LOW COST ALTERNATIVES IN COMMERCIAL MICROPROPAGATION OF BANANA (*Musa* spp.)

By

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THESIS

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2018

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I, hereby declare that this thesis entitled **'Low cost alternatives in commercial micropropagation of banana (*Musa* spp.)'** 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.

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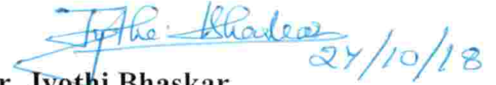

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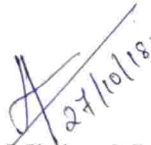

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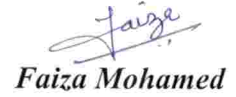

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ABBREVIATIONS

%	Percentage
μg	Microgram
AFLP	Amplified Fragment Length Polymorphism
ABA	Abscissic Acid
bp	Base pair
BAP	Benzyl amino purine
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EtBr	Ethidium bromide
g	Gram
ha	Hectare
ISSR	Inter Simple Sequence Repeats
IAA	Indole acetic acid
IBA	Indole butyric acid
Kb	Kilo base pairs
L	Litre
M	Molar
mg	Milligram
ml	Millilitre

mM	Milli mole
ng	Nanogram
NAA	1-Naphthaleneacetic acid
NCS-TCP	National Certification System for Tissue Culture Plants
^oC	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
P^H	Hydrogen ion concentration
PIC	Polymorphic Information Content
pM	Pico molar
PVP	Poly vinyl pyrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
TAE	Tris Acetate EDTA
TC	Tissue culture
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts
β	Beta
μl	Microlitre

Introduction

1. INTRODUCTION

Banana (*Musa* spp.) is an important fruit crop of tropical and sub-tropical countries and is known as poor man's apple. It belongs to Musaceae family and is one of the ancient fruits known to mankind. India is one among the major centres of origin of banana.

The leading banana cultivar of Kerala is Nendran (AAB group) belonging to the plantain subgroup, which contributes 58 per cent of production. It is the most valued plantain variety used in Kerala, fetching the best prize during festive occasions especially Onam, the regional festival of Kerala. The total area under banana cultivation in Kerala during 2016-17 was 57,158 ha with a production of 4.89 lakh tonnes (GoK, 2017).

Conventionally, bananas are propagated through suckers and the sword suckers are considered the best propagules. The annual requirement of planting material is very high in banana. The required number of planting material to cultivate an area of 57,158 ha will come to around 14 crores. Even if a 10 per cent substitution of planting material with tissue culture plants is aimed, the requirement of tissue culture plants is around 1.4 crores per year.

Tissue-culture banana plants have become a vital part of commercial banana production, leading to production of large number of true-to-type regenerated plants. Micropropagation played a crucial role in *Musa* improvement progammes worldwide (Vuylsteke *et al.*, 1996). Micropropagated banana plants establish faster and grow more vigorously than conventional propagules and they have uniform crop cycle and yield higher (Vuylsteke and Ortiz, 1996).

High unit production cost, poor multiplication and low survival rates during acclimatization are the major hindrances in micropropagation techniques (Kozai *et al.*, 1988). Small and medium-scale laboratories cannot access the benefits of plant tissue culture technology due to high cost of production. To make

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a commercial micropropagation unit viable, cost of production of plantlets should be minimum. The important cost factors in banana micropropagation include high energy consumption for maintaining required culture conditions like photoperiod, temperature and relative humidity and costly components in artificial media. The production costs can be minimized by using low cost alternatives for the above components.

The use of low cost options in plant tissue culture was reported by many scientists. Ganapathi *et al.* (1995) and Sharma and Singh (1995) reported the use of tap water for micropropagation of banana and ginger respectively. Autoclave for sterilization could be replaced with a pressure cooker with no detectable contamination (Gitonga *et al.*, 2010).

Refined white and unrefined brown sugars were found effective for coconut embryo culture in Philippines (Bonaobra *et al.*, 1994). In India, Ganapathi *et al.* (1995) reported that commercial grade sugar could replace analytical grade sucrose, with no significant change in the frequency of shoot formation in banana. Kodym and Arias (2001) reported that there was 90 per cent cost reduction when sucrose and gelrite was replaced with commercial sugar and a starch-gelrite mixture, respectively.

In many crops including banana, sago and isabgol were better gelling agents (Bhattacharya *et al.*, 1994, Babbar and Jain, 1998; Naik and Sarkar, 2001). Zimmerman *et al.* (1995) used a gelling mixture, consisting of 5 per cent corn starch and 0.05 per cent gelrite for the culture of strawberry and raspberry. For replacing high cost agar for tissue culture, isabgol derived from the seeds of *Plantago ovata*, might be a good alternative due to its colloidal and polysaccharide nature (Tyagi *et al.,* 2007).

Genotype dependant response in different stages of micropropagation is observed in banana. High genotypic difference in different banana cultivars viz. Attunendran, Nedunendran, Chengalikodan and Grand Naine in sprouting of explants, shoot proliferation, rooting and root growth was reported by Shylaja *et*

al. (2015). Cost-effective tissue culture media for large scale propagation of three commercial banana varieties viz. Rasthali, Grand Naine and Udhayam were developed by Saraswathi *et al.* (2016). They also reported genotypic difference in shoot proliferation in the different varieties. Hence, increasing the subculture passages without causing variability is another option to increase multiplication rate of plantlets and thereby to reduce the per plant cost of tissue culture plants.

Assessment of clonal fidelity of plantlets helps us to know the true-to-type nature of plantlets or variability induced in tissue culture cycle. The occurrence of genetic changes due to somaclonal variation in the regenerants was a serious concern in clonal micropropagation (Salvi *et al.*, 2001). Hence it is essential to establish genetic uniformity among the clones to confirm the quality of the plantlets for its commercial value.

In many crops, PCR techniques such as RAPD and ISSR are very useful in establishing the genetic stability of *in vitro* regenerated plantlets (Bhowmik *et al.*, 2016, Fayas *et al.*, 2018). Inter Simple Sequence Repeats (ISSR) markers were used to access genetic uniformity of tissue-cultured banana plantlets using low cost substitutes in three commercial varieties of banana by Saraswathi *et al.* (2016).

In this context, "Low cost alternatives in commercial micropropagation of banana (*Musa* spp.)" was taken up at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University with an objective to reduce cost of production in commercial micropropagation of banana. Adoption of these low cost alternatives in micropropagation protocols will reduce the production costs significantly thereby leading to area expansion and increased productivity in banana.

Review of Literature

2. REVIEW OF LITERATURE

The present investigations on "Low cost alternatives in commercial micropropagation of banana (*Musa* spp.)" focus on various low cost options in plant tissue culture. The relevant literature on various aspects of the investigations are reviewed in this chapter under different headings.

2.1 Origin and history

Bananas were familiar to the early Arabs and was also mentioned in the Qur'an as the 'tree of paradise'. The generic name *Musa* is stemmed from the Arabic word '*mouz*' (Robinson, 1996). Bananas were known from time immemorial in India from its mention in Ramayana (2020 BC), Koutilya's Arthasastra (300-400 BC) *etc*. Its historic importance for both consumptive and religious purposes can be depicted in the paintings and sculptures of Ajantha and Ellora caves (600 BC) (Uma *et al*., 2005). Edible bananas were native to Asia, originated perhaps somewhere in the mountainous region of Burma, Thailand, Assam or Indo-China. Then onwards it was spread to tropical parts of America, Africa, Australia, Philippines and Hawaii.

2.2 Botany

Banana is a herbaceous and monocotyledonous plant. The true stem is known as the 'rhizome' and it remains underground. Botanically, rhizome is a modified stem with buds (eyes) on the middle and upper parts of the rhizomes. Leaf sheaths known as 'pseudostem' comprise the portion 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. At the emergence from the pseudostem, the lamina appears as a tightly rolled cylinder which unfolds later from the top towards the base. About 40 leaves leaves are formed till flowering. At last, a bract like leaf which is short lived is formed which protects the bunch. The inflorescence initiates from the heart of the pseudostem. The peduncle varies with varieties and is often glabrous and

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pubescent. Female flowers are larger, with well developed ovary, long style and reduced stamens. Male flowers have well developed anthers, abortive ovary with slender style and stigma. Pollen in edible banana is rare. Male flowers abscise at the base of the ovary and are shed in a day after exposure. The bracts are usually reddish purple or violet in colour. The fruit is seedless, developed without pollination. Auxin stimulation assumed to take part in the development of banana fruit (vegetative parthenocarpy) (Robinson., 1996).

2.3 Genetics of banana

The modern method of classifying edible bananas was devised by Simmonds and Shepherd (1955) and Ortiz (1997). It originally came from two wild, seeded species, *Musa acuminata* Colla (A) *and Musa balbisiana* Colla (B) which are native to Southeast Asia. The major genomic groups were AA, AAA, AAAA, AB, AAB, ABB, BBB, ABBB, AAAB and AABB.

2.3.1 Cultivars used in the present investigations

2.3.1.1 Poovan (Rasthali)

Poovan is a table variety of choice and is priced high. It has medium bunch weight. The average bunch weight is 12 kg, contains five to seven hands with 60 to 80 fruits. The plant is tall and can be easily identified by the yellowish green stem with brownish blotches, reddish margins of the leaf sheath and petiole. Fruit is medium sized, thin skin, ivory-yellow in color, flesh firm, sweet with a pleasant apple flavour.

2.3.1.2 Njalipoovan

It is cultivated commercially in Karnataka and also grown as an intercrop in Kerala in coconut and arecanut gardens. Plants are medium sized, small fruit, flesh firm, pseudostem sender and yellowish with reddish petiole margin, sweet and fragrant. The average bunch weight is 10-12 kg. It is resistant to panama disease and leaf spot.

2.3.1.3 Grand Naine

Grand Naine is among the most well-known varieties of Cavendish group. It is a tall mutant of Dwarf Cavendish It belongs to AAA genotype which is a triploid variant of the species *M. acuminata.* It bears bunches weighing 25-30 kg with well spaced hands and uniform sized fingers. Fruit quality is similar to Dwarf Cavendish and requires propping. It has characteristic medium height and large fruit yields make it ideal for commercial cultivation. Susceptible to leaf spot and fusarium wilt.

2.3.1.3 Nendran

According to Shanmugavelu *et al.* (1992) Nendran which is also known as Rajeli (French Plantain) is an important group of bananas peculiar 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 the other preparations.

The nendran ecotypes used in the study are,

Attunendran

This is tall growing robust variety, a heavy yielder with 7 to 8 hands and about 70 fruits per bunch; a medium bunch weighs 12 kg. Fruit is long, slightly curved, apex tapering with a prominent long pointed beak unlike the Nananendran. Suitable for rain fed crop.

Chengalikodan

This variety has medium plant crop cycle (300-350 days) and is mainly characterised by pink purple bracts, fruit slightly curved, and perpendicular to the stalk at maturity.

Nedunendran

Nedunendran has medium plant crop cycle (300-350 days). This variety is mainly characterised by the fruit apex being lengthily pointed, first hand perpendicular to the axis or slightly drooping.

2.4 Tissue culture of banana

There are many reports on tissue culture in banana. *In vitro* adventitious buds formed from banana shoot apex after decapitation was firstly reported by Ma and Shii (1972) in Taiwan. These were soon followed by Berg and Bustamante (1974) who used meristem culture combined with thermotherapy for the production of virus free banana plants. Since then people are working on different aspects of banana tissue culture as a tool for maximizing banana production.

Many scientists have reported genotypic difference in tissue culture of banana. Influence of genotypes on the rate and type of proliferation was reported by Vuylsteke and De Langhe (1985) in which they observed higher proliferation was shown by B genome and lower in ABB when compared to AAB genome. Bhaskar (1991) reported the response of three cultivars in tissue culture belonging to AAB genome and AAA genome like Nendran, Palayankodan and Red banana. Cultivars showed in difference in the time taken for culture establishment and percentage of cultures established.

Embryogenic response of young male flowers of different banana genotypes were studied by Ganapathi *et al.* (1999). Rasthali (AAB) showed good embryogenic response compared to Shreemanti (AAA), Basrai (AAA), Lokhandi (AAA) and Trikoni (AAA).

Muhammad *et al.* (2004) studied multiplication in banana (*Musa* spp.) cv. Basrai *in vitro* in which they observed difference in multiplication rate among the explants of the same genotype and also shoot tips from different rhizomes behaved differently. Sapheera (2005) observed that the production of multiple shoots were lesser in diploid bananas when compared to triploid ones.

The difference in *in vitro* responses of diploid and triploid banana cultivars due to the effect of hormonal combinations in MS medium was reported by Resmi and Nair (2007).

An efficient culture medium for clonal mass propagation of two banana cultivars, Cavendish Dwarf and Valery was established by Farahani *et al.* (2008). They observed induction of multiple shoots on different combinations of growth regulators like TDZ and kinetin in Dwarf Cavendish and Valery.

High genotypic difference in different banana cultivars viz. Attunendran, Nedunendran, Chengalikodan and Grand Naine in sprouting of explants, shoot proliferation, rooting and root growth was reported by Shylaja *et al.* (2015). Saraswathi *et al.* (2016) reported genotypic difference in shoot proliferation in the different varieties studied. Higher number of shoots were produced by sago+ isabgol in Udhayam and Rasthali, whereas more number of shoots were observed in medium containing sago alone in Grand Naine.

In vitro culture techniques are now a requisite for the production of disease-free plants, quick multiplication of rare plant genotypes, transformation of plant genome, and production of plant-derived metabolites of important commercial value (Debnarh *et al.*, 2006; Altpeter *et al.*, 2016).

2.4.1 Tissue culture of banana using different explants and different routes

Many scientists have reported different routes through which banana is propagated *in vitro.*

Regeneration of meristems proceeds through organogenesis instead of somatic embryos which often results in production of chimeric plants (Hwang *et al.*, 1984, Banerjee and Sharma, 1988). Huang and Chi (1988) reported that vigorous development of banana callus was achieved only when the nutrient medium was supplemented with picloram and solidified with gelrite. Navarro *et al.* (1997) regenerated banana plants via somatic embryogenesis of diploid (*Musa acuminate* ssp. *malaccensis*) and triploid ("Grand Naine') bananas from immature zygotic embryos and male flower bud primordia.

Various explant sources can be used for culture initiation in banana. Shoot apices obtained from parental pseudostem, suckers, lateral buds and terminal inflorescence are the most commonly used explant sources. Axillary flower buds and floral apex exhibit morphogenetic plasticity in their juvenile stage and can be induced to revert to vegetative growth, resulting in *in vitro* production of multiple shoots. For clonal multiplication and maintenance of banana meristem culture is now commonly used.

Okole and Schulz (1996) reported that induction of callus and shoot bud is possible by using micro cross sections of banana and plantain leaf bases which can be used later for regeneration of healthy plants in three banana cultivars.

Ganapathi *et al.* (1992) reported that plants could be regenerated from encapsulated shoot tips isolated from multiple shoot cultures of banana cv. Basrai. Encapsulation was done in 3% sodium alginate solution containing various gel matrices and they observed that encapsulated shoot tips regenerated *in vitro* on different substrates. Regeneration of embryogenic cultures from female flowers of False Horn Plantain was reported by Grapin *et al.* (2000) This study demonstrated that somatic embryogenesis from immature flowers is suitable for genotypes of *Musa* with or without male buds.

A successful regeneration protocol was developed for the first time from cell suspension-derived protoplasts of the dessert banana cv. Grande Naine (AAA) via somatic embryogenesis by Assani *et al.* (2001).

Protocol for efficient and reproducible protoplast regeneration was developed by Assani *et al.* (2002) which resulted in the regeneration of seven new banana genotypes.

Kosky *et al.* (2002) initiated cell suspensions of the hybrid cultivar FHIA-18 (AAAB) which were established from sections of embryogenic tissue derived from male flowers. Jalil *et al.* (2003) established culture for plant regeneration from embryogenic suspension cultures of *Musa acuminata* cv. Mas (AA) from male inflorescence.

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There are reports on anthers being used as explant source for the production of haploid plants. Assani *et al.* (2003) initially reported the production of haploid plants of banana by inducing callus from anthers in which the most of the microspores were uninucleate.

Mahdavi *et al.* (2010) reported the potential of male inflorescences to be used as explants for rapid micropropagation of some Malaysian banana and plantain (*Musa* spp.) cultivars. An efficient regeneration protocol for wild banana (Pisang Jajee (AA)) from zygotic embryos at different maturity stages was developed by Uma *et al.* (2011). In this they emphasised on embryo ontogeny to determine the best maturity stage for embryo rescue, proper media and culture conditions (light and dark) for germination and regeneration. A micropropagation protocol for banana (*Musa* spp.) cv. Agnishwar was established by using shoot tip culture by Rahman *et al.* (2013).

2.5 Clonal micropropagation

Clonal micropropagation is the practice of rapid multiplication of stock plant material using modern plant tissue culture methods to produce large number of true to type plants. It is the vegetative propagation of plants under aseptic conditions using very small explants, which is impossible with conventional technique. Hence the technique is more valuable when limited tissue is available as explants. The target of micropropagation is to produce exact replica of the original plant selected for its desirable characters in many plant species (Bhojwani, 1980; Wang and Hu, 1982).

