MORPHOLOGICAL AND MOLECULAR ANALYSIS OF GENETIC STABILITY IN MICROPROPAGATED BANANA (*MUSA* SPP) VAR. NENDRAN

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **Morphological and molecular analysis of genetic stability in micropropagated banana (*Musa* spp.) var. Nendran"** is a bonafide record of research work done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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To my loving
family and
guide

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ABBREVIATIONS

%	Percentage
°Bx	Degree Brix
°C	Degree Celsious
>	Greater than
<	Less than
µg	Microgram
A	Ampere
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
BA	Benzyl adenine
BAP	Benzyl Amino Purine
BC	Before Christ
bp	Base pai
BSA	Bovine Serum Albumin
cDNA	complementary Deoxyribonucleic Acid
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
g	Gram
ha	Hectare
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
ISSR	Inter Simple Sequence Repeat
KAU	Kerala Agricultural University

Kb	Kilo basepairs
L	Litre
M	Molar
MBD	Male Bud Derived
mg	Milligram
ml	Millilitre
mM	Milli mole
MS	Murashige and Skoog's Medium
MSAP	Methylation Sensitive Amplification Polymorphism
NAA	Naphthalene Acetic Acid
ng	Nanogram
NHB	National Horticulture Board
°C	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
P^H	Hydrogen ion concentrati
pM	Pico molar
PVP	Poly vinyl pyrolidine
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Rotations per minute
SD	Sucker Derived
SRAP	Sequence Related Amplified Polymorphism
SSR	Simple Sequence Repeats
STMS	Sequence Tagged Microsatellite Sites
STR	Short Tandem Repeat
STS	Sequence Tagged Sites
TAE	Tris Acetate EDTA
TC	Tissue Culture

TE	Tris EDTA
TZD	Thiadiazuron
U	Unit
UBC	University of British Columbia
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USA	United States of America
UV	Ultra violet
V	Volts
β	Beta
µl	Microlitre

Introduction

1. INTRODUCTION

Banana (*Musa accuminata* L.) belongs to the family Musaceae. It is one of the oldest fruit known to mankind. It is widely grown in the tropics and subtropic regions of the world. India is recognized as one of the major centers of origin and diversity for *Musa*. India is the major producer of banana with an area of 8.3 lakh hectares and productivity of 35.9 metric tonnes per hectare; it covers 39.8 per cent of fruit production area in the country (NHB database, 2011).

Nendran (*Musa*, AAB group) is the leading banana cultivar of Kerala contributing 58 per cent of production, belonging to the plantain subgroup and is the most prized plantain variety used in Kerala, fetching a premium price during festive occasions especially Onam, which is the regional festival of Kerala. The pulp is firm and is suitable for the preparation of chips, banana figs and is also consumed as fresh ripe fruit. Steamed, fried and ripe nendran fruits are commonly used in Kerala. The unripe fruits are commonly used as a vegetable and also find place in baby food in dried powder form.

Bananas are propagated conventionally through suckers and the sword suckers are considered as the best propagules. *In vitro* propagation of banana has played a key role in obtaining a large number of homogeneous regenerated plants. Micropropagation has played a key role in *Musa* improvement programmes worldwide (Vuylsteke *et al.*, 1996). As compared to the conventional propagules, micropropagated banana plants establish faster and grow more vigorously. They yield higher in shorter duration with more uniform crop cycle (Vuylsteke and Ortiz, 1996). Maximum yield gain from *in vitro* derived plants range from 20 per cent in bananas to 70 per cent in plantains.

Somaclonal variation is usually observed when plants are regenerated from cultured somatic cells, mostly during callus formation and suspension culture. However, even in absence of de or redifferentiation stress, as during micropropagation, off-types are observed that reduce commercial value of resultant plants. Furthermore, most of the variants are inferior to the original cultivar from which they are derived (Smith and Drew, 1990). Dwarfism in 'Cavendish' bananas and inflorescence variations in plantains are often observed after micropropagation of respective mother genotypes. This genetic instability may be a risk associated with the application of *in vitro* culture techniques for germplasm handling and storage. Various factors have all been shown to influence both the quantity and the type of somaclonal variation in micropropagated banana (Smith and Hamill, 1993). These factors include genotype, origin of shoots *in vitro* (adventitious or axillary buds), number of subcultures, the choice of explants and the degree of dedifferentiation of the tissues in culture.

Somaclonal variation can be either genetic or epigenetic (i.e. non-heritable). There may be variability even among clonally propagated plants of a single donor clone. Somaclonal variations include point mutation, gene duplication, changes in number and the structure of chromosomes, transposable element movement and changes in DNA methylation (Jain, 2001). Epigenetic aspects of somaclonal variation involve mechanisms of gene silencing or gene activation that are not due to changes in sequence or chromosomal aberrations.

Changes in DNA methylation (addition of $-CH_3$ to cytosine) has been hypothesized as an underlying mechanism of tissue culture induced variation due to the high frequency of quantitative phenotypic variation, the activation of transposable elements, heterochromatin-induced chromosome breakage events *etc.* In order to estimate the level of DNA methylation in banana micropropagation, the Methylation-Sensitive Amplification Polymorphism (MSAP) technique has been used which is a relatively new modification of the Amplification Fragment Length Polymorphism

(AFLP) technique. The MSAP utilizes the restriction isoschizomer pair *Msp* I and *Hpa* II, whose ability to cleave at the sequence 5-C'CGG-3 is affected by the methylation state of the external or internal cytosine residues.

There is lot of data available on banana somaclonal variation at the phenotypic level in micropropagated bananas. However, the basis of this variation and its extent during multiple cycles of *in vitro* culture remains unknown. In the present investigation entitled "Morphological and molecular analysis of genetic stability in micropropagated banana (*Musa* spp) var. Nendran" attempts were made to study the genetic stability of regenerants in banana var. Nendran.

The present study has been undertaken with the following objective

To evaluate and characterize the variation in tissue culture derived banana plants regenerated in different subcultures through *in vitro* organogenesis using morphological and molecular (ISSR and MSAP) markers.

Review of Literature

2. REVIEW OF LITERARTURE

The present investigation on "Morphological and molecular analysis of genetic stability in micropropagated banana (*Musa* spp) *var.* Nendran" characterizes the variation in tissue culture derived banana plants regenerated in different subcultures through *in vitro* organogenesis. The relevant literature on various aspects of the investigation is reviewed in this chapter under different heads.

2.1 Origin and history

The generic name *Musa* is derived from the Arabic word *'mouz'*. Bananas were known to the early Arabs and appear in the Koran as the 'tree of paradise' (Robinson, 1996). In India, banana is known for its antiquity from its mention in Ramayana (2020 BC), Koutilya's Arthasastra (300-400 BC) *etc.* Its presence in paintings and sculptures of Ajantha and Ellora caves (600 BC) indicate its historic importance both for consummative and religious purposes (Uma *et al.*, 2005). The edible banana is indigenous to Asia, probably originated somewhere in the mountainous region of Assam, Burma, Thailand or Indo-China. From there it has spread to tropical parts of America, Africa, Australia, Philippines and Hawaii.

2.2 Botany

Banana is a monocotyledonous, herbaceous plant. The true stem remains underground which is known as the 'rhizome'. Botanically rhizome is a modified stem. There are buds or eyes on the middle and upper parts of the rhizomes. The portions above the ground are made up of leaf sheaths known as 'Pseudostem'. This is slightly swollen at the base (Shanmugavelu *et al.*, 1992). The top of the sheath is contracted into the petiole. The 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 later unfolds from the top towards the base. About 40 leaves are formed till flowering. In the last, a bract like leaf is formed which protects the bunch. It is short-

lived. The inflorescence of banana initiates from the heart of the pseudostem. The peduncle is often glabrous and pubescent and varies with varieties. Female flowers are larger, with well-developed ovary with a long style and reduced stamens. Male flowers have well developed anthers, abortive ovary with slender style and stigma. Pollen in edible bananas 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 (Robinson, 1996).

Green banana which becomes palatable after cooking is popularly referred as plantains and is a staple food in the coastal regions of the country especially in Kerala, while the fresh fruit consumed is referred to as dessert bananas (Uma *et al.*, 2005).

2.3 Genetics of banana

Musa species are grouped according to "ploidy" the number of chromosome sets they contain and the relative proportion of *Musa acuminata* (A) and *Musa balbisiana* (B) in their genome. Most familiar, seedless, cultivated varieties (cultivars) of banana are triploid hybrids (AAA, AAB, and ABB). Diploids (AA, AB and BB) and tetraploids (AAAA, AAAB, AABB and ABBB) are much rarer; the latter essentially being experimental hybrids.

Different varieties grouped under various genomics group are under cultivation. The Nendran variety fall under AAB group and it is a popular variety in Kerala where it is relished as a fruit as well as used for processing. Nendran is known to display considerable diversity in plant stature, pseudostem colour, presence or absence of male axis, bunch size, etc. Bunch has 5-6 hands weighing about 12-15 kg. Fruits have a distinct neck with thick green skin turning buff yellow on ripening. Fruits remain as starchy even on ripening. Shanmugavelu *et al.*, (1992) described different ecotypes of Nendran as follows:

Nana Nendran (Nendran Vazha, Nendran Bale, Thiruvadon, Chengazikodan, Ethakka).

This is a short stemmed and short duration variety of Nendran; a medium yielder with 4 to 5 hands, each with 7 to 9 long fruits and weighing about 12.5 cm on girth, with a slight curve at the middle or almost straight; irregularly five sided, apex short but distinct; skin thick, leathery, golden yellow colored, peeling off with difficulty from the pulp; pulp firm, pinkish in colour, core fairly conspicuous, flavor mildly pleasant, taste medium sweet, quality medium as a fruit; keeping quality very good.

Attu Nendran (Nendran, Nadu Nendran, Yethan)

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

Myndoli (Giant Plantain)

This variety differs from Attu Nendran in the number of hands and fruits in the bunch; this is a very heavy yielder, each bunch with about 10 hands and 120 fruits and weighing 25 Kg or more. The fruit is essentially the same as that of the above variety and is late maturing.

Menon and Aravindakshan, (1998) described different ecotypes of Nendran based on plant, bunch character and degree of development of the male phase.

Manjeri Nendran (a)

Identified at Kannara, was suitable for annual cropping besides displaying field tolerance to Sigatoka leaf spot and pseudostem borer. This variety is mainly characterised by purple brown bract colour, fruits straight at the distal end with double the number of fruits in I/II/III hand.

Manjeri Nendran (b)

Early maturing (300 days or below). This variety mainly characterised by short height, comparatively high yielding and I/II/III hand often have double the number of fruits.

Kaliethen

Kaliethen have short plant crop cycle (300-350 days). This variety mainly characterised by bunch position slightly angled, bunch lax, and fruit apex lengthily pointed. This variety is very common in Trivandrum district of Kerala.

Kottayam

Kottayam has short plant crop cycle (300 days or below). This variety is mainly characterised by bunch position slightly angled, bunch lax, and fruit apex lengthily pointed.

Changalikodan

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.

Nedu Nendran

Nedu Nendran has short 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.

Njockkon

It is an introduced variety from Africa having long plant crop cycle (above 350 days). This variety is mainly characterised by short pseudostem height, erect, overlapping leaf habit and is maintained in the banana germplasm at Banana Research Station, Kannara.

2.4 Tissue culture of banana

The first report of banana tissue culture came in early 1970's from Taiwan when Ma and Shii (1972) produced *in vitro* adventitious buds from banana shoot apex following decapitation. These were shortly 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.

2.4.1 Explant source for culture initiation

Cultures are initiated from different explant sources of banana plant. The most commonly used explant sources are shoot apices obtained from parental pseudostem, suckers, lateral buds and terminal inflorescence. The terminal floral apex and axillary flower buds manifest morphogenetic plasticity in their juvenile stage and can be induced to revert to vegetative growth, producing multiplying shoots *in vitro*. Banana meristem culture is now commonly applied for the clonal multiplication and maintenance of banana cultivars. Regeneration of meristems

proceeds through organogenesis instead of somatic embryogenesis due to which sometimes chimeric plants are obtained (Hwang *et al.*, 1984; Banerjee and Sharma, 1988)

Jalil *et al.* (2003) established culture for plant regeneration from embryogenic suspension cultures of *Musa acuminata* cv. Mas (AA) from male inflorescence while 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. Navarro *et al.* (1997) regenerated banana plants via somatic embryogenesis of diploid *(Musa acuminata* ssp. *malaccensis)* and triploid ('Grand Nain') bananas from immature zygotic embryos and male flower bud primordia.

Anthers from male flowers are another explant source for the production of *in vitro* banana plants. Assani *et al.* (2003) regenerated haploid banana plants of *Musa balbisiana* from anthers in which the majority of the microspores were at the uninucleate stage.

2.4.2 Disinfection of explants

For *in vitro* culture initiation, explants are normally collected from field grown plants, so the plant material is liable to be contaminated by microorganisms which must be disinfected before explants are transferred to *in vitro* conditions. Variations in sterilization procedures have been proposed by several researchers. Sodium hypochlorite is the most commonly used disinfectant for surface sterilization of banana explants (Sandra and Krikorian 1984; Mendes *et al.*, 1996).

For the explant disinfected after excision, a shorter treatment time and a lower hypochlorite concentration (0.0525 per cent) is also effective (Vessey and Rivera 1981). Banerjee and Sharma (1988) have replaced sodium hypochlorite with low concentrations of mercuric chloride. Double disinfection method has also been

adopted by some researchers, in which first large size explant is disinfected, followed by shoot tip excision and finally disinfection by some other chemical agent (Silva *et al.*, 1998). Sometimes explants are treated with fungicides and antibiotics to minimize the contamination in *in vitro* cultures (Van den Houwe *et al.*, 1998).

