Bioreactor

3.2.2.1 Assembly and setup of bioreactor

The Plantform bioreactor purchased from Sweden was used in the present study. Bioreactor culture vessels and different parts were connected according to the technical manual.

The bioreactor culture vessel with a size of 180×150×150 mm consists of a body made up of transparent polycarbonate. The exchange of gases is controlled by three inlets/outlets connected to the side of the body through nuts and clamps and sealed with heat resistant silicon o-rings. The exchange is possible through sterile filters connected to inlets. The middle filter connected with a plastic tube on the inner chamber goes through the basket. A basket present inside the chamber is used for keeping plant materials on to it. The basket is made up of small holes that allow a flow of nutrients. The body is closed by a lid which is airtight using a silicon seal.

After connecting different parts of the bioreactor the tubes were then joined to the electric valve. The clock was fixed according to the immersion time and immersion frequency by following the timer manual. The setup of the Plantform TIB system is presented in Plate 1.

3.2.2.2 Cleaning and autoclaving of bioreactor vessels and different parts of bioreactor

The vessels and different parts of the bioreactor were washed thoroughly and sterilized/autoclaved before use. The bioreactor vessels and connected parts were assembled the filters were covered with aluminum foil and the assembly was ready to use.

3.2.3 Experimental conditions

3.2.3.1 Transfer area and aseptic manipulation

The process of subculturing for multiplication, elongation, and rooting was carried out in the laminar air flow cabinet to maintain the aseptic condition. Prior to culturing, the surface of the laminar air flow cabinet was sterilized using 70 per cent alcohol. Then UV light was switched on for 20 min and the air was allowed to blow off for 15 min before working in the laminar air flow cabinet.

3.2.3.2 Culture conditions

The culture room was provided with an air conditioner and cultures were incubated at 25±2ºC with 16 h photoperiod (1000 lux). Humidity in the culture room varied from 60 to 80 per cent according to the prevailing climate.

3.2.4 Development of banana micropropagation protocol using Temporary Immersion Bioreactor

The standardized micropropagation protocol for banana cultivar Nendran developed at CPBMB (Shylaja *et al*., 2015) was followed for the development of micropropagation protocol using TIB. Based on preliminary optimization of TIB done at CPBMB, various treatments for the present study were fixed. Established cultures in the 5^{th} subculture stage received from the commercial micropropagation unit of CPBMB were utilized for the study. After every 21 days of inoculation, the cultures were transferred to the fresh medium. The different Clump size/ L of a medium, media for multiplication and rooting were optimized in bioreactor and compared with the conventional system. Three

Plate 1. Experimental setup with Plantform temporary immersion bioreactor

clump sizes/500 ml medium and three media each for multiplication and rooting based on already standardized media for banana micropropagation at CPBMB were compared. Immersion duration of one minute at three hourly intervals were fixed for all experiments. Comparison with conventional micropropagation protocol was done at multiplication, rooting, hardening and survival stages of plantlets. Growth parameters of plants viz. height of plants and number of leaves of plants produced by the two systems were also compared after two months of secondary hardening.

3.2.5 Hardening and acclimatization

After 21 days of incubation, the plantlets were taken out from the culture vessel using forceps. The plantlets from the solid medium were then washed under running tap water to remove agar. The primary hardening of plantlets was done in the small earthen pots filled with a potting mixture containing cocopeat, soil rite, and vermiculite in a 3:1:1 ratio and was kept in the hardening chamber in the shade net house. After two weeks of primary hardening, the plants were transferred to polyethene bags containing sand, soil, and cow dung in a 1:1:1 ratio and were kept for secondary hardening in the shade net house. The plants were observed for two months. Survival of plants after secondary hardening and growth parameters viz. plant height and number of leaves were recorded.

3.2.6 Clonal fidelity analysis using specific ISSR marker

The 5^{th} subculture stage cultures of banana cultivar Nendran received from CPBMB was used for the micropropagation studies in TIB and conventional systems. Plants rooted after passing through eight subculture passages both from TIB and conventional systems were analyzed for clonal fidelity using specific ISSR marker selected at CPBMB (UBC857) as reported by Rajitha *et al.* (2015). One hundred regenerants were planted out from each treatment, both from conventional and TIB, and clonal fidelity was assessed using DNA isolated from ten random plants each from the two systems.

The ISSR (Inter Simple Sequence Repeats) assay is a very simple, cheap, fast, highly discriminative, and reliable method for clonal fidelity analysis. The

assay requires a very small quantity of DNA sample (5-50 ng) per reaction. ISSR markers are widely distributed throughout the genome and also the design of primer is possible without knowing the sequence information. Hence, the ISSR marker system is suitable for clonal fidelity analysis of *in vitro* raised plantlets.

3.2.6.1 Genomic DNA isolation

Isolation of good quality genomic DNA from tissue culture derived banana plants is a very crucial step in ISSR assay. The extraction of nucleic acids was done using the CTAB method reported by Rogers and Bendich (1985). Young and tender leaves from tissue culture plants were collected and flash-frozen using liquid nitrogen. The leaves were then stored in a -80ºC deep freezer until isolation of genomic DNA.

Reagents (Details of the composition of reagents are provided in Annexure III)

1. 5X CTAB extraction buffer
    - -5 per cent CTAB (w/v)
    - -100 mM Tris (pH 8.0)
    - -20 mM EDTA (pH 8.0)
    - -1.4 M NaCl
    - -1 per cent PVP
2. CTAB (10%)
    - -10 per cent CTAB (w/v)
    - -0.7 M NaCl
3. TE buffer
    - -10 mM Tris (pH 8.0)
    - -1mM EDTA (reagent 1 and 3 autoclaved and stored at room temperature)
4. β- mercaptoethanol
5. Chloroform:isoamyl alcohol (24:1)
6. Isopropanol (chilled)
7. Ethanol (100 and 70 %)

8. Distilled water

9. RNase 1 per cent

3.2.6.1.1 Procedure for DNA isolation

Young and tender leaf tissue (0.25g) was ground in a pre-chilled mortar and pestle with liquid nitrogen and a pinch of PVP. 5X extraction buffer (1 ml) and β-mercaptoethanol (50µl) were added. The homogenized extract was then collected in a 2 ml autoclaved centrifuge tube. Then it was mixed properly and incubated at 65ºC for 30 min in a water bath with occasional mixing by gentle inversion followed by 10 min incubation on ice. The tubes were filled with an equal volume of chloroform: isoamyl alcohol (24:1v/v) and mixed by inverting the tubes. Centrifuged the tubes at 12000 rpm for 15 min at 4ºC. The extract was separated into three distinct phases. The Genomic DNA from the top aqueous phase was transferred into a fresh microcentrifuge tube. The RNase treatment was given by adding 1µl RNase at 37ºC for 45 min. After RNase treatment 1/10th volume of ten per cent CTAB buffer and an equal volume of chloroform: isoamyl alcohol were added and mixed thoroughly by inversion. Then the tubes were centrifuged at 12000 rpm for 15 min at 4ºC. Again a top aqueous phase was transferred into a new microcentrifuge tube. Chilled isopropanol 0.6 volume was added and mixed by quick gentle inversion till the DNA was precipitated. Incubated at -20ºC for 2 h or overnight and then the tubes were centrifuged at 10000 rpm for 10 min at 4ºC. The supernatant was discarded and the pellet containing DNA was washed first in 70 per cent ethanol and then by 100 per cent ethanol, spin for 3 min at 8000 rpm, and discarded the ethanol. Air-dried the pellet for 30 min, the pellet containing DNA was dissolved in 50 µl of TE buffer and stored at -20ºC.

3.2.6.2 Assessing the quality of DNA by electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis.

Materials for agarose gel electrophoresis

1) Agarose (SRL, Low EEO)

- 0.8 per cent (for genomic DNA)

- 1.8 per cent for ISSR assay

2) 50X TAE buffer (pH 8.0)

- Tris buffer
- Acetic acid
- 0.5 mM EDTA

3) 6X Loading/ Tracking dye

- 0.25 per cent bromophenol blue
- 0.25 per cent xylene cyanol
- 30 per cent glycerol

4) Ethidium bromide (0.5µg/ml)

5) Electrophoresis unit, power pack, gel casting tray, comb

6) UV transilluminator

7) Gel documentation and analysis system

The chemical composition of buffers and dye is given in Annexure IV. The procedure for agarose gel electrophoresis is given below:

The 50X stock solution of TAE was used to prepare1X TAE. Separation of genomic DNA on agarose gel was done by using 0.8 per cent agarose. For ISSR assay, 1.8 per cent agarose was used for the preparation of gel. Agarose was dissolved in 1X TAE buffer by melting in a microwave oven. Before solidifying, ethidium bromide (0.5µg ml^{-1}) was added and mixed well. The gel casting tray was fixed tightly. The comb was placed on a tray and poured the melted agarose into the tray. Then it was kept for 20-25 min to solidify and the comb was removed carefully. After removing the comb tray was kept in the electrophoresis unit. The tank was filled with 1X TAE buffer. DNA sample (4µl) and loading dye (1µl) were loaded into the wells using a micropipette. The tank was closed, ends of anode and cathode were connected to the power pack and the gel was run at 80V. When the gel loading dye reached e $2/3^{rd}$ length of the gel, the power was turned off.

The visualization of the gel was done by using a UV transilluminator for the presence or absence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented in a gel documentation

system. The gel profile was examined for the intactness and clarity of the DNA band.

3.2.6.3 Assessing the quality and quantity of DNA by NanoDrop method

NanoDrop^{R} ND-1000 spectrophotometer was used for the estimation of the quality and quantity of genomic DNA. First, by using 1µl autoclaved distilled water, the instrument was set to zero as blank. After setup of blank, 1 µl DNA sample was loaded on to the pedestal. The absorbance observed at 260 nm and 280 nm wavelength and the ratio of A_{260}/A_{280} were recorded to assess the purity of DNA.

Good quality DNA is obtained if the ratio of A_{260}/A_{280} is between 1.8 and 2.0. The quantity of DNA present in the sample was calculated using the following formula.

A_{260}=1 is equivalent to a 50 µg double-stranded DNA/ µl sample.

1 Absorbance at 260 nm=50 µg DNA/ml Therefore A_{260} x 50 gives the quantity of DNA in µg/ml.

3.2.6.4 Inter Simple Sequence Repeats (ISSR) assay

For analyzing clonal fidelity of the micro propagated plants of TIB and conventional systems, a specific ISSR marker standardised at CPBMB reported by Rajitha *et al*. (2015) was used.

Good quality genomic DNA (20-50 ng/µl) isolated from leaf samples of regenerants from TIB and conventional systems were amplified using the specific ISSR marker UBC 857, reported by Rajitha *et al*. (2015).

Polymerase Chain Reaction (PCR) was done for DNA amplification by using an Agilent thermocycler. A reaction mixture (20 µl) was prepared as shown below:

Composition of the reaction mixture for PCR

Materials	Quantity (µl)
a) Genomic DNA (20 ng/µl)	2.0
b) 10X *Taq* assay buffer B with $MgCl_2$	2.0
c) dNTP mix (10 mM each)	1.8
d) Taq DNA polymerase (3U)	0.4
e) Primer	2.0
f) Autoclaved distilled water	11.8
Total volume	20.0

The thermocycler was programmed as follows:

Initial denaturation	-	95ºC for 4 min	
Denaturation	-	94ºC for 45 seconds	36 cycles
Primer annealing	-	48ºC for 1 minute	
Primer extension	-	72ºC for 2 min	
Final extension	-	72ºC for 10 min	
4ºC for infinity to hold the sample			

The PCR products obtained after amplification of DNA was then electrophoresed to observe the banding pattern of ISSR assay. The gel was prepared by using 1.8 per cent agarose and 1X TAE buffer stained with ethidium bromide. After 2/3rd running of gel, it was stopped and visualized under a UV transilluminator and then the profile was documented and saved using a gel documentation unit. The polymorphism at the genetic level was examined according to the banding pattern of the ISSR assay.

3.2.6.5 ISSR data analysis

The banding pattern obtained after PCR amplification with the selected marker was compared with the source mother plant DNA and observed for polymorphism if any.

3.2.7 Statistical analysis

Software OPSTAT developed by Chaudhary Charan Singh Haryana Agricultural University was used for the statistical analyses. For comparing responses of different media in TIB and conventional system and growth of plants from TIB and conventional system, two-factor analyses were performed.

Results

4. RESULTS

The investigations on "Commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system" were conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during 2018 to 2020. The experiments were conducted to develop a micropropagation protocol in banana using the TIB system and to assess the clonal fidelity of micro propagated plants produced through the TIB system at a molecular level.

Studies were conducted in the banana cultivar Nendran (AAB) which is being produced commercially in the micropropagation unit of CPBMB. Established cultures in the 5^{th} sub culture stage of cultivar Nendran received from the micropropagation unit of CPBMB was utilized for the study. The results of the experiments are presented in the following sections.

4.1. Development of a banana micropropagation protocol using Temporary Immersion Bioreactor (TIB)

4.1.1 Effect of clump size on shoot proliferation of banana in different culture systems

Different clump sizes viz. 10, 15, and 20 clumps/ 500 ml of media were evaluated for shoot proliferation in a bioreactor. Shoot proliferation in different clump sizes using temporary immersion bioreactor were compared with conventional culture system. The different number of clumps were inoculated to 500 ml of liquid medium (MS + 5mg L^{-1} BA) in a Plantform TIB system and also in solid medium (MS + 5mg L^{-1} BA) for the conventional system. The experiment was carried out separately in both systems. After 21 days of inoculation, number of shoots proliferated from each clump size was observed in both systems. The effect of clump size on shoot proliferation in different culture systems is presented in Table 1.

Shoots proliferated in TIB and the conventional culture system showed significant differences. Irrespective of clump sizes, the conventional system recorded higher shoot proliferation.

The conventional culture system with 6.13 shoots proliferated showed better response as compared to 5.30 shoots proliferated in the bioreactor system. When different clump sizes were compared within culture systems, 20 clumps/ 500 ml medium recorded the highest (6.2) shoot proliferation in the Plantform TIB system. In a conventional system, 15 clumps/ 500 ml medium and 20 clumps/ 500 ml medium recorded the higher shoot proliferation, both the treatments were on par. Shoot proliferation in conventional system the 10 and 20 clumps/ 500 ml medium were also on par. In the TIB system, 10 and 15 clumps/ 500 ml medium showed no significant difference in shoot proliferation. The interaction effect of culture systems and clump sizes were also not significant. The effect of clump size on shoot proliferation in TIB is presented in Plate 2 and the conventional culture system is presented in Plate 3.

4.1.2. Effect of different multiplication media on shoot proliferation of banana in different culture systems

From the experiment on clump size optimization, 20 clumps/ 500 ml of media was selected as the best clump size. So for the experiment on the standardization of multiplication medium, 20 clumps/ 500 ml medium was inoculated. For shoot multiplication, three different media were evaluated and after three weeks of inoculation, shoot proliferation in different media in both the culture systems were observed. The effect of different multiplication media on multiple shoot formation in different culture systems is presented in Table 2.