In cultivated bananas, the natural regeneration through suckers is very slow due to hormone-mediated apical dominance of the mother plant. *In vitro* culture for commercial production of banana usually use shoot multiplication technique to increase the rate of plantlet production and improve the quality of regenerants such as uniformity and true to parental type.

Micropropagation aims at obtaining large number of plants that are genetically identical, developmentally normal with high potential to survive in the

ex vitro condition. Even though true-to-type plantlets are the anticipated goal of micropropagation, somaclonal variants are obtained many times due to involvement of tissue culture components and mode of regeneration.

Micropropagation is proved to be very efficient technique to speed-up the production of high quality pathogen-free plantlets, in terms of genetic and physiological uniformities (Sathish *et al.,* 2011; Supaibulwattana *et al.,* 2011)

2.5.1 Commercial micropropagation

Commercial application of plant tissue culture started in USA with micropropagation of orchids in 1970s. There was tremendous growth in the number of production units and the number of plants produced globally from 1985 to 1990. With an estimated global market of 15 billion US dollars per annum for tissue cultured products, the demand far exceeds production even with exponential growth in the industry, leaving enough possibility for development.

As an example protocol for commercial micropropagation, an efficient system was developed for *in vitro* mass propagation of the commercial cultivar *Musa acuminata* cv. Dwarf Cavendish by the cultivation of shoot apices by Rout *et al.* (2001).

Commercial production of banana cv. Rasthali (*Musa* spp.) from sword suckers by *in vitro* propagation of banana was reported by Govindaraju *et al.* (2012).

2.6 Low cost options in plant tissue culture

Micropropagation technology is more expensive than the conventional methods of plant propagation and requires several types of skills. It is a capital-intensive industry and in some cases the unit cost per plant becomes unaffordable. During the early years of the technology, there were difficulties in selling tissue culture plants because of the high cost as compared to the conventional planting material. This problem has been solved by inventing reliable and cost effective tissue culture methods without compromising on quality (Savangikar, 2004).

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The high cost of production has prevented laboratories with limited resources from accessing the benefits of plant tissue culture technology. A disadvantage of modern plant tissue culture methods is the relatively higher costs involved as compared to other methods (Sahu and Sahu, 2013). The important cost factors in banana micropropagation include high energy consumption for maintaining required culture conditions like photoperiod, temperature and relative humidity and costly components in artificial media. The use of low cost options in plant tissue culture was reported by many scientists.

2.6.1 Water sources

Ganapathi *et al.* (1995) and Sharma and Singh (1995) reported the use of tap water for micropropagation of banana and ginger respectively. Prabhuling *et al.* (2010) demonstrated a cheaper water source such as filtered (aquaguard) or even autoclaved potable tap water which can be used as low cost alternative water sources for successful micropropagation of banana 'Grande Naine'.

2.6.2 Carbon sources

Refined white and unrefined brown sugars were found effective for coconut embryo culture in Philippines (Bonaobra *et al.*, 1994). In India, Ganapathi *et al.* (1995) reported that commercial grade sugar could replace analytical grade sucrose, with no significant change in the frequency of shoot formation in banana. Kodym and Arias (2001) reported that when sucrose and gelrite was replaced with commercial sugar and a starch-gelrite mixture 90 per cent cost reduction was achieved. Raghu *et al.* (2007) reported the study of cost reduction alternatives in micropropagation of *Centella asiatica.* The use of household sugar instead of laboratory grade sucrose and tap water instead of double distilled water helped to reduce the cost on media ingredients and the use of liquid media in multiplication stage helped to reduce the cost on agar.

Table sugar as an alternative low cost medium component for *in vitro* micro-propagation of potato (*Solanum tuberosum* L.) was reported by Demo *et al.* (2008). They showed that table sugar not only enhanced micro-propagation but

also significantly lowered the production input costs by 34 to 51 per cent when compared with the analytical grade sucrose.

2.6.3 Gelling agents

In many crops including banana, sago and isabgol were better gelling agents (Bhattacharya *et al.*, 1994, Babbar and Jain, 1998; Naik and Sarkar, 2001). Zimmerman *et al.*, (1995) used a gelling mixture, which consisted of 5 per cent corn starch and 0.05 per cent gelrite for the culture of strawberry and raspberry. For replacing high cost agar for tissue culture, isabgol derived from the seeds of *Plantago ovata*, might be a good alternative due to its colloidal and polysaccharidic nature. (Tyagi *et al.,* 2007).

Cost reduction was achieved in *Curcuma longa* cv. Prathibha by using inexpensive carbon source and gelling agent (Tyagi *et al.*, 2007). Laboratory reagent grade sucrose was replaced by locally available commercial sugar (market sugar or sugar cubes) as carbon source and bacteriological grade agar by isabgol as gelling agent. No adverse effects on shoot regeneration and conservation on isabgol- gelled low cost media were observed.

Alternatives for agar for the micropropagation of African violet (*Saintpaulia ionantha*) was reported by Sharifi *et al.* (2010). They could find that the combination of starch, semolina and potato powder or combination of starch and agar can be low cost options for shoot induction in African violet. Cost-effective *in vitro* conservation of banana cv. Karpura Chakkarakeli (AAB; Mysore subgroup) using alternatives for gelling agent (isabgol) and carbon source (market sugar) without any adverse effects on cultures was reported by Agrawal *et al.* (2010). A combination of agar with potato starch or corn starch offered a firm support for plant tissues and can be successfully used for potato micropropagation was reported by Mohamed *et al.* (2010).

Cost reduction approaches for *in vitro* mass multiplication of potato (*Solanum tuberosum* L.) was developed by Venkatasalam *et al.* (2013) by using

commercial sugar, clean tap water and bacteriological agar or gelrite/ phytagel as low cost options.

Low cost tissue culture protocols were developed by Saraswathi *et al.* (2016) for commercial micropropagation of three banana varieties, mainly Rasthali, Grand Naine and Udhayam. The low cost water and carbon source used were table sugar (3 per cent) and reverse osmosis water. They tested six diverse gelling agent treatments: T1- sago alone, T2- isabgol alone, T3- sago + agar, T4- isabgol + agar, T5- sago + isabgol, and T6- agar alone as a control. In the two varieties of banana, Udhayam and Rasthali, sago and isabgol (T5) performed significantly good producing 10 shoots per explant in one cycle.

Esserti *et al.* (2017) reported the effect of seaweed extract (SE) from *Fucus spiralis* (Fs), *Cystoseira myriophylloides* (Cm) and *Laminaria digitata* (Ld) on *in vitro* plant tissue culture. They suggested that seaweed extract of *Fucus spiralis* (Fs) and *Cystoseira myriophylloides* (Cm) contain necessary nutrients and growth regulators to allow their use as medium for *in vitro* plant tissue culture.

2.6.4 Other low cost options

Tubular skylights were used as source for natural lighting for cost reduction in micropropagation of banana. When daylight was exploited instead of artificial light for the *in vitro* culture of banana, micropropagation rates were either the same or significantly higher than under artificial lighting (Kodym *et al.*, 2001, Kodym and Arias, 1999).

Autoclave for sterilization has been replaced with a pressure cooker with no detectable contamination (Gitonga *et al.*, 2010). Ogero *et al.* (2012) developed a low cost medium which can be used to propagate cassava from nodal cuttings. When locally available salts were used as sources of the Murashige and Skoog nutrients, it led to a significant reduction in the cost of production.

In micropropagation of potato, the main expenses are labour and equipment costs. To reduce the cost associated with media sterilization, Weber *et al.* (2015) used a disinfectant, sodium hypochlorite (NaOCl), in combination with

microwave heating, for media sterilization which controlled microorganism growth and maintained plantlet growth performance.

An efficient low cost medium "KFA and KFA plus" ("Flyash" as the main source of inorganic constituent; patented), which could replace the widely used expensive Murashige and Skoog's medium was reported by Biswas and Biswas (2017) for the micropropagation of *Lilium Asiatic*. The cost of media was reduced 10 times by using KFA plus as culture media as compared to MS ready media (Hi media, India) and encouraging results in growth and multiplication were obtained. Therefore the use of flyash media not only produce low cost plants but also the reduction of disposal problem of thermal power plant waste, leading to phytoremediation.

Kadam *et al.* (2018) evaluated the low cost alternative components of tissue culture media for their potential to supply nutrients and to support growing explant of banana cv. Cavendish *in vitro* for their cost effectiveness against conventional high cost media. The Murashige and Skoog (MS) salts and sucrose were replaced by vermicompost (50 g/L), table sugar (30 g/L) and coconut water (70 ml/L), and agar was replaced by filter paper, sand and wheat flour. The use of vermicompost and coconut water as nutrient source and that of filter paper as support matrix was found efficient alternative for conventional costly ingredients.

2.7 Number and duration of subculture cycles

The long period in culture increased the number of somaclonal variants observed in wheat regenerants was reported by Hartmann *et al.* (1989). It was reported that somaclonal variants appeared from the fifth subculture (1.3%) onwards and increased to 3.8% after 11 subcultures by Rodrigues *et al.* (1998).

The genetic stability may be affected by the rapid multiplication of a tissue leading to somaclonal variation (Israeli *et al.*, 1995). Petolino *et al.* (2003) reported that somaclonal variation is mostly apparent and higher in plants regenerated from long term cultures.

In fennel micropropagation, there was absence of genetic variation after long culture period of 17 months, suggesting a possible genotype effect (Bennici *et al.*, 2004). Bairu *et al.* (2006) reported that the rate of somaclonal variations are enhanced when the number of subculture and their duration are increased, especially cell suspension and callus cultures. A high rate of proliferation is achieved in relatively shorter periods during micropropagation and leads to more frequent subculturing.

There are also contrary reports on the effect of culture duration on somaclonal variation. Cases have been reported where multiple shoot culture of pea maintained over long period (24 years) remaining genetically stable and was comparable to the original genotype (Smykal *et al.*, 2007).

2.8 Clonal fidelity analysis using molecular markers

There are two types of molecular markers generally used for detection such as hybridisation based Restriction Fragment Length Polymorphisms (RFLP) (Botstein *et al.*, 1980) and Polymerase Chain Reaction (PCR) based molecular 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).

Polymerase Chain Reaction (PCR) based detection

Mullis and co-workers in 1983 invented the PCR, and it is based on enzymatic *in vitro* amplification of DNA (Weising *et al.*, 2005). In PCR, the DNA sequence is amplified with the help of primers and thermostable DNA polymerase.

During PCR, a small amount of DNA is amplified to a large extent as it passes through the process of denaturation, annealing and elongation. The major advantage of PCR based techniques is the requirement of only small quantity of

DNA samples. Various PCR based DNA markers used for genetic stability analysis are RAPD, SSR, ISSR and AFLP.

Random amplified polymorphic DNA (RAPD)

RAPD markers are generated based on the probability that a DNA sequence, which is homologous to a short, oligonucleotide primer (decamers for RAPDs) will occur at different sizes on opposite strands of a DNA template that is amplifiable by PCR (Williams *et al.*1990; Waugh and Powell 1992).

RAPD analysis is suitable for genotyping, phylogenetic analysis genetic diversity analysis and molecular selection [Williams *et al.*, 1990; Heun *et al.* 1994; Atak and Celik (2009)].

RAPD markers were used for screening the clonal fidelity of *in vitro*-raised bulblets of *Lilium sp.* (Asiatic hybrids) produced through adventitious mode of propagation. Only 14 primers gave clear reproducible bands out of the 20 primers used to screen the samples. When individual primers were analysed, it revealed that RAPD patterns produced were all shared by both the *in vitro*-raised bulblets and the mother bulb. There was no variation observed within the tissue culture-raised progenies (Varshney *et al.*, 2001).

Genetic stability of three economically important micropropagated banana (*Musa* spp.) cultivars of lower Indo-Gangetic plains, was assessed by RAPD and ISSR markers by Ray *et al.* (2006). Among the two marker systems used, ISSR fingerprinting detected more polymorphism than RAPD in 'Robusta' and 'Giant Governor' with most of the primers showing similar fingerprinting profile, whereas 'Martaman' revealed complete genetic stability.

RAPD markers were reported to be an effective and rapid technique for assessing the molecular stability of *in vitro* raised plants of gerbera at genomic level (Mandal *et al.*, 2010).

Genetic analysis of plantain ecotypes of banana using RAPD and ISSR markers was reported by Choudhary (2011). The study showed that variability exists among the different plantain ecotypes of Kerala.

Plants regenerated from petiole explants by direct shoot formation were assessed using RAPD and SSR primers, which produced clear, reproducible and scorable bands. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant. Similarity between the *in vitro* raised plants and the mother was 100 per cent, which confirmed the true-to-type nature of the *in vitro*-raised plants (Minerva *et al.*, 2012).

Genetic diversity of 12 accessions of gerbera was assessed through RAPD markers (Prajapati *et al.*, 2014).

Micropropagation and assessment of genetic fidelity of *Dendrocalamus strictus* (Roxb.) nees using RAPD and ISSR markers was done by Goyal *et al.* (2015). The ten RAPD decamers produced 58 amplicons and nine ISSR primers generated a total of 66 bands. All the bands generated were monomorphic.

The genetic fidelity of regenerated plants was evaluated by Bhowmik *et al.* (2016) by using two PCR-based DNA marker techniques, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) which detected no variability in the *in vitro* multiplied plantlets of *Alpinia calcarata* Rosc.

Inter Simple Sequence Repeats (ISSR)

Inter-simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences. These are easy to use, low-cost, and methodologically less demanding compared to other dominant markers (Ng and Tan, 2015).

ISSR markers are highly sensitive, highly reproducible and dominant, provides Mendelian segregation and has been successfully applied in genetic and evolutionary studies of many species. It can also be applied in studies involving

genetic identity, parentage, clone and strain identification and taxonomic studies of closely related species and useful in gene mapping studies (Zietkiewicz *et al.*, 1994; Godwin *et al.*, 1997) and diversity analysis.

When compared to RAPD primers (10-mers), they have high reproducibility possibly due to the use of longer primers (16- to 25-mers) which permits the subsequent use of high annealing temperature (45 to 60^0C) which leads to higher stringency (Reddy *et al.,* 2002). ISSR markers do not require any prior sequence data information for primer construction and the quantity of DNA sample required is very low (5-50 ng) as it is a PCR based marker (Kumar *et al.,* 2009). ISSR markers are randomly distributed over the entire genome.

Polymerase chain reaction (PCR) based techniques such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) are immensely useful in establishing the genetic stability of *in vitro* regenerated plantlets in many crop species (Lakshmanan *et al.*, 2007; Joshi and Dhawan, 2007).

Joshi and Dhawan (2007) employed Inter simple sequence repeat (ISSR) marker assay to validate the genetic fidelity of *Swertia chirayita* plantlets multiplied *in vitro* by axillary multiplication upto forty-two passages. A homogenous amplification profile was observed for all the micropropagated plants and results confirmed the clonal fidelity of the tissue culture-raised *S. chirayita* plantlets.

Gantait *et al.* (2010) reported the clonal fidelity study of micropropagated and sustained cultured clones of *Allium ampeloprasum* L. using ISSR marker system and ensured the continuous supply of quality propagules retaining genetic uniformity.

Bhatia *et al.* (2010) reported true-to-type clonal fidelity is one of the most important pre-requisites in micropropagation of crop species. Genetic fidelity of *in vitro* raised plants of gerbera derived from three different explants, *viz.*, capitulum, leaf and shoot tips was assessed by 32 ISSR markers, for their genetic

stability. Fifteen ISSR markers generated monomorphic banding pattern in all the clones.

Nadha *et al.* (2011) confirmed the true-to-type nature of the *in vitro* raised clones of *G. angustifolia* Kunth using DNA based RAPD and ISSR markers as they did not detect any variability in the tissue culture raised plantlets employing the axillary bud proliferation method for the commercial multiplication of *Guadua* without any risk of genetic instability.

Parida *et al.* (2011) assessed genetic fidelity of *in vitro* regenerated *Alpinia galanga* L. using DNA based molecular markers. They used RAPD and ISSR markers for assessment of genetic stability. All banding profiles from micropropagated plants were monomorphic and similar to the mother plant.

Borse *et al.* (2011) investigate clonal fidelity of banana (*Musa acuminata* cv. Grand Naine) regenerants from six different *in vitro* subculture generations and in the explant suckers by using ISSR and REMAP molecular markers. Both types of markers revealed high degree of monomorphism. Very low variation was observed up to the eighth subculture generation with polymorphic bands being low in both ISSR (0.96%) and REMAP (0.95%) markers.

Morphological and molecular analysis of genetic stability in micropropagated banana (*Musa* spp.) var. Nendran was reported by Amar (2012). The variation was relatively more in plants derived in later subculture (above 12) and the maximum variation was observed was 15 per cent for both male bud and sucker derived plants.

Khateeb *et al.* (2013) assessed the genetic stability of micropropagated plants using Inter-Simple Sequence Repeat (ISSR). The amplification products were monomorphic in all *in vitro* grown plants. No polymorphism was detected indicating the genetic integrity of *in vitro* propagated plants.