2.4.3 Culture initiation

After the excision of shoot tips, explants are cultured *in vitro* for shoot initiation. At this stage, the explant consists of shoot apical meristem, covered by 3 to 6 leaf primordia and supported on a small base of rhizome tissue. This shoot tip is cultured on the medium intact, wounded or fragmented into pieces. Ma and Shii (1972) decapitated shoot tips before culture initiation. A number of vertical cuts into the meristamatic dome were applied by Vessey and Rivera (1981) in order to facilitate better multiple shoot emergence. The number of incisions ranged from 2 to 10 and were made in such a way that the base of the explant was kept intact.

Different media were reported for culture initiation. Some investigators initiated the cultures on the same media as later used for multiplication while others used low concentration of hormones for culture initiation. Most common salt mixture used for culture initiation of banana was the MS medium (Murashige and Skoog, 1962) with some modifications as reported by Drew *et al.*, 1989. Assani *et al.* (2003) initiated cultures from anthers on MS medium containing vitamins of Morel supplemented with 500 mg l^{-1} casein hydrolysate, 4.4 µM BAP, and 2.3 µM IAA.

When kinetin was used as sole cytokinin, adenine sulphate was also added in the medium as a conducive agent to shoot initiation (Nandwani *et al.*, 2000). Some investigators used only single cytokinin for culture initiation (Cronauer and Krikorian, 1984), while others used mixture of cytokinins (Nandwani *et al.*, 2000;

Rahman *et al.*, 2002). A combination of cytokinin and auxin was also used for banana culture initiation by a number of researchers (Hwang *et al.*, 1984).

2.4.4 *In vitro* multiplication

Plant growth regulators are essential media components for the manipulation of growth and development of explants *in vitro*. Their concentration and ratio in the medium often determines the pattern of development *in vitro*. Cytokinins and auxins are used as growth regulators for *Musa* tissue culture. The most widely used and the most effective cytokinin for this purpose is the adenine based cytokinin BAP.

Sandra and Krikorian (1984) reported 9.1 shoots per explant during *in vitro* multiplication of 'Grande Naine' on a modified Murashige and Skoog (1962) medium supplemented with 5.0 mg l^{-1} 6-benzylarninopurine (BAP). On the other hand Rahman *et al.* (2002) achieved 4.52 shoots per explant on the same concentration of BAP on MS medium during *in vitro* multiplication of cv. Bari-l, indicating the genotypic response towards cytokinins.

Highest shoot length (3.62 cm) was achieved when M.S medium was supplemented with 1.5 mg l^{-1} NAA. Mendes *et al.* (1996) used 4.5 mg l^{-1} BAP in MS medium during the study of the behavior of vegetative apices coming from different rhizomes. They reported that shoot tips coming from different rhizomes behave differently under *in vitro* conditions, some being highly productive and others producing a much smaller number of plants under similar culture conditions.

Abdullah *et al.* (1997) used MS medium with 20 µM BAP for shoot proliferation of cultures initiated from clones of desert banana *(Musa* spp.). Nor-Aziah and Khalid (2002) used higher concentration of BAP during regeneration of *in vitro* banana plants from scalps and whole meristems. Scalps were

induced on MS supplemented with coconut water and high concentration of BAP.

Venkatachalam *et al.* (2006) achieved direct shoot regeneration from leaf sheaths of silk banana (AAB) when cultured on medium containing 22.4 µM BA. Some researchers have reported that a combination of BAP and auxin enhanced shoot proliferation and shoot length during tissue culture of banana. Bhagyalakshmi and Singh (1995) used MS medium with 8.9 M benzyladenine and 0.98 M indolebutyric acid during shoot culture of three cultivars of banana, Cavendish, Bluggoe and Silk.

There are studies in which more than one cytokinin with or without auxin were used together to enhance the shoot proliferation during banana tissue culture. Thiadiazuron (TDZ) is a phenyl urea based cytokinin frequently used in tissue culture. The effects of BAP (5, 10, 20 and 30 M) and thidiazuron (0.4, 1, 2 and 3 M) were tested alone or with 1M IAA for shoot multiplication of three banana types by Hamide and Mustafa (2004). They found that in all three types shoot proliferation and elongation were significantly greater with TDZ than with BAP.

Kinetin is another synthetic PGR used in banana tissue culture and was so named because of its ability to promote cytokinesis (cell division). Hwang *et al.* (1984) added 2 mg l^{-1} kinetin and 2 mg l^{-1} indole acetic acid in MS medium during meristem culture of banana and the population of buds was increased by five times per month. Wong (1986) compared kinetin and 6-benzylaminopurine (BA) along with indolebutyric acid (IBA) during *in vitro* multiplication of banana *(Musa* spp.) and found that BA was more effective than kinetin.

Arinaitwe *et al.* (2000) used MS modified medium supplemented with various equimolar concentrations (16.8, 20.8, 24.8 and 28.8 mM) of BAP, TDZ, ZN, 2-iP and KN to determine suitable concentration ranges of the

cytokinins for micropropagation of banana cultivars. The results showed that shoot proliferation was dependent on cytokinin type, its concentration and the banana cultivar.

During micropropagation of banana, blackening and of tissues is commonly observed which interfere with plant growth. Martin *et al.* (2007) controlled tissue necrosis of cvs. Grande Naine (AAA), Dwarf Cavendish (AAA), Nendran (AAB) and Quintal Nendran (AAB) by the addition of 50-100 mg/l calcium chloride in the MS medium. Titov *et al.* (2006) controlled oxidation of phenolic compounds secreted by flowers, by washing these in 0.125 per cent potassium citrate solution before culturing.

2.4.5 *In vitro* rooting

In vitro multiplication of banana is normally carried out in the presence of high cytokinin levels which inhibit root formation and elongation. Moreover during *in vitro* multiplication shoots may lack roots and are growing in the form of bunches which cannot be transferred directly to field conditions. Prior to transfer in free living conditions, individual shoots are separated from cluster and grown on root induction media. The concentration of cytokinin in the rooting medium should be lower than auxins in the multiplication medium, so that cytokinin/ auxin ratio becomes low which is favorable for root induction as reported by (Gupta, 1986; Wong, 1986). However most of the investigators omit cytokinins entirely from the rooting medium. The most frequently incorporated auxins in rooting medium were NAA, IAA and IBA.

Hwang *et al.* (1984) regenerated roots from *in vitro* plants of *Musa sapientum* L. on MS medium to which 1 gm/l activated charcoal was added. After four weeks of incubation, the plants developed numerous roots and were ready for transfer in the field. However Sandra and Krikorian *et al.* (1984) added IAA, NAA or IBA @ 1mg l^{-1} along with 0.025 per cent activated charcoal.

Indole butyric acid (IBA) was effective for root induction of *in vitro* raised banana plants and frequently used for this purpose. Dore-Swamy *et al.* (1983) first time used IBA during banana tissue culture. Banerjee and Sharma (1988) achieved rooting on semi-solid medium with 0,2 mg/l IBA during plant regeneration from long- term banana cultures. Molla *et al.* (2004) reported that a good number of healthy roots were produced on half MS containing 0.4, 0.5 or 0.6 mg/l IBA. Madhulatha *et al.* (2006) used IBA and NAA in combination during optimization of liquid pulse treatment for production of *in vitro* rooted plants cv. Nendran (*Musa* spp. AAA)

Naphthalene acetic acid (NAA) is another auxin used frequently at lower concentrations for root induction of *in vitro* raised banana plants. Cronauer and Krikorian (1984) found 1 mg l-1NAA to be satisfactory in AAA and AAB bananas respectively. Arinaitwe *et al.* (2000) achieved rooting on MS medium containing 1.2 µM NAA during the study of proliferation rate effects of cytokinins on Kibuzi, Bwara and Ndizwemiti banana cultivars.

There are reports that roots can be induced without growth regulators (Albany *et al.*, 2005; Silva *et al.*, 1998) but most of the authors agreed with the inclusion of growth regulators for root induction.

2.4.6 Hardening of *in vitro* raised banana plants

Micropropagated plants are delicate plants because they are produced in a closed, sterile environment and grown on nutrient-rich artificial media under controlled conditions with high humidity and low light intensity. The transfer of rooted plantlets from aseptic culture conditions directly to the external environment can result in significant loss of plants. When removed from the tissue culture environment, micropropagated plants must be allowed to adjust to the outside environment with its varying light levels, changing temperature, reduced humidity, lower nutrient availability and presence of

pathogens.

Tissue-cultured plants generally have poor in cuticle; therefore evaporates water rapidly upon transfer to natural conditions. Moreover, due to limited space and presence of excess carbon source, their photosynthetic apparatus was not fully resulting in fulfillment of their energy demands are met by reserves of starch accumulated during culture. These reserves thus deplete creating emerging crises during hardening.

The effects of triazoles on *in vitro* hardening and acclimatization of banana regenerated from floral apices was studied by Murali and Duncan (1995). Banana shoots were grown on culture media with 0, 1, 2 or 4 mg/l of triazoles (triadimefon or uniconazole) for one month. The resulting plantlets were then transferred to a peat moss and sand (1:1, v/v) potting mixture. *In vitro* triadimefon treatment acted as a conditioning agent and obviated the need for hardening or weaning the plantlets. Triadimefon-treated plants were turgid and healthy as compared with control plants.

Growth and development of *in vitro* raised plants of cv. Pioneira *(Musa* sp. AAAB) during hardening was studied by Silva *et al.* (1998). *In vitro* rooted plantlets were transferred to plastic bags containing organic substrate. Different parameters of growth were recorded in green house, humid chamber in green house, screen house, humid chamber in screen house, under tree canopy, humid chamber under tree canopy and in field conditions. All treatments showed 100 per cent plantlet growth, except for the direct field planting (39.7 per cent).

Jasrai *et al.* (1999) developed protocols for hardnening of *in vitro* derived banana plants without greenhouse facilities. *In vitro* raised plants were transferred in polythene bags which were perforated six cm from the base. The bags containing the plants were placed inside a plastic tray. High humidity was maintained by spraying water after every two hours. On an average 92 per cent

of the plantlets survived.

2.4.7 Field performance of tissue culture banana plants

The advantages of *in vitro* micropropagated banana plants included higher rates of multiplication, production of disease free planting material and small space required to multiply the large numbers of plants.

Robinson *et al.* (1993) achieved 20.4 per cent higher yield than conventional plants, due to larger bunches and a shorter cycle to harvest. On the other hand *in vitro* derived plants of plantain *(Musa* spp. AAB) did not show any higher yield (Vuylsteke and Ortiz 1996) and more phenotypic variation was observed in tissue culture plants.

Field performance of *in vitro* raised plants was compared with true-to-type plantains to evaluate their horticultural traits (Vuylsteke *et al.,* 1996). Significant variation was observed for plant and fruit maturity, leaf size, yield and its components, but not for leaf number, plant height, or suckering. Three of the four somaclonal variants were horticulturally inferior to the original clone from which they were derived. Only one variant which resembled an existing cultivar, out yielded the true-to-type clone. However, its fruit weight and size were lower.

Optimum plantlet size for tissue culture banana plants was studied by Fraser and Eckstein (1998). They reported that small plants of 100 mm took three weeks longer to harvest and had six per cent lower yields as compared to 300 mm size. Plants of 500 mm size showed slightly lower yields as compared with 300 mm plants. It was advisable that 200 mm plants should be planted at least 100 mm below soil surface, preferably in furrow.

2.5 Somaclonal variation in plants

Plant tissue culture has become one of the fundamental tools of plant science research. It is extensively employed in the production, conservation and improvement of plant resources. The presence of somaclonal variation in populations derived from tissue culture is affecting the use of tissue culture negatively and has remained a major problem. Conversely, it is a source of new desirable clones/variants with better agronomic traits.

2.5.1 Definitions and background

Somaclonal variation is defined as variation originating in cell and tissue cultures (Larkin and Scowcroft, 1981). Presently, the term somaclonal variation is universally used for all forms of tissue culture derived variants (Bajaj, 1990); however, other names such as protoclonal, gametoclonal and mericlonal variation are often used to describe variants from protoplast, anther and meristem cultures, respectively (Karp, 1994). Some scientists added another aspect to the definition and require that somaclonal variation can be heritable through a sexual cycle. Unfortunately, it is not always possible to demonstrate heritability because of complex sexual incompatibilities, seedlessness, polyploidy or long generation cycles. Therefore, explaining the heritable nature of somaclonal variation for these types of plants could be difficult and almost impossible (Skirvin *et al.*, 1994).

Since the first observation and report of somaclonal variation by Braun (1959), it remains one of the major problems of many tissue cultured plants. The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process, involving only mitotic division of the cell and theoretically, it should not cause any variation. Ideally, clonal multiplication of genetically uniform plants is the expectation (Larkin, 1998). The occurrence of uncontrolled and random spontaneous variation during the culture process is, therefore, an unexpected and mostly undesired phenomenon (Karp, 1994).

Contrary to these negative effects however, its usefulness in crop improvement through creation of novel variants are also well documented. Induced somaclonal variation can be used for genetic manipulation of crops with polygenic traits (Brar and Jain, 1998). It can also be an important tool for plant breeding via generation of new varieties that could exhibit disease resistance and improvement in quality as well as better yield (Unai *et al.*, 2004).

2.5.2 Origin and sources of somaclonal variation

Spontaneous heritable variation was known to plant growers before the science of genetics was established and the art of plant breeding practiced. The commencement of the domestication of plants coincided with the occurrence of "sports", "bolters", "off-types" and "freaks" in vegetatively propagated plants such as sugarcane, potato and banana. Some of the successful cultivars based on spontaneous mutation such as the naval orange, dwarf bananas, colored and striped sugarcane as well as several potato cultivars are comparable to somaclonal variants and are frequently cultivated (Ahloowalia, 1986).