Irrespective of the media tried, TIB system exhibited significantly higher shoot proliferation (13.00) than the conventional system (7.67). Murashige and Skoog (MS) medium supplemented with 5mg L^{-1} BA recorded highest shoot proliferation in both the culture systems. In the TIB, the medium with 5mg L^{-1} BA recorded 14.71 shoots while in the conventional system it recorded 8.35 shoots. In both the culture systems, shoot proliferation in MS medium with 3mg L^{-1} and 4mg L^{-1} BA were on par. The interaction effects of culture systems and media were not significant. The effect of different multiplication media on multiple shoot formation in TIB is presented in Plate 4 and the conventional culture system is presented in Plate 5.

Table 1. Effect of clump size on shoot proliferation in different culture systems

Culture systems	No. of clumps/ 500 ml medium			Mean No. of shoots proliferated/ clump/culture system
	10	15	20	
	*Mean No. of shoots proliferated/ clump			
Temporary immersion bioreactor	4.83	4.86	6.20	5.30
Conventional micropropagation	5.48	6.72	6.21	6.13
CD (0.05)	Factor A (Culture system)	Factor B (Clump size)	Factor(A X B)	
	0.656	0.804	NS	
SE (m)	0.219	0.268	0.380	

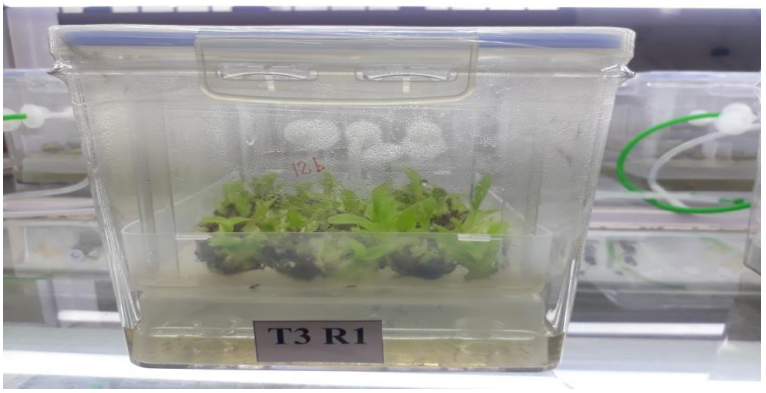
*Mean of different clump sizes, No. of replications- 4

Table 2. Shoot proliferation in multiplication media in different culture systems

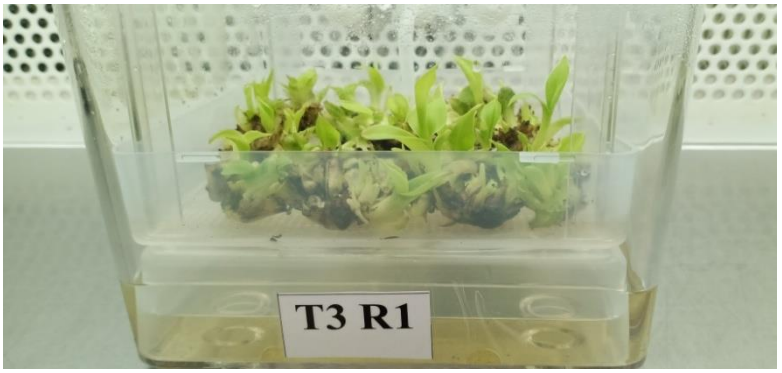
Culture systems	**Multiplication media**			**Mean No. of shoots proliferated/ clump/ culture system**
	MS + 3mg L^{-1} BA	MS + 4mg L^{-1} BA	MS + 5mg L^{-1} BA	
	***Mean No. of Shoots proliferated/clump**			
Temporary immersion bioreactor	11.81	12.47	14.71	13.00
Conventional micropropagation	6.79	7.88	8.35	7.67
CD (0.05)	Factor A (Culture system)	Factor B (Multiplication media)	Factor (A X B)	
	1.263	1.547	NS	
SE (m)	0.422	0.517	0.731	

*Mean of 20 observations / replication, No. of replications - 4

After one week of inoculation (20 clumps/ 500 ml medium)



After two weeks of inoculation (20 clumps/ 500 ml medium)



After three weeks of inoculation (20 clumps/ 500 ml medium)

Plate 2. Effect of clump size on shoot proliferation in temporary immersion bioreactor

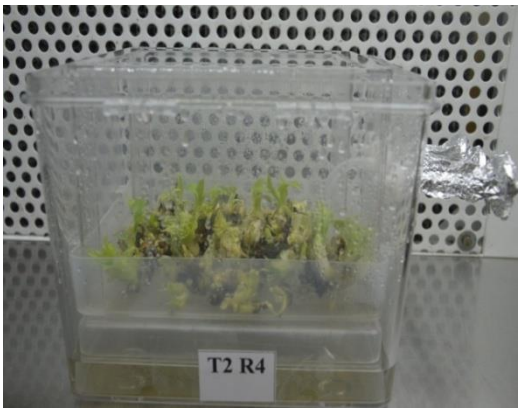
After one week of inoculation (20 clumps/ 500 ml medium)

After two weeks of inoculation (20 clumps/ 500 ml medium)

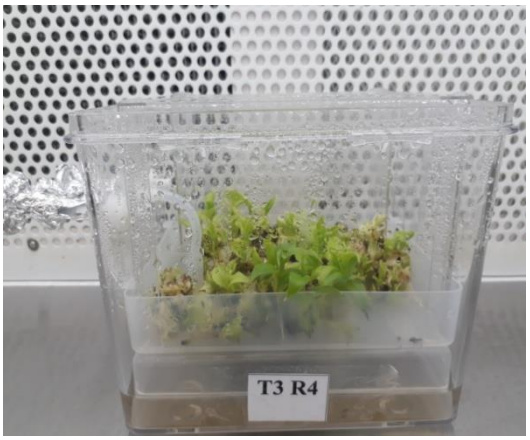
After three weeks of inoculation (20 clumps/ 500 ml medium)

Plate 3. Effect of clump size on shoot proliferation in conventional micropropagation system

MS + 3mg L^{-1} BA

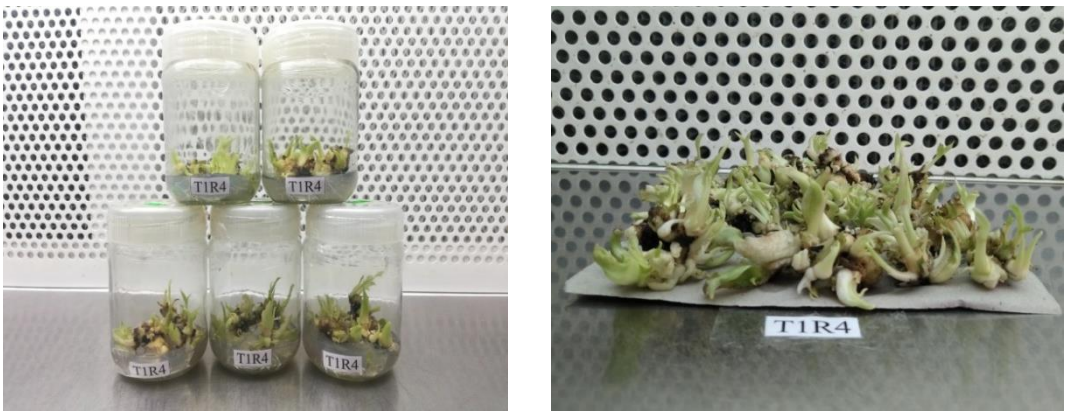


MS + 4mg L^{-1} BA



MS + 5mg L^{-1} BA

Plate 4. Effect of different multiplication media on shoot proliferation in temporary immersion bioreactor



MS + 3mg L^{-1} BA

MS + 4mg L^{-1} BA

MS + 5mg L^{-1} BA

Plate 5. Effect of different multiplication media on shoot proliferation in conventional micropropagation system

4.1.3 Effect of different rooting media on rooting and root characters of plantlets from different culture systems

Three different rooting media ware studied in both the culture systems. The media tried were MS + 1mg L^{-1} IBA and MS + 2mg L^{-1} IBA with 2 per cent sucrose and MS + 2mg L^{-1} IBA with 3 per cent sucrose. Twenty five shoots were kept for rooting in each replication. In both the culture systems, in the three different media tried, 99-100 per cent rooting was observed. The effect of different rooting media on rooting in different culture systems is shown in Table 3.

4.1.3.1 Root characters of plantlets from temporary immersion bioreactor and conventional micropropagation system in different rooting media

Days taken for root initiation, number of roots/plantlet, length of roots, plant height, and number of leaves/plantlet were recorded after three weeks of inoculation in different rooting media. The effect of different rooting media on root characters of plantlets from TIB is shown in Plate 6 and from the conventional micropropagation system in Plate 7.

Irrespective of rooting media tried, the conventional system recorded significantly early initiation of roots (5.04 days) as compared to the TIB system (5.97 days). Murashige and Skoog medium supplemented with 3 per cent sucrose recorded early initiation of roots in a bioreactor. However, in the conventional system days taken for root initiation was found on par in the three media tried. Culture systems and media interaction effects were not significant. Days taken for root initiation in different rooting media in different culture systems are shown in Table 4.

Plantlets from the temporary immersion bioreactor system recorded a significantly higher number of roots (10.29) as compared to the conventional culture system (4.70). The number of roots/ plantlet in different rooting media in different culture systems is shown in Table 5. With respect to the number of roots in different media, MS medium supplemented with 2mg L^{-1} IBA with 3 per cent sucrose recorded a higher number of roots in both the culture systems. The number of roots produced in MS media supplemented with 1mg L^{-1} IBA and 2mg L^{-1} IBA with 2 per cent sucrose was found on par in both the culture systems. The interaction effects were also not significant.

Table 3. Rooting of plantlets in different rooting media in different culture systems

Culture systems	Rooting media	No. of shoots kept for rooting	No. of shoots rooted	Rooting (%)
Temporary immersion bioreactor	MS + 2mg L^{-1} IBA+ 2% Sucrose	100	100	100
	MS + 2mg L^{-1} IBA+ 3% Sucrose	100	100	100
	MS + 1mg L^{-1} IBA+ 2% Sucrose	100	100	100
Conventional micropropagation	MS + 2mg L^{-1} IBA+ 2% Sucrose	100	99.16	99.16
	MS + 2mg L^{-1} IBA+ 3% Sucrose	100	100	100
	MS + 1mg L^{-1} IBA+ 2% Sucrose	100	100	100

MS + 2mg L^{-1} IBA+ 2 per cent Sucrose

MS + 2mg L^{-1} IBA+ 3 per cent Sucrose

MS + 1mg L^{-1} IBA+ 2 per cent Sucrose

Plate 6. Effect of different rooting media on root characters in plantlets of temporary immersion bioreactor

MS + 2mg L^{-1} IBA+ 2 per cent Sucrose

MS + 2mg L^{-1} IBA+ 3 per cent Sucrose

MS + 1mg L^{-1} IBA+ 2 per cent Sucrose

Plate 7. Effect of different rooting media on root characters in plantlets of conventional micropropagation system

Table 4. Days taken for root initiation in different rooting media in different culture systems

Culture systems	Rooting media			Mean No. of days taken for root initiation/ culture system
	MS + 2mg L^{-1} IBA+ 2% Sucrose	MS + 2mg L^{-1} IBA+ 3% Sucrose	MS + 1mg L^{-1} IBA+ 2% Sucrose	
	***Mean No. of days taken for root initiation**			
Temporary immersion bioreactor	6.12	5.36	6.43	5.97
Conventional micropropagation	5.05	4.83	5.23	5.04
CD (0.05)	Factor A (Culture system)	Factor B (Rooting media)	Factor(A X B)	
	0.345	0.422	NS	
SE (m)	0.115	0.141	0.199	

*Mean of 25 observations / replication, No. of replications - 4

Table 5. Number of roots/plantlet in different rooting media in different culture systems

Culture systems	Rooting media			Mean No. of roots/ plantlet/ culture system
	MS + 2mg L^{-1} IBA+ 2% Sucrose	MS + 2mg L^{-1} IBA+ 3% Sucrose	MS + 1mg L^{-1} IBA+ 2% Sucrose	
	***Mean No. of roots/ plantlet**			
Temporary immersion bioreactor	10.04	11.71	9.11	10.29
Conventional micropropagation	4.07	5.86	4.17	4.70
CD (0.05)	Factor A (Culture system)	Factor B (Rooting media)	Factor(A X B)	
	0.837	1.025	NS	
SE (m)	0.279	0.342	0.484	

*Mean of 25 observations / replication, No. of replications: 4

The length of roots in plantlets in different rooting media in two culture systems is shown in Table 6. A conventional culture system produced significantly longer roots as compared to the TIB system. The root length of plantlets in three different media tried was found on par in the two culture systems.

The shoot length of plantlets in different rooting media in different culture systems is shown in Table 7. Irrespective of the media tried, plantlets from the TIB recorded significantly higher shoot length (10.88 cm) as compared to the conventional system (8.94 cm). Length of shoots was found on par in the different media tried in the two culture systems and ranged from 10.48 - 11.32 cm in TIB and 8.92-8.98 cm in the conventional system.

The number of leaves/ plantlet in different rooting media in the two culture systems is shown in Table 8. Plantlets from TIB recorded a significantly higher number of leaves (4.84) as compared to the conventional system (4.39). The number of leaves were on par in the three media tried in the two different culture systems and ranged from 4.70 - 4.96 in TIB and 4.31- 4.49 in the conventional system. The interaction effect between culture systems and media were found nonsignificant.