Datta (2014) studied on micropropagation of gerbera and genetic stability of plantlets was assessed using ISSR assay. Results of genetic stability studies showed that mother plant and regenerants derived from flower buds were uniform

to the extent of 80 per cent. Plants regenerated through indirect pathway showed higher variation (40%).

Choudhary *et al.* (2015) observed a homogeneous amplification profile for all micropropagated plantlets of commercial banana cultivar Robusta when amplified using 20 ISSR primers

In vitro regeneration and assessment of genetic fidelity of acclimated plantlets by using ISSR markers in PPR-1 (*Morus* sp.) was reported by Rohela *et al.* (2018). The *in vitro* regenerated PPR-1 mulberry plantlets were confirmed as clonally uniform and genetically stable.

ISSR-assisted analysis of clonal fidelity supported with SEM and histology using *in vitro* propagated plants of *Moringa peregrina* (Forssk.) which is an endangered desert tree was reported by Fayas *et al.* (2018). The clonal fidelity of the *in vitro* plantlets developed through direct organogenesis was assessed using ISSR marker. The similarity indices between the parental plants and their progenies were above 98.2 per cent and indicated that the progenies were highly similar to the mother plant.

Simple sequence repeats (SSR) or Microsatellites markers

Simple Sequence Repeats (SSR) are tandem repeats of DNA sequence of only a few base pairs (1-6 bp) in length. They are also known as microsatellite markers, Short Tandem Repeats (STR) or Simple Sequence Length Polymorphism (SSLP). The term microsatellite was introduced to characterize the simple sequence stretches amplified by PCR (Hearne *et al.*, 1992).

Microsatellite sequences are abundant, dispersed throughout the genome and are highly polymorphic in plant genomes, even among closely related cultivars, due to mutations causing variation in the number of repeating units in genomes (Condit and Hubbell, 1991). The most abundant is the dinucleotide repeat (McCouch *et al.*, 2002).

In *in vitro* cultures, microsatellite markers act as highly sensitive markers for monitoring genetic variations (Lopes *et al.,* 2006).

The genetic homogeneity of *in vitro* raised plants of grape vine cultivar Crimson Seedless was confirmed by Nookaraju and Agrawal (2012) using microsatellite markers. Allelic composition of 23 *in vitro* raised plants and the mother plant at 5 SSR loci did not show any polymorphism. Their study confirmed the genetic uniformity among the *in vitro* raised plants and demonstrated the reliability of *in vitro* propagation system used for the cultivar.

Brito *et al.* (2010) tested SSR primers for micropropagated plants of olive species *Olea maderensis* and *O. europaea* ssp. *europaea* var. *sylvestris* confirmed its genetic purity with that of the mother plant.

Pandey *et al.* (2012) reported the genetic fidelity testing of micro-shoots of sugarcane based on SSR analysis which indicated a strong genetic purity similar to the parent genotype. Lack of variation confirmed the genetic purity of tissue culture plantlets of sugarcane raised through direct organogenesis in young whorl leaf roll explants and also the suitability of overall regeneration protocol.

The efficiency of microsatellite marker development depends on the abundance of repeats in the target species and the ease with which these repeats can be developed into helpful markers (Alizadeh *et al.,* 2015).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Low cost alternatives in commercial micropropagation of banana (*Musa* spp.) was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during September 2016 – July 2018, with the objective to reduce the cost of production in commercial micropropagation of banana. The study mainly focused on the following aspects:

1) Cost reduction in plant tissue culture medium: a) Substitution of sucrose with common sugar b) Substitution of agar with isabgol/ sago 2) Increasing subculture cycles and clonal fidelity analysis using specific ISSR marker to reduce per plant cost. The materials used and methodologies adopted are furnished in this chapter.

3.1 Materials

3.1.1 Source of culture

Studies were conducted in six banana cultivars viz. Attunendran (AAB), Nedunendran (AAB), Chengalikodan (AAB), Poovan (Rasthali- AAB), Njalipoovan (AB) and Grand Naine (AAA) which are commercially produced at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, in the commercial micropropagation unit.

Established cultures of six banana cultivars in the 5th subculture cycle received from the commercial micropropagation unit of CPBMB were utilized for the study.

3.1.2 Laboratory chemicals, glassware and plasticwares

The chemicals used for the study were of good quality (AR grade) from various agencies like Merck India Ltd., HIMEDIA and SISCO Research Laboratories. Common sugar (granules) was purchased from supermarket. Isabgol was supplied by Abhyudhay industries, Gujarat. Sago was purchased from

supermarket. The *Taq* DNA polymerase, dNTPs, Taq buffer and molecular marker (λDNA/*Hind*III + *Eco*RI double digest) were supplied by Bangalore Genei Ltd. All the plasticwares used were obtained from Axygen and Tarson India Ltd. The ISSR primer was supplied by Sigma Aldrich Chemicals Pvt. Ltd.

3.1.3 Equipment and machinery

The present research work was carried out using plant tissue culture and molecular biology facilities available at CPBMB, College of Horticulture. For micropropagation studies, all the aseptic manipulations were carried out in laminar air flow (LABLINE INDUSTRIES). Media sterilization was done in autoclave (Nat steel equipment Pvt. Ltd.). Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500). The quality and quantity of DNA was estimated using NanoDrop^R ND-1000 spectrophotometer. DNA amplification reaction was carried out in AB thermocycler. For agarose gel electrophoresis, horizontal gel electrophoresis system (BIO-RAD, USA) was used. For imaging and documenting the agarose gel profile, BIO-RAD Gel Doc system was used. The details are given in Annexure I.

3.2 Methods

3.2.1 Cost reduction in plant tissue culture medium

3.2.1.1 Substitution of sucrose with common sugar

Sucrose (laboratory grade) was substituted with common sugar at 30 gL^{-1} in MS medium. The response of six different cultivars for shoot multiplication and rooting were studied by substituting sucrose (control) with common sugar to know the genotypic difference in response in different media. Separate experiments were conducted for different cultivars.

3.2.1.2 Substitution of laboratory grade agar with marine agar

Substitution of agar (laboratory grade) with marine agar was done in multiplication stage in one of the cultivar Chengalikodan. Agar (laboratory grade) was substituted with marine agar at 7.5 gL^{-1} in MS medium. The response of the

cultivar for shoot multiplication was studied by substituting agar with marine agar to know the genotypic difference in response in the two media.

3.2.1.3 Substitution of agar with isabgol/ sago

Substitution of agar (marine agar) with isabgol/ sago was done in rooting and multiplication media in the six different cultivars. . Gelling property of MS media was tested using different proportions of sago/ isabgol [T1- Sago (100%), T2- Isabgol (100%), T3- Sago (50%) + Isabgol (50%), T4- Agar (50%) + Isabgol (50%), T5- Agar (50%) + Sago (50%), T6- Agar (100%)]. The response of six different cultivars at multiplication and rooting stage was observed in the different media tried. The experiment was conducted in CRD with required number of replications. Separate experiments were conducted for different cultivars.

3.2.2 Culture media

3.2.2.1 Preparation of MS medium

Standard procedures were followed for the preparation of MS (Murashige and Skoog, 1962) plant tissue culture medium. Five stock solutions of major and minor nutrients were prepared and stored in pre-cleaned glass bottles under refrigerated conditions. Stock III was stored in amber colored bottle. Stock solutions of various growth regulators were also stored under refrigerated conditions.

A clean beaker, rinsed with distilled water was used to prepare the medium. After taking distilled water 1/3rd the volume, aliquots from all stock solutions were pipetted in proportionate volume in the beaker. For preparing MS medium of full strength, 20 ml was pipetted out 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.

Required quantity of agar/ isabgol/ sago was added as solidifying agent after making up the volume of the medium. The medium was stirred and heated in

a microwave oven to melt the agar/ other solidifying agents. Then 15-20 ml of hot medium was poured into 25 x 150 mm pre-sterilized glass culture tubes and plugged with non-absorbent cotton or 50 ml to culture bottles and autoclaved. Chemical compositions of MS medium is given in Annexure II.

3.2.2.2 Growth regulators

Auxins (IBA) and cytokinins (BA) were incorporated in the media at various stages of culture for multiplication and rooting of cultures.

3.2.2.3 Autoclaving

The test tubes or culture bottles with nutrient media were autoclaved at a pressure of 15 lbs/ sq.inch (121^0C) for 20 minutes. After autoclaving, the tubes were removed and allowed to cool. The inoculation was done 4-5 days after media sterilization to ensure that tubes were free of microbial contamination.

3.2.3 Experimental conditions

3.2.3.1 Transfer area and aseptic manipulation

All the aseptic manipulations such as subculturing for multiplication, elongation and rooting were carried out in the laminar air flow cabinet. The work table of laminar air flow cabinet was sterilized by swabbing with 70 per cent alcohol. Then UV light was switched on for 20 minutes to achieve aseptic environment inside the cabinet and air was allowed to blow off for 15 minutes before working in the laminar air flow cabinet.

3.2.3.2 Culture conditions

The cultures were incubated at 26 ± 2^0C in an air conditioned culture room with 16 hours photoperiod (1000 lux) from fluorescent tubes. According to the prevailing climate, humidity in the culture room varied from 60 to 80 per cent.

3.2.4 Micropropagation protocol

The micropropagation protocols optimized for the six different cultivars at CPBMB (Shylaja *et al.*, 2015) were followed for production of plantlets.

Regular subculturing was followed at 21 days interval. The effect of different media on shoot proliferation, rooting and root characters were studied. The number of shoots proliferated in each subculture cycle for the different cultivars were observed to assess the shoot proliferation in each medium. Similarly, percentage of rooting, days taken for root initials to appear, number of roots and root length were observed in different cultivars in the different media combinations.

3.2.5 Hardening and acclimatization

The *in vitro* rooted plantlets were taken out of the culture vessels using forceps after soaking the culture in water for five minutes. Plantlets were washed in running tap water to remove the solidified medium. It was then treated with 0.1 per cent Bavistin for five minutes. The plantlets were planted out in small earthen pots filled with potting mixture containing coco peat, soilrite and vermiculite in 3:1:1 ratio and were kept in net house for primary hardening. After two weeks, the plants were transferred to polythene bags containing sand, soil and cow dung in 1:1:1 ratio and were kept for secondary hardening.

The plants were observed for one month and survival rate was recorded.

3.2.6 Increasing subculture cycles and clonal fidelity analysis using specific ISSR marker

Subculturing of 5th stage cultures received from CPBMB were done using protocol optimised for the different cultivars at CPBMB (Shylaja *et al.*, 2015). Plants derived from subculture passages 7 to 11 in different cultivars were analysed for clonal fidelity using specific ISSR primer optimized at CPBMB as reported by Rajitha *et al.* (2015). Fifty regenerants were planted out in six cultivars, stage wise and clonal fidelity was assessed using DNA isolated from 10

plants of each stage per cultivar. For one cultivar, fifty DNA isolations were done and altogether 300 DNA isolations were done for six cultivars.

3.2.6.1 Clonal fidelity studies using ISSR marker

For analysing the clonal fidelity of micropropagated plants, ISSR assay was carried out. The main advantage of ISSR (Inter Simple Sequence Repeats) markers is very simple, fast, cost-effective, highly discriminative and reliable. They do not need any prior sequence information and require only a small quantity of DNA sample (5-50 ng per reaction. ISSR markers have wide distribution throughout the genome. Hence, ISSR markers are suitable for assessment of clonal fidelity of *in vitro* regenerated plants.

3.2.6.1.1 Genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important pre-requisites for doing ISSR analysis. The CTAB procedure reported by Rogers and Bendich (1994) for the extraction of nucleic acids was used for the extraction of genomic DNA from tissue culture derived banana plants. Young tender leaves collected from healthy plants early in the morning were used for genomic DNA isolation.

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

    -1.4M NaCl

    -1 per cent PVP

2. CTAB (10 per cent)

    -10 per cent CTAB (w/v)

-0.7M 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 per cent)

8. Distilled water

9. 1% RNase

Procedure for DNA isolation

Young and tender leaf tissue (0.25g) was weighed and ground in a pre-chilled mortar and pestle in the presence of liquid nitrogen and a pinch of PVP. 1 ml of 5X extraction buffer and 50 µl β-mercaptoethanol were added to it. The homogenized sample was transferred to an autoclaved 2 ml centrifuge tube. It was mixed thoroughly and the mixture was incubated at 65°C for 30 minutes with occasional mixing by gentle inversion. The mixture was incubated on ice for 10 minutes. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and the mixture was mixed by inversion to emulsify. Then it was centrifuged at 12000 rpm for 15 minutes at 4°C. The contents got separated into three distinct phases. The top aqueous phase containing DNA was transferred into a fresh micro centrifuge tube. 1µl RNase was added into it and it was incubated at 37°C for 45 minutes. Then 1/10th volume of ten per cent CTAB buffer and equal volume of chloroform: isoamyl alcohol was added and mixed thoroughly by inversion. It was centrifuged at 12000 rpm for 15 minutes at 4°C. The aqueous phase was transferred into a clean microcentrifuge tube and 0.6 volume of chilled

isopropanol was added and mixed by quick gentle inversion till the DNA was precipitated. It was incubated at -20°C for 2 hours. Then the mixture was cntrifuged at 10000 rpm for 10 minutes at 4°C. Supernatant was poured off gently. The DNA pellet was washed with 70 per cent ethanol followed by100 per cent ethanol, spun for 3 minutes at 8000 rpm and decanted the ethanol. Air dry the pellet for 30 minutes and the pellet was dissolved in 50 µl of TE buffer or autoclaved distilled water and stored at -20°C.

3.2.6.1.2 Assessing the quality of DNA by electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.,* 1989).

Materials for agarose gel electrophoresis

1) Agarose (SRL, Low EEO)

- 0.8 per cent (for genomic DNA)
- 1.8 per cent for ISSR

2) 50X TAE buffer (pH 8.0)

- Tris buffer
- Acetic acid
- 0.5 mM EDTA

3) 6X Loading/ Tracking dye

- 0.03% bromophenol blue
- 0.03% xylene cyanol
- 60% 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 are given in Annexure IV. The procedure for agarose gel electrophoresis is given below:

From 50X stock solution, 1X stock solution was prepared. Agarose (0.8% for genomic DNA and 1.8% for ISSR) was weighed and dissolved in 1X TAE buffer by melting. When the temperature was bearable, ethidium bromide (0.5 μgml^{-1}) was added and mixed well. The open end of the gel casting tray was sealed with a cello tape and was kept on a horizontal levelled surface. The comb was desirably placed and the melted agarose was poured into the tray. The gel was allowed to set for 20-25 minutes. Then the comb was removed carefully. The tray was kept in the electrophoresis unit with well side directed towards the cathode. 1X TAE buffer was added into the tank. DNA sample (4 μl) along with tracking dye (1 μl) was loaded into the wells using a micropipette carefully. λDNA/ *Eco*RI+*Hind*III double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (80V) and current (50A). When the tracking dye reached $2/3^{rd}$ length of the gel, the power was turned off.

The gel was taken out from the electrophoresis unit and viewed under UV transilluminator for presence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented in gel documentation system. The gel profile was examined for intactness and clarity of DNA band.

3.2.6.1.3 Assessing the quality and quantity of DNA by NanoDrop method

The quality and quantity of genomic DNA was estimated using NanoDrop^R ND-1000 spectrophotometer. Before taking sample readings, the instrument was set to zero by taking 1 μl autoclaved distilled water as blank. One micro litre of nucleic acid sample was measured at a wavelength of 260 nm and 280 nm and A_{260}/A_{280} ratios were recorded to assess the purity of DNA.

A ratio of 1.8 to 2.0 for A_{260}/A_{280} indicated good quality of DNA. The quantity of DNA in the pure sample was calculated using the formula $A_{260}=1$ is equivalent to 50 µg double stranded DNA/ µl sample.

1 Absorbance at 260 nm= 50 µg DNA/ml

Therefore A_{260} x 50 give the quantity of DNA in µg/ml.

3.2.6.1.4 Molecular Marker assay

For analyzing clonal fidelity of the micropropagated plants, specific ISSR primer optimized at CPBMB as reported by Rajitha *et al.* (2015) was used.

Inter Simple Sequence Repeats (ISSR) analysis

ISSR assay was performed to detect the polymorphism in amplification patterns in the region between two SSR's. This was carried out by amplifying the DNA by using specific primers relating to the SSR region flanking the ISSR.

Good quality genomic DNA (20 to 50 ng/µl) isolated from banana leaf samples were subjected to ISSR assay. ISSR primers supplied by 'Sigma Aldrich Chemical Pvt. Ltd.' with good resolving power were used for amplification of DNA.

ISSR primer selected for the study

Specific ISSR primer optimized at CPBMB which was reported by Rajitha *et al.* (2015) was used for the clonal fidelity analysis of tissue culture banana plants regenerated after subculture passages of 7 to 12. The ISSR primer was checked for amplification using bulked DNA.

Amplification of DNA by Polymerase Chain Reaction (PCR) was done in Agilent thermocycler. It was performed in a 20 µl reaction mixture 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 (10mM 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	-	95°C for 4 minutes	
Denaturation	-	94°C for 45 seconds	36 cycles
Primer annealing	-	48°C for 1 minute	
Primer extension	-	72°C for 2 minutes	
Final extension	-	72°C for 10 minutes	

4°C for infinity to hold the sample

The amplified PCR products were run on 1.8 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA/ *Eco*RI+*Hind*III double digest). The profile was visualized under UV transilluminator and documented and saved using gel documentation unit. The documented ISSR profiles were carefully examined for polymorphism.