In contrast to spontaneous mutations *in vivo, in vitro* generated variations seem to occur more frequently (Yang *et al.*, 2010) and are detected easily because variants can be readily spotted in a limited space and within a short time (Ahloowalia, 1986). The exposure of unprotected genetic material to chemicals in the medium and survival of the resulting variants in a non-selective environment increases the mutation rate several fold over that in glasshouse or field grown plant populations (Skirvin *et al.*, 1994). Even if the rate of mutagenesis are the same in cell and tissue cultures as in field grown plants, the sheer number of occurrences in a cell population would make accumulation of mutants far greater than in field grown plants. Therefore, somaclonal variants can be detected more frequently in cell cultures than mutations in field grown populations (Ahloowalia 1986; Larkin, 1998).

The *in vitro* culture of plant material can induce or reveal variation between cells, tissues and organs thereby creating variation within cultures or among the somaclones. Some or all of the somaclones may be physically different from the stock plants from which the culture was derived (Skirvin *et al.,* 1994). Variability of this kind, which usually occurs spontaneously and is largely uncontrolled or directed, can be of two different kinds viz changes caused by cells having undergone persistent genetic change and those caused by temporary changes to cells or tissues, which are either genetically or environmentally induced (Pierik, 1987; Karp, 1994). Generally though, somaclonal variation *in vitro* can be the result of individuals exhibiting one or more of the following changes; physical and morphological changes in undifferentiated callus; differences in the ability to organize and form organs *in vitro*; changes manifested among differentiated plants and chromosomal changes (Skirvin *et al.,* 1993).

Somaclonal variants may differ from the source plant permanently or temporarily. Temporary changes result from epigenetic or physiological effects and are nonheritable and reversible (Kaeppler *et al.,* 2000). However, permanent variants referred to somaclonal variants are heritable and often represent an expression of pre-existing variation in the source plant or are due to the de novo variation via an undetermined genetic mechanism(s) (Larkin and Scowcroft, 1981).

As a result, the causes of somaclonal variation are not always well understood and have not been fully elucidated. Although it has been studied extensively, the causes remain largely theoretical or unknown (Skirvin *et al.,* 1993). Generally, variation in tissue culture could either be pre-existing or tissue culture induced (George, 1993).

2.5.3 Pre-existing variation

Heritable cellular variation could result from mutations, epigenetic changes or a combination of both mechanisms (Kaeppler *et al.,* 2000). The distinction between

the two mechanisms is an important one because genetic mutations are essentially irreversible and are likely to persist in the progeny of regenerated plants, whereas epigenetic changes are not transmitted by sexual reproduction (Kaeppler *et al.*, 2000). Variation in ploidy level (Bright *et al.*, 1983), tissue culture induced chromosome aberrations and rearrangement (Phillips *et al*, 1988), mechanisms regulating the cell cycle (Beemster *et al.*, 2003), activation of transposable elements (Peschke *et al.*, 1987) are some of the factors thought to induce pre-existing variations.

2.5.3.1 Chromosome aberration and rearrangements

Thorough characterization and classification of tissue culture induced chromosome aberrations have led to a better understanding of somaclonal variation. Variation in chromosome number and structure has been observed among tissue cultured somaclones (Hao and Deng, 2002; Mujib *et al.*, 2007). Detailed studies have indicated that structural chromosome changes most accurately reflect the frequency and extent of karyotypic changes (Lee and Phillips, 1988). In tissue cultured cells, the predominant type of aberration is the result of changes in chromosome structure. Therefore events leading to chromosome breakage and in some instances subsequent exchange or reunion of fragments appear to be of fundamental importance (Lee and Phillips, 1988).

2.5.3.2 The role of the cell cycle

The regulatory mechanism of the cell cycle can play a direct role in plant growth and morphogenesis. Kaplan (1992) proposed two opposing views named the 'cell theory' and the 'organismal theory' to address the function of the cell cycle in the growth process. The former considers cells as the building blocks of an organism such that any increase in cell number causes growth while the latter considers cell division as a consequence rather than cause of growth. This suggests that any effect on a cell or organ could possibly induce an effect to the whole organism.

2.5.3.3 Transposable elements activation

Transposable elements are mobile DNA sequences in a genome that can induce gene mutations and contribute to genome rearrangements. Transposons account for significant portions of most plant genomes and were first discovered in maize culture by McClintock (1950). Activation of cryptic transposable elements is another source of chromosome based somaclonal variation. Chromosome breakage is a means for initiating activity of maize transposable elements (Peschke *et al.*, 1987).

The discovery of activation of maize transposable elements in tissue culture suggested a possible relationship between somaclonal variation and mobile elements. Genetic evidence also suggests that certain unstable mutants may be explained by transposable elements and the tissue culture environment probably provides a conducive environment for DNA sequence transposition (Larkin and Scowcroft, 1981). For instance, the induction of callus followed by subsequent shooting and rooting would disrupt normal cell function and may activate transposable elements, stress-induced enzymes or other products (Pietsch and Anderson, 2007).

Recently, Gao *et al.* (2009) observed that the new insertions of transposons in a rice cultivar regenerated through tissue culture was responsible for somaclonal variation. Therefore, it has been suggested that transpositional events such as activation of transposable elements and the putative silencing of genes and a high frequency of methylation pattern play a major role in somaclonal variation (Barret *et al.*, 2006). However, the extent of that role and the mechanism of the process have not been elucidated and is poorly understood (Kaeppler *et al.*, 2000).

2.5.4. Tissue culture induced variation

During *in vitro* culture, the propagation methods, genotype, nature of tissue used as starting material, type and concentration of growth regulators, number as well as the duration of subcultures are some of the factors that determine the frequency of

variation (Pierik, 1987). The effects of some of these mentioned factors on the occurrence of somaclonal variations are discussed below.

2.5.4.1 *In vitro* propagation method used

The presence of a disorganized growth phase in tissue culture is considered as one of the factor that causes somaclonal variation (Rani and Raina, 2000). *In vitro* growth conditions can be extremely stressful on plant cells and may instigate highly mutagenic processes (Shepherd and Dos Santos, 1996). Cellular organisation is also important in terms of describing the origin and cause of somaclonal variation. Tissue culture involves disorganised growth at various levels, ranging from those systems which least disturb cellular organization such as meristem tip culture to systems such as protoplasts and non-meristem explant cultures where regeneration is achieved through the formation of adventitious shoots after a phase of disorganised callus or cell suspension culture (Scowcroft, 1984).

Systems subject to instability and disorganised growth demonstrated that cellular organization is a critical feature and that somaclonal variation is related to disorganised growth (Sivanesan, 2007). Generally, the more the organizational structure of the plant is broken down, the greater the chance of mutations occurring (Cooper *et al.,* 2006). Although the direct formation of plant structures from cultured plant tissue, without any intermediate callus phase, minimizes the chance of instability, the stabilizing influence of the meristem is usually lost when plants are grown in culture (Karp, 1994).

2.5.4.2 Types of tissue or starting material used

Highly differentiated tissues such as roots, leaves and stems generally produce more variants than explants from axillary buds and shoot tips which have pre-existing meristems (Sharma *et al.,* 2007). There are however, some exceptions where more organized tissues like shoot-tips cause more variation compared to somatic

embryogenesis as reported in bananas (Israeli *et al.*, 1996). The use of undifferentiated tissue such as the pericycle, procambium and cambium as starting material for tissue culture reduces the chance of variation (Sahijram *et al.*, 2003). Gross changes in the genome including endo-polyploidy, polyteny and amplification or diminution of DNA sequences could also occur during somatic differentiation in normal plant growth and development (D'Amato, 1977).

Tissue source therefore can affect the frequency and nature of somaclonal variation (Kawiak and Łojkowska, 2004). The processes of de-differentiation and re-differentiation may involve both qualitative and quantitative changes in the genome and different DNA sequences may be amplified or deleted during these changes in the state of the cell that is related to the original tissue source and regeneration system (Lee and Phillips, 1988). Somaclonal variation therefore can arise from somatic mutations already present in the donor plant.

2.5.4.3 Type and concentration of applied plant growth regulators (PGR)

Optimal concentration and precise ratios of auxins and cytokinins is essential for efficient micropropagation (Skoog and Miller, 1957). The primary events, controlled by exogenously applied plant growth regulators (PGRs) that trigger morphogenesis via cell-cycle disturbance might induce variability (Peschke and Phillips, 1992). PGRs also preferentially increase the rate of division in cells already genetically abnormal (Bayliss, 1980). The genetic composition of a cell population can therefore, be influenced by the relative levels of both auxins and cytokinins (D'Amato, 1975). Cells of normal ploidy are often seen to be at an advantage in media where these chemicals are present in low concentrations or totally absent. Evidence for direct mutagenic action of growth regulators is somewhat inconclusive and most evidence points to a more indirect effect through stimulation of rapid disorganized growth (Karp, 1994).

The presence of a relatively high concentration (15 mg/l) of BA was implicated in the increase in chromosome number in a somaclonal variant CIEN BTA- 03 derived from the banana cultivar 'Williams' (Gimenez *et al.,* 2001). High levels of BA (30 mg/l) also greatly increased the genetic variability of rice callus cultures compared to that found in cultures incubated with 2 mg/l BA (Oono, 1985). Diphenylurea derivatives were implicated in incidence of somaclonal variation in bananas (Roels *et al.,* 2005), calamondin (Siragusa *et al.,* 2007) and soybean (Radhakrishnan and Kumari, 2008). Auxins used during cultures of unorganised calli or cell suspension were found to increase genetic variation by increasing the rate of DNA methylation (Loschiavo *et al.,* 1989). Likewise, the synthetic auxin 2, 4-D that is frequently used in callus and cell cultures, is often associated with genetic abnormalities such as polyploidy and the stimulation of DNA synthesis that may result in endoreduplication (Mohanty *et al.*, 2008).

The possibility of unbalanced concentrations of auxins and cytokinins inducing polyploidy was also highlighted (Swartz, 1991). Induction of callus using 2, 4-D at high concentration has been implicated as cause of somaclonal variation in strawberry (Nehra *et al.*, 1992), soybean (Gesteira *et al.,* 2002) and cotton (Jin *et al.,* 2008). Hence, sub and optimal levels of PGRs, synthetic hormones to a greater extent in the culture media have been linked with somaclonal variation (Martin *et al.,* 2006).

Conversely, Reuveni *et al.* (1993) indicated that variation in other medium components including high levels of cytokinins did not directly affect the rate of somaclonal variation in 'Cavendish' bananas, instead the main effect was that of genotype. Leaf-colour variants observed in somaclones of Caladium varied with auxin type more than with concentration of specific auxins. Using equal molar concentration of different auxins, the number of variants was higher on media containing either 2, 4, 5-T or 2, 4-D than NAA or IBA (Ahmed *et al.,* 2004). However, PGRs did not influence the genetic stability and uniformity of organogenesis and somatic embryogenesis-derived fennel (Bennici *et al.,* 2004).

Similarly, the exposure of the banana cultivar 'Nanjanagudu Rasabale' to relatively high concentrations of two cytokinins (BA: 53.28 lM; KN: 55.80 lM) caused no somaclonal variation (Venkatachalam *et al.,* 2007). In view of these contradictory reports, the role of type and concentration of PGRs particularly cytokinins on incidence of somaclonal variation in different plant species remains a subject for debate and warrants further stringent experiments.

2.5.4.4 Number and duration of subcultures

Increasing the number of subculture and their duration enhances the rate of somaclonal variations, especially cell suspension and callus cultures (Bairu *et al.,* 2006). During micropropagation, a high rate of proliferation is achieved in relatively shorter periods and leads to more frequent subculturing. Rodrigues *et al.* (1998) showed that somaclonal variants appeared from the fifth subculture (1.3%) onwards and increased to 3.8% after 11 subcultures. The rapid multiplication of a tissue may affect genetic stability leading to somaclonal variation (Israeli *et al.,* 1995). Also, studies have shown that somaclonal variation is particularly apparent and higher in plants regenerated from long term cultures (Petolino *et al.,* 2003). For example, the long period in culture increased the number of somaclonal variants observed in wheat regenerants (Hartmann *et al.,* 1989). Similarly, Bairu *et al.* (2006) observed an increase in the rate of occurrence of variants with progressive sub-culturing of micropropagated bananas.

Contrary reports on the effect of culture duration on somaclonal variation have also been made. For instance, multiple shoot culture of pea maintained over a long period (24 years) remained genetically stable and was comparable to the original genotype (Smykal *et al.,* 2007). Absence of genetic variation was also observed after a long culture period (17 months) of fennel micropropagation (Bennici *et al.,* 2004), suggesting a possible genotype effect.

The increase of the variant rate as a function of the length of the culture period and observations of different variant rates among lines cultured for the same lengths of time under strictly identical culture conditions are two apparently confusing experimental features often reported in tissue culture (Muller *et al.,* 1990; Podwyszynska, 2005). For better understanding of this problem as well as the variant rate evolution in tissue culture, Cote *et al.* (2001) proposed a statistical model for predicting the theoretical mutation rate with the number of multiplication cycles as the primary parameter. Two main conclusions derived from the model: that a variant rate increase can be expected as an exponential function of the number of multiplication cycles and secondly, after a given number of multiplication cycles, variable off-type percentages can be expected.

2.5.4.5 Effect of stress and genotype

Stress during tissue culture can also induce somaclonal variation. Different genomes however, respond differently to this stress-caused variation indicating that somaclonal variation has genotypic components. The differences in stability are related to differences in genetic make-up whereby some components of the plant genome make them unstable during the culture process. This could be better explained by the repetitive DNA sequences, which can differ in quality and quantity between plant species (Lee and Phillips, 1988).