Table 6. Length of roots in plantlets in different rooting media in different culture systems

Culture systems	**Rooting media**			**Mean root length/plantlet/ culture system (cm)**
	MS + 2mg L^{-1} IBA+ 2% Sucrose	MS + 2mg L^{-1} IBA+ 3% Sucrose	MS + 1mg L^{-1} IBA+ 2% Sucrose	
	***Mean Root length/ plantlet (cm)**			
Temporary immersion bioreactor	8.04	8.12	7.66	7.93
Conventional micropropagation	8.69	8.11	9.66	8.82
CD (0.05)	Factor A (Culture system)	Factor B (Rooting media)	Factor(A X B)	
	0.879	NS	NS	
SE (m)	0.294	0.360	0.509	

(*Mean of 25 observations / replication, No. of replications - 4)

Table 7. Length of shoot in plantlets in different rooting media in different culture system

Culture systems	**Rooting media**			**Mean length of shoot in plantlet / culture system (cm)**
	MS + 2mg L^{-1} IBA+ 2% Sucrose	MS + 2mg L^{-1} IBA+ 3% Sucrose	MS + 1mg L^{-1} IBA+ 2% Sucrose	
	*** Mean Length of shoot in plantlet (cm)**			
Temporary immersion bioreactor	10.84	10.48	11.32	10.88
Conventional micropropagation	8.92	8.98	8.93	8.94
CD (0.05)	Factor A (Culture system)	Factor B (Rooting media)	Factor(A X B)	
	0.548	NS	NS	
SE (m)	0.183	0.224	0.317	

*Mean of 25 observations / replication, No. of replications- 4

Table 8. Number of leaves/ plantlet in different rooting media in different culture systems

Culture systems	Rooting media			Mean number of leaves/ plantlet/culture system
	MS + 2mg L^{-1} IBA+ 2% Sucrose	MS + 2mg L^{-1} IBA+ 3% Sucrose	MS + 1mg L^{-1} IBA+ 2% Sucrose	
	***Mean Number of leaves/ plantlet**			
Temporary immersion bioreactor	4.70	4.96	4.86	4.84
Conventional micropropagation	4.31	4.49	4.38	4.39
CD (0.05)	Factor A (Culture system)	Factor B (Rooting media)	Factor(A X B)	
	0.272	NS	NS	
SE (m)	0.091	0.111	0.157	

*Mean of 25 observations / replication, No. of replications: 4

4.1.4 Hardening and acclimatization of plantlets

After three weeks of the rooting experiment, the plantlets were planted out treatment wise separately from TIB and conventional micropropagation systems. The primary hardening was done by using a mixture of coco peat, soil rite, and vermiculite in a 3:1:1 ratio in small earthen pots and were kept in the net house in chambers with high humidity for two weeks, and the survival percentage was recorded. Primary hardened plants from TIB are presented in plate 8 and the plants from the conventional micropropagation system are presented in Plate 9. The primary hardened plants after two weeks of plant out were transferred to polythene bags containing sand, soil, and cow dung in a 1:1:1 ratio for secondary hardening. The plants were observed for two months in the net house and the survival rate was recorded. The plants after two months of secondary hardening from the TIB system are shown in Plate 10 and from the conventional system in Plate 11.

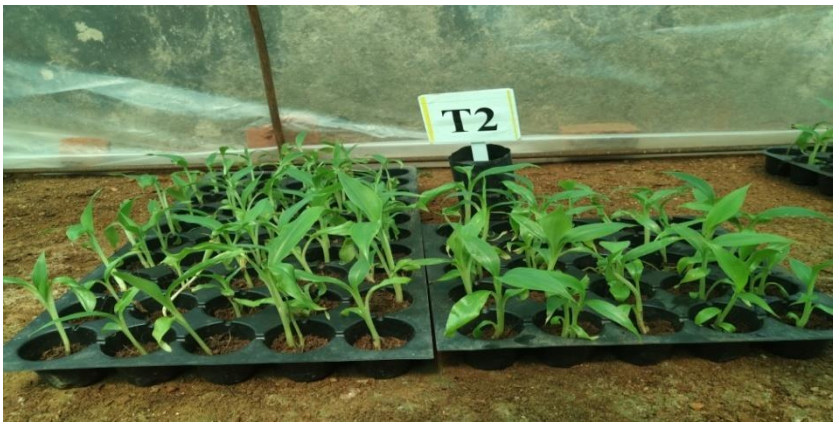
Plants from TIB and conventional system registered almost uniform survival. The survival percentage was uniform in plants from different media in both the culture systems. The survival of plants after primary hardening from different culture systems is shown in Table 9.

The survival of the secondary hardening was 90-91 per cent. The survival was found uniform in two culture systems. Plants from different rooting media of two culture systems registered uniform survival after secondary hardening. The survival of plants after secondary hardening from different culture systems is shown in Table 10.

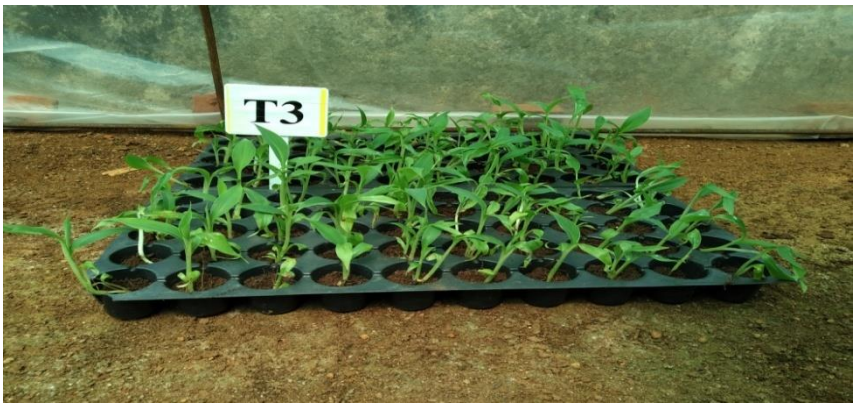
Table 9. Survival of plants after primary hardening

Culture systems	Rooting media	No. of plantlets planted out	No. of plants survived after two weeks	Survival (%)	Survival (%) in different culture systems
Temporary immersion bioreactor	MS + 2mg L^{-1} IBA+ 2% Sucrose	100	99	99	98.22
	MS + 2mg L^{-1} IBA+ 3% Sucrose	75	74	98.66	
	MS + 1mg L^{-1} IBA+ 2% Sucrose	100	97	97	
Conventional micropropagation	MS + 2mg L^{-1} IBA+ 2% Sucrose	100	97	97	97.66
	MS + 2mg L^{-1} IBA+ 3% Sucrose	100	98	98	
	MS + 1mg L^{-1} IBA+ 2% Sucrose	100	98	98	

MS + 2mg L^{-1} IBA+ 2 per cent Sucrose

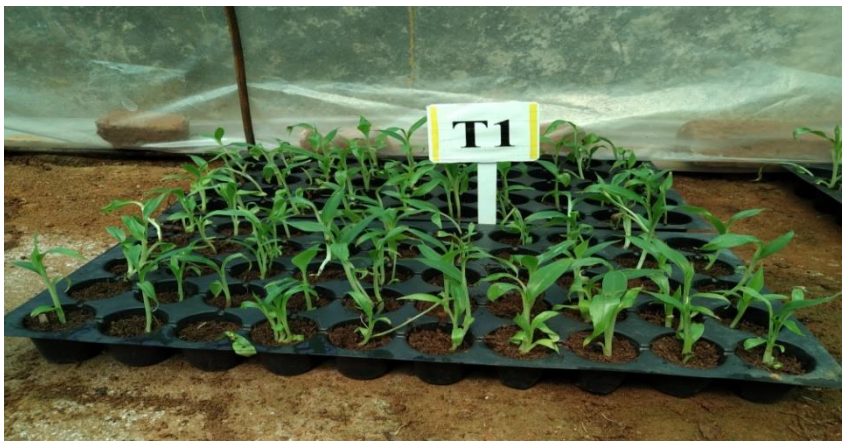


MS + 2mg L^{-1} IBA+ 3 per cent Sucrose

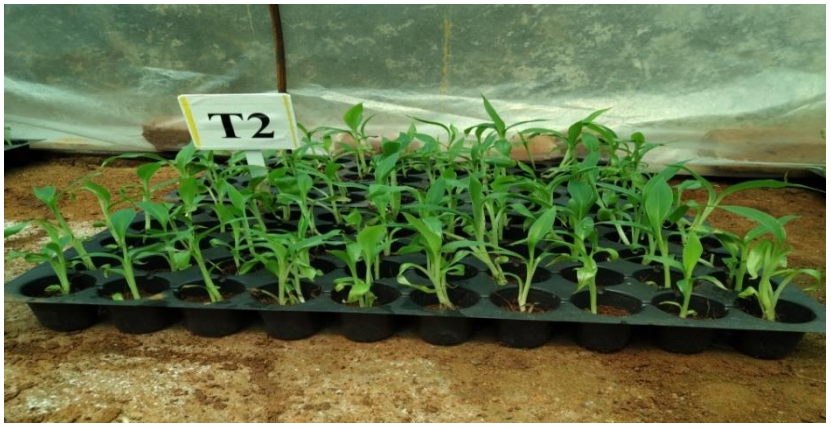


MS + 1mg L^{-1} IBA+ 2 per cent Sucrose

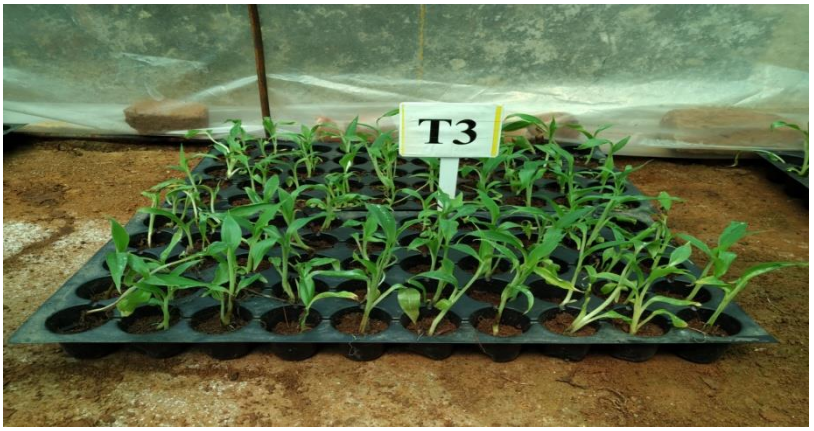
Plate 8. Plants from temporary immersion bioreactor after two weeks of primary hardening



MS + 2mg L^{-1} IBA+ 2 per cent Sucrose



MS + 2mg L^{-1} IBA+ 3 per cent Sucrose



MS + 1mg L^{-1} IBA+ 2 per cent Sucrose

Plate 9. Plants from conventional micropropagation system after two weeks of primary hardening

Table 10. Survival of plants after secondary hardening

Culture systems	Rooting media	No. of plants planted after primary hardening	No. of plants survived after two months	Survival (%)	Survival (%) in different culture systems
Temporary immersion bioreactor	MS + 2mg L^{-1} IBA+ 2% Sucrose	99	91	91.91	91.51
	MS + 2mg L^{-1} IBA+ 3% Sucrose	74	68	91.89	
	MS + 1mg L^{-1} IBA+ 2% Sucrose	97	88	90.72	
Conventional micropropagation	MS + 2mg L^{-1} IBA+ 2% Sucrose	97	88	90.72	90.10
	MS + 2mg L^{-1} IBA+ 3% Sucrose	98	90	91.83	
	MS + 1mg L^{-1} IBA+ 2% Sucrose	98	86	87.75	

4.1.4 .1 Plant characters after secondary hardening

After successful secondary hardening for two months, morphological parameters were recorded in plants produced from two culture systems. The number of leaves and plant height were recorded from both of the culture systems in all the treatments.

The height of TC plants derived from different rooting media and culture systems (sixty days after secondary hardening) is shown in Table 11. Culture systems, rooting media and media x culture system interaction effects were found significant with respect to plant height. The plants from TIB recorded significantly higher plant height as compared to the conventional system. In TIB and conventional system, plants from different rooting media exhibited significantly different plant height. In TIB system, highest plant height was recorded in medium (MS + 2mg L^{-1} IBA+ 2% sucrose) followed by MS + 2mg L^{-1} IBA+ 3 per cent sucrose and MS + 1mg L^{-1} IBA+ 2 per cent sucrose. In a conventional system, MS medium with 2mg L^{-1} IBA+ 3 per cent sucrose recorded the highest plant height, followed by 1mg L^{-1} IBA+ 2 per cent sucrose and 2mg L^{-1} IBA+ 2 per cent sucrose, both of them were on par. Plant height recorded in plants of MS + 1mg L^{-1} IBA+ 2 per cent sucrose of TIB and conventional systems were found on par while plants from other media showed significant difference.

The number of leaves in TC plants derived from different rooting media and culture systems is shown in Table 12. Culture systems, rooting media and media x culture system interaction effects were found significant with respect to the number of leaves also. Plants from the TIB system produced a significantly higher number of leaves (6.53) as compared to the conventional system (5.89). In the TIB system, plants from the three different media recorded a significantly different number of leaves. The highest number of leaves was recorded in MS + 2 mg L^{-1} IBA+ 3 per cent sucrose (6.8) followed by MS + 1mg L^{-1} IBA+ 2 per cent sucrose (6.5) and MS + 2 mg L^{-1} IBA+ 3 per cent sucrose (6.3). In the conventional system, MS + 2 mg L^{-1} IBA+ 2 per cent sucrose and MS + 1mg L^{-1} IBA+ 2 per cent sucrose were on par with respect to number of leaves.

MS + 2mg L^{-1} IBA+ 2 per cent Sucrose

MS + 2mg L^{-1} IBA+ 3 per cent Sucrose

MS + 1mg L^{-1} IBA+ 2 per cent Sucrose

Plates 10. Plants from temporary immersion bioreactor after two months of secondary hardening

MS + 2mg L^{-1} IBA+ 2 per cent Sucrose

MS + 2mg L^{-1} IBA+ 3 per cent Sucrose

MS + 1mg L^{-1} IBA+ 2 per cent Sucrose

Plates 11. Plants from conventional micropropagation system after two months of secondary hardening

Table 11. Height of TC plants derived from different rooting media and culture systems (sixty days after secondary hardening)

Culture systems	Rooting media			Mean plant height (cm)/culture system
	MS + 2mg L^{-1} IBA+ 2% Sucrose	MS + 2mg L^{-1} IBA+ 3% Sucrose	MS + 1mg L^{-1} IBA+ 2% Sucrose	
	*Mean Plant height (cm)			
Temporary immersion bioreactor	36.05	34.25	29.85	33.39
Conventional micropropagation	29.80	36.00	30.00	31.93
CD (0.05)	Factor A (Culture system)	Factor B (Rooting media)	Factor(A X B)	
	0.706	0.864	1.222	
SE (m)	0.236	0.289	0.408	

*Mean of 25 observations / replication, No. of replications - 4

Table 12. Number of leaves in TC plants derived from different rooting media and culture systems (Sixty days after secondary hardening)

Culture systems	Rooting media			Mean No. of leaves/ plant/ culture system
	MS + 2mg L^{-1} IBA+ 2% Sucrose	MS + 2mg L^{-1} IBA+ 3% Sucrose	MS + 1mg L^{-1} IBA+ 2% Sucrose	
	*Mean Number of leaves/ plant			
Temporary immersion bioreactor	6.30	6.80	6.50	6.53
Conventional micropropagation	5.70	6.30	5.65	5.89
CD (0.05)	Factor A (Culture system)	Factor B (Rooting media)	Factor(A X B)	
	0.071	0.086	0.122	
SE (m)	0.024	0.029	0.041	

*Mean of 25 observations / replication, No. of replications: 4

4.2 Analysis of clonal fidelity using specific ISSR marker

4.2.1 Isolation of genomic DNA

4.2.1.1 Source of DNA

For the isolation of genomic DNA, the rooted plantlets after 8th subculture passage obtained from TIB and the conventional system were planted out and maintained in the net house. The DNA was extracted from randomly selected 10 plants of each treatment. The parental DNA of mother plants available at CPBMB was received for clonal fidelity analysis studies.