3.2.6.1.5 ISSR data analysis

Amplification profile of the clones in each stage was compared to the mother plant with the selected primer. Bands of DNA fragments were scored manually as (1) for presence or (0) for absence. The DNA amplification pattern in different stages were analyzed and variability at DNA level was calculated.

3.2.7 Statistical analysis

Statistical analyses were done using WASP 2.0 software developed by ICAR. For comparing sucrose with common sugar in multiplication and rooting media,'t' test was used. For comparing the different gelling agents, CRD was used for analysis.

3.2.8 Analysis of cost of MS medium with low cost alternatives

The cost for one litre MS medium with the low cost additives and standard additives was worked out. The current market price of standard and low cost additives like common sugar, isabgol, sago and marine agar were considered for working out the cost. The per cent cost reduction was estimated by comparing the cost of standard additives in one litre MS medium with the cost of low cost additives in one litre MS medium.

Results

4. RESULTS

The results of the investigations on "Low cost alternatives in commercial micropropagation of banana (*Musa* spp.) conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period September 2016 – July 2018 are presented in this chapter.

Studies were conducted in six cultivars of banana viz. Attunendran (AAB), Nedunendran (AAB), Chengalikodan (AAB), Poovan (Rasthali- AAB), Njalipoovan (AB) and Grand Naine (AAA) which are being commercially produced at CPBMB in the micropropagation unit.

Established cultures of six different cultivars in the 5th subculture cycle received from the commercial micropropagation unit of CPBMB were utilized for the study.

4.1 Cost reduction in plant tissue culture medium

4.1.1 Substitution of sucrose with common sugar

In the MS shoot multiplication medium, laboratory grade sucrose (30 gL^{-1}) was substituted with common sugar (30 gL^{-1}) and shoot proliferation in different cultivars were studied. The cultures received from commercial unit at CPBMB were subcultured to MS shoot multiplication medium containing common sugar at 30 gL^{-1} or sucrose (30 gL^{-1}) as the carbon source. The experiment was conducted separately for each cultivar. Shoot proliferation in 7th subculture cycle in different cultivars are presented in Table 1.

The shoot proliferation was observed after three weeks of inoculation in all the six cultivars. No significant difference was observed in shoot proliferation when sucrose was substituted with common sugar in all the six cultivars. So

common sugar was found equally effective as that of sucrose and not influencing the shoot proliferation in different banana cultivars.

Table 1: Shoot proliferation in different cultivars when sucrose was substituted with common sugar in MS medium

Cultivars	Shoot proliferation in one culture cycle of 21 days in 7th subculture cycle		t (0.05)
	Sucrose (30 gL^{-1})	Common sugar (30 gL^{-1})	
Nedunendran[1]	14.42	12.83	2.074 (NS)
Chengalikodan[1]	11.83	10.66	2.074 (NS)
Attunendran[2]	6.25	7.33	2.074 (NS)
Poovan[2]	0.41	1.41	2.201 (NS)
Njalipoovan[2]	1.0	0.16	2.074 (NS)
Grand Naine[2]	3.41	4.58	2.074 (NS)

1-culture bottle 2-culture tube

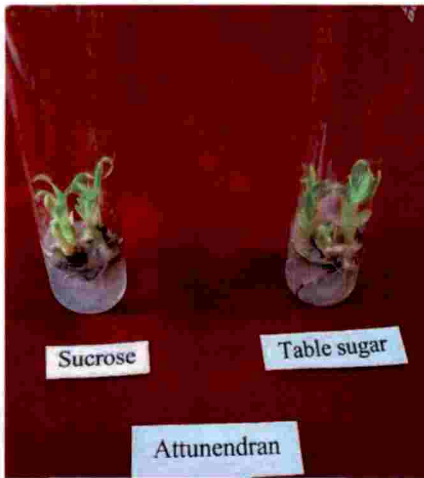
4.1.2 Substitution of sucrose with common sugar in MS rooting medium

4.1.2.1 Rooting in different cultivars when sucrose was substituted with common sugar in MS medium

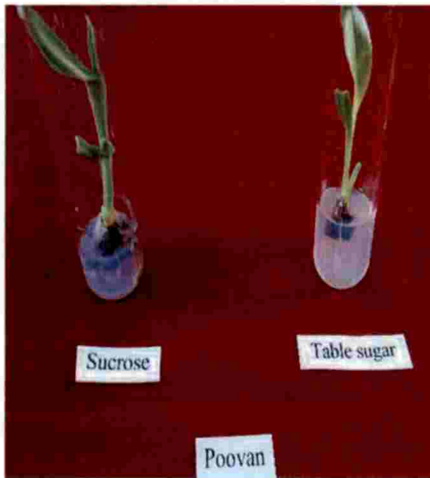
Sucrose substituted with common sugar recorded 100 per cent rooting in MS rooting medium in all the six cultivars studied. Common sugar was found equally good to sucrose in inducing roots in the rooting medium. The percentage of rooting of different cultivars in MS rooting medium when sucrose was substituted with common sugar is shown in Table 2.

a) Nedunendran

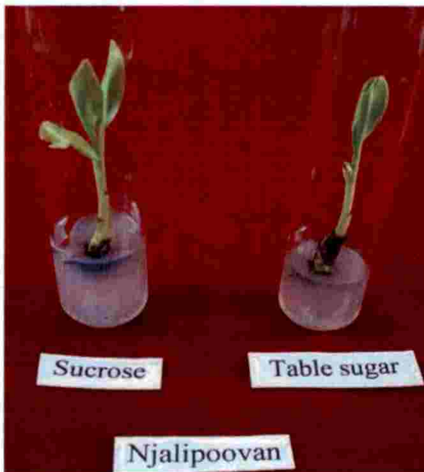
b) Chengalikodan



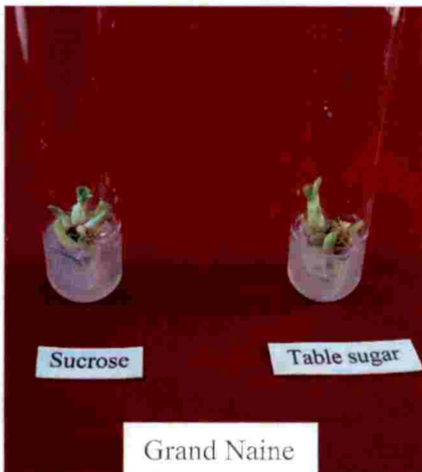
c) Attunendran



d) Poovan



e) Njalipoovan



f) Grand Naine

Plate 1: Shoot proliferation in different cultivars in MS medium when sucrose was substituted with common sugar

Table 2: Rooting (%) in different cultivars in MS rooting medium when sucrose was substituted with common sugar

Cultivars	Rooting (%)	
	Sucrose	Common sugar
Nedunendran	100	100
Attunendran	100	100
Chengalikodan	100	100
Poovan	100	100
Njalipoovan	100	100
Grand Naine	100	100

4.1.2.2 Root characters of different cultivars in MS rooting medium when sucrose was substituted with common sugar

Substitution of sucrose with common sugar had no significant influence in the days taken for root initials to appear. For number of primary roots produced, there was no significant difference in all the cultivars except Attunendran in which medium with common sugar produced more number of primary roots (8.41) than sucrose (6.75). Root length was generally higher in medium with common sugar in the cultivars studied. Medium with common sugar produced more number of secondary roots in some of the cultivars like Chengalikodan while medium with sucrose produced more number of secondary roots in cultivars like Attunendran and Njalipoovan (Table 3).

In Nedunendran, the days taken for root initials to appear did not show any significant difference when sucrose was substituted with common sugar in the MS rooting medium. Similarly, the number of primary roots produced, root length, number of secondary roots in sucrose and common sugar supplemented MS rooting medium also did not show any significant difference.

For Attunendran, the days taken for root initials to appear and root length did not show significant difference between sucrose and common sugar. In the case of number of primary roots, medium with common sugar (8.42) was significantly superior to sucrose (6.75). The number of secondary roots recorded were highest in sucrose (25.5) medium compared to the common sugar medium (17.67).

In Chengalikodan also, the days taken for root initials to appear and number of primary roots were not significantly different when sucrose was substituted with common sugar. Root length recorded was the highest in common sugar supplemented medium with 9.35 cm which was significantly different from root length in sucrose medium (6.95 cm). The number of secondary roots were highest in common sugar (7.33) while it was 2.83 in sucrose medium. Hence, in Chengalikodan, the root length and number of secondary roots were observed highest when common sugar was used as carbon source in MS medium.

In Poovan, the days taken for root initials to appear, root length, number of primary roots and number of secondary roots were not significantly different in sucrose and common sugar treatments. In Njalipoovan, the days taken and number of primary roots was not significantly different when sucrose was substituted with common sugar. The root length recorded was highest in common sugar medium (27.19) and number of secondary roots were highest in sucrose medium (19.10). Hence, in Njalipoovan the number of secondary roots recorded were the highest when sucrose was used as carbon source in MS medium and root length recorded was the highest when common sugar was used.

In the case of Grand Naine, root length was significantly different in sucrose and common sugar. Common sugar substituted medium showed highest root length of 14.79 cm which was significantly different with root length in sucrose (12.5 cm). Hence, in Grand Naine root length recorded was the highest when common sugar was used as carbon source in MS rooting medium. The other characters were not significantly different in both the treatments.

Table 3: Root characters in different cultivars in MS medium when sucrose was substituted with common sugar

Cultivars	Days taken for root initials to appear			Number of primary roots			Root length (cm)			Number of secondary roots		
	Sucrose	Common sugar	t (0.05)	Sucrose	Common sugar	t (0.05)	Sucrose	Common sugar	t (0.05)	Sucrose	Common sugar	t (0.05)
Nedunendran	4.25	4.75	2.07 NS	6.25	5.66	2.07 NS	8.95	8.93	2.20 NS	3.41	4.66	2.07 NS
Attunendran	4.08	4.00	2.07 NS	6.75	8.41	2.07 **S**	15.04	15.04	2.07 NS	25.5	17.66	2.07 **S**
Chengalikodan	3.5	3.583	2.07 NS	6.83	7.83	2.07 NS	6.95	9.35	2.07 **S**	2.83	7.33	2.20 **S**
Poovan	4.75	4.83	2.07 NS	7.00	5.58	2.07 NS	15.76	18.48	2.07 NS	22.5	23.41	2.07 NS
Njalipoovan	5.7	5.6	2.10 NS	6.6	5.4	2.10 NS	18.69	27.19	2.10 **S**	19.1	11.00	2.10 **S**
Grand Naine	5.00	4.91	2.07 NS	6.91	6.33	2.07 NS	12.5	14.79	2.07 **S**	16.66	21.08	2.07 NS

a) Nedunendran

b) Attunendran

c) Chengalikodan

d) Poovan

e) Njalipoovan

f) Grand Naine

Plate 2: Root characters in different cultivars in MS medium when sucrose substituted with common sugar

4.1.3 Shoot proliferation in different cultivars in MS medium when laboratory grade agar was substituted with marine agar

In the MS shoot multiplication medium, laboratory grade agar (7.5 gL^{-1}) was substituted with marine agar (7.5 gL^{-1}) and shoot proliferation in the cultivar Chengalikodan was observed. The shoot proliferation was observed after three weeks of inoculation. No significant difference was observed in shoot proliferation when laboratory grade agar was substituted with marine agar in the cultivar Chengalikodan (Table 4). So marine agar was found equally effective as that of laboratory grade agar and it did not influence shoot proliferation in Chengalikodan.

Table 4: Shoot proliferation in cultivar Chengalikodan in MS medium when laboratory grade agar was substituted with marine agar

Cultivar	Shoot proliferation in one culture cycle of 21 days in 7th subculture cycle		t (0.05)
	Laboratory grade agar	Marine agar	
Chengalikodan	20.08	17.16	2.07(NS)

4.1.4 Gelling property of MS medium by substitution of agar with isabgol/ sago

Gelling property of MS medium was tested in the following six treatments. T1- Sago (100%), T2- Isabgol (100%), T3- Sago (50%) + Isabgol (50%), T4- Agar (50%) + Isabgol (50%), T5-.Agar (50%) + Sago (50%), T6- Agar (7.5 gL^{-1}- 100%) and the results are presented in Table 5.

Table 5: Standardization of gelling property of MS medium by substitution of agar with isabgol/ sago

Treatments	Gelling property
T1- Sago (100%)	Not solidified
T2- Isabgol (100%)	Not solidified
T3- Sago (50%) + Isabgol (50%)	Not solidified
T4- Agar (50%) + Isabgol (50%)	Solidified
T5- Agar (50%) + Sago (50%)	Solidified
T6- Agar (100%).	Solidified

Out of the six treatments, T4, T5 and T6 exhibited solidification and hence they were used for further studies. T6 was used as control. Substitution of agar to the extent of 100 per cent with other gelling agents like sago and isabgol was not found effective. When 50 per cent substitution of agar with other solidifying agents was done, there was good solidification of the medium.

4.1.5 Shoot proliferation in different cultivars in MS medium when agar was substituted with isabgol/ sago

Shoot proliferation in different cultivars were studied in the MS shoot multiplication medium with selected two combinations of gelling agents [agar (50%)+ isabgol (50%) and agar (50%)+ sago (50%)]. Agar (100%) is kept as control. The experiment was conducted separately for each cultivar. The results on shoot proliferation showed that there was no significant variation in different cultivars when half of agar was substituted with isabgol/ sago. Hence, agar the costly component in tissue culture medium can be successfully substituted partially with either isabgol or sago.

Shoot proliferation in the selected medium showed no significant variation in all the cultivars except for Chengalikodan in which agar (50%)+ sago (50%) showed highest proliferation (4.22) which was significantly different from the other two treatments (Table 6).

a) T1- Sago (100%)

b) T2- Isabgol (100%)

c) T3- Sago (50%) + Isabgol (50%)

d) T4- Agar(50%)+ Isabgol (50%)

e) T5- Agar (50%)+ Sago (50%)

f) T6- Agar (100%)

Plate 3: Gelling property of MS medium by substitution of agar with isabgol and sago

When compared to Chengalikodan, other Nendran types exhibited higher shoot proliferation. In the cultivars studied, lowest shoot proliferation was observed in Njalipoovan and highest in Attunendran. When the overall effect of gelling agents was observed, agar (50%)+sago (50%) was found to give higher proliferation irrespective of the cultivars studied.

Table 6: Shoot proliferation in different cultivars in MS medium when agar was substituted with isabgol/ sago

Cultivars	Shoot proliferation in one culture cycle of 21 days in 7th subculture cycle			CD (0.05)
	Agar (100%)	Agar(50%)+ Isabgol(50%)	Agar(50%)+ Sago(50%)	
Nedunendran	4.06	4.21	4.22	NS
Attunendran	4.67	6.12	6.76	NS
Chengalikodan	2.37	3.16	4.22	1.094
Poovan	2.47	1.33	1.58	NS
Njalipoovan	0.08	0.25	0.25	NS
Grand Naine	3.81	2.78	3.37	NS

4.1.6 Substitution of agar with isabgol/ sago in MS rooting medium

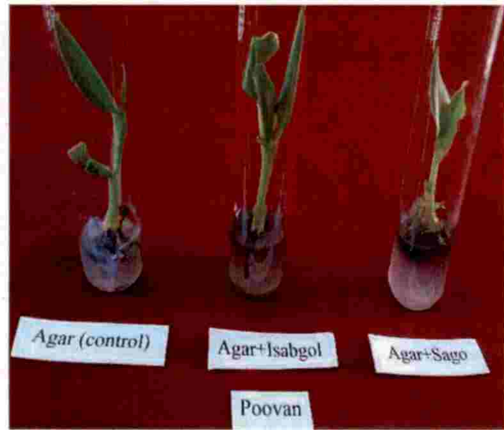
4.1.6.1 Rooting in different cultivars when agar was substituted with isabgol/ sago in MS rooting medium

Partial substitution of agar (50%) with isabgol/ sago was not influencing rooting and the combinations were found equally good to agar (100%). When agar was substituted half with isabgol/ sago, the percentage of rooting was found 100 per cent in all the six cultivars studied. The percentage of rooting in different cultivars when agar was substituted with isabgol/ sago in MS rooting medium is shown in Table 7.

a) Nedunendran

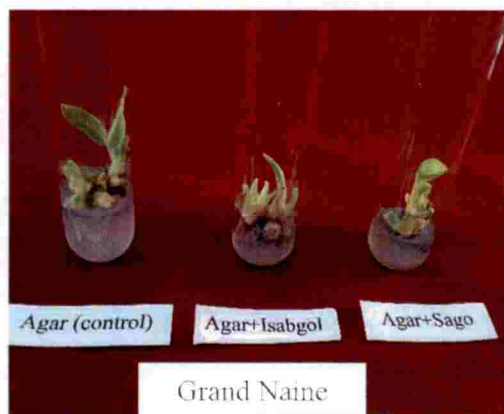
b) Attunendran

c) Chengalikodan



d) Poovan

e) Njalipoovan



f) Grand Naine

Plate 4: Shoot proliferation in different cultivars in MS medium when agar was substituted with isabgol/ sago

Table 7: Rooting (%) in different cultivars when agar was substituted with isabgol/ sago in MS rooting medium

Cultivars	Rooting (%)		
	Agar (100%)	Agar(50%)+ isabgol(50%)	Agar(50%)+ sago(50%)
Nedunendran	100	100	100
Attunendran	100	100	100
Chengalikodan	100	100	100
Poovan	100	100	100
Njalipoovan	100	100	100
Grand Naine	100	100	100

4.1.6.2 Root characters in different cultivars when agar was substituted with isabgol/ sago in MS rooting medium

In the rooting medium also, agar can be substituted partially with isabgol/ sago. Substitution of 50 per cent agar with isabgol/ sago showed no significant difference in days taken for root initials to appear, number of primary roots, number of secondary roots and root length in majority of the cultivars studied (Table 8).