Inherent instability of a cultivar was a major factor that influenced dwarf off-type production in banana tissue culture. For instance cv. 'New Guinea Cavendish' had a higher level of instability *in vitro* than cv. 'Williams'. Similarly, dwarf off-types remained stable during *in vitro* culture, and the tissue culture conditions that induced dwarfism in normal plants did not induce reversion of the dwarf off-type trait (Damasco *et al.,* 1998). Embryogenic cell suspension age and genotype affected the frequency and phenotype of variants produced significantly in *Coffea arabica* (Etienne and Bertrand, 2003). Mehta and Angra (2000) indicated that there was

variation in disease resistance among somaclones of wheat cultivars due to inherent conditions of the explants. Similarly, the genotype and type of explant strongly influenced occurrence of somaclonal variation in callus cultures of strawberry (Popescu *et al.*, 1997). In Musa *spp.*, the type and rate of variation was specific to the genotype (Israeli *et al.*, 1991), interaction between the genotype and the tissue culture environment (Martin *et al.*, 2006) and genome composition (Sahijram *et al.*, 2003). These and other related phenomenon has continued to stimulate interest to study factors governing and/or influencing genetic stability in an *in vitro* environment.

2.6 Methods of detecting somaclonal variants

High rates of somaclonal variation during micropropagation of many plants remain a major problem, especially in large-scale commercial operations. Early detection and elimination of variants is therefore essential to reduce the losses to growers. Efficient detection of variants can also be used to spot variants with useful agronomic traits. Somaclonal variants can be detected using various techniques which are broadly categorized as morphological, physiological/biochemical and molecular detection techniques. Each of these techniques has their peculiar strengths and limitations.

2.6.1 Morphological detection

Morphological characters have long been used to identify species, genera, and families in plants. Variants can be easily detected based on characters such as difference in plant stature, leaf morphology and pigmentation abnormality (Israeli *et al.*, 1991). For example, banana off-types can be visually detected during acclimatization in the green house before transplanting to the field. In the field, it is also possible to detect dwarf off-types by observing the stature and leaf index (leaf length/width) 3–4 months after establishment (Rodrigues *et al.*, 1998). Similarly, in date palm, the production of bastard offshoots, excessive vegetative growth, leaf

whitening and variegation are common morphological traits used in detecting somaclonal variants (Zaid and Kaabi, 2003).

However, morphological traits are often strongly influenced by environmental factors and may not reflect the true genetic composition of a plant (Mandal *et al.*, 2001). In addition, morphological markers used for phenotypic characters are limited in number, often developmentally regulated and easily affected by environmental factors (Cloutier and Landry, 1994). Likewise, Jarret and Gawel (1995) raised concerns about the irregular responses of the genomes under *in vitro* manipulation that can result in over or under estimations of the degree of closeness among somaclones. For example, major changes to the genome, as a result of *in vitro* manipulation may not be expressed as an altered phenotype and vice versa. Furthermore, the detection of variants using morphological features is often mostly feasible for fully established plants either in the field or greenhouse. This is not an ideal technique for commercial application due to cost implications (Israeli *et al.*, 1995).

2.6.2 Physiological/biochemical detection

Relative to morphological detection, the use of physiological responses and/or biochemical tests for detecting variants is faster and can be carried out at juvenile stages to lower the possible economic loss (Israeli *et al.*, 1995). The response of plants to physiological factors such as hormones and light can be used as basis to differentiate between normal and variant somaclones (Peyvandi *et al.*, 2009). For instance, gibberellic acid regulates growth and influences various developmental processes such as stem elongation and enzyme induction. Therefore, disturbances in gibberellic acid metabolism and level have been suggested as one of the possible indicators of somaclonal variation in higher plants (Sandoval *et al.*, 1995).

Based on responses to exogenous gibberellic acid, dwarfs of many plant species have been classified as either gibberellic acid-responsive or gibberellic acid-non responsive (Graebe, 2003). For example, Damasco *et al.* (1996) observed that normal banana plants showed significantly greater leaf sheath elongation in response to gibberellic acid treatment than dwarfs and elongation in normal banana plants was twofold greater than the dwarfs. Similarly, Sandoval *et al.* (1995) reported a hormonal analysis based on endogenous gibberellins which could be useful to understand and characterize the inter-varietal differences linked to height in *Musa spp*. However, this technique is mostly useful and has been proved to be very effective for plantlets at the de-flasking stage (Damasco *et al.,* 1996).

Light as energy source for photosynthesis and temperature are essential prerequisites for plant growth and development. Plants have developed mechanisms to adapt and acclimate to variations in light regime from deep shade to extremely bright light (Long *et al.,* 1994). Photoinhibition is a state of physiological stress and is expressed as a decline in photosynthetic capability of oxygen evolving photosynthetic organisms due to excessive illumination and plays an important role in plants (Adir *et al.,* 2003). Generally, shade tolerant plants are more susceptible to photoinhibition than sun-loving plants (Long *et al.,* 1994). Senevirathna *et al.* (2008) suggested a mild shade environment in which the level of photosynthetic photon flux density (PPFD) is high enough to saturate carbondioxide assimilation and low enough to induce shade acclimation will help to optimize the photosynthetic productivity in plants. Based on photoinhibition, Damasco *et al.* (1997) evaluated the responses of tissue cultured normal and dwarf off-type Cavendish bananas to suboptimal temperatures under field and controlled environmental conditions. The dwarf off-types showed improved tolerance to low temperature and light compared to the normal somaclones.

Many authors have used different biochemical tests to distinguish amongst somaclones. This approach has been very useful to quantify some interesting somaclonal variants (Daub and Jenns 1989; Kole and Chawla 1993; Thomas *et al.*, 2006). Carbon dioxide assimilating potential was higher in white popinac variants than normal somaclones (Pardha and Alia, 1995). The synthesis of pigment such as chlorophyll, carotenoids, anthocyanins can be used as basis for detecting somaclonal variants (Shah *et al.*, 2003). For example, Mujib (2005) observed that pineapple variants showed significantly lower chlorophyll than normal regenerants. Similarly, the total carotenoid content varied greatly between normal and variant somaclones (Wang *et al.*, 2007). However, the application of most biochemical tests is complex and requires high expertise. Beside, most of the biochemical tests are done on small scale in the laboratory using *in vitro* techniques. Most favorable results obtained have not been successfully implemented for commercial purposes (Daub, 1986).

2.6.3 Cytological methods

Variation in ploidy or chromosomal number and structure is direct and strong evidence of a high likelihood of change in genetic composition of an organism (Al Zahim *et al.*, 1999). Chromosomes as well as other nuclear components such as RNA and DNA content variations are important somaclonal variation detection techniques that have widely been used (Nakano *et al.*, 2006; Fiuk *et al.*, 2010).

Karyological analysis and observation of chromosomal aberration using light microscopy, oil immersion or other complex microscopy techniques have been successfully employed for detecting somaclonal variation in *in vitro* regenerants (Raimondi *et al.*, 2001; Mujib *et al.*, 2007), however, it is time-consuming and often tedious especially where chromosomes are difficult to observe (Dolezel, 1997). As a result, flow cytometry is now widely used for counting and examining chromosomes (Dolezel *et al.*, 2004). This involves preparation of aqueous suspensions of intact nuclei whose DNA is stained using a DNA fluorochrome. The nuclei are classified

according to their relative fluorescence intensity or DNA content. This is achieved by suspending the prepared samples in a stream of fluid under an electronic detecting apparatus (Dolezel and Bartos, 2005). Because the sample preparation and analysis is convenient and rapid, this technique has been employed to assess the ploidy stability in cork oak (Loureiro *et al.,* 2005), Juniperus (Loureiro *et al.,* 2007) as well as detection of somaclonal variants in strawberry (Nehra *et al.,* 1992), bananas (Gimenez *et al.,* 2001) and potato (Sharma *et al.,* 2007).

Nevertheless, the role of cytosolic compounds interfering with quantitative DNA staining is not well understood. Another problem is the absence of a set of internationally agreed DNA reference standards causing limitations in the use of flow cytometry (Dolezel and Bartos, 2005).

2.6.4 Proteins and isozymes

Proteins are the most abundant organic molecules in cells with diverse functions. Isozymes are multiple molecular forms of enzymes. Isozymes are different variants of the same enzymes which differ in amino acid sequence but catalyze the same metabolic reactions (Hunter and Merkert, 1957). It is well known that morphological variation is a result of biochemical variation which is expressed as variation among proteins. As a result, the discriminating property of proteins and isozymes is a function of the number of polymorphic loci that can be identified and genetically characterized in an organism (Jarret and Gawel, 1995).

Until recently, isozymes were one of the most widely used molecular markers for studying genetic variation in most organisms (Weising *et al.,* 2005). Historically, proteins and isozymes such as peroxidase, malate dehydrogenase and superoxide dismutase have been extensively used to study variation in sugarcane (Srivastava *et al.,* 2005), beans (Gonzalez *et al.,* 2010) and Musa *spp.* (Rivera, 1983). Mandal *et al.* (2001) also used both salt-soluble polypeptide and four isozymes for varietal identification of various banana cultivars.

Variations in somaclones can be detected by analysing clones for protein and enzyme polymorphism. Freshly prepared tissue extracts mostly from the leaf part are loaded on a nondenaturing starch or polyacrylamide gel. Proteins of these extracts are separated by their net charge and size during electrophoresis and incubation with specific isozymes. Thereafter, the position of a particular enzyme in the gel is detected after incubation with a dye (Jarret and Litz, 1986). Depending on the number of loci, their state of homo or heterozygosity and the specific isozymes used, one or several bands are visualized and the polymorphism of the bands reveals variation (Weising *et al.*, 2005).

Although, analyses of isozymes patterns of specific enzymes provide a convenient method for detection of genetic changes, the technique is subjected to ontogenic variations as well as other environmental factors. In addition, the number of isozymes are limited and only DNA regions coding for soluble proteins can be sampled (Venkatachalam *et al.*, 2007). For instance, total protein and isozymes did not reveal variations between normal and dwarf off-types as well as closely related cultivars of Musa *spp.* (Bhat *et al.*, 1992). Mandal *et al.* (2001) also reported the limitation of salt soluble peptide as molecular markers for varietal identification for banana cultivars. Subsequently, many authors no longer rely on this technique to detect variants and instead other more sensitive techniques are now employed.

2.6.2 Molecular detection

Molecular techniques are valuable tools used in the analysis of genetic fidelity of *in vitro* micropropagated plants. At the molecular level, variations in tissue culture derived plants arise from changes in chromosome number or structure, or from more subtle changes in the DNA (Gostimsky *et al.*, 2005). Visible morphological variation is known to occur at a much lower frequency than at the DNA level (Evans *et al.*, 1984). As a result, it is necessary to examine for potential variation at the molecular level in order to determine locations and extent of deviance from the true-to-type

clone plant (Cloutier and Landry, 1994). This may be conducted at an early growth stage, while still in tissue culture and prior to the considerable time and expense of achieving full regeneration. The work by Botstein *et al.* (1980) on the construction of genetic maps using Restriction Fragment Length Polymorphism (RFLP) was the first reported molecular marker technique in the detection of DNA polymorphism.

Presently, a number of molecular techniques are available to detect sequence variation between closely related genomes including differences between source plants and somaclones. These techniques involve the use of molecular markers which are useful in comparing the DNA from different samples for the differentiation in plants due to sequence variation by identifying random polymorphisms (Cloutier and Landry, 1994). DNA extracts from the leaf part are mostly used in investigations because of the ease of acquisition and preparation (Jarret, 1986).

As a result of the high specificity of DNA, molecular markers are able to identify a particular fragment of DNA sequence that is associated to a part of the genome and comparisons are usually made on the basis of the presence or absence of a DNA band. Besides, the use of isozymes which are relatively quicker and cheaper, direct DNA sequencing, single nucleotide polymorphisms and microsatellites are now available for more informative marker systems. RFLPs and isozymes share common advantages over morphological markers such as co-dominance, absence of pleiotropic effects and Mendelian inheritance (Havey and Muehlbauer, 1989). The underlining principles, similarity and differences as well as strength and limitations of the various molecular detection methods are briefly discussed below.

2.6.2.1 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism is a technique used for genome analysis of organisms, thereby providing a molecular basis for any observed differences. It involves the digestion of the crude DNA of the organism with restriction endonuclease. Restriction endonucleases are enzymes produced by a

variety of prokaryotes and are naturally used to destroy invading foreign DNA molecules by recognizing and cutting specific DNA sequence motifs (Weising *et al.*, 2005). Endonuclease recognition sites are usually 4–6 base pairs in length and the shorter the recognition sequence, the greater the number of resultant fragments. In RFLP, extracted DNA is digested with restriction enzymes and the resultant fragments are separated by gel electrophoresis according to their sizes. Molecular variations in the organisms are revealed by the resultant length of the fragments after digesting with the restriction enzymes. This differential profile is generated due to nucleotide substitutions or DNA rearrangements like insertion or deletion or single nucleotide polymorphisms (Agarwal *et al.*, 2008).

RFLP was one of the first techniques used to study somaclonal variation and has been widely used in several species. For example, Jaligot *et al.* (2002) described methylation-sensitive RFLP markers that differentiated between normal and abnormal embryogenic calli of oil palm. The markers were useful for the early detection of somaclonal variation. Generally, RFLP markers are relatively highly polymorphic, co-dominantly inherited and highly reproducible (Agarwal *et al.*, 2008). Although RFLP markers are useful for sampling various regions of the genome and are potentially unlimited, the technique is time consuming, costly and a large amount of plant tissue is required for analyses (Piola *et al.*, 1999).

In addition, it involves the use of radioactive/toxic reagents and is technically demanding. For example, it requires the development of cDNA or genomic DNA probes when heterologous probes are unavailable. These limitations led to the development of new set of less technically complex methods which are Polymerase chain reaction (PCR)-based.