4.2.1.2 Isolation and purification of DNA

The young tender leaves from the plantlets produced in TIB and conventional culture systems were used for isolation of genomic DNA. Isolation of DNA was done following the CTAB method reported by Roger and Bendich (1994). The procedure was slightly modified due to RNA contamination and pure DNA was obtained by giving RNase treatment after DNA isolation.

4.2.1.3 Quantification of DNA

The quality and quantity of isolated DNA were analyzed. Agarose gel electrophoresis was used to view the quality of the DNA sample, and the quantity of the DNA was estimated by using the NanoDrop spectrophotometer. Pure quality non-degraded DNA with intact clear bands or without smear was observed on gel under UV light. Absorbance (A_{260}/A_{280}) ratio in a range between 1.7-2.0 was recorded after the NanoDrop spectrophotometer analysis (Table 13). The Good quality DNA was further diluted and used as a template for ISSR assay.

Table No. 13. Quantity analysis of DNA isolated from regenerants4.2.2 ISSR analysis

Temporary immersion bioreactor system			Conventional system		
Sample No.	**Concentration (ng/µl)**	**Absorbance Ratio (A260/280)**	**Sample No.**	**Concentration (ng/µl)**	**Absorbance Ratio (A260/280)**
1	87.28	1.7	1	107.5	1.7
2	213.65	1.9	2	93.40	1.8
3	140.83	2.0	3	251.31	1.8
4	144.77	2.0	4	149.69	1.7
5	138.12	2.0	5	155.59	2.0
6	104.54	2.0	6	70.14	1.7
7	119.43	2.0	7	185.88	1.8
8	162.14	2.0	8	382.61	2.0
9	148.29	1.8	9	246.36	1.9
10	188.06	2.0	10	446.07	2.0

Clonal fidelity analysis was done using a specific ISSR primer, UBC857 reported by Rajitha *et al.* (2015) at CPBMB. Isolated genomic DNA from mother plant and plants from TIB and conventional system were subjected to ISSR analyses.

Gel electrophoresis was carried out to run the PCR product and gel pictures were scored to observe the total number of amplicons, number of polymorphic and monomorphic amplicons. The amplification pattern obtained in plants from different culture systems is presented in Plate 12.

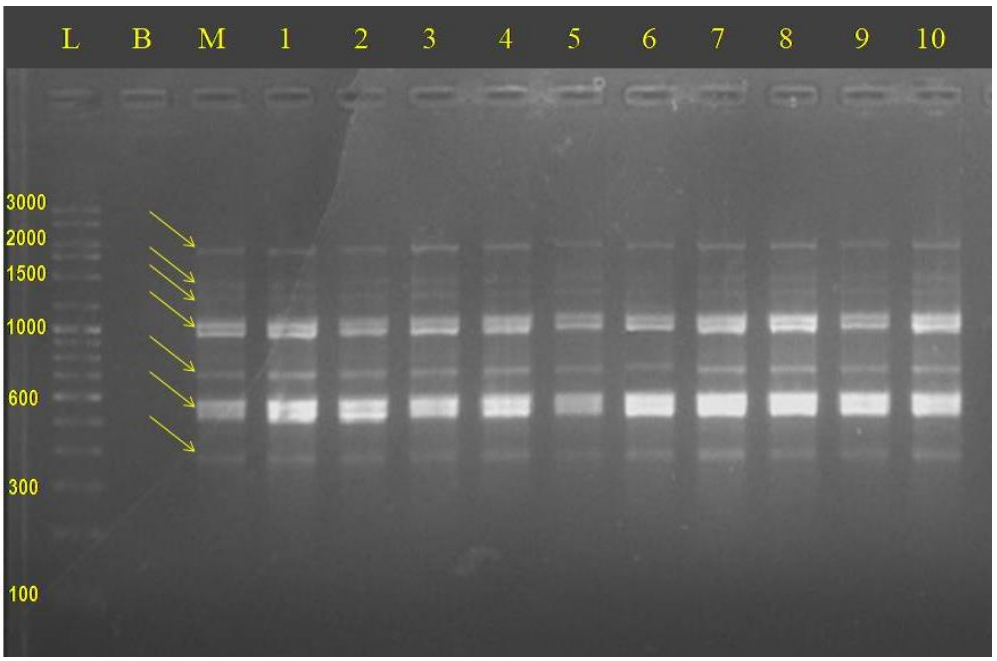
Amplification with the UBC 857 primer generated clear amplicons. The bands visualized were in the size range of 300 to 2000 bp. There were no polymorphic amplicons observed in the ISSR amplification profiles in plants of cultivar Nedunendran after the 8^{th} subculture cycle in different culture systems (Table 14).

Table: 14 (a): DNA amplification pattern in plants of different culture systems using specific ISSR primer

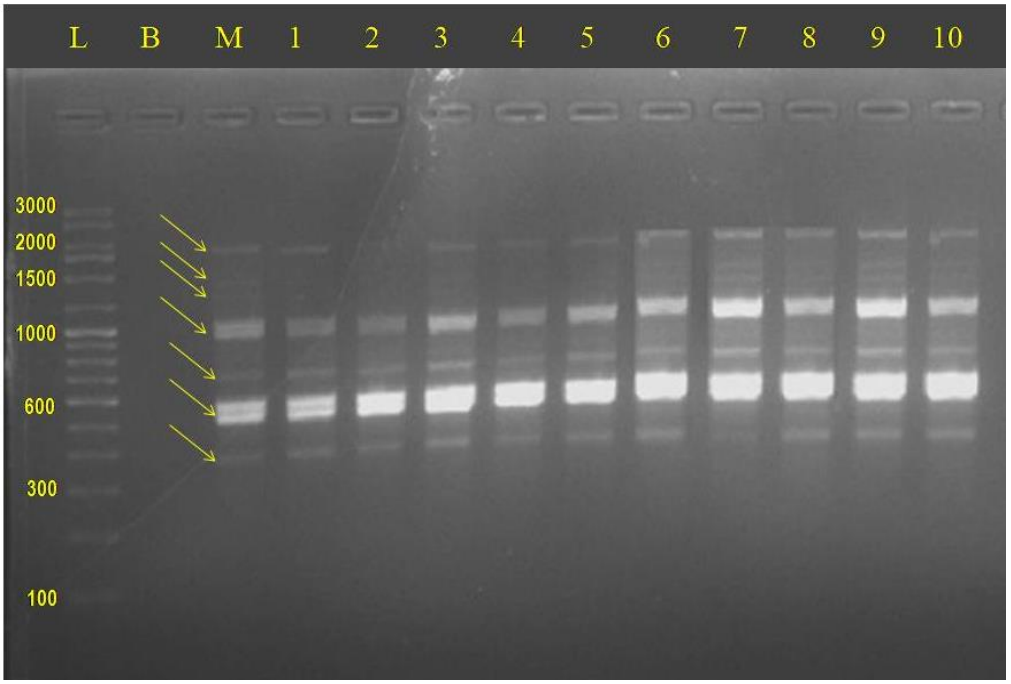
Mother plant/ regenerants	Total No. of amplicons	No. of monomorphic amplicons	No. of polymorphic amplicons
Source mother plant (Nedunendran)	7	-	-
Regenerants from temporary immersion bioreactor system			
1	7	7	0
2	7	7	0
3	7	7	0
4	7	7	0
5	7	7	0
6	7	7	0
7	7	7	0
8	7	7	0
9	7	7	0
10	7	7	0

Table: 14 (b): DNA amplification pattern in plants of different culture systems using ISSR primer

Mother plant /regenerants	Total No. of amplicons	No. of monomorphic amplicons	No. of polymorphic amplicons
Source mother plant (Nedunendran)	7	-	-
Regenerants from conventional system			
1	7	7	0
2	7	7	0
3	7	7	0
4	7	7	0
5	7	7	0
6	7	7	0
7	7	7	0
8	7	7	0
9	7	7	0
10	7	7	0



Temporary immersion bioreactor



Conventional micropropagation system

L: Molecular weight marker (3kb), B: blank, M: mother plant, 1-10: regenerants

Plate 12. DNA amplification pattern in plants from different culture systems

5. DISCUSSION

The present research work on "Commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system" was carried out to to develop an efficient commercial micropropagation protocol in banana using a temporary immersion bioreactor (TIB) system. The experimental results of the investigations are discussed in this chapter.

Studies were conducted in the cultivar Nedunendran (AAB) which is being produced commercially at the micropropagation unit of CPBMB. Established cultures of cultivar Nedunendran in the 5^{th} subculture cycle received from the commercial micropropagation unit of CPBMB were utilized for the study. The clonal fidelity analysis standardized using a specific ISSR marker at CPBMB was used to analyze the clonal fidelity of plants after the 8^{th} subculture passage. The Plantform temporary immersion bioreactor designed by Professor Margareta Welander, Sweden and Dr. Abdullah J Sayegh, Ireland was used in the present study.

5.1 Development of banana micropropagation protocol using temporary immersion bioreactor

5.1.1 Effect of clump size on shoot proliferation of banana in different culture systems

Number of clumps/litre of medium is an important physical parameter that influences growth of culture. The total number of shoots proliferated generally depends on clump density. Clumps under *in vitro* conditions compete with each other for a constant amount of water and nutrients from growth medium (Yildiz, 2011). More clump densities may also cause limited nutritional supply which result in less growth of culture. Therefore to get maximum multiplication from a defined volume of the nutritional medium, number of clumps inoculated needs special attention. Keeping this in mind an experiment was conducted to find out the effect of different clumps sizes (number of clumps/ 500 ml medium) on shoot proliferation.

In the present study, three different clump sizes viz. 10, 15, and 20 clumps/ 500 ml of media were evaluated for shoot proliferation in a TIB and compared with the conventional micropropagation system. Murashige and Skoog (MS) medium supplemented with 5mg L^{-1} BA was used to initiate shoot multiplication in both the systems. However the medium was solidified using agar for the conventional system, while for TIB liquid medium without agar was used. After 21 days of inoculation, the clumps from the conventional culture system showed higher shoot proliferation (6.13 shoots) as compared to the TIB system (5.3 shoots), irrespective of the number of clumps inoculated.

Clump size of 20 clumps/ 500 ml medium recorded the highest shoot (6.2) proliferation in TIB followed by 15 clumps/ 500 ml medium (4.86) and 10 clumps/ 500 ml medium (4.83) which indicates the positive effect of higher clump density on shoot proliferation in a TIB.

In conventional micropropagation system, 15 clumps/ 500 ml medium and 20 clumps/ 500 ml medium recorded higher shoot proliferation of 6.72 and 6.21 respectively, both of them were on par. The number of shoots proliferated was 5.48 from clump size of 10 clumps/ 500 ml medium.

According to Son *et al*. (1999), high inoculum density (20±30%) is important to achieve a minimal lag phase and a significant growth of plant cells. The effect of explant density on biomass increase varies with the culture systems, plant species, and required culture period (Peak *et al*. 2001).

For the multiplication of plantain, five intact shoots in 150 ml medium was reported by Cejas *et al*. (2011), which is in conformity with the result of the present investigations, in which 20 clumps/ 500 ml medium recorded the highest shoot proliferation in bioreactor and 15-20 clumps/ 500 ml medium recorded higher shoot proliferation in conventional system. In banana to find out the optimum quantity of medium required for shoot proliferation, Prabhuling and Sathyanarayna (2017) inoculated 1-5 multiple buds in each culture bottle with 40 ml of MS semisolid medium. Multiple bud density of four/culture bottle was found best with higher total shoot production/L (291.94), shoot length (3.22

cm), number of leaves/shoot (2.81), and lower cost per shoot (Rs. 1.175) in the conventional system.

Hahn and Paek (2005) also suggested that a high explant density did not inhibit the multiplication of shoots in Chrysanthemum if volume of culture vessel used was large. To know the optimal explant density of Chrysanthemum in a bioreactor, different number of single node explants (20 or 40 or 60 or 80) were tested. The 80-node inoculation was found as the best explant density for multiplication of shoots in bioreactors, with the least number of branches and shoot length.

The success of micropropagation in TIS mainly depends on the inoculums density, quantity of liquid medium and volumes of the culture vessel. A wide range of published literature is available in this regard. The highest multiplication of Eucalyptus shoots was achieved when cultures were initiated with 50 buds per RITA® vessel (McAlister *et al*., 2005). Silva *et al*. (2007) used 10 pineapple buds per 300 ml medium in 1 L flask. For potato microtuber production, 60 explants in 3.5 L medium was used by Perez-Alonso *et al*. (2007). For elephant's ear, 30 corms per 1 L medium in 3 L flasks and for *E. globulus,* five microshoots per 200 ml medium in 600 ml flask were reported by Gonzales *et al*. (2011).

Agarwal *et al.* (2012) observed, significantly higher number of proliferated shoots in the *Eucalyptus tereticornis* culture when larger shoot clumps (15- 20 shoots) were inoculated, compared to smaller shoot clumps (4-5 shoots).

Another study by Garcia-Ramirez *et al*. (2016) showed that increased number of shoots, length of shoots, and leaf number were observed with an inoculum density of 12 explants in *B. Vulgaris* out of three different inoculum densities (6, 12 and 18 explants/SIT) tried.

These results indicate that the more number of clumps/litre of medium gave maximum shoot production if optimum nutrient medium and large volume culture vessel are provided. For culture vessel of size 180×150×150 cm in Plantform TIB, 20 clumps/ 500 ml medium were found optimal.

5.2. Effect of different multiplication media on shoot proliferation of banana in different culture systems

Among the three different clump sizes evaluated 20 clumps/ 500 ml of medium was observed as the best. Hence clump size of 20 clumps/ 500 ml medium was fixed to evaluate different media for shoot multiplication in both the culture systems.

In the present investigations, it was observed that the TIB system showed a significantly higher number of shoot proliferation (13.00) as compared to the conventional culture system (7.67). In different media tried, the medium MS+5mg L^{-1} BA was found as the best recording high shoot proliferation in both the culture systems (Figure.1). In the bioreactor, the medium with 5mg L^{-1} BA recorded shoot proliferation of 14.71 shoots/ clump while in the conventional system it recorded 8.35 shoots/ clump.