In the varieties Attunendran, Njalipoovan and Grand Naine the days taken for root initials to appear, the number of primary roots produced, root length, number of secondary roots did not show any significant difference when agar was substituted with agar (50%)+ isabgol (50%) and agar (50%)+ sago (50%) in the rooting medium.

In Nedunendran, the days taken for root initials to appear and root length were not significantly different in all the three treatments. Number of primary roots and number of secondary roots showed significant differences among the

treatments. Highest number of primary roots were observed in agar (8.58) and agar (50%)+ sago (50%) (8.25) and both of them were on par. Lowest number of primary roots was observed in agar (50%)+ isabgol (50%) (5.41). In the case of number of secondary roots, highest number was observed in agar (19.41) and agar (50%)+ sago (50%) (18.58) which were on par with each other. Lowest number of secondary roots were observed in agar (50%)+ isabgol (50%) (10.08).

In Chengalikodan, the days taken for root initials to appear did not show any significant difference among the three treatments but the number of primary roots, root length and number of secondary roots showed difference. Highest number of primary roots was observed in agar (8.25) and lowest in agar (50%)+ isabgol (50%) (6.41) and agar (50%)+ sago (50%) (6.16) and both of them were on par. Root length recorded was highest in agar (50%)+ sago (50%) (15.87) and lowest in agar (50%)+ isabgol (50%) (11.30) and agar (12.65) and both of them were on par. Highest number of secondary roots was observed in agar (50%)+ sago (50%) (22.08) and lowest was observed in agar (50%)+ isabgol (50%) (13.66). Number of secondary roots observed in agar (16) was on par with agar (50%)+ isabgol (50%) (13.66) and agar (50%)+ sago (50%) was superior (22.08).

In Poovan, the days taken for root initials to appear and number of secondary roots showed no significant difference among the three treatments whereas the number of primary roots and root length showed significant differences among the treatments. Highest number of primary roots (7.25) was shown by agar (100%) which was significantly superior to agar (50%)+ sago (50%) (4.66) and agar (50%)+ isabgol (50%) (3.83). The highest root length was observed in agar (50%)+ isabgol (50%) (20.43), which was significantly superior to agar (15.77) and agar (50%)+ sago (50%) (15.56). However, agar (100%) and agar (50%)+ sago (50%) were on par with respect to agar (50%)+ isabgol (50%) (20.43).

Table 8: Root characters in different cultivars when agar was substituted with isabgol/ sago

Cultivars	Days taken for root initials to appear				No. of primary roots				Root length (cm)				No. of secondary roots			
	Agar	Agar+ isabgol	Agar+ sago	CD (0.05)	Agar	Agar+ isabgol	Agar+ sago	CD (0.05)	Agar	Agar+ isabgol	Agar+ sago	CD (0.05)	Agar	Agar+ isabgol	Agar+ sago	CD (0.05)
Nedunendran	4.25	4.41	4.16	NS	8.58	5.41	8.25	1.50	11.11	10.95	11.55	NS	19.41	10.08	18.58	5.50
Attunendran	4.08	4.00	4.00	NS	5.08	5.58	6.58	NS	11.91	11.79	13.65	NS	·11.5	16.41	18.33	NS
Chengalikodan	3.75	4.16	3.91	NS	8.25	6.41	6.16	1.64	12.65	11.30	15.87	1.64	16	13.66	22.08	6.25
Poovan	5.08	5.16	5.00	NS	7.25	3.83	4.66	2.04	15.76	20.43	15.55	3.43	22.5	14.08	11.75	NS
Njalipoovan	5.8	5.6	5.7	NS	6.6	6.00	6.8	NS	18.59	18.25	15.6	NS	19.1	13.1	13.4	NS
Grand Naine	5.16	5.08	5.16	NS	6.91	6.75	7.33	NS	14.15	14.24	14.69	NS	17.75	15.41	16.91	NS

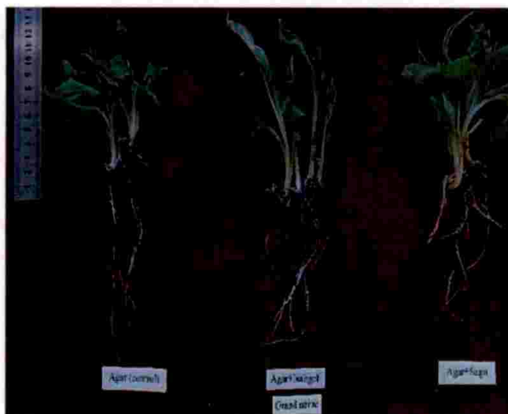
a) Nedunendran

b) Attunendran

c) Chengalikodan

d) Poovan

e) Njalipoovan



f) Grand Naine

Plate 5: Root characters in different cultivars in MS medium when agar was substituted with isabgol/ sago

4.1.7 Hardening and acclimatization of plantlets

The rooted plantlets were planted out in small earthen pots filled with potting mixture containing coco peat, soilrite and vermiculite in 3:1:1 ratio and were kept in net house for primary hardening. After two weeks, the plants were transferred to polythene bags containing sand, soil and cow dung in 1:1:1 ratio and were kept for secondary hardening. The plants were observed for one month and survival rate was recorded. The survival of plantlets when sucrose was substituted with common sugar is shown in Table 9.

The survival of the plantlets were similar in regenerants from MS medium with sucrose or common sugar in all the six cultivars studied.

Table 9: Survival of plantlets when sucrose was substituted with common sugar

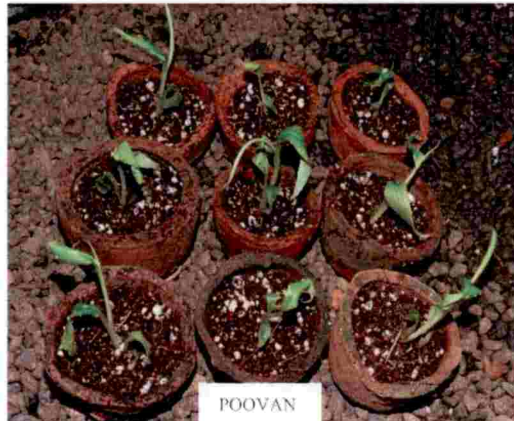
Cultivars	Survival of plantlets (%)	
	Sucrose	Common sugar
Nedunendran	84.21	80.95
Attunendran	100	100
Chengalikodan	85	83.33
Poovan	100	100
Njalipoovan	100	100
Grand Naine	100	100

When agar was partially substituted with isabgol/ sago in the rooting medium, agar (50%)+ sago (50%) performed equally good as that of agar in all the six cultivars. In the case of agar (50%)+ isabgol (50%), it showed less survival when compared to agar (50%)+ sago (50%) and agar in Attunendran and Njalipoovan. The survival of plantlets when agar was substituted with isabgol/ sago is shown in Table 10.

a) Nedunendran

b) Attunendran

c) Chengalikodan



d) Poovan

e) Njalipoovan

f) Grand Naine

Plate 6: Hardening and acclimatization of plantlets of different cultivars

Table 10: Survival of plantlets when agar was substituted with isabgol/ sago

Cultivars	Survival of plantlets (%)		
	Agar (100%)	Agar(50%)+ isabgol(50%)	Agar(50%)+ sago(50%)
Nedunendran	90.9	100	100
Attunendran	100	88.23	100
Chengalikodan	95.45	100	100
Poovan	100	100	100
Njalipoovan	100	88.88	100
Grand Naine	100	100	100

4.1.8 Analysis of cost of MS medium with low cost alternatives

The cost reduction for one litre MS medium using low cost additives such as common sugar, marine agar, isabgol and sago and laboratory grade sucrose and agar are presented in Table 11.

When laboratory grade sucrose was substituted with common sugar in MS medium, the cost of additives for one litre MS medium was Rs. 15.5 (sucrose) and Rs. 1.0 (common sugar) respectively and the per cent cost reduction for one litre MS medium was 96.6 per cent. When laboratory grade agar was substituted with marine agar in MS medium, the cost of additives for one litre MS medium was Rs. 62.4 (agar) and Rs. 17.5 (marine agar) respectively and the per cent cost reduction for one litre MS medium is 72 per cent. The cost of isabgol for one litre MS medium was Rs. 3.75 and cost of sago for one litre MS medium was Rs. 0.30.

When marine agar (50%)+ isabgol (50%) was used as low cost additives, the cost of additives for one litre MS medium was Rs. 10.6 and the cost reduction for one litre MS medium was 39.4 per cent when compared to marine agar (100%). When marine agar (50%)+ sago (50%) was used as low cost additives, the cost of additives for one litre MS medium was Rs. 8.9 and the cost reduction

for one litre MS medium was 48.3 per cent when compared to marine agar (100%).

When the standard additives like laboratory grade sucrose and laboratory grade agar were used, the cost of additives for one litre MS medium was Rs. 77.9. When low cost carbon source and gelling materials [common sugar+ marine agar (50%)+ sago (50%)] standardized in the present investigations were used in different banana cultivars, there was a cost reduction of 87 per cent as compared to standard laboratory grade additives.

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Table 11: Analysis of cost of MS medium with low cost alternatives

SI no.	Components	Cost/kg (Rs)	Required qty/ L (g)	Cost of additives for 1L MS medium (Rs)	% cost reduction
1	Sucrose (laboratory grade)	516	30	15.5	96.6
2	Common sugar	34	30	1.0	
3	Agar (laboratory grade)	8316	7.5	62.4	72
4	Marine Agar	2330	7.5	17.5	
5	Isabgol	500	7.5	3.75	-
6	Sago	40	7.5	0.30	-
7	Marine Agar (50%)+ Isabgol (50%)	-	7.5	8.75+1.875=10.6	39.4% with marine agar
8	Marine Agar (50%) + Sago (50%)	-	7.5	8.75+0.15=8.9	48.3% with marine agar
9	Agar Himedia+ Sucrose Himedia	-	-	62.4+15.5=77.9	87.29
10	Marine Agar (50%)+Sago(50%)+ Common sugar	-	-	8.9+1.0=9.9	

4.2 Clonal fidelity analysis using specific ISSR marker

4.2.1 Genomic DNA isolation

4.2.1.1 Source of DNA

For the isolation of genomic DNA, the plantlets planted out in each subculture passage were maintained at CPBMB net house. DNA was isolated from these plants from 7th subculture to 11th subculture passage. In the present study DNA was isolated from randomly selected 10 plants per stage per cultivar. The DNA of mother plants of all cultivars were received from CPBMB.

4.2.1.2 Isolation and purification of DNA

The genomic DNA which was isolated through CTAB method reported by Roger and Bendich (1994) was not pure and had slight RNA contamination. RNase treatment was carried out after DNA isolation to get high quality DNA.

4.2.1.3 Quantifiation of DNA

The quality and quantity of isolated DNA was analysed using both electrophoresis and NanoDrop spectrophotometer. Intact clear bands indicated good quality non- degraded DNA. Spectrophotometric analysis gave ratio of UV absorbance (A_{260}/A_{280}) between 1.8 and 2.0. The DNA after appropriate dilution was used as template for ISSR analysis.

4.2.2 ISSR analysis

Clonal fidelity was analysed using specific ISSR primer optimized at CPBMB as reported by Rajitha *et al.* (2015). Genomic DNA isolated from mother plants and regenerants were subjected to ISSR analyses.

The gel pictures were scored for total number of amplicons, number of monomorphic and polymorphic bands and per cent polymorphism was worked out and it is presented in Table 12 (a) and (b). The amplification pattern obtained for

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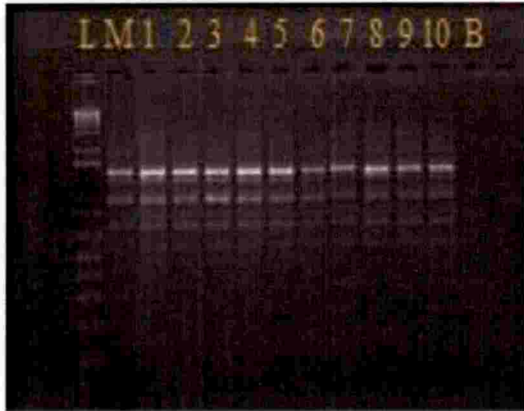
plantlets of different subculture passages of different cultivars are presented in Plates 7-12.

Amplification with the UBC 857 primer generated ten clear amplicons. The bands visualized were in the size range 300-2000 bp.

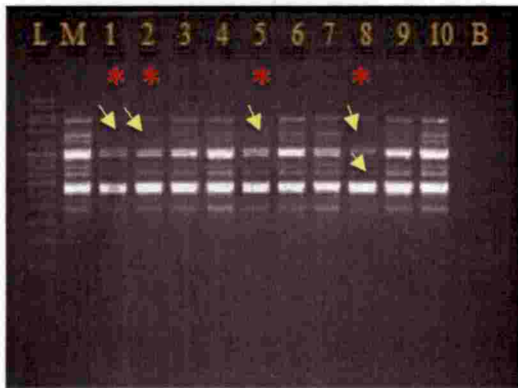
DNA amplification pattern observed in plantlets of different subculture passages of different cultivars is presented in Table 12 (a) and (b). Polymorphic bands were observed as subculture progressed in cultivars like Nedunendran, Attunendran and Grand Naine. In Chengalikodan, up to 10th subculture passage no polymorphic bands were observed and in subculture 11, two bands were found as polymorphic.

In highly multiplying cultivars like Attunendran, Nedunendran and Grand Naine, subculturing above 8th stage was found to induce variation. In low proliferating cultivars like Chengalikodan, Poovan and Njalipoovan, subculturing beyond 8th stage up to S10 was not found to show any variation. Hence, it can be concluded that 1 or 2 subculture cycles can be done extra for cultivars with lower shoot proliferation like Chengalikodan, Poovan and Njalipoovan while the subculture cycles should be restricted to eight in cultivars like Grand Naine, Nedunendran and Attunendran.