2.6.2.2 Polymerase Chain Reaction based techniques

Polymerase chain reaction was invented by Mullis and coworkers in 1983, and is based on the enzymatic *in vitro* amplification of DNA (Weising *et al.*, 2005). In PCR, a DNA sequence of interest is exponentially amplified with the aid of primers and a thermostable DNA polymerase. The reaction involves repeated cycles, each consisting of denaturation, primer annealing and elongation steps. Usage of random primers eradicated the limitation of prior sequence for PCR analysis (Agarwal *et al.*, 2008). PCR based analytical techniques using various molecular markers provide an essential tool needed to reveal polymorphism at the DNA sequence level and solve the problems of introgression as well as lineage (Simmons *et al.*, 2007).

2.6.2.3 Random Amplified Polymorphic DNA

Random amplified polymorphic DNA involves the use of single short primers of arbitrary nucleotide sequence to reproducibly amplify segments of target genomic DNA. These short primers referred to as genetic markers are used to reveal polymorphisms among the amplification products which are seen as visible bands with the aid of ethidium bromide-stained agarose gel electrophoresis (Williams *et al.*, 1990).

Arbitrary primed PCR (AP-PCR), arbitrary amplified DNA (AAD) and DNA amplification fingerprinting (DAF) are other variants of random amplified polymorphic DNA (RAPD). For example, in AP-PCR, a single primer (10–15 nucleotides long) is used and involves amplification for initial two cycles at low stringency. Subsequently, the remaining cycles are performed at higher stringency by increasing the annealing temperature (Welsh and Mcclelland, 1990). Although AP-PCR was not widely accepted because it involves the use of autoradiography, it has been simplified and fragments can now be fractionated with the use of agarose gel electrophoresis (Agarwal *et al.*, 2008). With the DAF technique, shorter single

arbitrary primers (less than 10 nucleotides) is used for amplification and the fragments are analysed using polyacrylamide gel coated with silver staining (Caetano-Anolles and Bassam, 1993). Technically, RAPD has been described as the simplest version of PCR with arbitrary primers used for detecting DNA variation and for convenience, all RAPD variants are commonly referred to as RAPD (Weising *et al.*, 2005).

Rout *et al.* (1998) used RAPD marker to evaluate the genetic stability of micropropagated plants of Zingiber officinales cv. V3S18. Fifteen arbitrary decamers were used to amplify DNA from in vivo and *in vitro* plant material to assess the genetic fidelity. All RAPD profiles from micropropagated plants were monomorphic and similar to those of field grown control plants. No variation was detected within the micropropagated plants.

An efficient micropropagation protocol produced large number of plants of the three elite banana (Musa spp.) cultivars Robusta (AAA), Giant Governor (AAA) and Martaman (AAB) from shoot tip meristem. The genetic relationships and fidelity among the cultivars and micropropagated plants was assessed by random amplified polymorphic DNA (Ray *et al.*, 2006).

Lattoo *et al.* (2006) assessed the genetic fidelity of *in vitro* derived plants of an endangered medicinal herb Chlorophytum arundinaceum Baker. A study was conducted to assess genetic relationships among 'Sukali Ndizi' clones collected from 16 different localities in Uganda using RAPD (Pillay *et al.*, 2003).

Twelve decamer random amplified polymorphic DNA (RAPD) primers were used to study somaclonal variation among micropropagated *Phalaenopsis bellina* (Khoddamzadeh *et al.*, 2010)

Banana plantlets were analyzed for their genetic stability using randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR)

markers. A total of 50 RAPD and 12 ISSR primers resulted in 625 distinct and reproducible bands showing homogeneous RAPD and ISSR patterns. Band intensity histogram of each gel confirmed their monomorphic nature with no genetic variation among the plantlets analyzed (Venkatachalam *et al*, 2007)

Genetic variations and relationships among 21 commercially important banana cultivars of South India were evaluated using 50 decamer RAPD primers and 12 ISSR primers. The primers were selected after a preliminary screening of several such primers for their ability to produce clear and reproducible patterns of multiple bands. The analyses resulted in the amplification of totally 641 bands of 200– 3100 bp, of which 382 bands were polymorphic, corresponding to nearly 60% genetic diversity. The RAPD and ISSR surveys between pairs of 21 cultivars revealed 60.15% and 56.73% of polymorphic bands, respectively (Venkatachalam *et al.*, 2008).

2.6.2.4 Inter Simple Sequence Repeats (ISSR)

Many molecular marker techniques are available today. PCR-based approaches are useful because of their simplicity and requirement for only small quantities of sample DNA. The choice of a molecular marker technique depends on its reproducibility and simplicity. ISSRs are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Amplification in the presence of nonanchored primers also has been called microsatellite-primed PCR, or MP-PCR, (Meyer *et al.*, 1993). Such amplification does not require genome sequence information and leads to multilocus and highly polymorphous patterns (Zietkiewicz *et al.*, 1994 and Tsumara *et al.*, 1996). Each band corresponds to a DNA sequence delimited by two inverted microsatellites. Like RAPDs, ISSRs markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers.

ISSR markers linked to the traits of agronomic importance have been sequenced and used as STS markers in markers aided selection. An attractive possibility is thus the use of ISSR as probes for *in-situ* hybridization for physical mapping of homologous chromosome sites (Pasakinsene *et al.*, 2000). Another advantage in the use of ISSR markers lies in their linkage to SSR loci. Although micro satellites themselves are probably non functional and selectively neutral, they are known to be linked to coding regions, so that ISSRs are likely to be linked to coding region, and hence ISSRs are likely to mark gene rich regions (Kojima *et al.*, 1998).

ISSR markers are effective multilocus markers for applications such as diversity analysis, fingerprinting and genome mapping, gene tagging and marker assisted selection, phylogeny studies, and crop conservation. As no prior sequence knowledge is required, they are more rapidly applied than SSR markers, and they are more reliable and robust than RAPD markers, mainly due to the method of detection, and possibly also to the fact that primers are longer, and hence PCR condition are more stringent (Zietkiewicz *et al.*, 1994).

Racharak and Eiadthong, (2007) selected ISSR marker to investigate the genetic relationship among subspecies of *Musa acuminate* and A-genome consisting of cultivated bananas and to examine the genetic variation of subspecies of *M. acuminata* that are naturally distributed in Thailand. Sasikumar and Syamkumar, (2007) developed molecular genetic fingerprints of 15 *Curcuma* species using ISSR and RAPD markers to elucidate the genetic diversity/relatedness among the species.

Chandrika *et al.*, (2008) demonstrated that ISSR markers could be applied to evaluate the genetic stability of regenerants of *in vitro* grown *Dictyospermum ovalifolium* for the conservation of genetic resources of Western Ghats of India. Bhatia *et al.*, (2009) assessed the clonal fidelity of micropropagated gerbera plants by ISSR markers. The clones derived from capitulum and shoot tip explants did not

show any genetic variation, whereas, one of the leaf-derived clones exhibited some degree of variation.

Chandrika and Rai, (2009) determined genetic fidelity in micropropagated plantlets of *Ochreinauclea missionis* an endemic; threatened and medicinal tree using ISSR markers and it proved to be a reliable method for assessing genetic stability of micropropagated plants. The developed *in vitro* plants which ascertained stability could be reintroduced back to its original habitat for conservation purpose.

Poerba and Ahmad, (2010) studied the genetic variability among 18 cultivars of cooking bananas and plantains by RAPD and ISSR markers. RAPD primers produced 63 amplified fragments varying from 250 to 2500 bp in size and 96.82 per cent polymorphic bands. ISSR primers produced 26 amplified fragments varying from 350 bp to 2000 bp in size and 92.86 per cent polymorphic bands. The range of genetic distance of 18 cultivars was from 0.06-0.67.

Lu *et al.,* (2011) revealed the molecular assessment of genetic identity and genetic stability in banana cultivars (*Musa* spp.) from China using ISSR markers. Pestanana *et al.,* (2011) were the first to use combined data of agronomic and molecular characterization to evaluate the genetic variability of gamma ray induced banana (*Musa* AAB sp.) mutants using a multivariate statistical algorithm.

2.6.2.5 Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism is a PCR-based tool used in genetics research, DNA fingerprinting, and in the practice of genetic engineering. It was developed in the early 1990s to overcome the limitation of reproducibility associated with RAPD. Theoretically, AFLP represent the ingenious combination power of RFLP and flexibility of PCR-based technology (Agarwal *et al.,* 2008). It provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity. In addition, cDNA AFLP and three endonuclease AFLP (TE-

AFLP) which are used to quantify differences in gene expression levels and to detect transposable element mobility respectively are variations of AFLP technique (Weising *et al.*, 2005).

In AFLP-PCR, genomic DNA is digested using two restriction endonucleases, typically one with a 6-bp recognition sequence (usually *Eco*RI) and the other with a 4-bp recognition sequence (usually *Mse*l). Thereafter, adapters of known sequence are ligated to complementary double stranded adaptors of the ends of the restriction fragments. A subset of the restriction fragments are then amplified using two primers complementary to the adaptor and restriction site fragments for two successive rounds of selective PCR amplification. The first round of PCR (preselective amplification) uses primers that match the adapters on the EcoRI end and Msel end of the fragments plus one extra nucleotide. The second round (selective amplification) has an additional two nucleotides. These rounds of selective amplification reduce the resulting pool of DNA fragments to a size more manageable for analysis (Vos *et al.*, 1995). The fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies (Weising *et al.*, 2005). This shows that AFLP is a very sensitive and reliable marker technique that could be useful for detecting specific genomic alterations associated with tissue culture variation and identifying slightly different genotypes.

AFLP analysis was performed in order to assess DNA variation among Arabidopsis thaliana plants regenerated by organogenesis from roots. Fifty-one regenerated plants were subjected to AFLP analysis using 12 primer combinations. The plants were obtained from root explants excised from five different seedlings. From the studied plants, 66.6% showed at least one variation when compared against the regenerated plants obtained from the same explant (Polanco and Ruiz, 2002).

Vendrame *et al.*, (1999) evaluated the applicability of AFLP analysis for the assessment of genetic variability in somatic embryos of pecan [*Carya illinoinensis* (Wangenh.) C. Koch] and made comparisons between and within embryogenic culture lines. AFLP readily detected differences between culture lines, with 368 polymorphic loci identified.

The genetic diversity of Musa balbisiana was assessed by the amplified fragment length polymorphism (AFLP) fingerprinting in 15 populations of China. Four primer pairs produced 199 discernible loci. High levels of genetic diversity were detected. (Wang *et al.*, 2007)

Vroh-Bi *et al.* (2011) used AFLP to analyze accessions of the AA, BB, AB, AAA, AAB, and ABB groups of *Musa*, and polymorphic regions were sequenced to characterize candidate genes.

2.6.2.6 Microsatellite markers

In contrast to all the PCR-based techniques explained above which are arbitrarily primed or non-specific, microsatellite-based marker techniques are sequence targeted. They are also known as; simple sequence repeats (SSRs), short tandem repeats (STRs), sequence-tagged microsatellite sites (STMS) and simple sequence length polymorphisms (SSLP) (Hautea *et al.*, 2004). Microsatellites consist of tandemly reiterated short DNA (one to five) sequence motifs which are abundant and occur as interspersed repetitive elements in all eukaryotic (Tautz and Renz, 1984) as well as in many prokaryote genomes (Van Belkum *et al.*, 1998). Microsatellite marker techniques use the intra- and inter-individual variation in microsatellites or simple sequence repeat regions for fingerprinting analyses (Agarwal *et al.*, 2008). Polymorphism results from differences in the number of repeat units between individuals at a particular microsatellite locus and is believed to be due to unequal crossing over or slippage of DNA polymerase during replication of repeat tracts (Levinson and Gutman, 1987; Coggins and O'prey, 1989).

However, variation in the number of tandemly repeated units has been reported to be mainly due to strand slippage during DNA replication where the repeats allow matching via excision or addition of repeats (Schlotterer and Tautz, 1992). As slippage in replication is more likely than point mutations, microsatellite loci tend to be hypervariable and are implicated for the extensive interindividual length polymorphisms observed in microsatellite assays (Agarwal *et al.*, 2008).

Most importantly, studies have showed that the PCR-amplified microsatellite markers are inherited in a Mendelian fashion (Litt and Luty, 1989). For the microsatellite assay, the sequence information of repeat-flanking regions is used to design locus-specific PCR primer pairs and amplified. These PCR loci-specific primers are either unlabelled primer pairs or primer pairs with one radio labelled or fluoro-labelled primer. Subsequently, amplified PCR products are separated on denaturing polyacrylamide gel and visualized by autoradiography, fluorometry or staining with silver or ethidium bromide (Weising *et al.*, 2005). Although relatively expensive, the use of fluorescent labelled microsatellite primers and laser detection in genotyping procedures has significantly improved the throughput and automation of this technique (Wenz *et al.*, 1998).

Microsatellite markers have been found immensely useful in establishing genetic stability of several micropropagated plants such as sorghum (Zhang *et al.*, 2010), trembling aspen (Rahman and Rajora 2001), bananas (Hautea *et al.*, 2004; Ray *et al.*, 2006). grapevine (Welter *et al.*, 2007), wheat (Khlestkina *et al.*, 2010) and sugarcane (Singh *et al.*, 2008). In comparison to AFLP and RFLP, the microsatellite marker technique such as ISSR is cost efficient, overcomes hazards of radioactivity and requires lesser amounts of DNA (Zietkiewicz *et al.*, 1994).

In addition, these markers are highly reproducible and becoming more popular due to their co-dominant inheritance nature, high abundance in organisms, enormous extent of allelic diversity as well as the ease of assessing microsatellite size

variation using PCR with pairs of flanking primers (Agarwal *et al.*, 2008). However, a major drawback for the use of microsatellites is that the development of primers is time-consuming (Squirrell *et al.*, 2003).