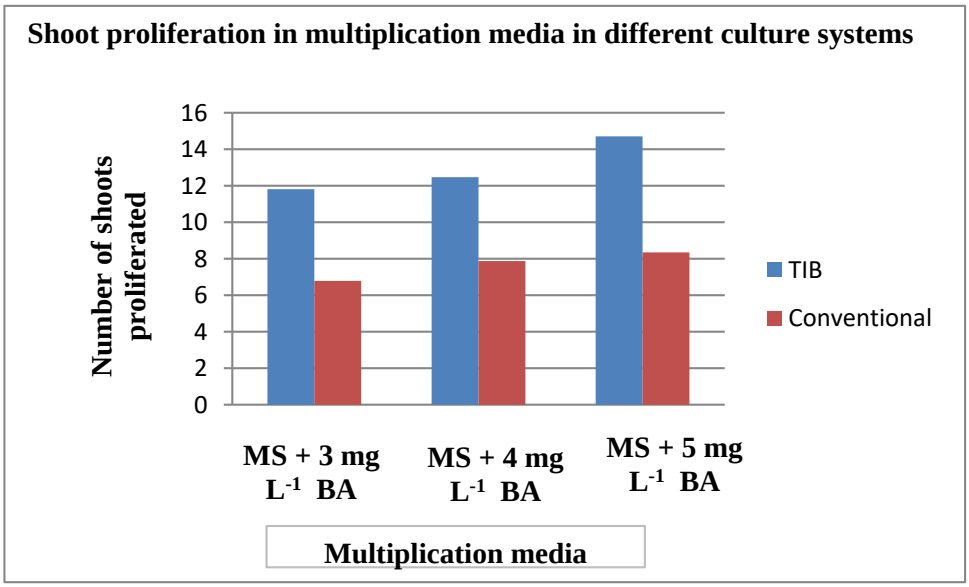


Figure. 1 Shoot proliferation in multiplication media in different culture systems

According to the first report on the use of the TIS system for banana micropropagation by Alvard *et al*. (1993), in the cultivar Grand Naine, the TIS system recorded shoot multiplication of 5.2 shoots per explant. Later Roels *et al*. (2006) noted higher number of shoots (6.4 shoots/explant) in cv. CEMSA 3/4 (AAB) using TIB. A similar trend of increased multiplication rate of banana

in TIB was reported by Farahani and Majd (2012) and Aragon *et al*. (2014). Wilken *et al*. (2014) also used 5000 ml volume TIS and found increased shoot number (7 shoots/explant) in cv. Grand Naine. Similarly, higher number of shoot proliferation (7.3 shoots/explants) in TIS was reported by Bello-Bello *et al*. (2019) as compared to the semi-solid medium, and liquid medium.

Among the available published literature, the study by Thanakornpaisarn (2017) reported the highest number of shoots (9.8±0.33 shoots) and buds (2.25±0.26) per explant in banana cv. Mali-Oung (ABB genome) inoculated in MS + 5 mg L^{-1} BA + 10 mg L^{-1} chitosan with the 1 minute of immersion time, 3 times per day in bioreactor.

Ferdous *et al*. (2015) used MS medium supplemented with different concentrations of BAP (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg L^{-1}) to study *in vitro* shoot regeneration in Amritasagar and Sabri banana cultivars. Highest single shoot formation was reported in 5.0 mg L^{-1} BA. Similar observations were recorded by Molla *et al*. (2017) reported highest number of shoots (7.0) per explant in MS medium supplemented with BAP 5.0 mg L^{-1}.

Higher multiplication in MS medium supplemented with BAP 5.0 mg L^{-1} observed in the present investigation is in conformity with the results of Ferdous *et al*. (2015), Thanakornpaisarn (2017) and Molla *et al*. (2017).

Shoot multiplication reported in the present investigations in TIB is 14.71 shoots/ clump which was found higher than the available reports which may be due to the use of advanced stage cultures for multiplication and the type of bioreactor. Ayenew *et al*. (2013) reported the highest shoot number (13.17±1.72) in pineapple using TIB compared to a plastic jar (9.83±1.94) and glass jam jar (6.33±1.03).

As in the present investigations, superiority of TIS over other conventional systems was reported by several authors in other species such as, sugarcane (Lorenzo *et al*., 1998); pineapple (Escalona *et al*., 1999); apple (Zhu *et al*., 2005); eucalyptus (Mc-Alister, 2005); teak (Quiala *et al*., 2012); pistachio (Akdemir *et al*., 2014); bamboo (Ribeiro *et al*., 2016) and sugarcane (Silva *et al*.,

2015; Matoso *et al*., 2017), which support the present finding.

The new TIB system, Plantform showed good adaptability and better growth rate with higher shoot proliferation than the conventional and other bioreactor systems which was reported by various authors such as, Vidal *et al*. (2015) in chestnut, Benelli and Carlo (2018) in *Olea europaea* L., and Aka *et al*. (2019) in myrtle.

Using the TIS system 5-7 times greater multiplication than culture from the semisolid system was reported in a hybrid of *E. Grandis* × *E. Urophylla* (Businge *et al*., 2017) and in *Anthurium andreanum* (Martinez-Estrada *et al*., 2019). These findings support our multiplication rate of 14.71 shoots/ clump in MS medium supplemented with BAP 5.0 mg L^{-1} in TIB which was 76 per cent higher, over the conventional system in the same medium.

Moreover, in TIS, availability of ventilation and the use of liquid medium provides easy uptake of nutrients (Etinne and Berthouly, 2002; Schonherr, 2006). The easy uptake of nutrients and phytohormones by the plant surfaces improve the growth and quality of plantlets in TIS (Quiala *et al*., 2006; Akdemir *et al*., 2014). Another benefit of using TIB is that the removal of ethylene like volatile compounds is possible within the culture container due to ventilation (Roels *et al*., 2006). Increased functional activity of stomata in the leaves compared to semisolid environment (Afreen, 2008) and carbon dioxide recirculation for photosynthesis process (Aragon *et al*., 2014) are the other advantages of TIB. These factors could explain the increased rate of multiplication and growth of plants in TIB system.

5.3 Effect of different rooting media on rooting and root characters of plantlets from different culture systems

The effect of the three different rooting media on rooting and root characters of plantlets from Plantform TIB and conventional system were studied. The rooting was observed as 100 per cent for the Plantform system in the different rooting media tried, and in the solid medium rooting percentage was 99-100 per cent.

Waman *et al*. (2014) studied the effect of carbon source on rooting in banana and reported that there was 100 per cent rooting in shoots obtained from a medium containing 3 per cent sucrose. Similarly, 100 per cent rooting in different banana cultivars viz. Attunendran, Nedunendran, Chengalikkodan, Poovan, Njalipoovan and Grand Naine was reported by Mohammed (2018) in a study conducted at CPBMB.

Welander *et al*. (2014) reported 100 per cent rooting in *Digitalis lutea* x *purpurea, Echinacea purpurea, and Rubus idaeus* micropropagated in Plantform bioreactor.

Ramirez-Mosqueda *et al*. (2016) reported 100 per cent rooting of TIS derived shoots in *Stevia rebaudiana*. Aka *et al*. (2019) also evaluated Plantform TIS system and solid medium for myrtle culture and reported 100 per cent rooting of shoots from Plantform bioreactor. These results support the present finding of 100 per cent rooting in shoots from Plantform TIS system.

5.3.1 Root and shoot characters of plantlets from TIB and conventional culture system in the different rooting medium

In the present study, plantlets derived from Plantform were better in root characters than the solid medium. Plantlets from TIB recorded significantly higher number of roots, root length, and more number of leaves. In the conventional system, early initiation of roots and higher root length than TIB were noted.

The roots were initiated in the media about 5 days of inoculation. Solid medium recorded significantly early root initiation (5.04 days) than Plantform system (5.97 days). In TIB, Murashige and Skoog medium supplemented with 3 per cent sucrose recorded early root initiation. However, in the conventional system, root initiation was on par in the three different media tried.

Plantlets produced using Plantform TIB recorded the highest number of roots (10.29) whereas number of roots produced in the conventional solid medium was only 4.70. With respect to the number of roots in different media tried, the medium MS+2mg L^{-1} IBA+ sucrose (3%) recorded higher number of

roots in both the culture systems (Figure. 2).

The root length was found lower in Plantform system (7.93 cm) compared to the conventional solid medium (8.82 cm). However, there was no significant difference in root length in the three different media tried in the two culture systems.

Plantform system recorded significantly higher shoot length in plantlets. The average shoot length in PlantFom bioreactor was 10.88 cm while, in conventional system the shoot length was only 8.94 cm. Different media tried recorded shoot length on par in both the culture systems (Figure-3). The number of leaves in the plantlets from Plantform bioreactor was significantly higher (4.84) when compared to conventional system (4.39). Average numbers of leaves from different rooting media in both the culture systems were graphically represented in Figure 4.

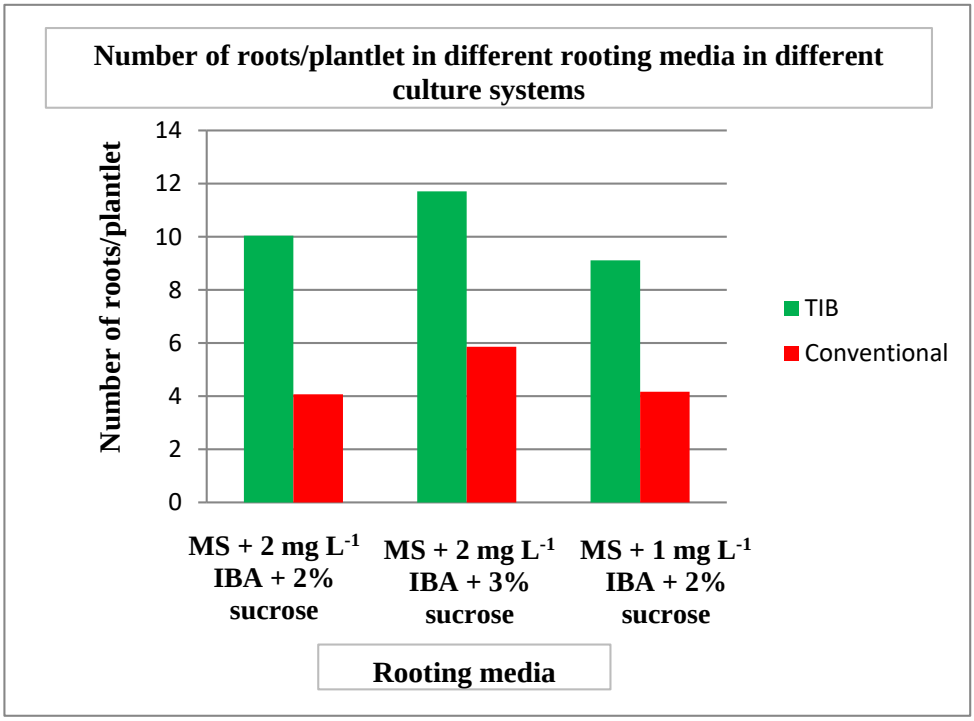


Figure 2. Number of roots/ plantlet in different rooting media in different culture systems

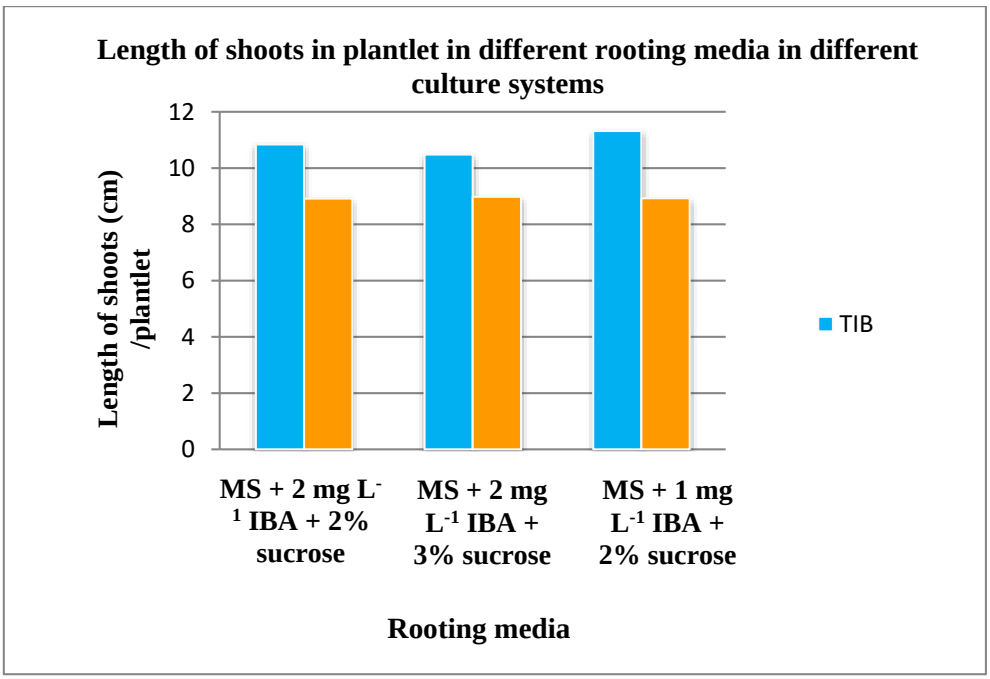


Figure 3. Length of shoots in plantlet in different rooting media in different culture systems

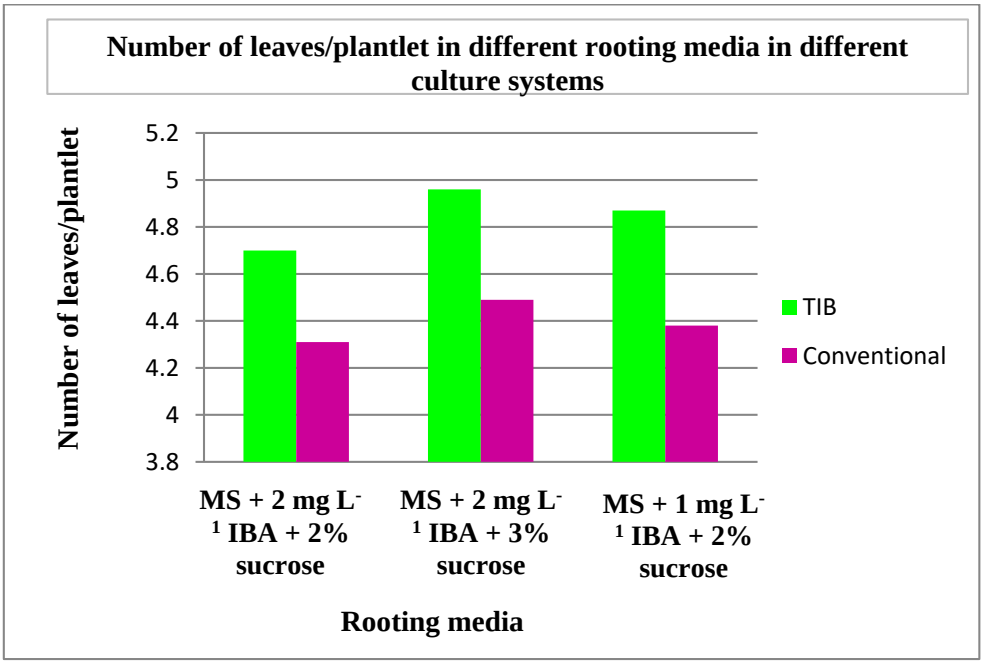


Figure 4. Number of leaves/plantlet in different rooting media in different culture systems

Rahman (2002) reported that among the different IBA concentrations tried 2 mg L-1 IBA was found most effective for *in vitro* rooting in banana. He reported increased number of roots (7.00) and root length/ plantlet (4.26 cm) with increasing concentration of IBA up to 2mg L^{-1} IBA and thereafter root length declined up to 5 mg L^{-1} IBA.