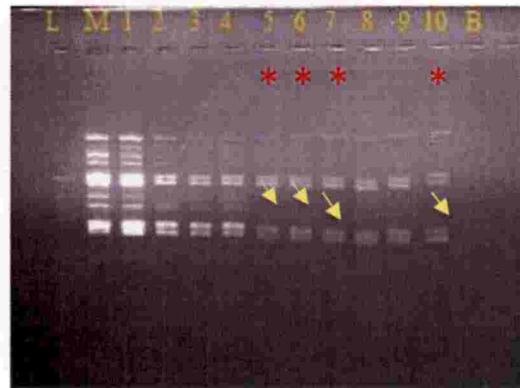


a) S-7

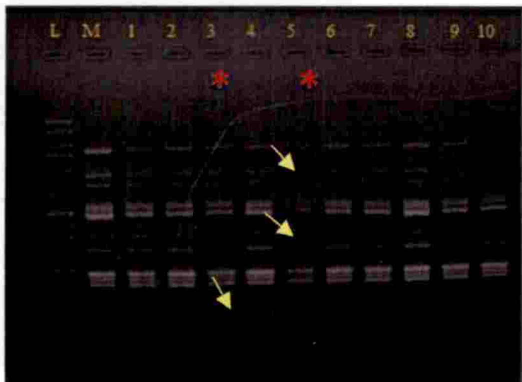
b) S-8



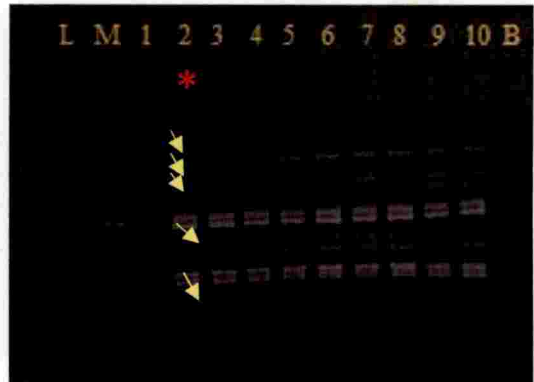
c) S-9



d) S-10



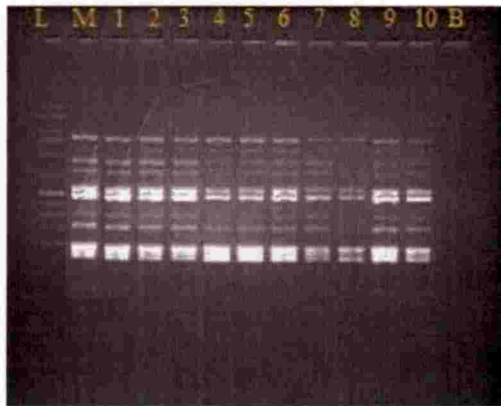
e) S-11



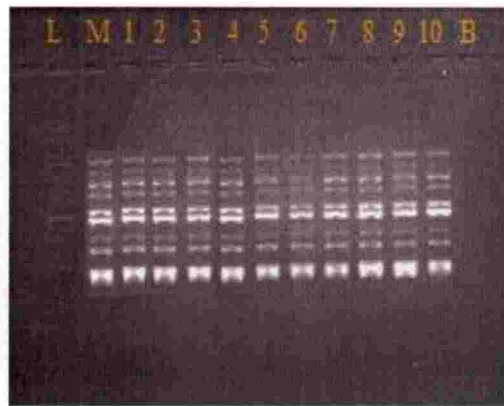
f) S-12

L: ladder (1kb), M: mother plant, 1-10: regenerants, B: Blank

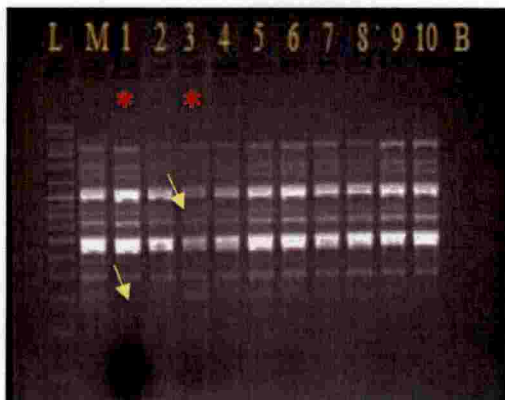
Plate 7: DNA amplification pattern in regenerants from different subculture passages of cv. Nedunendran



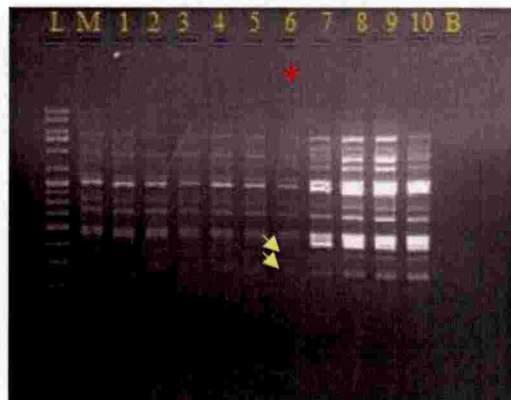
a) S-7



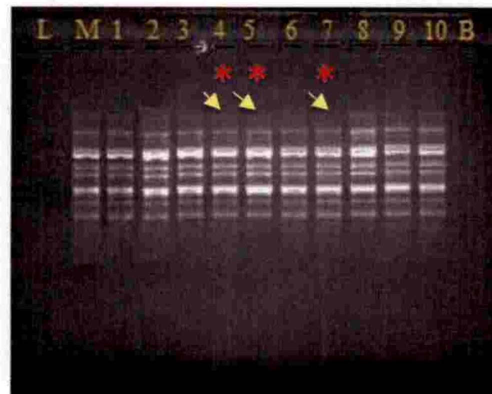
b) S-8



c) S-9



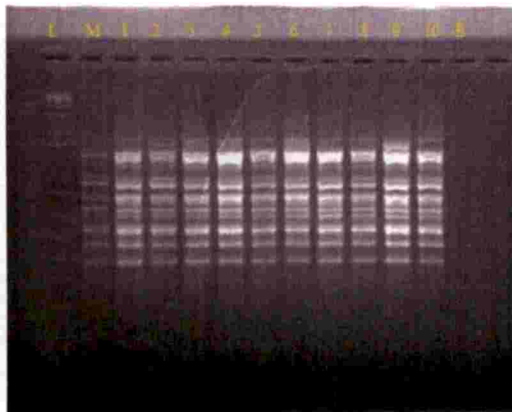
d) S-10



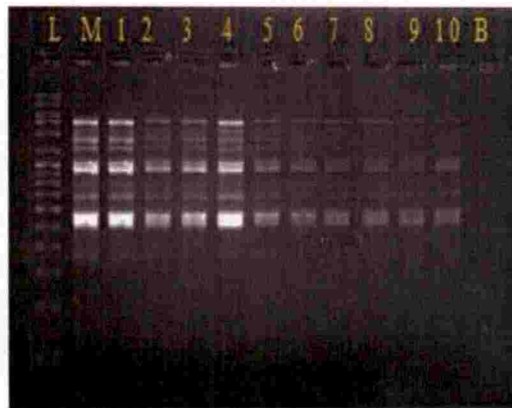
e) S-11

L: ladder (1kb), M: mother plant, 1-10: regenerants, B: Blank

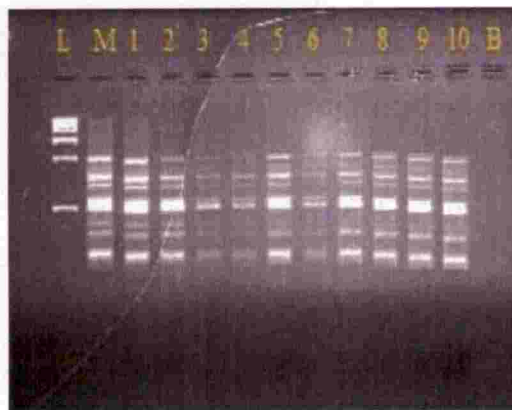
Plate 8: DNA amplification pattern in regenerants from different subculture passages of cv. Attunendran



a) S-7

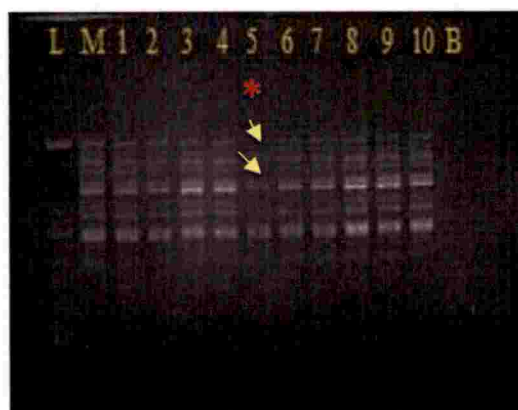


b) S-8



c) S-9

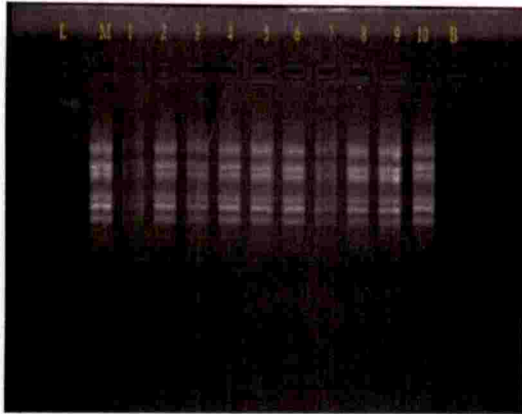
d) S-10



e) S-11

L: ladder (1kb), M: mother plant, 1-10: regenerants, B: Blank

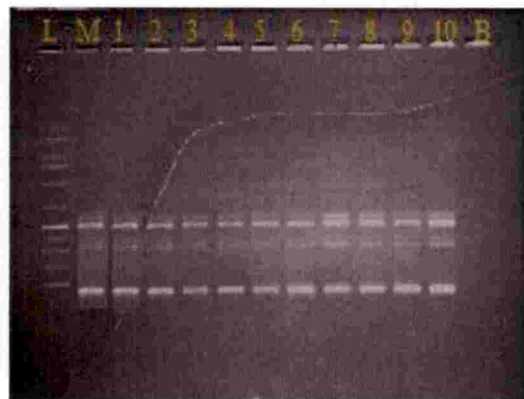
Plate 9: DNA amplification pattern in regenerants from different subculture passages of cv. Chengalikodan



a) S-7

b) S-8

c) S-9

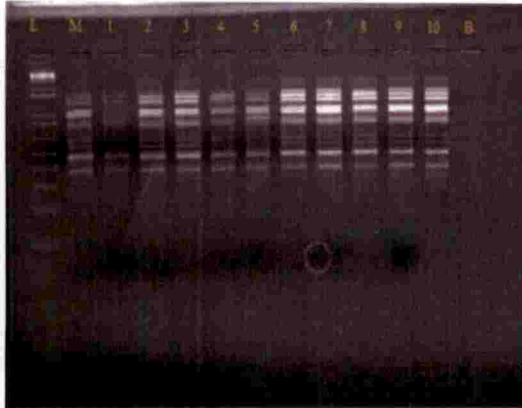


d) S-10

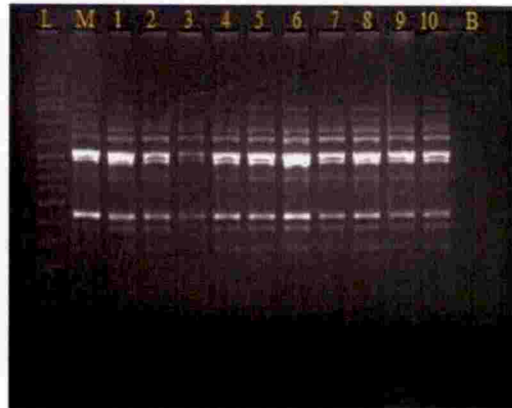
e) S-11

L: ladder (1kb), M: mother plant, 1-10: regenerants, B: Blank

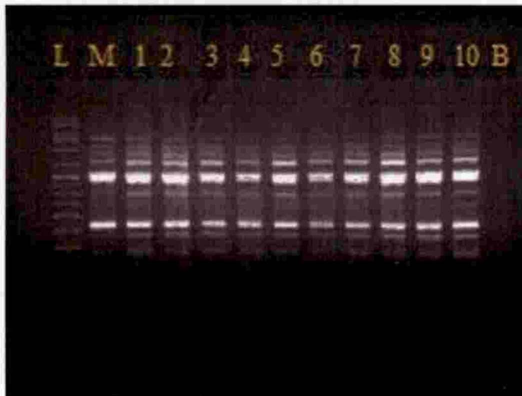
Plate 10: DNA amplification pattern in regenerants from different subculture passages of cv. Poovan



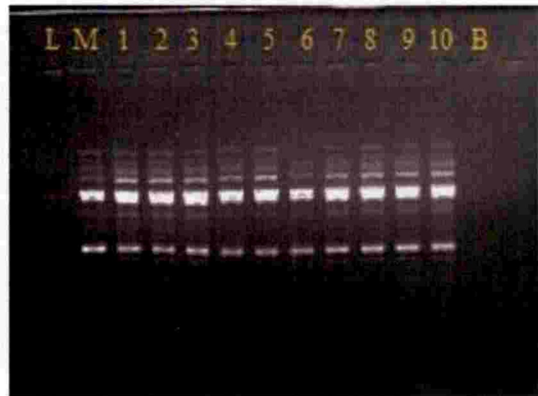
a) S-7



b) S-8



c) S-9

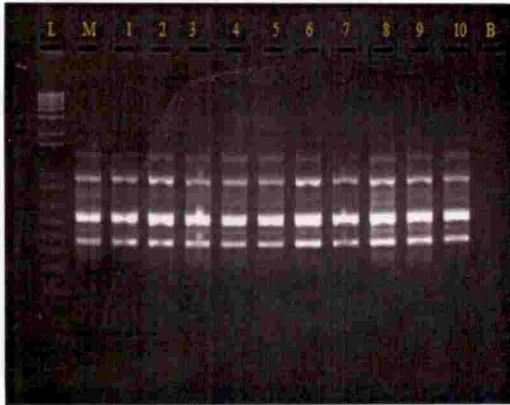


d) S-10

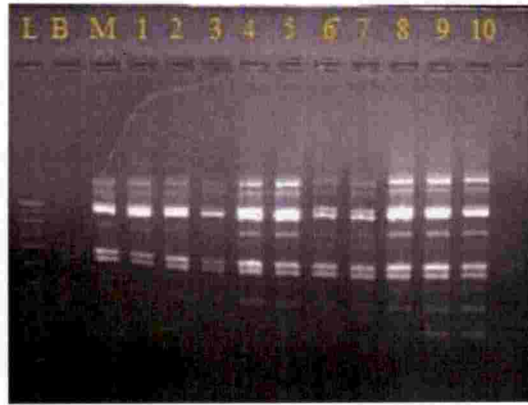
e) S-11

L: ladder (1kb), M: mother plant, 1-10: regenerants, B: Blank

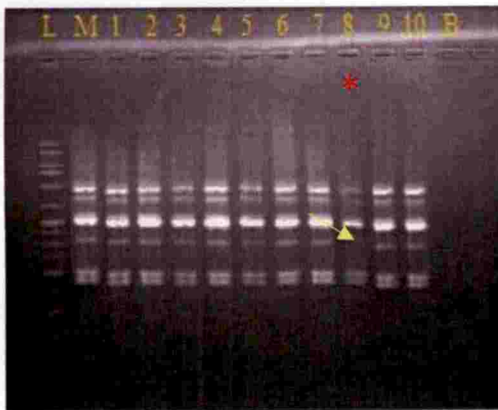
Plate 11: DNA amplification pattern in regenerants from different subculture passages of cv. Njalipoovan



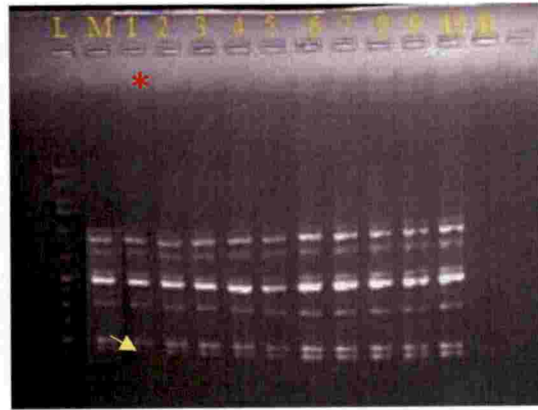
a) S-7



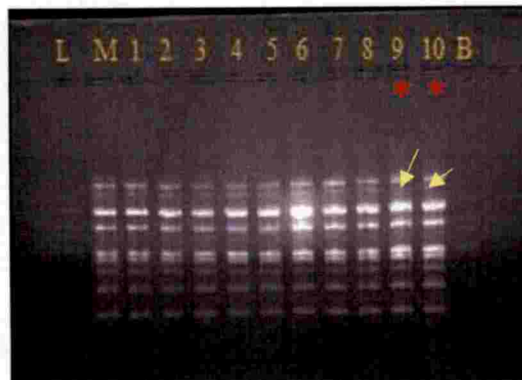
b) S-8



c) S-9



d) S-10



e) S-11

L: ladder (1kb), M: mother plant, 1-10: regenerants, B: Blank

Plate 12: DNA amplification pattern in regenerants from different subculture passages of cv. Grand Naine

Table: 12 (a): DNA amplification in regenerants of different subculture passages in different banana cultivars using ISSR primer

Sl No.	Variety and stage		Total no. of amplicons in mother plant	Mean no. of monomorphic amplicons in 10 regenerants	Mean no. of polymorphic amplicons in 10 regenerants	Polymorphism (%)
1	Nedunendran	S-7	10	10	0	0
		S-8	10	10	0	0
		S-9	10	5	0.5	5
		S-10	10	6	0.4	4
		S-11	12	9	0.3	2.5
		S-12	12	7	0.5	4.16
2	Attunendran	S-7	9	9	0	0
		S-8	9	9	0	0
		S-9	9	7	0.2	2.22
		S-10	11	9	0.2	1.81
		S-11	10	7	0.3	3
3	Chengalikodan	S-7	12	12	0	0
		S-8	9	9	0	0
		S-9	9	9	0	0
		S-10	9	9	0	0
		S-11	10	8	0.2	2

Table: 12 (b): DNA amplification in regenerants of different subculture passages in different banana cultivars using ISSR primer

Sl No.	Variety and stage		Total no. of amplicons in mother plant	Mean no. of monomorphic amplicons in 10 regenerants	Mean no. of polymorphic amplicons in 10 regenerants	Polymorphism (%)
4	Poovan	S-7	7	7	0	0
		S-8	8	8	0	0
		S-9	11	11	0	0
		S-10	6	6	0	0
		S-11	11	11	0	0
5	Njalipoovan	S-7	11	11	0	0
		S-8	11	11	0	0
		S-9	11	11	0	0
		S-10	9	9	0	0
		S-11	10	10	0	0
6	Grand Naine	S-7	8	8	0	0
		S-8	8	8	0	0
		S-9	7	6	0.1	1.42
		S-10	7	6	0.1	1.42
		S-11	9	7	0.2	2.22

Discussion

5. DISCUSSION

The present investigations on "Low cost alternatives in commercial micropropagation of banana" were carried out in order to reduce cost of production in commercial micropropagation of banana. The results of the experiments are discussed in this chapter.