2.7 Epigenetics

Epigenetics is a relatively new field of molecular biology dealing with regulatory mechanisms of gene activity and inheritance that are independent of changes in the nucleotide sequence of DNA. Studies of recent years have shown that gene expression changes during differentiation and these changes are transmitted to daughter cells through mitosis. Similarly, changes in gene expression arising during ontogeny can be inherited through generations i.e., transmitted through meiosis. In both cases these changes are not related with changes in DNA sequence. Thus, epigenetics includes two interrelated fields. One investigates hereditable mechanisms in regulation of gene expression during development, and the other elucidates mechanisms of hereditary transmission of this regulation for individual genes by germ line cells. In both cases the hereditable mechanisms that determine gene expression are not related with changes in the DNA text. (Tchurikov, 2005)

The term "epigenetics" has had multiple definitions which have generated confusion regarding its use and meaning. "Epigenetics" was first used by C. H. Waddington in 1942 to describe the genetics of epigenesis, the differentiation of cells from their initial totipotent state in embryonic development. Since Waddington created the term before the physical nature of genes was understood to be transmitted by DNA, the term was initially a conceptual model of how genes might interact to produce a phenotype. Later, epigenetics began to describe the mechanisms of temporal and spatial control of gene activity during the development and "epigenetics" began to imply any aspect other than the DNA sequence *per se* that influenced an organism's development. Thus, the current meaning of epigenetics implies heritable changes to gene expression patterns due to modifications acting

upon the DNA that may be potentially reversible, yet are independent of genetic mutations or DNA sequence changes (Holliday, 2006)

Epigenetic modifications affect not only DNA (*e.g.* cytosine methylation) but also include modifications of the nucleosome, an octamer formed by a central H3/H4 tetramer between two H2A/H2B dimers and wrapped by approximately 150 basepairs (bp) of DNA which is the core subunit of chromatin (Jiang and Pugh, 2009). Once stabilized, nucleosomes are organized into chromatin fibers, referring to the combination of DNA, RNA, and protein that together eventually comprise the chromosome. The contrasting states of euchromatin and heterochromatin have been classically described "active" and "silent" chromosomal regions, respectively, and are generally associated with specific combinations of epigenetic modifications (Fransz *et al.*, 2003).

2.7.1 Methylation Sensitive Amplification Polymorphism (MSAP)

The methylation-sensitive amplification polymorphism (MSAP) technique is a relatively new and modification of the amplified fragment length polymorphism (AFLP) technique. It was first developed to determine DNA methylation events in dimorphic fungi and later adapted for the detection of cytosine methylation in the rice genome. The MSAP technique utilizes the restriction isoschizomer pair *Msp* I and *Hpa* II (instead of *Mse* I as in the original protocol) whose ability to cleave at the sequence 5-CCGG-3 is affected by the methylation state of the external or internal cytosine residues. *Hpa* II is inactive if one or both cytosines are fully methylated (both strands methylated) but cleaves the hemimethylated sequence (only one DNA strand methylated), whereas Msp I cleaves CmCGG but not mCCGG.

DNA methylation, especially methylation of cytosine in eukaryotic organisms, has received considerable attention in recent years. In animals and human beings, numerous studies suggest that DNA methylation has both epigenetic and mutagenic effect on various cellular activities such as differential gene expression,

cell differentiation, chromatin inactivation, genomic imprinting and carcinogenesis (Gonzalgo and Jones, 1997).

In higher plants, DNA methylation plays a role in gene expression (Meyer *et al.*, 1994) as actively transcribed sequences are more often less methylated than the promoters and certain coding regions of silent genes (Finnegan *et al.*, 1993). DNA methylation plays an essential role in regulating plant development. It has been demonstrated that DNA methylation plays an integral role in timing of flowering and in endosperm development in Arabidopsis (Finnegan *et al.*, 2000). Significant differences in the level of cytosine methylation have been observed among various tissue types in some plant species such as tomato (Messeguer *et al.*, 1991), maize (Lund *et al.*, 1995) and rice (Dhar *et al.*, 1990).

DNA methylation is found to be associated with induction of mutation and novel genetic variation (Rasmusson and Phillips, 1997), leading to the development of a separate biological field called 'epigenetics', which is based on the changes in DNA other than the changes in its nucleotide sequence (Holliday, 2002).

In the past, nucleotide modified by methylation was not considered to be part of primary nucleotide sequence of an individual. However, since DNA methylation occurs at defined target sequences and not all target sites are methylated, it represents a potentially important form of polymorphism. In this way, epigenetic information systems, like DNA methylation, could generate epigenetic variation that had never been considered as the cause of phenotypic variation (Tsaftaris and Polidoros, 1993). DNA methylation, in addition to being the cause of epigenetic variation, is also cause of mutation and generation of genetic variation. Methylated cytosine frequently deaminates to thiamine, thus 5 methyl cytosines are hot spots for mutations, providing an interconnection between epigenetic and genetic variation.

Portis *et al.* (2003) studied the analysis of cytosine methylation during pepper seed germination using an adaptation of the AFLP technique called methylation-sensitive amplified polymorphism (MSAP). Notable changes in MSAP profiles of genomic DNA obtained from embryo tissues of dry seeds and germinating seeds were detected. The changes were mainly: (i) fragments not detected in dry seeds were present after digestion with both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I at a certain stage during germination; (ii) fragments present after both digestions in dry seeds were no longer detected upon germination.

Bardini *et al.* (2003) carried out a study to to determine the involvement of the antibiotic kanamycin, commonly used as a selective agent in transformation protocols, in the phenomenon of somaclonal variation. The approach used was MSAP to assess methylation changes at CCGG sequences. They found that use of kanamycin as a selective agent caused extensive methylation changes in the genome with both hyper and hypomethylation events.

Guo *et al.* (2007) investigated possible alterations in level and pattern of cytosine methylation at the CCGG sites in the same set of regenerants relative to the donor plant, by the MSAP method. A total of 1,674 MSAP profiles were resolved using 39 primer combinations. Of these, 177 (10.5%) profiles were polymorphic among the regenerants and/or between the regenerant(s) and the donor plant, in *Eco*RI + *Hpa*II or *Eco*RI + *Msp*I digest but not in both, indicating alteration in cytosine methylation patterns of specific loci.

Gao *et al.* (2010) assessed the genetic and epigenetic stability in regenerated plantlets of Freesia hybrid. MSAP analysis revealed that tissue culturing of the flower, induced DNA cytosine methylation alterations in both CG and CNG levels and patterns compared with the donor plant. The variation rate was 1.1 and 1.3 per cent for the direct and indirect embryogenesis pathways, respectively. The findings

show that tissue culture of flowering plants is a form of stress which can induce some heritable epigenetic variations.

Li *et al.* (2007) found that the epigenetic diversity among the individual genotypes of wild barley *spp.* based on the MSAP data was higher than the genetic diversity based on the other marker data.

Chakrabarty *et al.* (2003) assessed the extent and pattern of cytosine methylation during somatic embryogenesis in *Eleuterococcus senticosus* , using 12 pairs of MSAP primers and they found that 16.99 per cent of 5'-CCGG-3' sites in the genome of non-embryogenic callus were cytosine methylated, where as 11.20 per cent were methylated in case of embryogenic callus tissue. Hypermethylation of DNA in non-embryogenic callus compared with embryogenic callus reflects the marked expression of this molecular feature, which may well contribute to the developmental gene expression.

Yanli *et al.* (2008) adopted MSAP to compare the levels of DNA cytosine methylation at CCGG sites in tassel, bracteal leaf and ear leaf from maize inbred lines, 18 White and 18 Red, respectively and also examined specific methylation patterns of the three tissues. The results were like methylation level were the highest in bracteal leaf, the lowest in tassel and full methylation of internal cytosine was the dominant type in the maize genome. These results clearly demonstrated the power of the MSAP technique for large-scale DNA methylation detection in the maize genome and the complexity of DNA methylation change during plant growth and development.

MSAP analysis was used to investigate the genome of two sibling tobacco cultivars, Yunyan85 and Yunyan87, their parent K326. However, the methylation extent had different alteration between the sibling tobacco cultivars relative to their parent; it rose to 42.97 and 36.55 per cent in Yunyan85 and Yunyan87, respectively (Yang *et al.*, 2010).

MSAP analysis revealed the differences in the DNA methylation patterns in the normal and variant *Doritaenopsis* plants which were correlated with phenotypic variation. Using 12 combination of primers, a total of 36 (11.6%), 77 (22.9%), 73 (19.9%), and 47 (13.7%) sites were found to be methylated at cytosine in the genomes of a normal and three variant plants (Park *et al.*, 2009).

Yi (2010) carried out a study to detect cause of phenotypic variation of Jatropha population in China using the methylation sensitive florescence AFLP (MfAFLP) method. However their results suggested possible involvement of epigenetics in jatropha development.

Qi *et al.* (2010) investigated genetic and epigenetic changes from a genome-wide perspective by using 43 pairs of selective primers of MSAP marker in four sets of newly synthesized allotetraploid wheat lines. They suggested utility of MSAP to detect significant epigenetic variation among allotetraploid wheat lines.

MSAP technique has been employed on somatic embryo-derived oil palms (*Elaeis guineensis* Jacq.) to identify methylation polymorphisms correlated with the "mantled" somaclonal variation. Overall 64 selective primer combinations were used and they have amplified 23 markers exhibiting a differential methylation pattern between the two phenotypes (Jaligot *et al.*, 2002)

The genetic and epigenetic stability of the *in vitro* hop plants was analyzed with MSAP technique. No genetic variation among control and treated plants was found, even after 12 cycles of micropropagation. Epigenetic variation was detected, first, when field and *in vitro* samples were compared (Peredo *et al.*, 2009)

MSAP technique was adapted to screen a photoperiod responsive gene of rice KDML105. Six out of thirty-two primer combinations gave twelve *Eco*RI/*Hpa*II generated MSAP markers from the genomic DNA of KDML105 after exposure to short-day (SD) photoperiod. These MSAP fragments were cloned and used as probes

to hybridize to MSAP fingerprints. Positive fragments generating the signals correlated to the MSAP fingerprints were sequenced and aligned to the database. Ten out of twelve MSAP markers showed 93 to 100 per cent homology to database sequences and the best homology fragment (F1) was chosen for complete gene cloning, sequencing and alignment (Thanananta *et al.*, 2006).

Wang *et al.* (2009) studied the genome structure changes and DNA methylation adjustments at CCGG sites in a genome-wide level were analyzed in the three different autoploidy watermelons by the ISSR and MSAP methods.

2.7.1.1 MSAP analysis in banana

Two amplified fragment length polymorphism (AFLP)-derived techniques were Both the amplified fragment length polymorphism (AFLP) and the methylation-sensitive amplification polymorphism (MSAP) DNA fingerprinting techniques were used to evaluate the extent of DNA polymorphisms in both conventionally propagated and micropropagated 'Cavendish' banana plants. Both techniques detected significant levels of DNA polymorphisms in micropropagated banana plants whereas no polymorphisms could be detected in banana plants propagated using the conventional technique of corm division. Using both AFLP and MSAP techniques it was found that the extent of DNA polymorphisms was higher in micropropagated banana plants derived from male floral apical explants than in plants regenerated from vegetative apical explants (suckers). Global differences in methylation patterns were found between micro- and conventionaly-propagated plants. Micropropagated plants were found to be relatively hypermethylated when compared to those propagated by conventional means (James *et al.*, 2000).

MSAP were used in banana to survey cytosine methylation status at CCGG sites in order to obtain an alternative source of diversity data. A higher degree of polymorphism was revealed allowing the classification of the samples into three

clusters. No correlation was observed between the phenotypic classification and methylation diversity (Noyer *et al.*, 2005).

The MSAP technique was adapted in this study for the detection of differential cytosine methylation in the dwarf banana 'Curare enano' compared with its normal-sized *in vitro*-generated off-type. Results show typical patterns containing about 100 nicely separated fragments (Engelborghs *et al.*, 1999).

The extent of DNA methylation polymorphism was evaluated in micropropagated banana (*Musa* AAA cv. 'Grand Naine') derived from either the vegetative apex of the sucker or the floral apex of the male inflorescence using MSAP technique. In all, 465 fragments, each representing a recognition site cleaved by either or both of the isoschizomers were amplified using eight combinations of primers. A total of 107 sites (23%) were found to be methylated at cytosine in the genome of micropropagated banana plants. In plants micropropagated from the male inflorescence explant 14 (3%) DNA methylation events were polymorphic, while plants micropropagated from the sucker explant produced 8 (1.7%) polymorphisms. No DNA methylation polymorphisms were detected in conventionally propagated banana plants. These results demonstrated the usefulness of MSAP to detect DNA methylation events in micropropagated banana plants and indicate that DNA methylation polymorphisms are associated with micropropagation (Peraza-Echeverria *et al.*, 2001).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Morphological and molecular analysis of genetic stability in micropropagated banana (*Musa* spp) var. Nendran" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, during the period from 2010 to 2012, with the objective of evaluation and characterization of the variation in tissue culture derived banana plants regenerated in different subcultures through *in vitro* organogenesis. Description of the materials used and the methodology adopted in the study has been furnished in this chapter.

3.1 Chemicals, Glassware and Plastic Wares

The chemicals used for the present study were of good quality (AR grade) from various agencies including MERCK, SRL and HIMEDIA. Molecular biology enzymes and buffers were supplied by Bangalore GeNei Ltd. All the plastic wares used were obtained from Axygen and Tarson India Ltd. ISSR primers were obtained from Sigma Aldrich Chemical Pvt. Ltd. and MSAP reagents and primers were obtained from Promega Corporation, Fitchburg (USA).