Similar trend of rooting characters were reported by Bello-Bello *et al*. (2019) who evaluated different culture systems for *in vitro* banana culture and reported that plants produced using SETIS™ bioreactor (TIB) formed more number of roots per plant (5.33±0.33), followed by the partial immersion (3.66±0.33) and Ebb-and-Flow systems (3.66±0.66). Lower number of roots per plant was obtained in the semisolid medium. They reported that there was no significant difference in root length in the SETIS™, partial immersion, and semi-solid culture system, with an average length of 6 cm. Also, the longest shoots were reported in SETIS™ followed by partial immersion, with 13.16± 0.33 and 11.83±0.60 cm in height, respectively. With respect to the number of leaves, the SETIS™ and partial immersion systems had the highest leaf number (6 leaves/plant) followed by the semisolid culture system (5 leaves/plant). The higher number of roots, higher shoot length, and more number of leaves recorded in TIB is in similarity with our findings.

Shukla *et al*. (2019) also cultured *Musa* spp. in static liquid culture as well as rocker based temporary immersion system and compared with a commonly practised semisolid medium. Using liquid medium, significantly earlier root initiation than semi-solid medium was reported. The rocker based TIS generally performed better than the static liquid. The highest root number, root length, and shoot length were recorded in TIS.

Ayenew *et al*. (2013) compared the TIB system with plastic jar and glass jam jar and reported the highest shoot length (8.67±1.02 cm) more roots (16.33±1.03) and number of leaves (10.17±1.83) in TIB. Also recorded highest root length (6.27 cm) in TIB which is not matched with our results and may be due to the difference in between design of the container used in TIB system.

The findings of the present investigation is in line with Aka *et al*. (2019) who evaluated Plantform and solid medium for myrtle culture and reported significant differences in the number of roots, root length, plant length, and number of leaves. The plantlets from the Plantform bioreactor recorded the highest root number (5.40), root length (8.10 cm), plant length (8.80 cm), and the number of leaves (6.70) as compared to the solid medium. Our results are matching with all parameters except root length and this may be due to change in evaluated species. Abahmne (2020) reported the highest shoot and root length (17.17) in date palm plantlets produced by TIS compared to the gelled medium.

5.4 Acclimatization

Plantlets with well developed roots from Plantform bioreactor and culture bottles were transferred to plastic pots. Plants were successfully hardened stepwise in a greenhouse. The positive outcome after the acclimatization was the better growth performance of the plantlets from the Plantform bioreactor. Plantlets from both the culture systems recorded uniform survival. In the present study, no variation could be observed in survival of plantlets after hardening in the two culture systems. Also, the plantlets from different rooting media combinations did not show any statistical difference in the survival of plants after two months of secondary hardening in both the culture systems. The survival after secondary hardening was 90-91 per cent.

Higher survival rate of plants derived from liquid culture in TIS was reported by several authors. In banana 95.71 to 99.80 per cent survival of plans produced through the TIS system was reported by Aragon *et al.* (2010). Similarly higher survival of acclimatized banana plants produced through TIS was demonstrated by Aragon *et al*. (2014), who compared TIB to semi-solid medium and reported more survival rate in TIB. Shukla *et al*. (2019) also reported 100 per cent survival of *Musa* spp. plantlets derived from rocker based TIS.

The present result is similar with Bello-Bello *et al*. (2019) who reported that different culture systems had no effect on the survival percentage after four

months of hardening.

In comparison with previous studies dealing with *the ex vitro* performance of TIS derived plants, a similar result was reported by Lorenzo *et al*. (1998) who compared TIS with conventional culture systems and reported there was no significant difference in plant survival. Plantlets grown in TIS exhibited 100 per cent survival rate was reported in *Kalopanax septembolus* by Kim *et al*. (2011). According to Persson (2012), the result after four weeks of acclimatization trial showed 98 per cent plantlet survival from both Plantform bioreactor system and semisolid medium in *D. lutea purpurea, and Rubus idaeus.*

More than 90 per cent *ex vitro* plant survival in TIS was reported in pineapple (Scherer *et al*., 2012), Raspberry (Debnath, 2014), *Stevia rebauidiana* (Ramriz-Mosqueda *et al*., 2016), carnation (Marzieh *et al*., 2017), and *Anthurium andreanum* (Martinez-Estrada *et al*. 2019).

There was 95 to 100 per cent survived plantlets of *Digitalis lutea x purpurea, Echinacea purpurea, and Rubus idaeus* micropropagated in Plantform bioreactor as reported by Welander *et al*. (2014), a similar trend of plantlets survival after two months of acclimatization was noticed in date palm (Abahmne, 2020) also, which supports the present finding of survival of plants produced using Plantform bioreactor.

5.4 .1 Plant characters after secondary hardening

After successful secondary hardening for two months, morphological parameters such as plant height and number of leaves were recorded in plants produced from two culture systems. After two months of hardening, the plants from TIB recorded higher plant height and more number of leaves than conventional system. The increased growth observed for plants from TIB may be due to the high percentage of closed stomata, lower stomatal index, lower rate of transpiration and higher rate of photosynthesis in plants from TIB as reported by Chakrabarty *et al*., 2007; Aragon *et al*., 2014; Vieira *et al*., 2015 and Martinez-Estrada *et al*., 2019.

In bioreactor, highest plant height (36.05 cm) was recorded in media (MS

+ 2mg L^{-1} IBA+ 2% sucrose). In conventional system, MS medium with 2mg L^{-1} IBA+ 3 per cent sucrose (36.00 cm) recorded the highest plant height. When used more sucrose in rooting medium, plants from TIB recorded higher plant height (Figure 5).

A significantly higher number of leaves (6.53) was observed in Plantform as compared to the conventional system (5.89). In bioreactor plants, number of leaves produced was significantly different in the three media studied. The highest number of leaves was recorded in MS + 2mg L^{-1} IBA+ 3 per cent sucrose (6.8) followed by MS + 1mg L^{-1} IBA+ 2 per cent sucrose (6.5) (Figure 6). The lowest number of leaves (5.6) per plant was observed in plantlets from the conventional system in the medium containing MS + 1mg L^{-1} IBA+ 2 per cent Sucrose.

Vasane and Kothari (2007) hardened tissue culture-derived banana plants and reported that there was an average of 5-6 number of leaves per plant. Waman *et al.* (2014) studied the effect of carbon source on banana micropropagation and reported the highest number of leaves (6.2) per plant after 45 days of secondary hardening.

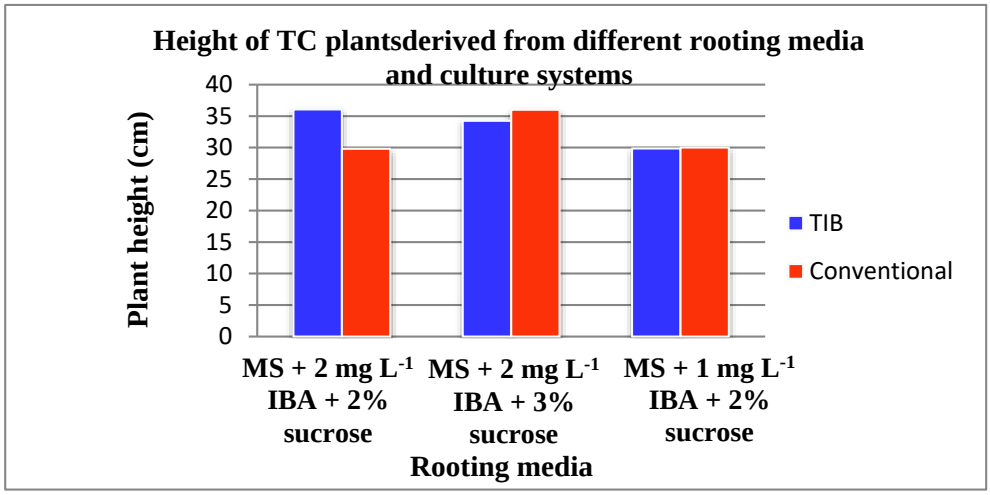


Figure 5. Height of TC plants derived from different rooting media and culture systems (sixty days after secondary hardening)

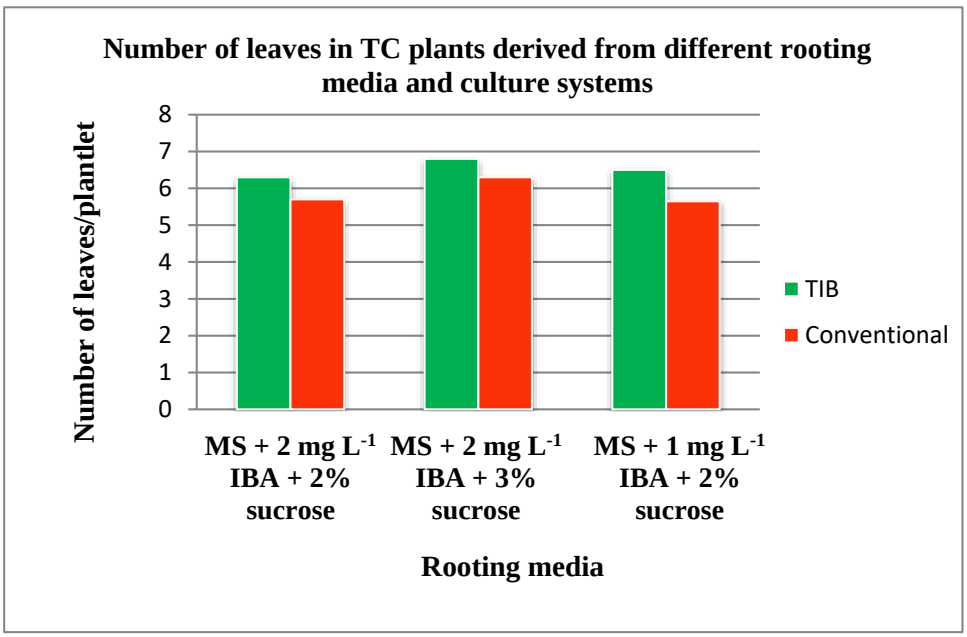


Figure 6. Number of leaves in TC plants derived from different rooting media and culture systems (Sixty days after secondary hardening)

5.5 Analysis of clonal fidelity using specific ISSR marker

The genomic stability may be affected by the rapid multiplication of a tissue leading to somaclonal variation (Israeli *et al*., 1995). In the previous studies reported, morphological (Krikorian *et al*., 1993; Martin *et al*., 2006) and genomic (Ray *et al*., 2006) variation in micropropagated plantain and banana have been detected. Bairu *et al*. (2006) reported that the rate of somaclonal variation is enhanced when the rate of proliferation is high and when the subculturing is more frequent. In this contrast, the genetic fidelity of *in vitro* developed plantlets was analyzed in the present study.

5.5.1 Genomic DNA isolation

The 5^{th} subculture stage cultures received from CPBMB were subcultured up to the 8^{th} stage using the protocol optimized for Nendran cultivars at CPBMB (Shylaja *et al*., 2015).

DNA was isolated from randomly selected 10 plants of each treatment using protocols reported by Rogers and Bendich, (1994) with a 5X CTAB extraction buffer. Good quality DNA extracted after electrophoresis exhibited distinct bands without shearing.

Nucleic acid degradation and the effects of secondary metabolites can be prevented by freezing the tissues in liquid nitrogen (Hernandez and Oyarzum, 2006). The problem of polyphenols can be avoided by using β- mercaptoethanol and polyvinyl pyrrolidone (PVP) along with the extraction buffer. The β-Mercaptoethanol disrupted the protein disulfide bond and initiated degradation of the protein. The addition of polyvinyl pyrrolidone (PVP) removed polyphenols (Matasyoh *et al*., 2008).

The detergent present in the extraction buffer called CTAB helped to release nucleic acids into the buffer by disrupting the cell membrane. The protection of the released DNA from the action of the DNase enzyme was done by the action of EDTA. Chloroform: isoamyl alcohol helped in the separation of the organic mixture and aqueous phase of the isolated DNA. The EDTA was also a major component of TE buffer in which the DNA was dissolved and stored, reported by Sambrook *et al*. (1989).

High molecular weight band with a low amount of RNA indicates good quality DNA in the sample (Wettasinghe and Peffley, 1998). Several authors (Wettasinghe and Peffley, 1998; Raval *et al*., 1998) reported the removal of RNA contamination from DNA using the RNase. In the present work, good quality DNA was obtained using RNase treatment.

5.5.2 Quality and Quantity analysis of genomic DNA

The yield and purity of isolated DNA samples varied with species to species. In the present study 87.28, ng/µl to 213.65 ng/µl yield, and 1.7 to 2.0 purity (A_{260}/A_{280}) of DNA were obtained. Isolated good quality DNA from mother plants were used for comparison of ISSR amplification profile of tissue culture plants rooted after 8^{th} subculture stage.

5.5.3 ISSR analysis

In the present study, specific ISSR primer optimized at CPBMB was used for clonal fidelity analysis as reported by Rajitha *et al.* (2015). Isolated genomic DNA from mother plants and regenerants after 8^{th} subculture stage was subjected to ISSR analysis.

To detect genomic variation, several DNA based markers can be used. Among them, Inter Simple Sequence Repeats (ISSRs) are 100-3000 bp long DNA fragments, located between adjacent, oppositely oriented microsatellite regions. These fragments are amplified by PCR using microsatellite core sequences as primers (16-18 bp). Assessment of the genetic integrity among *in vitro* developed clonal plants using ISSR marker was reported by several authors in different species (Zietekiewicz *et al.,* 1994). The long primer sequence stable at higher annealing temperature than those of RAPD primers accounts for the higher reproducibility of the ISSR marker system (Pradeep *et al.,* 2002).

ISSR marker assay requires very less amount of DNA and also it uses simple and faster procedure and does not involve any radioactivity tests. These characteristics of ISSR make it more efficient marker technique and have been widely used to know the genetic differences between micropropagated plantlets (Carvalho *et al.,* 2004). Such markers were successfully used by many authors (Frederic *et al*., 2007; Guo *et al*., 2007; Kumar *et al*., 2010).

The presence of variability among the regenerants is one of the major limiting factors in the commercial micropropagation. Plantlet produced by *in vitro* has to be assured that they are genetically stable. Molecular markers can be utilized for this and it has been reported that ISSR markers are a reliable marker system.

In the present study, the banana plantlets produced using different culture systems were subjected to clonal fidelity analyses after 8^{th} subculture stage using the specific ISSR primer optimized at CPBMB.

Amplification with the UBC 857 primer generated clear amplicons. The bands visualized were in the size range 300-2000 bp. DNA amplification pattern observed in plans of different culture systems of cultivar Nedunendran revealed monomorphic bands in all the tested samples. The results obtained demonstrate genetic stability and uniformity for all the tested *in vitro*

regenerated plantlets after 8th subculture stage in both the culture systems.