Studies were conducted in six cultivars of banana viz. Attunendran (AAB), Nedunendran (AAB), Chengalikodan (AAB), Poovan (AAB), Njalipoovan (AB) and Grand Naine (AAA) which are being commercially produced at CPBMB in the micropropagation unit.

Established cultures of six different cultivars in the 5th subculture cycle received from the commercial micropropagation unit of CPBMB were utilized for the study. The clonal fidelity analysis standardized using specific ISSR marker at CPBMB was used to analyse the clonal fidelity of regenerants from S7 to S11 stage.

4.1 Cost reduction in plant tissue culture medium

4.1.1 Substitution of sucrose with common sugar

The most commonly used carbon source in micropropagation is sucrose and it adds considerably to the media cost. Use of common sugar reduces the cost of the medium.

Common sugar which is locally available has been successfully used for micropropagation of banana by several scientists like Ganapathi *et al.* (1995) and Kodym and Zapata-Arias (2001).

In the present study, in MS shoot multiplication medium, sucrose ($30gL^{-1}$) was substituted with common sugar ($30gL^{-1}$) and shoot proliferation in different cultivars were observed after 21 days. There was no significant difference in shoot

proliferation when sucrose ($30gL^{-1}$) was substituted with common sugar ($30gL^{-1}$) in all the cultivars studied. This might be because of the presence of 96 to 97 per cent sucrose in table sugar in comparison to 99.98 per cent in laboratory grade sucrose (Tyagi *et al.*, 2007). There was a cost reduction of 96.6 per cent when laboratory-grade sucrose was replaced with commercially available common sugar in medium preparation. Table sugar was used in the tissue culture medium in many research labs (Kaur *et al.*, 2005).

Demo *et al.* (2008) observed that the mean number of nodes per plantlet in potato was significantly higher in brown sugar when compared to refined sugar for cultivars Kenya Sifa and Asante and variation in growth performance among the cultivars was observed.

Venkatasalam *et al.* (2013) reported that sulphur less sugar significantly enhanced the microplant height (10.4 cm) in potato which was found on par with commercial sugar (10.0 cm) and standard control sucrose (9.5 cm), while galactose noted the minimum plant height (2.1 cm). This report is comparable with the results obtained in the present investigations. Also, Gupta *et al.* (2011), obtained best results on shoot multiplication in ginger in the medium containing common sugar ($30g\ L^{-1}$) which justified the results of the present investigations.

4.1.2 Rooting and root characters of different cultivars in MS rooting medium when sucrose was substituted with common sugar

In the present study, sucrose ($30gL^{-1}$) substituted with common sugar ($30gL^{-1}$) recorded 100 per cent rooting in MS rooting medium in all the six cultivars studied. Common sugar was found equally good to sucrose in inducing roots in the rooting medium.

The root characters showed significant difference among the cultivars. Substitution of sucrose with common sugar had no significant influence in the days taken for root initials to appear. For number of primary roots produced, there was no

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significant difference in all the cultivars except Attunendran. Root length was generally higher in medium with common sugar in the cultivars studied. Medium with common sugar produced more number of secondary roots in cultivar Chengalikodan while medium with sucrose produced more number of secondary roots in cultivars like Attunendran and Njalipoovan.

Laboratory grade sucrose was compared with white and brown sugars in potato by Demo *et al.* (2008). Higher mean number of roots per plantlet was obtained in brown sugar in all the three cultivars. Hundred per cent survival was obtained after 14 days of culturing in the three carbon sources. No significant differences in survival of plantlets were obtained after transplanting for the cultivars Asante and Kenya Sifa but survival was significantly lower for cultivar Tigoni in analytical grade sucrose medium. Hence, they concluded that table sugar not only enhanced multiplication and rooting but also significantly lowered the input costs by 34 to 51 per cent when compared with the analytical grade sucrose.

Agrawal *et al.* (2010) reported that there was no significant effects of market sugar on shoot (1.0–1.3 shoots/ shoot explant) and also root (1.5–2.0 roots/shoot explant) regeneration observed.

Shylaja *et al.* (2015) reported high genotypic difference in different banana cultivars viz. Attunendran, Nedunendran, Chengalikodan and Grand Naine in sprouting of explants, shoot proliferation, rooting and root growth. In the work conducted by Saraswathi *et al.* (2016), Rasthali and Grand Naine showed significant differences in plant height, number of roots and root length in three banana varieties.

4.1.3 Hardening and acclimatization of different cultivars in MS rooting medium when sucrose was substituted with common sugar

In the present study, the survival of the plantlets were similar in regenerants from MS medium with sucrose or common sugar in all the six cultivars studied. According to Demo *et al.* (2008), there was no significant differences observed for

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survival of transplants of potato cultivars obtained from three different carbon sources namely sucrose, refined sugar and brown sugar. According to Kubota *et al.* (2001), *in vitro* plant growth was promoted by supplying sugar to the culture medium which increased photosynthetic ability and thereby survival rates of tissues. Hence, an initial source of carbon and energy should be supplied to the potato plantlets through medium until they can use CO_2 as their chief carbon source for effective metabolism. Kaur *et al.* (2005) reported that survival of micropropagated strawberry plants was 100 per cent in the field. The plants exhibited good adaptability when table sugar, tapioca (*Manihot esculenta*) granules and tap water were used as low cost media additives.

4.1.4 Gelling property of MS medium by substitution of agar with isabgol/ sago

Although starches from numerous sources like barley, corn, potato, rice and wheat are used as alternative gelling agents in plant tissue culture medium, a major drawback of starch being used as a gelling agent was that starch gelled medium was weakly solidified. Therefore, starches were used in combination with other solidifying agents like agar, agarose, gelrite, *etc.* (Zimmerman *et al.* (1995).

Gelling property of MS medium was tested in the following six treatments. T1- Sago (100 %), T2- Isabgol (100 %), T3- Sago (50 %) + Isabgol (50 %), T4- Agar (50 %) + Isabgol (50 %), T5- Agar (50 %) + Sago (50 %), T6- Agar (7.5 gL^{-1}- 100 %).

Out of the six treatments tried, only T4 [Agar (50 %) + Isabgol (50 %)] and T5 [Agar (50 %) + Sago (50 %)] exhibited solidification and hence they were used for further studies. T6 [Agar (100 %)] was used as control. 100 per cent substitution of agar with other gelling agents like sago and isabgol was not found effective. When 50 per cent substitution of agar with other solidifying agents was done, there was good solidification of the medium. Saraswathi *et al.* (2016) reported that for Rasthali,

Grand Naine and Udhayam banana varieties, sago and agar, sago and isabgol, and isabgol and agar combinations were found optimal.

4.1.5 Shoot proliferation in different cultivars in MS medium when agar was substituted with isabgol/sago

Shoot proliferation in different cultivars were studied in the MS shoot multiplication medium with selected two combinations of gelling agents [agar (50 %)+ isabgol (50 %) and agar (50 %)+ sago (50 %)]. Shoot proliferation in the selected medium showed no significant variation in all cultivars except for Chengalikodan in which agar (50 %)+ sago (50 %) showed highest proliferation. This is in agreement with the results obtained by Saraswathi *et al.* (2016). They observed that when compared to the controls in Udhayam (8 shoots/ explant) and Rasthali (7.0 shoots per explant), treatment T5 (sago + isabgol) produced the maximum number of shoots per explant (10.0). In Grand Naine, T1 (sago alone) produced the highest number of shoots per plant (6.0) which was followed by control (T6 agar; 5.0).

In many crops including banana, sago performed better as gelling agents (Babbar and Jain, 1998; Naik and Sarkar, 2001; Bhattacharya *et al.* 1994). The results obtained in the present investigations are also in agreement with the results of Nene and Sheila (1996). They showed that sago exhibited better performance under *in vitro* conditions, apart from the opaqueness of medium which reduced the visibility to check for fungal contaminations. Naik and Sarkar (2001) reported that sago starch can be used as a substitute for agar in potato *in vitro* culture and it substantially reduced the medium cost. Sharifi *et al.* (2010) reported that agar is the best gelling agent for shoot induction in African violet but due to the high price of this compound, the combination of starch, semolina and potato powder or combination of starch and agar can be low cost options.

Isabgol husk is chemically comprised of arabinoxylan (arabinose 17–22.6 %, xylose 50–74.6 %) and galacturonic acid (5–8%) and husk contains complex

polysaccharides which increase its viscosity (Fischer *et al.* 2004; Craeyveld *et al.* 2009). Eberhard *et al.* (1989) reported that the role of arabinose and xylose was found to promote the induction of shoots and flower in tobacco and embryogenesis in microspore cultures of wheat (Letarte *et al.* 2006). Morphological data analysis of field-grown plants suggests no adverse effects of isabgol on growth and development of *in vitro* conservation-derived plants. Gupta *et al.* (2011) obtained best response among different gelling agents in ginger for shoot multiplication in the medium solidified with Isabgol ($30gL^{-1}$) with over five shoots per culture (5.84) followed by medium with agar (5.01). In the present study, there was no significant difference among the treatments tried for all cultivars except Chengalikodan for shoot proliferation whereas Gupta *et al.* (2011) proved that isabgol was better gelling agent.

Bhattacharya *et al.* (1994) used isabgol for micropropagation of Chrysanthemum cv. Birbal Sahni and also isabgol was used for micropropagation of *Syzygium* cuminii by Babbar and Jain (1998) and they reported that the seeds of *Syzygium cuminii* started germinating 3 weeks after inoculation and the growth of shoots of the seedlings was similar when cultured on agar or isabgol media.

Kuria *et al.* (2008) reported that number of nodes in potato plantlets cultured on different gelling agents like cassava starch, cassava starch+ agar or on liquid medium were not significantly different in three generations of subculturing which is comparable to the results obtained in the present investigations. Dalvi *et al.* (2011) used cotton as low cost support matrix in potato micropropagation. The plant height increased significantly with cotton as support matrix as compared to agar *i.e.* 33 per cent increase in plant height. Number of nodes increased significantly with cotton as support matrix as compared to agar i.e. 53 per cent increase in number of internodes and 40 per cent increase in number of leaves was observed.

Venkatasalam *et al.* (2013) reported that when compared to the control, gelrite considerably increased the number of leaves (4.6) and nodes (5.0) in potato and it was

found on par with bacteriological grade agar. Even if the unit cost of gelrite/ phytagel is higher than agar but the quantity of gelrite/ phytagel used for solidifying unit quantity of media is very less (25%) and will lead to save 43 to 52 % cost on solidifying agent. Saraswathi *et al.* (2016) reported that the effect of low-cost gelling agents in Grand Naine was non-significant and it might be due to the high multiplication rate in Grand Naine when compared to the other varieties.

4.1.6 Rooting and root characters of different cultivars in MS rooting medium when agar was substituted with isabgol/ sago

In the present study, partial substitution of agar (50 %) with isabgol/ sago did not influence rooting and the combinations were found equally good to agar (100 %). When agar was substituted half with isabgol/ sago, the percentage of rooting was found 100 per cent in all the six cultivars studied. Similarly, Saraswathi *et al.* (2016) observed that root initiation and penetration was not inhibited by different gelling agents and they obtained a rooting greater than 95 per cent irrespective of variety and gelling agent. Substitution of 50 per cent agar with isabgol/ sago showed no significant difference in days taken for root initials to appear, number of primary roots, number of secondary roots and root length in cultivars like Attunendran, Njalipoovan and Grand Naine. Babbar and Jain (1998) reported that the growth of roots of the seedlings of seeds of *Syzygium cuminii* when cultured on agar or isabgol media were similar which is in agreement with our results.

In the present study, in Nedunendran, Chengalikodan and Poovan, root characters were significantly different for root length, number of primary roots and number of secondary roots which may be due to genotypic differences. Genotypic difference in response among different banana cultivars is observed in banana micropropagation. High genotypic difference in different banana cultivars viz. Attunendran, Nedunendran, Chengalikodan and Grand Naine in rooting and root growth was reported by Shylaja *et al.* (2015).

4.1.7 Hardening and acclimatization of plantlets

In the present study, when agar was partially substituted with isabgol/ sago in the rooting medium, agar (50 %)+ sago (50 %) performed equally good to agar in cultivars like Attunendran, Njalipoovan and Grand Naine. Bhattacharya *et al.* (1994) reported that the performance of the plantlets during hardening stage and survival of the plants in the field were similar even if the plantlets exhibited variation in growth characters when grown on different gelling agents.

Gupta *et al.* (2011) reported that there was 92 per cent survival rate in plants derived from isabgol gelled medium followed by agar (90 per cent) and sago gelled medium (76 per cent) after one month of hardening and also the number of roots were almost double when plantlets were raised on isabgol gelled medium. In the present study, agar (50 %)+ sago (50 %) performed better than agar (50 %) + isabgol (50 %) in per cent survival. Kuria *et al.* (2008) reported that potato plantlets cultured on different gelling agents like cassava starch and agar showed no significant differences in survival.

4.1.8 Increasing subculture cycles and clonal fidelity analysis using specific ISSR marker

Increasing number of subculture cycles

Subculturing of 5th stage cultures received from CPBMB was done using protocol optimised for the different cultivars at CPBMB (Shylaja *et al.*, 2015) to know any variation occurring in latter subcultures. The long period in culture increased the number of somaclonal variants in wheat regenerants as reported by Hartmann *et al.* (1989). It was reported that somaclonal variants appeared from the fifth subculture (1.3 per cent) onwards in micropropagated Nanico (*Musa* spp., AAA) and increased to 3.8 per cent after 11 subcultures by Rodrigues *et al.* (1998).

The genetic stability may be affected by the rapid multiplication of a tissue leading to somaclonal variation (Israeli *et al.*, 1995). Petolino *et al.* (2003) reported that somaclonal variation is mostly apparent and higher in plants regenerated from long term cultures.

Bairu *et al.* (2006) reported that the rate of somaclonal variations are enhanced when the number of subculture and their duration are increased, especially in cell suspension and callus cultures. A high rate of proliferation is achieved in relatively shorter periods during micropropagation and leads to more frequent subculturing and more somaclonal variation.

Genomic DNA isolation

DNA was isolated from young tender leaves of the plantlets planted out in each subculture passage. The protocols reported by Rogers and Bendich, (1994) with 5X CTAB extraction buffer generated good quality DNA. The DNA after electrophoresis exhibited distinct bands without shearing. During DNA extraction the homogenisation, pulverisation and uniformity of grinding of plant tissues were essential.

For the homogenization of the leaf tissue, excess liquid nitrogen was used. Liquid nitrogen aided in preserving the frozen tissue, preventing nucleic acid degradation and effect of secondary metabolites and also it is a better mechanical disruption of tissues (Hernandez and Oyarzum, 2006). Addition of β-mercaptoethanol and poly vinyl pyrrolidone (PVP) along with the extraction buffer helped to overcome the problem of polyphenols. β- Mercaptoethanol disrupted the protein disulphide bond and was capable of initiating protein degradation. To get good quality DNA, poly vinyl pyrrolidone (PVP) was added and it removed polyphenols and inhibited co-precipitation of polysaccharides, previously confirmed by Matasyoh *et al.* (2008).

CTAB, the detergent present in the extraction buffer, helped to release nucleic acids into buffer by disrupting the cell membrane. EDTA present in the extraction buffer helped to protect the released DNA from the action of DNase enzyme. It is a chelating agent, which efficiently blocks Mg^{2+}, the major cofactor of DNase enzyme. The DNA isolated by CTAB method was made free of chlorophyll by using chloroform: isoamyl alcohol that helped in the separation of organic mixture and aqueous phase of the DNA isolation. EDTA was also a major component of TE buffer in which the DNA was dissolved and stored, reported by Sambrook *et al.* (1989).

A DNA sample is of good quality when it appears as a band of high molecular weight with a low amount of RNA (Wettasinghe and Peffley, 1998). Several workers (Wettasinghe and Peffley, 1998; Raval *et al.*, 1998; Gallego and Martinez, 1996) reported the use of RNase for removal of RNA contamination from the isolated DNA. In the present study, RNase treatment was performed and it yielded good quality DNA.

Quality and Quantity analysis of genomic DNA

Good quality DNA isolated from mother plants and regenerants were used for ISSR marker analysis. The quality and quantity of DNA was calculated using the absorbance ratio at 260/280 for the various samples using Nanodrop^{R} spectrophotometer. Those samples with ratio between 1.8- 2.0 were considered to be of high quality DNA. If the value goes beyond 2.0, it indicates RNA contamination and if less than 1.8, it indicates protein contamination.

ISSR analysis

In the present study, clonal fidelity was analysed using specific ISSR primer optimized at CPBMB as reported by Rajitha *et al.* (2015). Genomic DNA isolated from mother plants and regenerants were subjected to ISSR analyses. In the present

study, PCR-based technique, ISSR was adopted to characterise the *in vitro* regenerants.

Inter Simple Sequence Repeats (ISSRs) are DNA fragments, 100-3000 bp long located between adjacent, oppositely oriented microsatellite regions. These are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). ISSR primers are efficient in assessing the genetic integrity among clonally propagated plants as reported by many workers in different species (Zietekiewicz *et al.,* 1994; Bhatia *et al.,* 2011; Vanijajiva, 2012). The use of longer primers allowing for higher annealing temperatures than those of RAPDs accounts for high reproducibility with ISSR technique (Pradeep *et al.,* 2002).