3.1.1 Equipment and Machinery

The present research work was carried out using the molecular biology facilities and equipment items available at CPBMB, College of Horticulture. Quantification of DNA was done by NanoDrop^R ND-1000 spectrophotometer. The PCR was carried out in the thermal cycler of model Eppendorf Master Cycler (Eppendorf, USA) and the horizontal gel electrophoresis system (BIO-RAD, USA) was used for agarose gel electrophoresis. Gel Doc- BIO-RAD was used for imaging and documenting the agarose gel. Acrylamide and bis-acrylamide were obtained from Merck & Co., Inc. (USA). The details are given in Annexure I.

3.2 Preparation of MS medium

Standard procedures were followed for the preparation of plant tissue culture media. Stock solutions of major and minor nutrients were prepared and stored in pre cleaned glass bottles in refrigerated conditions. Stock III was stored in amber colored bottle.

A clean glass vessel, rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volume in the vessel. For preparing media of full strength, 20 ml pipetted from 50 X stocks and 10 ml from 100 X stocks. A small volume of distilled water was added to it and later on, required quantities of sucrose and inositol were added and dissolved in it. 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.

Agar was added as solidifying agent at 0.75 per cent (w/v) concentration, after adjusting pH. The medium was stirred and heated to melt the agar; poured when hot into culture vessels and were plugged with absorbent cotton. For solid media, test tubes (15 cm x 2.5 cm) and conical flasks (100, 250, 500 ml) were used as culture vessels. Vessels containing the media were sterilized in an autoclave at 121°C in 15 psi for 20 min. The medium was allowed to cool to room temperature and stored in culture room until used.

3.2.1 Growth regulators

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

3.2.2 Antibiotics

The stock solutions of the antibiotics were prepared freshly under sterile conditions. Aliquots were taken from them and were added to sterilized media. The solid media was first melted, cooled to 40°C and then desired quantities of antibiotics were added to them. Only cefotaxim was used in the MS medium for killing the bacteria.

3.2.3 Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants, subsequent subculturing and preparation of antibiotic media were all carried out under laminar air flow cabinet.

3.2.4 Culture room

The cultures were incubated at 26 ± 2°C in an air conditioned culture room with 16 hrs light photoperiod (1000 lux) from florescent tubes. Humidity in the culture room varied from 60 to 80 per cent according to the climate prevailing.

3.3 *In vitro* multiplication

In vitro multiplication of banana was done using male bud and sucker as explant source.

3.3.1 Source of the explants

Explants were collected from the stock plants planted in the backyard of the Centre for Plant Biotechnology and Molecular Biology (CPBMB). Male bud and sucker were used as explant source for the study.

3.3.2 Preparation of explants and culture establishment

Surface sterilization of explant was done in order to make the explants free of microorganisms. Surface sterilization was carried out under aseptic conditions in a laminar airflow cabinet. The outer sheaths of male bud and sucker were peeled out to get the inner core (3 to 5 cm size) as explant. The explants were sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) for 10 min. For the sucker explants bavistin treatment (15 min) was given in order to remove fungal contamination. After the surface sterilization the solution was drained off, explants were washed for four times using sterile distilled water. Then explants were drained carefully by transferring them onto filter paper pieces on a sterile petridish.

The media used of the culture establishment of male bud explants was:

½ MS, 2, 4-D (3 mg l^{-1}), NAA (1 mg l^{-1}), IAA (1 mg l^{-1}), Biotin (1 mg l^{-1}), Sucrose (3%), Phytagel (0.2 %).

The media used for the culture establishment of sucker derived explant was

Full MS, BA (5 mg l^{-1}), Sucrose (3%), Agar (0.75%).

The chemical compositions of the Murashige and Skoog (MS) medium are given in Annexure II.

The cultures of banana using male bud and sucker as explant source were already established in the tissue culture laboratory of the Centre for Plant Biotechnology and Molecular Biology (CPBMB). Those established cultures which have started producing multiple shoots were used for subsequent subculturing for the study.

3.3.3 Propagule multiplication

Multiplication media standardized earlier (Amin *et al.*, 2009) were used without any further modifications.

The composition of the multiplication media used was

Full MS, Adenine sulphate (3 mg l^{-1}), BA (5 mg l^{-1}), Sucrose (3%), Agar (0.75%).

Regular subculturing was followed at 25 days interval. During the sub culturing of the banana cultures; rooting and plant out was followed only for the subculture number 3, 6, 8, 10, 12, 14 and 16 as envisioned in the research programme.

3.3.4 Rooting

The shoots obtained from multiplication media were excised using sterile blade and inoculated in the following media for rooting.

½ MS, NAA (2 mg l^{-1}), Charcoal (0.25 %), Sucrose (3%), Agar (0.75%).

Earlier standardized rooting media composition (Amin *et al.*, 2009) were used without any further modifications.

3.3.5 Hardening

The *in vitro* rooted plantlets were taken out of the culture vessels using forceps, after soaking the media in water for five minutes. The solidified media from the plantlets was washed out under running tap water. The plantlets were then planted in small earthen pots filled with sterilized sand and kept in net house. After one month plants were transferred to large polythene bags containing potting mixture.

3.3.6 Field planting

After two months of hardening in net house plants were transferred to the field. Field was prepared by digging the pits of recommended size (50 x 50 x 50 cm). Spacing allowed between two plants was 2 x 2 m. Eight tissue cultured banana plantlets of each subculture specified were planted in each row. While planting carbofuran was applied in soil surrounding the plantlet. Male bud derived plants were planted from February 2011 onwards and the sucker derived plants were planted from September 2011 onwards. All the plants were maintained as per package of practices, Kerala Agricultural University (KAU, 2010).

3.4. Morphological characterization

Morphological observations were recorded at two months after plantout; four and eight months after field planting for both sucker and male bud derived plants. Out of eight plants per row five plants were subjected for morphological characterization. The major morphological characters for which observations were taken include:

3.4.1 Vegetative characters

1. **Plant height (cm)**

   Recorded from the base of pseudostem to the base of the last leaf

2. **Number of leaves**

   All green leaves persisting except the young folded one were recorded.

3. **Leaf length and breadth (cm)**

   Recorded for the third, fully unfolded leaf counting down from the top of the plant.

4. **Girth of the plant (cm)**

Circumference of the psedostem one meter above the ground level.

3.4.2. Fruit characteristics

The following elements were recorded for the bunches obtained during the course of the study.

Total weight of the bunch (Kg), number of hands , number of fruits per hand, weight of each hand (Kg), weight of single fruit (Kg), size of single fruit (cm) and the Total Soluble Solids (TSS °Bx).

3.4.3 Morphological data analysis

Statistical analysis of entire morphological data recorded was done by Duncun's test

3.4.3.1 Duncun's test:

All values are mean±S.E.M. obtained from morphological data from different subcultures. For statistical analysis, one-way ANOVA with Duncan's variance (SPSS 17) was used to compare the groups. In all the cases a difference was considered significant when p was <0.05.

3.5 Molecular analysis

Molecular analysis of plants derived from different subcultures of indirect and direct organogenesis was carried out with two molecular markers ISSR and MSAP.

3.5.1 Genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important pre requisites for doing ISSR and MSAP analysis. The CTAB procedure reported by Rogers and Bendich (1994) for the extraction of nucleic acids was used for the isolation of genomic DNA from tissue culture derived banana plants. The young

unfurled leaves from healthy plants were collected early in the morning and used for the genomic DNA isolation.

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

1. 2 X CTAB Extraction buffer
2. CTAB solution (10 per cent)
3. β- mercaptoethanol
4. Chloroform: Isoamyl alcohol (24:1, v/v)
5. Isopropanol (chilled)
6. Ethanol (70 per cent)
7. TE buffer

3.5.1.1 Protocol

Young and tender leaf tissue (1g) was weighed and ground in liquid nitrogen using mortar and pestle along with 50 µl of ß- mercaptoethanol and a pinch of Poly Vinyl Pyrrolidone (PVP). The sample was ground into fine powder using excess of liquid nitrogen, 4ml of extraction buffer (2x) and the mixture was transferred to a sterile 50 ml centrifuge tube containing 3 ml of pre-warmed extraction buffer (total 7 ml). The homogenate was incubated for 30 minutes at 65° C with intermittent mixing. Equal volumes of chloroform and isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 15 minutes at 4°C. The contents got separated into three distinct phases. The aqueous phase containing DNA was pipetted out into a fresh 50 ml Oakridge tube. One tenth volume of 10 per cent CTAB solution was added followed by equal volume of chloroform: isoamyl alcohol (24:1) mixture and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was collected, 0.6 volume of chilled isopropanol was added and incubated at -20 °C for 30 minutes. The

precipitated DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4 °C. The pellet was collected and washed first with 70 per cent alcohol. It was air dried for 30 minutes at room temperature and dissolved in 100 µl of TE buffer.

3.5.2 DNA Purification

The DNA isolated would contain RNA as contaminant and hence was purified by phenol precipitation and RNase treatment (Sambrook *et al.*, 1989).

Reagents

1. Phenol : chloroform mixture (1:1,v/v)
2. Chloroform: Isoamyl alcohol (24:1,v/v)
3. Chilled isopropanol
4. 70% Ethanol
5. TE buffer
6. 1% RNase

The RNase- A from Sigma, USA was used to prepare RNase. One per cent solution was prepared by dissolving RNase- A in TE buffer at 100 ^{0}C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20 ^{0}C. The procedure followed for DNA purification was as follows:

RNase solution (2 µl) was added to 100 µl DNA sample and incubated at 37 ^{0}C in dry bath (GeNei, Thermocon) for 1 hour. The volume was made up to 250 µl with distilled water and equal volumes of phenol: chloroform (1:1) mixture was added. This was then centrifuged at 12,000 rpm for 10 minutes at 4 ^{0}C. The aqueous phase was collected in a fresh micro centrifuge tube and added equal volumes of chloroform: isoamyl alcohol (24:1). Again it was centrifuged at 12,000 rpm for 10

minutes at 4 ^{0}C. The above two steps were repeated and finally DNA was precipitated from the aqueous phase with 0.6 volume of chilled isopropanol. The mixture was then incubated at -20 ^{0}C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4 ^{0}C. The pellet of DNA obtained was washed with 70 per cent ethanol. The pellets were air dried and dissolved in 100 µl TE buffer.

3.5.3 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)

2) 50X TAE buffer (pH 8.0)

3) 6X Loading/ Tracking dye (0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol)

4) Electrophoresis unit, power pack (BIO-RAD), casting tray, comb

5) Ethidium bromide solution (0.5 µg/ml)

6) UV transilluminator (Wealtec)

7) Gel documentation and analysis system (BIO-RAD)

Chemical composition of buffers and dye are given in Annexure IV. The procedure followed for agarose gel electrophoresis was as follows:

1X TAE buffer was prepared from the 50 X TAE stock solutions. Agarose (0.8 per cent) was weighed and dissolved in TAE buffer by boiling. Then ethidium bromide was added at a concentration of 0.5 µg ml^{-1} and mixed well. The open end of gel casting tray was sealed with a cellotape and kept on a horizontal levelled surface.

The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 20-25 minutes after which the comb was removed carefully. The tray was kept in the electrophoresis unit with the well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (4µl) along with the tracking dye (1µl) 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 (100 V) and current (50 A). The power was turned off when the tracking dye reached 2/3rd length of the gel.

Then the gel was taken from the electrophoresis unit and viewed under UV light in a transilluminator. The DNA fluoresces under UV light due to ethidium bromide dye. The quality of DNA was judged by the clarity of DNA band. The image was documented and saved in the gel documentation system.

3.5.4 Assessing the quality and quantity of DNA by NanoDrop method

The quantity and quality of genomic DNA was estimated using Nano Drop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to zero by taking 1µl autoclaved distilled water as blank. One micro litre from each sample was quantified and was measured in ng/µl. The absorbance of nucleic acid samples was measured at a wavelength of 260 nm and 280 nm and OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios were recorded to assess the purity of DNA. A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated good quality DNA. The quantity of DNA in the pure sample was calculated using the formula $OD_{260} = 1$ is equivalent to 50 µg double stranded DNA/ µl sample.

1OD at 260 nm = 50 µg DNA/ml

Therefore OD_{260} X 50 gives the quantity of DNA in µg/ml.

3.6. Molecular marker assay

Inter Simple Sequence Repeats (ISSR) and Methylation-Sensitive Amplification Polymorphism (MSAP) were the two molecular markers used for the study.

3.6.1. Inter Simple Sequence Repeats (ISSR) assay

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

The good quality genomic DNA isolated from leaf samples of the banana plants of selected subcultures was subjected to ISSR assay as per the procedure reported by Zietkiewicz *et al.* (1994). ISSR primers obtained from UBC (University of British Columbia) primer set 100, 9 with good resolving power were used for amplification of DNA. ISSR primers were selected after an initial primer screening.

3.6.1. Screening of ISSR primers

Fourteen ISSR primers screened by Rakeshkumar (2011) were used for this study. Out of fourteen ten good quality primers were selected. List and sequence information of screened and selected ISSR primers is provided in Table 1 and 2.