Genetic uniformity of banana tissue culture plants similar to donor mother plants using ISSR marker assay was reported by many authors. According to National Certification System for Tissue Culture Plants (NCS-TCP) promoted by Department of Biotechnology, Govt. of India, subculturing up to 8th passage was recommended in banana to ensure production of genetically stable quality tissue culture plants. Lakshmanan *et al*. (2007) reported that there were no polymorphic bands in the tissue-cultured banana plants as compared to donor plants when screened with ISSR and RAPD markers. In a report by Borse *et al.* (2011), clonal fidelity of banana cv. Grand Naine was evaluated using ISSR and REMAP molecular markers and reported very low per cent of variation in ISSR (0.96) and REMAP (0.95) assays after the 8th subculture generations. For highly multiplying banana cultivars like Attunendran, Nedunendran, and Grand Naine, subculturing for multiplication up to the 8th subculture stage is recommended (Mohamed, 2018). Similarly Nandkumar *et al*. (2018) also reported monomorphic banding pattern in all the tested samples of cv. Grand Naine and Rasthali using ISSR marker system.

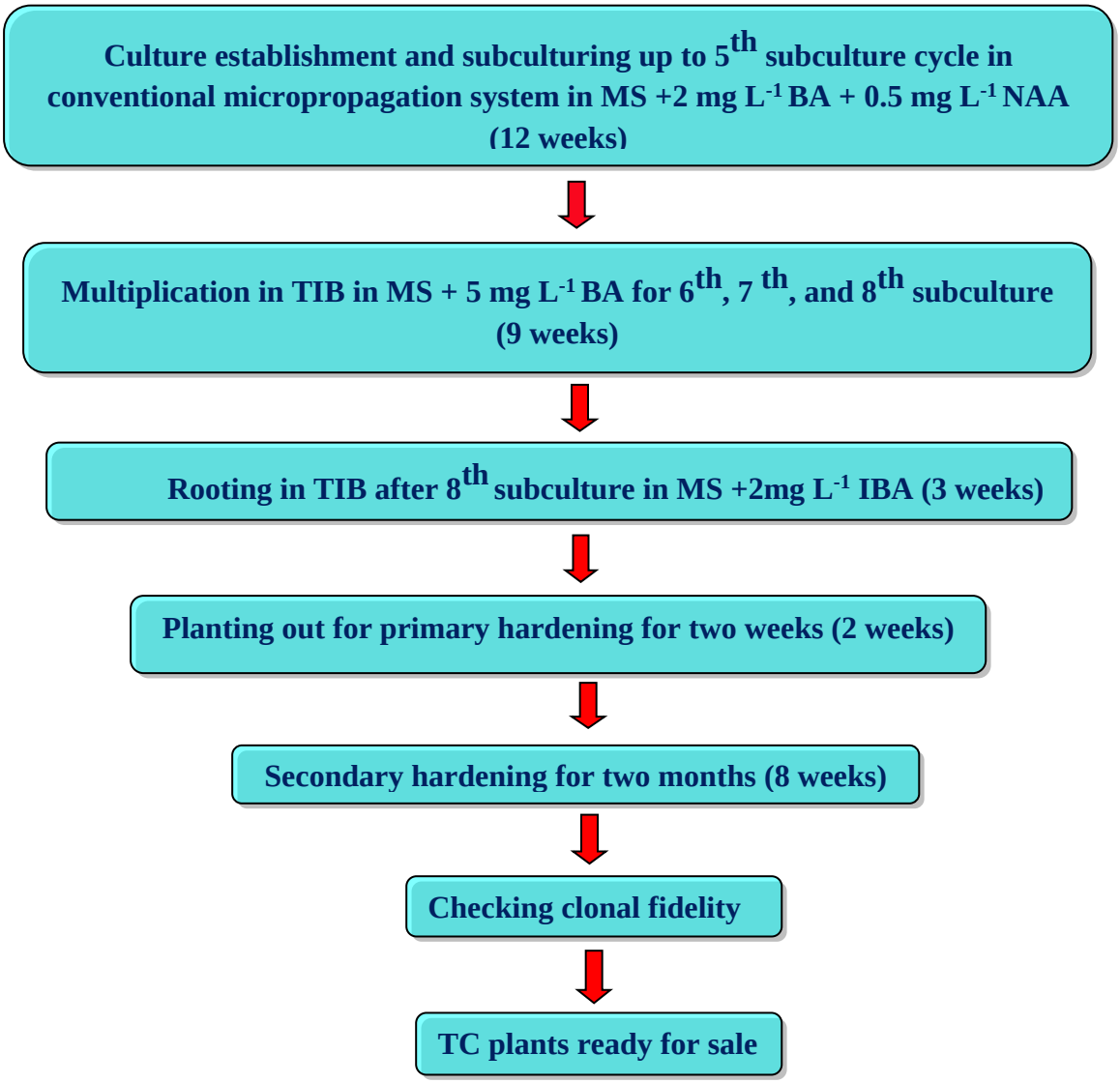
Debnath (2009) compared different culture systems (bioreactor, gelled medium, and conventional runner cuttings) for micropropagation of bounty strawberry and reported that ISSR marker assay produced homogenous amplification pattern in the tissue culture and donor control plants confirming the clonal fidelity of micropropagated plants. Similarly, EST-PCR analysis carried out by Debnath (2011) indicated that bioreactor derived blueberry plants showed 100 per cent similarity with donor plant when tested with 14 primers.

Homogenous amplification profiles with ISSR and SSR marker systems in the tissue culture and donor control plants confirmed the clonal fidelity of bioreactor-derived micropropagated plants in several species such as, raspberry (Debnath 2014), Myrobolan (Nasri *et al*., 2019), and lingonberry (Arigundam *et al*., 2020).

5.6 Development of a micropropagation protocol for banana using Temporary Immersion Bioreactor

Conventional micropropagation methods are not always reliable and scaling-up is not always easy. Nevertheless, application of tissue culture in horticulture, agriculture, and forestry is currently still expanding day by day. Using conventional micropropagation only high demand and high value crops is now propagated due to high cost of production. (Chu, 1995; Kitto, 1997). In order to overcome the limitations of conventional micropropagation in banana, micropropagation using Plantform temporary immersion bioreactor is developed in the present investigation (Figure 7).

Figure 7. Development of a micropropagation protocol using Plantform temporary immersion bioreactor



5.7 Comparison of micropropagation protocol using Plantform temporary immersion bioreactor with conventional micropropagation system

The advantages of Plantform TIB system include higher rate of multiplication, faster growth of cultures, less production cost, less time and laboratory space. High rate of multiplication and growth are primarily due to the effect of liquid medium (Levin *et al*., 1997) and availability of ventilation in culture vessel. In the present investigation Plantform TIB system recorded 5.35 times more plants than the conventional system after multiplication of shoots for 6^{th}, 7^{th} and 8^{th} subculture cycles in bioreactor. Projected multiplication of 12,000 plantlets from a single explant in conventional micropropagation of banana was reported by Singh *et al*., (2011).

The use of Plantform TIB system requires less space for media preparation, incubation, vessel storage and washing as compared to conventional micropropagation system. Reduced number of vessels needs less lighting facility in the bioreactors. Minimum autoclaving simplified the medium preparation, washing of vessels and handling of the cultures.

The use of liquid media in Plantform bioreactor provide close contact with plant tissue and easy absorption of nutrients by the cultures, also if new nutrients are required during a cycle, changing of medium of the vessel without subculturing the culture is also possible . In contrast, in the conventional system, each single shoot has to be handled in each subculture stage.

Similar advantages of TIB system over the conventional methods was already discussed by Mehrotra *et al*. (2007) and Akin-Idowu *et al*. (2009).

However the physiological disorders like hyperhydricity in shoots cultured using liquid medium in bioreactor was reported by many authors (Businge *et al*., 2017; Chakrabarty *et al*., 2007; Gonzalez *et al*., 2011; Oliveira *et al*., 2011b; Marzieh *et al.,* 2017). In the present study no such kind of hyperhydricity was observed in cultures from TIB.

5.8 Comparison of media cost in Plantform bioreactor and conventional system for production of a plantlet

Comparison of media cost in Plantform bioreactor and conventional system is presented in Table 15. As the major saving is in media cost, other capital and running costs are not calculated for production of a TC plantlet. Media cost is calculated using the present cost of chemicals required for MS media and growth regulators to be supplemented followed at commercial micropropagation unit of CPBMB to produce the plants as per the multiplication rate shown in flow chart (Figure 8) for both the systems. Calculations are based on data obtained from the present study. The experiment started with 20 initial clumps from the 5th subculture stage for both the systems. With eight subculture passages, 54,850 plants can be produced with the Plantform TIB system as per the shoot multiplication observed in the bioreactor (14.71 rounded to 14.0). In the conventional system, only 10,240 plants can be produced with the multiplication observed (8.35 rounded to 8.0) The use of liquid media reduces the cost of media by elimination of agar, the costly component for solidification of tissue culture media in conventional system. Media dispensing time is also very much reduced in Plantform TIB. As the multiplication rate is more and media cost is less, a plantlet could be produced with a cost of Rs.0.07/ plantlet and Rs.0.02/ plantlet using laboratory grade and commercial grade chemicals respectively in Plantform TIB. In conventional system, due to lower multiplication rate and higher media cost, the media cost is worked out as Rs.0.58/plantlet and Rs.0.15/ plantlet using laboratory grade and commercial grade chemicals respectively.

Fig 8. Possible shoot multiplication and production of rooted plantlets in Plantform temporary immersion bioreactor and conventional system

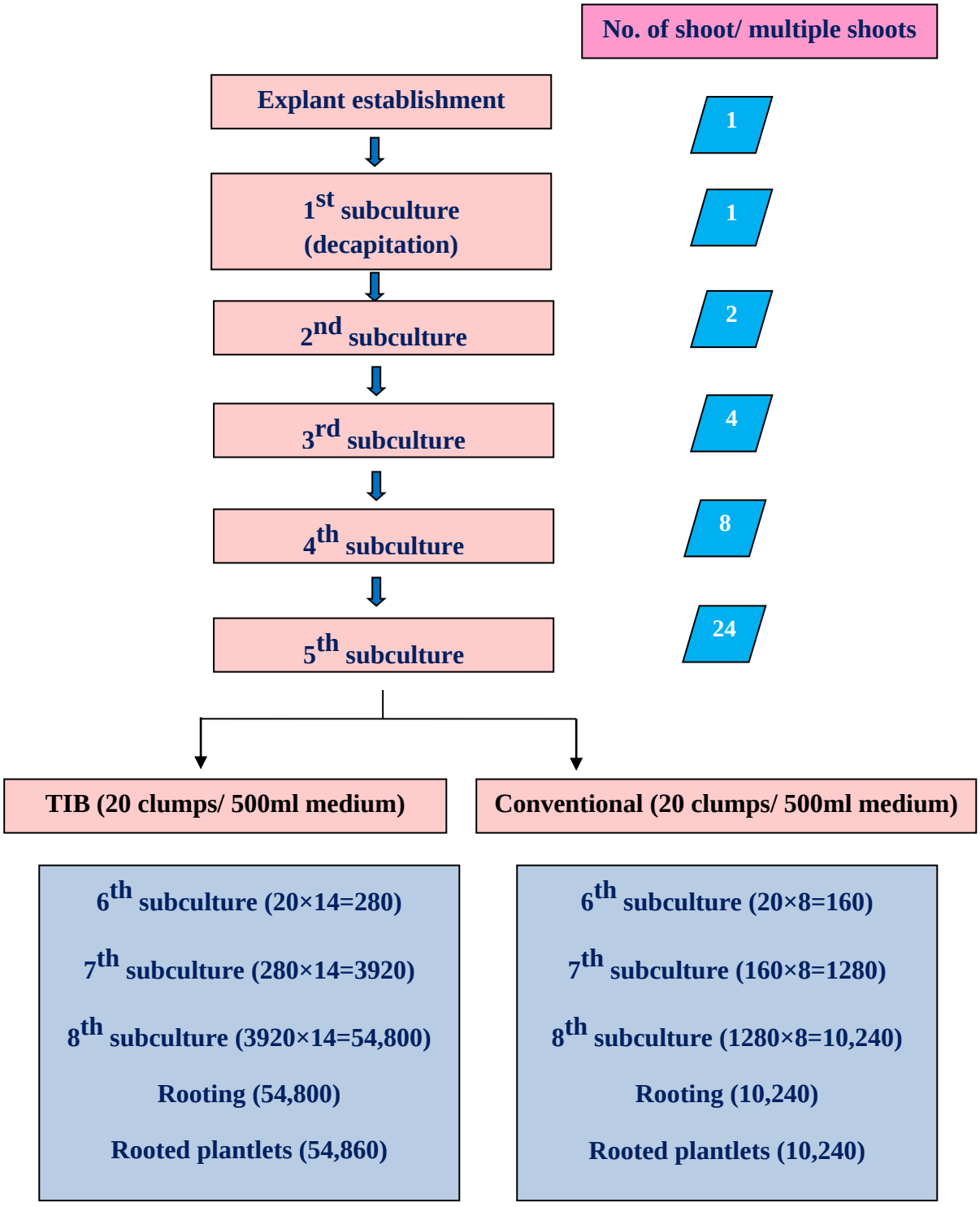


Table. 15 Comparison of media cost in Plantform bioreactor and conventional micropropagation system

*Media cost / plantlet in Plantform TIB					**Media cost/ plantlet in conventional micropropagation system				
Subculture	**Multiple shoots**	**Media required (L)**	**Media Cost (Rs.)**		**Subculture**	**Multiple shoots**	**Media required (L)**	**Media Cost (Rs.)**	
			Laboratory grade chemicals	**#Commercial grade chemicals**				**Laboratory grade chemicals**	**##Commercial grade chemicals**
5^{th} (***)	24	0.5	43	11	5^{th}	24	0.5	43	11
6^{th}	280	0.5	9.5	3	6^{th}	160	0.5	43	11
7^{th}	3920	7.0	133	42	7^{th}	1280	4.0	344	88
8^{th}	54,850	98	1862	588	8^{th}	10,240	32	2752	704
Rooting	54,850	98	1862	588	Rooting	10,240	32	2752	704
Total			3910	1232				5934	1518
Media cost/plantlet			0.07	0.02				0.58	0.15

* Cost of MS medium with laboratory grade chemicals for TIB – Rs.19/ L

** Cost of MS medium with laboratory grade chemicals for conventional micropropagation - Rs.86/ L

Cost of MS medium with commercial grade chemicals for TIB - Rs.6/ L

Cost of MS medium with commercial grade chemicals for conventional micropropagation - Rs.22/ L

***up to 5^{th} subculture using conventional system

In conventional system, 50 ml of semisolid medium is dispensed to each culture bottle. Less number of culture vessels, use of liquid media free of agar in Plantform TIB system helps for easy cleaning and washing the containers and reduce the cost of autoclaving for each transfer. Also fresh media can be added to the bioreactor vessels without subculturing. The subculturing can be done quickly in the Plantform TIB system as the shoots can be cut and 20 clumps can be dropped into the vessel. But in the conventional semisolid medium, as each culture bottle to be opened and clumps to be placed with care, more time is required for subculturing for multiplication and rooting.