ISSR markers are simple as well as faster, require only slight amount of DNA sample and does not involve any radioactivity tests. Therefore ISSR markers have been successfully used to distinguish the genetic similarities or dissimilarities in micropropagated plantlets in various plants (Carvalho *et al.,* 2004).

A major limiting factor in the commercial utilization of *in vitro* propagation is the presence of variability among the mother plant and the clonal regenerants (Rahman and Rajora, 2001). Henceforth regenerants produced through any *in vitro* regeneration protocol has to be assured that they are true to type. Molecular markers can be utilized for this and it has been reported that ISSR markers are really a reliable marker system that gives reproducible and accurate variability information. It has been used to access the clonal fidelity of clonal regenerants of many crops (Chavan *et al.*, 2014; Devi *et al.*, 2014; Thorat *et al.*, 2017; Fayas *et al.*, 2018; Rohela *et al.*, 2018).

In the present study, the six different cultivars of banana from S7 to S11 were subjected to clonal fidelity analyses using the specific ISSR primer optimized at

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CPBMB. Amplification with the UBC 857 primer generated ten clear amplicons. The bands visualized were in the size range 300-2000 bp.

DNA amplification pattern observed in plantlets of different subculture passages of different cultivars revealed polymorphic bands as subculture progressed above S8 in cultivars like Nedunendran, Attunendran and Grand Naine. In Chengalikodan, up to 10th subculture passage, no polymorphic bands were observed and in subculture 11, two bands were found polymorphic.

In Grand Naine also, variability was observed from S9 stage onwards. In cultivars like Poovan and Njalipoovan which exhibited low multiplication rate, no variability was observed in regenerants up to S11.

In Nedunendran the variability ranged from 2.5 to 5 per cent from S9 stage onwards. In Attunendran, the polymorphism observed was 1.81 to 3 per cent. In Grand Naine, the polymorphism observed was 1.42 to 2.22 per cent from S9 stage and in Chengalikodan polymorphism observed was only 2 per cent in S11 stage.

Larkin and Scowcroft (1981) reported that gross and cryptic chromosomal changes, or extensive changes in chromosome number, occur early during induction in an *in vitro* culture. Likewise, the rapid multiplication of a tissue or long-term cultures may affect genetic stability and thereby leading to somaclonal variation (Reuveni *et al.*, 1993).

Genetic variation observed was low in plants regenerated from adventitious shoots from axillary buds or from other well developed meristematic tissue (Joshi and Dhawan, 2007). Hence, it becomes vital to regularly check the genetic purity of the micropropagated plants in order to produce clonally uniform progeny while using diverse techniques of micropropagation.

Joshi and Dhawan (2007) have employed ISSR marker assay to validate the genetic fidelity of *Swertia chirayita* plantlets multiplied *in vitro* by axillary

multiplication up to forty two passages. Regenerated plants derived from organ cultures, calli, protoplasts and somatic embryos sometimes can show phenotypic and genotypic variation due to the mutations occurring in *in vitro* culture conditions (Orbovic *et al.*, 2008). Clonal fidelity of micropropagated and sustained cultured clones of *Allium ampeloprasum* L. using ISSR primers was reported by Gantait *et al.* (2010).

In a report by Borse *et al.* (2011) clonal fidelity of banana (*Musa acuminata* cv. Grand Naine) regenerants from six different *in vitro* subculture generations and in the explant suckers were evaluated by using ISSR and REMAP molecular markers. Very low variation was observed up to the eighth subculture generations with polymorphic bands both ISSR (0.96per cent) and REMAP (0.95per cent) markers system. Similar results were observed in DNA amplification pattern observed in plantlets in the present investigations, in cultivars like Nedunendran, Attunendran and Grand Naine wherein polymorphic bands were observed as subculture progressed above S8 stage.

Parida *et al.* (2011) assessed genetic fidelity of *in vitro* regenerated *Alpinia galanga* L. subjected to RAPD and ISSR markers to assess genetic stability. Nadha *et al.* (2011) confirmed the genetic uniformity using RAPD and ISSR markers in *in vitro* raised clones of *G. angustifolia* Kunth.

In a work conducted by Choudhary (2011), a total of 111 ISSR amplicons were generated of which 58 were found polymorphic, thus giving an average of seven polymorphic bands per primer. The ISSR assay confirmed the existence of considerable variation at the DNA level in the plantain ecotypes studied.

Amar (2012) reported that polymorphism was observed in plants derived after 8th subculture which is in agreement with our results. In male derived plants, maximum number of polymorphic amplicons were observed in 14th and 16th subcultures. The underlying mechanism of tissue culture induced variation has been

hypothesized as changes in DNA methylation (addition of –CH3 to cytosine) due to high frequency of quantitative phenotypic variation, activation of transposable elements, heterochromatin induced chromosome breakage events, etc.

Khateeb *et al.* (2013) assessed genetic stability of micropropagated plants of *Moringa peregrina* using ISSR marker. In all reported studies, they found the monomorphic amplification in different explants regenerants and ensured true-to-type nature of micropropagated plants.

Thorat *et al.* (2017) reported that ISSR markers, Co 86032 did not show any polymorphism and in the ISSR marker Q117, 92.18 per cent true-to-type plantlets were found. These results confirmed that somaclonal variation occured during the process of indirect organogenesis and RAPD and ISSR marker based molecular analysis is a suitable method for an early detection of variation in sugarcane and it justified the results obtained. The clonal fidelity of the *in vitro* plantlets of *Moringa peregrina* developed through direct organogenesis was assessed using ISSR marker by Fayas *et al.* (2018). The similarity indices between the parental plants and their progenies were above 98.2 per cent and it indicated that the progenies were highly similar to their mother plant.

National Certification System for Tissue Culture Plants (NCS-TCP) is a system of Department of Biotechnology (DBT) to ensure production and distribution of quality tissue culture planting materials. NCS-TCP is a unique quality management system, first of its kind in the world, which ensures the distribution of true-to-type disease free planting materials. NCS-TCP guidelines were established for different crops in which micropropagation is commercially practised. Selection of good quality mother plants, use of quality inputs, correct subculture passages, virus indexing and clonal fidelity analyses are mandatory in the system.

The salient outcome from the present investigations are standardization of low cost alternatives in plant tissue culture media and higher number of subculture cycles

that can be undertaken in low multiplying banana cultivars to increase multiplication rate and to reduce per plant cost in commercial micropropagation.

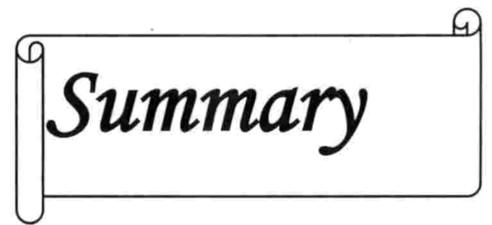
Common sugar was found equally good to laboratory grade sucrose and the media with common sugar was not influencing shoot proliferation and rooting in different cultivars. Marine agar was found equally good to laboratory grade agar and it did not influence shoot proliferation. Fifty per cent of marine agar can be substituted with either isabgol or sago and the low cost gelling agents did not influence shoot proliferation, rooting and survival in different banana cultivars. There was 87 per cent reduction in media cost when low cost additives were used instead of laboratory grade additives.

In a commercial micropropagation cycle of cultivar Grand Naine involving eight subculture passages and one rooting culture cycle, approximately 18,000 plants can be produced from one shoot tip explant which requires 203 L MS media. MS media with laboratory grade sucrose and agar will come to Rs. 77.9/ L and Rs. 15,813.70 for 203 L. In the new media with low cost additives like marine agar (50%)+ sago (50%)+ common sugar developed in the present study, the cost of media will come to Rs. 9.9/ L and Rs. 2009.7 for 203 L. The media cost for production of one plantlet using laboratory grade chemicals is Rs. 0.88 and in the new low cost media the cost of one plantlet is Rs. 0.11 with a saving of 87 per cent for media cost for production of one plantlet. Other costs to be worked out for calculating cost of production of tissue culture plantlets are cost of explant+ electricity+ equipment maintenance+ wages of skilled/ supervisory labour+ potting media for hardening+ other inputs and irrigation costs which will be the same in both the cases.

In clonal fidelity analysis using specific ISSR marker, polymorphic amplicons were observed in highly multiplying cultivars like Attunendran, Nedunendran and Grand Naine when subculturing for multiplication progressed above 8th subculture passage while no such polymorphism was observed in low multiplying cultivars like

Poovan and Njalipoovan. In the cultivar Chengalikodan, no polymorphic amplicons were observed upto 10th subculture passage. So for highly multiplying cultivars like Attunendran, Nedunendran and Grand Naine subculturing for multiplication up to 8th subculture stage is recommended. In Chengalikodan, subculturing for multiplication can be advanced up to 9th subculture passage and in Poovan and Njalipoovan multiplication can be advanced up to 10th subculture passage in the protocol standardized at CPBMB.

However, the commercial feasibility of the findings and working out the economics of production is possible only by large scale adoption of media components and subculture pattern in commercial micropropagation protocol. Standardization of media for high shoot proliferation in cultivars like Chengalikodan, Njalipoovan and Poovan, standardization of other direct regeneration protocols for high plantlet recovery and optimization of media and culture conditions for scaling up multiplication using bioreactors are the other future research areas to reduce the cost of production.


Summary

6. SUMMARY

The study entitled "Low cost alternatives in commercial micropropagation of banana (*Musa* spp.) was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during September 2016 – July 2018, with the objective to reduce the cost of production in commercial micropropagation of banana. The study mainly focused on the following aspects:

1) Cost reduction in plant tissue culture medium: a) Substitution of sucrose with common sugar b) Substitution of agar with isabgol and sago 2) Increasing subculture cycles and clonal fidelity analysis using specific ISSR marker to reduce per plant cost.

Studies were conducted in six banana cultivars viz. Attunendran (AAB), Nedunendran (AAB), Chengalikodan (AAB), Poovan (AAB), Njalipoovan (AB) and Grand Naine (AAA). The cultures of six different cultivars in S5 stage received from commercial micropropagation unit of CPBMB formed the experimental material. The micropropagation protocols optimized for different banana cultivars at CPBMB was used for the study. The clonal fidelity analysis standardized using specific ISSR marker at CPBMB was used to analyse the clonal fidelity of regenerants from S7 to S11 stage.

The salient findings of the study are listed below:

- Common sugar (30 gL^{-1}) was found equally good to laboratory grade sucrose (30 gL^{-1}) and it did not influence the shoot proliferation in different banana cultivars in MS shoot multiplication medium
- Common sugar (30 gL^{-1}) was also found equally good to laboratory grade sucrose (30 gL^{-1}) in the MS rooting medium

- Substitution of sucrose with common sugar in the rooting medium had no significant influence in the days taken for root initials to appear in all the cultivars studied
- There was no significant difference for number of primary roots produced, in cultivars like Nedunendran, Chengalikodan, Poovan, Njalipoovan and Grand Naine when sucrose or common sugar was used as carbon source in MS rooting medium
- Root length was higher in MS rooting medium with common sugar in cultivars like Chengalikodan, Njalipoovan and Grand Naine
- MS rooting medium with common sugar produced more number of secondary roots in Chengalikodan while medium with sucrose produced more number of secondary roots in cultivars like Attunendran and Njalipoovan
- The survival of the plantlets were similar in regenerants from MS medium with sucrose or common sugar in all the six cultivars studied
- There was good solidification of MS medium when fifty per per cent of agar was substituted with solidifying agents like isabgol and sago
- No significant variation was observed in shoot proliferation in different cultivars when half of agar was substituted with isabgol and sago in MS multiplication medium
- Nendran types like Attunendran and Nedunendran exhibited higher shoot proliferation than Chengalikodan. Lowest shoot proliferation was observed in Njalipoovan and highest in Attunendran
- Agar (50%) +sago (50%) was found to give higher shoot proliferation in cultivar Chengalikodan than.agar (50%)+ isabgol (50%) and agar (100%)

- Partial substitution of agar with isabgol and sago in MS rooting medium did not influence rooting and the combinations were found equally good to agar (100%)
- Substitution of 50 per cent agar with isabgol and sago showed no significant difference in days taken for root initials to appear, number of primary roots, number of secondary roots and root length in cultivars like Attunendran, Njalipoovan and Grand Naine.
- Agar (50%)+ sago (50%) performed equally good as that of agar (100 %) in per cent survival of plantlets in Attunendran, Njalipoovan and Grand Naine
- In clonal fidelity analysis, amplification with the ISSR primer generated ten clear amplicons. The bands visualized were in the size range 300- 2000 bp
- Polymorphic bands were observed from S9 stage as subculture progressed in cultivars like Nedunendran, Attunendran and Grand Naine
- In Chengalikodan, up to 10th subculture passage no polymorphic bands were observed and in subculture 11, two bands were found as polymorphic
- In cultivars like Poovan and Njalipoovan which exhibited low multiplication rate, no variation was observed in regenerants up to S11
- In highly multiplying cultivars like Attunendran, Nedunendran and Grand Naine, subculturing above 8th stage was found to induce variation
- For cultivars with lower shoot proliferation like Chengalikodan, Poovan and Njalipoovan, 1 or 2 subculture cycles can be done extra while the subculture cycles should be restricted to eight in highly proliferating cultivars like Grand Naine, Nedunendran and Attunendran

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Appendices

ANNEXURE I

List of laboratory equipments used for the study

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 media

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
	KI	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
100 mg/ L myo-inositol		
6-9 g/ L Agar		
7.5 g/ L semisolid		
30 g/ L sucrose		
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 0°C 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.

Low cost of alternatives in commercial micropropagation of banana (*Musa* spp.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Tissue culture banana plants have become an integral part of commercial banana production. Banana micropropagation is hampered by high unit cost of production, poor multiplication and low survival rates during acclimatization. To make a commercial micropropagation unit viable, cost of production of plantlets should be brought to minimum.

The investigations on 'Low cost alternatives in commercial micropropagation of banana' was hence taken up at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture from 2016-2018. The aim of the investigations was to reduce the cost of production in commercial micropropagation of banana. Studies were conducted in different cultivars like Attunendran (AAB), Nedunendran (AAB), Chengalikodan (AAB), Poovan (AAB), Njalipoovan (AB) and Grand Naine (AAA) which were commercially produced at CPBMB in the micropropagation unit. Established cultures of six cultivars in the 5th subculture cycle received from the commercial micropropagation unit of CPBMB were utilized for the study. The micropropagation protocols optimized for the six different cultivars at CPBMB were followed for production of plantlets. The clonal fidelity analysis standardized using specific ISSR marker at CPBMB was used to analyse the clonal fidelity of regenerants from S7 to S11 stage.

Sucrose was substituted with common sugar @ 30gL^{-1} in MS multiplication and rooting media and shoot proliferation, rooting and root characters in different cultivars were studied. Common sugar was found equally good to sucrose and it did not influence shoot proliferation in different banana cultivars. Common sugar was also found equally good to sucrose in the rooting media recording 100 per cent rooting in all the six cultivars studied. Substitution of sucrose with common sugar had no significant influence in the days taken for root initials to appear. For number of primary roots produced, there was no significant difference in all the cultivars in the two media except Attunendran in

which medium with common sugar produced more number of primary roots (8.41) than sucrose (6.75). Root length was higher in MS rooting medium with common sugar in cultivars like Chengalikodan, Njalipoovan and Grand Naine. Medium with common sugar produced more number of secondary roots in cultivar Chengalikodan while medium with sucrose produced more number of secondary roots in cultivars like Attunendran and Njalipoovan.

When fifty per cent of agar was substituted with other solidifying agents like sago or isabgol, there was good solidification of MS medium. There was no significant variation in shoot proliferation in different cultivars when half of agar was substituted with isabgol or sago in the MS multiplication medium. Fifty per cent substitution of agar with isabgol or sago was found equally good to agar (100%) in rooting medium. Hundred per cent rooting was observed in the three different media combinations in all the cultivars. Substitution of 50 per cent agar with isabgol or sago showed no significant difference in days taken for root initials to appear, number of primary roots, number of secondary roots and root length in cultivars like Attunendran, Njalipoovan and Grand Naine. There was no difference in survival of plantlets in treatments with different low cost additives. When low cost carbon source and gelling materials were used instead of standard additives, there was 87 per cent reduction in media cost.

Clonal fidelity was analysed using specific ISSR marker optimized at CPBMB as reported by Rajitha *et al.* (2015). Polymorphic amplicons were observed as subculture progressed from S9 – S11 in cultivars like Nedunendran, Attunendran and Grand Naine. In Chengalikodan, up to 10th subculture passage no polymorphic bands were observed. In cultivars like Poovan and Njalipoovan which exhibited low multiplication rate, no polymorphic bands were observed in regenerants up to S11 stage.

For highly multiplying cultivars like Attunendran, Nedunendran and Grand Naine subculturing for multiplication up to 8th subculture stage is recommended. In Chengalikodan, subculturing for multiplication can be advanced

up to 9th subculture passage and in Poovan and Njalipoovan multiplication can be advanced up to 10th subculture passage in the protocol standardized at CPBMB.

However, the commercial feasibility of the findings and working out the economics of production is possible only by large scale adoption of media components and subculture pattern in commercial micropropagation protocol.

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