The Plantform vessel is available with special designs that require less space than the semisolid system. The smaller size of the laboratory and the less number of skilled workers reduce air-conditioning needs that ultimately affect reduction of energy costs. Increased rate of multiplication in the Plantform vessel was achieved in a smaller space compared to the semisolid system. The initial cost required for the Plantform TIB vessels is high as many sets have to be established to achieve the multiplication as shown in Table 15, but the vessels are reused and this high cost is soon offset with the higher multiplication rates and total turnover of plants produced. Overall the total production costs were reduced in the temporary immersion system.

Similar advantages of TIB was discussed by other researchers in different plants species. Doubled multiplication rate and up to 46 per cent reduction in production cost was reported in sugarcane micropropagation using temporary immersion bioreactor by Lorenzo *et al*. (1998), while 20 per cent cost reduction in pineapple was reported by Escalona *et al*. (1999). Significant cost reduction by eliminating labour intensive stage and reduced production time for large scale micropropagation of coffee using temporary immersion system compared to standard conventional method was reported by Etienne (2000). Levin and Tanny (2004) also reported significant cost reduction using bioreactors. More than double production of plants using TIB system within half of the time period required for conventional semisolid medium reduces the production cost at commercial level as reported by McAlister *et al*. (2005) for production of *Eucalyptus*.

Another salient observation recorded in the present investigations is the high growth rate observed for plants from Plantform TIB. After two months of secondary hardening, the plants from TIB recorded higher plant height and more number of leaves than conventional system. Also, plants from the rooting medium with low sucrose recorded better growth parameters. The possibility of reducing the secondary hardening period and saving of sucrose in rooting media are the other advantages noted

The total duration shown in the protocol developed in the present investigations after two months of secondary hardening of TC plants from a single explant is 34 weeks which can be modified by starting early stage multiplication in bioreactor by bulking shoots from different explants and reducing the multiplication cycles in bioreactor. To avoid contamination of cultures in bioreactor vessels, cleanliness and strict hygiene in all the steps of micropropagation and in the maintenance of bioreactor laboratory should be followed.

An efficient commercial micropropagation protocol using a Plantform TIB was thus developed for banana in the present study. Temporary immersion bioreactor system exhibited high shoot multiplication, better root and shoot characters for plantlets and better growth of plants after hardening. Due to the high rate of multiplication in TIB, production of plantlets was more in TIB and use of liquid media considerably reduced the media cost. Clonal fidelity analysis revealed that the plantlets produced after 8^{th} subculture stage were true to type. Field evaluation of plants from both the culture systems and comparison of yield and quality of plants have to be taken up further.

6. SUMMARY

The study entitled "Commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during 2018 - 2020, with the objective to develop an efficient commercial micropropagation protocol in banana using a temporary immersion bioreactor (TIB) system. The study mainly focused on the following aspects:

1) Development of a micropropagation protocol for banana using TIB system.
2) Clonal fidelity analysis of micropropagated plants from TIB using specific ISSR marker.

Studies were conducted in banana cultivar Nedunendran (AAB) which is being commercially micropropagated at CPBMB. Established cultures of the cultivar received from the commercial micropropagation unit of CPBMB at the 5th subculture cycle were utilized for the study. The micropropagation protocol standardized at CPBMB for the cultivar Nedunendran was followed for production of plantlets. The protocol was optimized for bioreactor micropropagation and at each stage of propagation, it was compared with conventional micropropagation system. Number of clumps/ 500 ml. of medium to initiate shoot multiplication, media for multiplication and rooting were standardized for TIB and compared with conventional micropropagation system. Immersion duration of one minute at three hourly intervals were fixed for all the experiments. The root characters, survival of plantlets after hardening and growth of plants after secondary hardening were also compared for plants from both the culture systems. The clonal fidelity analysis standardized at CPBMB for banana using specific ISSR marker was used to analyze the fidelity of rooted plants from the two culture systems after 8th subculture passage.

The salient findings of the study are summarised below:

- Different clump sizes (10, 15, and 20 clumps /500 ml media) were evaluated for shoot multiplication in bioreactor and conventional system of micropropagation. In bioreactor, highest shoot proliferation (6.2 shoots/clump) was recorded for 20 clumps/ 500 ml medium. In conventional system, 15 clumps/ 500 ml medium and

20 clumps/ 500 ml medium recorded higher shoot proliferation (6.7 and 6.2 shoots/clump respectively), both of them were on par.

- Temporary immersion bioreactor system exhibited significantly higher shoot proliferation (13.00) than the conventional system (7.67), irrespective of multiplication media tried.

- Murashige and Skoog (MS) multiplication medium supplemented with 5mg L^{-1} BA recorded highest shoot proliferation in both the culture systems. In the bioreactor, the medium with 5mg L^{-1} BA recorded 14.71 shoots/ clump while in conventional system it recorded 8.35 shoots/ clump.

- In both the culture systems, in the three different rooting media tried (MS + 1mg L^{-1} IBA with 2 % sucrose , MS + 2mg L^{-1} IBA with 2 % sucrose and MS + 2mg L^{-1} IBA with 3 % sucrose), 99-100 per cent rooting was observed.

- Conventional culture system recorded significantly early initiation of roots (5.04 days) as compared to the bioreactor system (5.97 days), irrespective of rooting media tried. Murashige and Skoog medium supplemented with 2mg L^{-1} IBA with 3 per cent sucrose recorded early initiation of roots in bioreactor. In the conventional system, days taken for root initiation was found on par in the three media tried.
- Plantlets from temporary immersion bioreactor system recorded significantly higher number of roots (10.29) than the conventional culture system (4.70). The MS medium supplemented with 2mg L^{-1} IBA with 3 per cent sucrose recorded higher number of roots in both the culture systems.

- Conventional culture system produced significantly longer roots (8.82 cm) as compared to bioreactor system (7.93 cm), irrespective of rooting media tried. The root length of plantlets in the different rooting media tried was found on par in both the culture systems and ranged from 7.66-8.12 cm in bioreactor and 8.11-9.66 cm in conventional system.

- Plantlets from the bioreactor recorded significantly higher shoot length (10.88 cm) than the conventional system (8.94 cm), irrespective of rooting media tried. Length of shoots was found on par in the different media tried in the two culture systems and ranged from 10.48 - 11.32 cm in bioreactor and 8.92-8.98 cm in

conventional system.

- Plantlets from bioreactor system recorded significantly higher number of leaves (4.84) than the conventional system (4.39). Numbers of leaves were on par in the three media tried in the two culture systems and ranged from 4.70 - 4.96 in bioreactor and 4.31- 4.49 in conventional system.

- Plants from bioreactor and conventional system registered almost uniform survival of 90-91 per cent after two months of secondary hardening. The survival percentage was uniform in plants rooted in different rooting media in both the culture systems.

- Culture systems, rooting media and media x culture system interaction effects were found significant with respect to growth of plants 60 days after secondary hardening.
- The plants from bioreactor recorded significantly higher plant height (33.39 cm) than conventional system (31.93 cm), irrespective of rooting media tried. Highest plant height (36.5 cm) was recorded for plants rooted in medium MS + 2mg L^{-1} IBA+ 2 per cent sucrose in bioreactor and in MS + 2mg L^{-1} IBA+ 3 per cent sucrose (36.00 cm) in the conventional culture system.

- Plants from TIB system produced significantly higher number of leaves (6.53) than conventional system (5.89). The number of leaves in bioreactor was in the range of 6.30-6.80 while in conventional system it was in the range of 5.65-6.30.

- In clonal fidelity analysis, DNA amplification profiles of rooted plants after 8^{th} subculture stage, amplified by ISSR primer UBC857 were analyzed. The bands visualized were in the size range of 300 to 2000 bp.

- There was no polymorphic amplicons in the ISSR amplification profile in plants rooted after the 8^{th} subculture cycle in both the culture systems when compared with the amplification profile of source mother plant. The plants rooted after the 8^{th} subculture stage from both the culture systems are thus true to type.

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Appendices

ANNEXURE I

List of laboratory equipments used for the study

Plantform Bioreactor	: Plantform, Sweden
Refrigerated centrifuge	: Kubota, Japan
Horizontal electrophoresis system	: Biorad, USA
Thermal cycler	: Veriti Thermal Cycler (Applied Biosystem, USA)
Gel documentation system	: Biorad, USA
Nanodrop® ND-1000 spectrophotometer	: Nanodrop® Technologies lnc. USA

ANNEXURE II

Chemical composition of the Murashige and Skoog medium

Stock	Chemical	mg/ Litre	Stock concentration	Stock
I	$(NH_4)NO_3$	1,650	50 X	82.5 g/l
	KNO_3	1,900		95.0 g/l
	KH_2PO_4	170		8.5 g/l
	$MgSO_4.7H_2O$	370		18.5 g/l
II	$CaCl_2.2H_2O$	440	50 X	22.0 g/l
	(Prepare the stock separately or it may precipitate)			
III	Na_2EDTA	37.3	100 X	3.7 g/l
	$FeSO_4.7H_2O$	27.8		2.8 g/l
	(Remember to prepare this as described under stock solution preparation)			
IV	$MnSO_4.4H_2O$	22.3	100X	2.23 g/l
	$ZnSO_4.7H_20$	8.6		860 mg/l
	H_3BO_3	6.2		620 mg/l
	Kl	0.83		83.0 mg/l
	$Na_2MoO_4.2H_2O$	0.250		25.0 mg/l
	$CuSO_4.5H_2O$	0.025		2.5 mg/l
	$CoCl_2.6H_2O$	0.025		2.5 mg/l

V	Vitamins	100X	
	Glycine	2.0	200 mg/l
	Nicotinic acid	0.5	50 mg/l
	Pyridoxine- HCL	0.5	50 mg/l
	Thiamin- HCI	0.1	10 mg/l
	Myo- inositol	100	
	Agar	7.5 g/l	
	sucrose	30 g/l	
	pH	5.7- 5.8	

ANNEXURE III

Reagents required for DNA isolation

Reagents:

1. 5X CTAB extraction buffer (100 ml)

CTAB : 5g

(Cetyl trimethyl ammonium

bromide) Tris HCl : 1.21 g

EDTA : 0.745 g

NaCl : 8.18 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. CTAB (10 %, 100 ml)

CTAB : 10 g

NaCl : 4.09 g

3. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. Chilled isopropanol

Isopropanol was stored in refrigerator at 0ºC and was used for the study.

5. Ethanol (70 %)

To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100 ml) Tris HCl (10 mM) :

0.1576 g EDTA (1 mM)

: 0.0372 g

The solution was prepared, autoclaved and stored at room temperature

ANNEXURE IV

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

Tris base : 242 g

Glacial acetic acid : 57.1 ml

0.5M EDTA (pH 8.0) : 100 ml

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in a dark bottle.

Commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system

By

Waghmare Vaibhav Gautam

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ABSTRACT OF THE THESIS

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Kerala Agricultural University

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR – 680 656
KERALA, INDIA
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ABSTRACT

Banana (*Musa* spp.) is an important fruit crop cultivated worldwide. Tissue culture banana plants are now widely used as planting materials for commercial banana cultivation. High cost of production, poor multiplication rate and less survival of plantlets during acclimatization are some of the problems experienced in conventional micropropagation of banana. As there is high demand for tissue culture banana plants, the conventional tissue culture techniques need to be modified to increase the multiplication rate and to reduce the unit cost of production of plantlets. Use of temporary immersion bioreactor (TIB) is a very good option to increase multiplication rate and reduce cost of production.

The investigations on 'Commercial micropropagation of banana (*Musa* spp.) using a temporary immersion bioreactor system' was hence taken up at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture from 2018-2020. The aim of the investigations was to develop an efficient commercial micropropagation protocol in banana using a temporary immersion bioreactor system.

Established cultures of the cultivar Nedunendran (AAB) received from the commercial micropropagation unit of CPBMB at the 5th subculture cycle were utilized for the study. The micropropagation protocol standardized at CPBMB for the cultivar Nedunendran was followed for production of plantlets. The protocol was optimized for bioreactor micropropagation and at each stage of propagation, it was compared with conventional micropropagation system. Number of clumps/ 500 ml. of medium to initiate shoot multiplication, media for multiplication and rooting were standardized for TIB and compared with conventional micropropagation system. Immersion duration of one minute at three hourly intervals were fixed for all the experiments. The root and shoot characters, survival of plantlets after hardening and growth of plants after hardening were also compared for plants from both the culture systems. The clonal fidelity analysis standardized at CPBMB for banana using specific ISSR marker was used to analyze the fidelity of rooted plants from the two culture systems after 8th subculture passage.

Higher clump size (20 clumps/ 500 ml media) was found good to initiate shoot

multiplication in TIB with multiplication of 6.2 shoots/clump while in conventional system 15 and 20 clumps/ 500 ml of medium were found on par and recorded multiplication of 6.7 and 6.2 shoots/ clump respectively. Temporary immersion bioreactor system exhibited significantly higher shoot proliferation (13.00 shoots/clump) than the conventional system (7.67 shoots/ clump). Murashige and Skoog (MS) multiplication medium supplemented with 5mg L^{-1} BA recorded highest shoot proliferation in the both culture systems, recording 14.71 shoots/ clump in TIB and 8.35 shoots/ clump in conventional system. In both the culture systems, in the three different rooting media tried (MS + 2mg L^{-1} IBA+ 2% Sucrose, MS + 2mg L^{-1} IBA+ 3% Sucrose, MS + 1mg L^{-1} IBA+ 2% Sucrose), 99-100 per cent rooting was observed. Plantlets from TIB recorded significantly higher number of roots (10.29) than the conventional system (4.70). The root length of plantlets was more in conventional system (8.82 cm) as compared to TIB system (7.93 cm). Plantlets from bioreactor recorded significantly higher shoot length (10.88 cm) and more number of leaves (4.84) while plantlets from conventional system recorded less shoot length (8.94 cm) and less number of leaves (4.39). Survival of the plantlets after hardening was 90-91 per cent in both the culture systems.

After sixty days of secondary hardening, plants from TIB exhibited better growth recording more plant height and more number of leaves than the conventional system. Plants from the rooting medium MS + 2mg L^{-1} IBA+ 2 per cent sucrose recorded plant height of 36.05 cm in TIB while plants from conventional system recorded plant height of 29.80 cm and the number of leaves was 6.3 in bioreactor plants and 5.7 in conventional system.

Clonal fidelity analyzed using specific ISSR marker, UBC857 revealed that there was no polymorphism in the ISSR amplification profiles of rooted plants after the 8^{th} subculture cycle when compared with the amplification profile of source mother plant in both the culture systems and the plants produced are true to type.

An efficient commercial micropropagation protocol for banana using a TIB was thus developed in the present study. Temporary immersion bioreactor system exhibited high shoot multiplication, better shoot and root characters for plantlets and better growth of plants after hardening. Due to the high rate of multiplication

in TIB, there is more production of plantlets and use of liquid media considerably

reduced the media cost. Clonal fidelity analysis revealed that the plantlets produced after 8th subculture stage were true to type. Field evaluation of plants from both the culture systems and comparison of yield and quality of plants have to be taken up further.