The amplification reaction was carried out in an Eppendorf Master Cycler (Eppendorf, USA). A master mix without the template DNA was prepared using the reaction mixture for the required number of reactions. From this master mix, 18 µl was pipetted out into each PCR tube and finally 2 µl of template DNA was added. Thus PCR amplification was performed in 20 µl reaction mixture as constituted below:

Table 1. Details of the 14 ISSR primers used for the initial screening

Sl.No.	Primer	Nucleotide sequence (5'–3')
1	UBC 814	$(CT)_8$ A
2	UBC 820	$(GT)_8$ C
3	UBC 834	$(AG)_8$ YT
4	UBC 835	$(AG)_8$ YC
5	UBC 836	$(AG)_8$ YA
6	UBC 843	$(CT)_8$ RA
7	UBC 844	$(CT)_8$ RC
8	UBC 845	$(CT)_8$ RG
9	UBC 848	$(CA)_8$ RG
10	UBC 857	$(AC)_8$ YG
11	UBC 868	$(GAA)_6$
12	UBC 890	VHV $(GT)_7$
13	ISSR 3	$(CT)_8$ TG
14	ISSR 6	$(GT)_6$ CC

Table 2. Details of the primers selected for ISSR assay

Sl.No.	Primer	Nucleotide sequence (5'–3')
1	UBC 814	$(CT)_8$ A
2	UBC 820	$(GT)_8$ C
3	UBC 835	$(AG)_8$ YC
4	UBC 836	$(AG)_8$ YA
5	UBC 843	$(CT)_8$ RA
6	UBC 845	$(CT)_8$ RG
7	UBC 848	$(CA)_8$ RG
8	UBC 868	$(GAA)_6$
9	UBC 890	VHV $(GT)_7$
10	ISSR 3	$(CT)_8$ TG

Note- Single letter abbreviations for mixed base positions

R = (A,.G); Y = (C, T); H = (A, C, T); V = (A, C, G).

Composition of the reaction mixture for PCR (20.0 µl)

a. Genomic DNA (50 ng)	2.0 µl
b. 10X *Taq* buffer B	2.0 µl
c. dNTPs mix (10mM)	1.0 µl
d. MgCl2	2.0 µl
e. Primer (10pM)	1.5 µl
f. Taq DNA polymerase (3U)	0.3 µl
g. Autoclaved distilled water	11.2 µl
Total volume	= 20.0 µl

The PCR tubes were kept in the thermal cycler and the reaction run with the following programme:

Step 1: 94°C for 5 min.	- Initial denaturation	
Step 2: 94°C for 45 sec.	- Denaturation	} 35 cycles
Step 3: 45-50°C for 1 min.	- Annealing	
Step 4: 72°C for 1.30 min.	- Extension	
Step 5: 72°C for 7 min.	- Final extension	
Step 6: 4°C for 10 min.	- For infinity to hold the sample	

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer and stained with ethidium bromide along with marker (λDNA/*Eco*RI+ *Hind*III double digest). The profile was visualized under UV transilluminator and documented using gel documentation system BIO-RAD. The documented ISSR

profiles were carefully examined for polymorphism. Number of bands produced by each primer were counted and tabulated. Those primers, which gave good amplification with more than four bands, were selected for further studies.

3.6.2. ISSR with selected Primers

ISSR assay was performed with the DNA samples isolated from specific subcultures using 10 selected primers with same PCR reaction mixture compositions and programme.

3.6.3 ISSR data analysis

Amplification profiles of different subcultures with different primers were compared with each other and bands of DNA fragments scored manually as (1) or (0) depending on the presence or absence of a particular band respectively. The data was analysed using 1) Numerical Taxonomy System of Multivariate Statistical Programme (NTSYS) (Rohlf, 1998); software package. The dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) as per Sneath and Sokal (1973).

3.7 Methylation Sensitive Amplification Polymorphism (MSAP) assay

The method was adapted from Reyna-Lopez *et al.* (1997) who modified the protocol for AFLP described by Vos *et al.* (1995) to incorporate the use of methylation sensitive restriction enzymes. The modified protocol involved the use of the isoschizomers, *Hpa*II and *Msp*I in place of *Mse*I as the frequent cutter while the rare cutter *Eco*RI was unchanged. Both *Hpa*II and *Msp*I recognize the same tetra nucleotide sequence (5'-CCGG-3'), but display differential sensitivity to DNA methylation. *Hpa*II is inactive when any of the two cytosines is fully methylated (both strands methylated), but cuts the hemi-methylated (only one DNA strand methylated) whereas, *Msp*I cuts when an internal cytosine is methylated (5'-C^{m}CGG-3') but not when external cytosine is methylated (5'-^{m}CCGG-3'). The MSAP assay is

described here under the following heads *viz.*, restriction digestion, adapter ligation, pre-amplification, selective amplification and polyacrylamide gel separation followed by silver staining.

3.7.1 Restriction digestion of genomic DNA

The first step in preparing samples for MSAP analysis was to cut the genomic DNA with restriction enzyme pair (*Eco*R1 + *Msp*I and *Eco*R1 + *Hpa*II)

1. Following components were added to a microcentifuge tube

Components	Tube 1	Tube 2
Genomic DNA (250 ng)	1 µl	1 µl
10 x buffer B	5 µl	5 µl
BSA (10 µg/ul)	0.5 µl	0.5 µl
Enzyme	*Eco*R1 + *Msp*I (1 µl each)	*Eco*R1 + *Hpa*II (1 µl each)
Distilled water	41.5 µl	41.5 µl
Total	**50 µl**	**50 µl**

2. Mixed gently and centrifuged. Incubated the mixture for 5 hours at 37°C
3. Restriction inactivated at 65°C for 15 min.
4. Kept on ice for 5 to 7 min.

Getting the Adapters ready

For each enzyme used, there is an adapter pair that will be ligated to the sticky ends. The adapters come as single strands, so the two strands of each adapter must be annealed to each other before they can be used.

1. For *Eco*RI adapter pair (final concentration of 5μM):

Mix: 5 μl of *Eco*RI forward adapter (@ 100μM)
5 μl of *Eco*RI reverse adapter (@ 100μM)
90 μl of TE buffer

100 μl total

For *Msp*I adapter pair (final concentration of 50μM):

Mix: 50 μl of *Msp*I forward adapter (@ 100μM)
50 μl of *Msp*I reverse adapter (@ 100μM)

100 μl total

2. After mixing the adapters, heated at 95°C for 5 min to denature. Then slowly cooled them to room temperature.

Programme: 95°C for 2 min.
95°C Decreasing to 25° C by 1°C per min intervals
4°C Hold

3.7.2 Ligation of adaptors

The next step after restriction digestion was ligation of adapters to the overhanging sticky ends produced by the restriction enzymes. Double-stranded adaptors ligated to the ends of the DNA fragments, generating template DNA for subsequent polymerase chain reaction (PCR) amplification.

1. Added 10 µl of ligation solution to the digested sample

Composition of ligation solution was as follows:

Components	Tube 1	Tube 2
1X T4 DNA ligase buffer	2 µl	2 µl
10 U T4 DNA ligase	3 µl	3 µl
5 Pmol *Eco*R1 Adapter	2.5 µl	2.5 µl
50 Pmol *Msp*I-*Hpa*II adapter	2.5 µl	2.5 µl
Total	**10 µl**	**10 µl**

2. Gently mixed and centrifuged. Then incubated at 23°C for 5 hours. Reaction stopped at 65°C for 10 min.
3. Placed on ice for few minutes

3.7.3. Pre-amplification reaction

The sequences of the adaptors and the restriction site serve as primer binding sites for a subsequent pre-amplification of the restricted fragments. Pre-amplification uses primers that match the adapter sequence and have only one additional

"selective" base. Only those genomic fragments that have an adaptor on each end amplify exponentially during PCR amplification.

Composition of the reaction mixture for Pre-amplification reaction (50.0 µl) was as follows:

Components	**Tube 3**	**Tube 4**
Ligated product	5 µl from tube 1	5 µl from tube 2
*EcoR*1 + 1 primer	3 µl	3 µl
*Msp-Hpa*II +0 primer	3 µl	3 µl
MgCl2	3 µl	3 µl
dNTP	2 µl	2 µl
Taq polymerase	1 µl	1 µl
Taq buffer B	5 µl	5 µl
Distilled water	28 µl	28 µl
Total	**50 µl**	**50 µl**

The amplification reaction was carried out in an Eppendorf Master Cycler (Eppendorf, USA). A master mix without the ligated product was prepared using the reaction mixture for the required number of reactions. From this master mix, 45 µl was pipetted out into each PCR tube. Ligated product (5 µl) was added to it. PCR amplification was performed.

The PCR tubes were kept in the thermal cycler and the reaction run with the following programme.

Step 1: 94°C for 1 min. - Denaturation

Step 2: 56°C for 1 min. - Annealing } 20 cycles

Step 3: 72°C for 2 min. - Extension

Step 4: 4°C for 10 min. - Cooling of samples

3.7.4 Selective amplification

The pre-amplified product was used as template for selective amplification and selective amplification primer had two additional nucleotide bases.Pre amplified product diluted to 1:25 with TE buffer and further used for selective amplification.

Composition of the reaction mixture for selective amplification reaction (20.0 µl) was as follows.

Components	Tube 5	Tube 6
Diluted pre amplified product	5 µl from tube 3	5 µl from tube 4
*Eco*R1 + 2 primer	4 µl	4 µl
*Msp*I-*Hpa*II primer	2 µl	2 µl
MgCl2	1.5 µl	1.5 µl
dNTP	1 µl	1 µl
Taq polymerase	2 µl	2 µl
1X PCR buffer	2 µl	2 µl
Distilled water	2.5 µl	2.5 µl
Total	**20 µl**	**20 µl**

The amplification reaction was carried out in an Eppendorf Master Cycler (Eppendorf, USA). A master mix without the pre amplified product was prepared using the reaction mixture for the required number of reactions. From this master mix, 15 µl was pipetted out into each PCR tube. Pre amplified product (5 µl) was added. PCR amplification was performed in a 20 µl reaction mixture as constituted below:

PCR progamme for selective amplification

1 cycle of - 94°C for 30 s

65°C for 30 s

72°C for 1 min

Annealing temperature lowered by 0.7°C for 12 cycles

24 cycles of- 94°C for 30 s

56°C for 1 min

72°C for 2 min

List and sequence information of adapters, pre amplification and selective amplification primers used for MSAP analysis is provided in Table 3.

3.7.5 Polyacrylamide gel separation and silver staining

Procedure:

- The work area was cleaned (Wiped with D/W & alcohol).
- The bind & repel plate was cleaned with D/W & alcohol.
- Bind silane was applied on the Glass plate & Repel silane on IP plate respectively.

Bind silane: bind silane 5µl + 1ml ethanol acetic acid mix (199:1).

Table 3. List of MSAP adapters, pre-amplification and selective amplification primers used and their sequences

Adapter

Si. No.	Name	Nucleotide sequence (5'–3')
1	*Eco*RI -adapter	CTCGTAGACTGCGTACC
2	*Hpa*II-*Msp*I-adapter	CGACTCAGGACTCAT

Pre amplification primers

Si. No.	Name	Nucleotide sequence (5'–3')
1	*Eco*RI + A	GACTGCGTACCAATTCA
2	*Hpa*II/MspI + 0	GATGAGTCCTGAGTCGG

Selective amplification primer pairs

Si no.	Name	Sequence	Name	Nucleotide sequence (5'–3')
1	E + AG	GACTGCGTACCAATTCAG	MH + ATG	GATGAGTCCTGAGTCGGATG
2	E + AT	GACTGCGTACCAATTCAT	MH + CA	GATGAGTCCTGAGTCGGCA
3	E + AC	GACTGCGTACCAATTCAC	MH + ATG	GATGAGTCCTGAGTCGGATG
4	E + AG	GACTGCGTACCAATTCAG	MH + ATG	GATGAGTCCTGAGTCGGATG

Repel silane: repel silane 0.5ml + 1ml ethanol acetic acid mix (199:1).

- Spacer was placed along the sides of the IP plate.
- Glass plate was placed over it (Bind silane applied surface towards the IP plate).
- Black frames were placed along the side of the plates & locked it by folding the lever on the back towards the plate.
- The entire unit was kept in the bottom frame vertically & locked it using the white knobs on both sides.
- The entire unit was kept parallel to the work area and comb was adjusted at the top of the plate in inverted position.
- The acrylamide solution (4%) was carefully injected without any bubbles using syringe through the opening in the bottom frame.
  (50ml Acrylamide/bis-acrylamide + 0.5ml 10% APS (ammonium persulphate) + 50µl TEMED).
- Let it for solidification (20Mins).
- The bottom frame was removed & transferred it to electrophoresis tank containing
  0.5X TBE buffer
- The inner chamber of the IP plate was filled with TBE buffer.
- The comb was removed and the upper surface of the gel was cleaned using pipette.
- The power pack was connected properly.
- Kept it for pre run (1500v, 45Mins).
- Then the power pack was switched off & the upper surface of the gel was cleaned using pipette to remove bubbles.
- Comb was placed to unit and samples were loaded carefully to the well (Sample: DNA denatured at 94°C for 5Mins) with formamide dye.

- Connected it to the power pack. (Set the volt & time according to the requirement, pressed run). 1200V, 3hrs
- Switched off power.
- Plates were removed from the unit and ice was applied on the surface of the plate for some time.
- Gel plate was removed with spatula and also removed spacer & comb.
- The plate was kept in a tray containing 10% acetic acid. Placed it on the rocker for 20 mins for fixation.
- The plate was kept in D/W for washing out the acetic acid traces (7 min for 3 times).
- The plate was kept in 0.1% silver nitrate solution with intermittent shaking.
- Quick wash in D/W was followed & transferred the plate into developing solution

  (45gm sodium carbonate + 2ml formaldehyde + 300µl sodium thiosulphate (10%) in ice cold distilled.water) until the bands developed. Bands were appeared as brown in colour. The plate was transferred to 10% acetic acid (10mins.).

- Finally the plate was kept in D/W for 5 Mins and documented after drying.

3.7.6 MSAP Data analysis:

3.7.6.1 Scoring and calculation of methylation levels

The scoring of differential methylation pattern was based on the presence and absence of bands, assuming each band represented a restriction site of 5'CCGG3'. Since *Hpa*II is sensitive to full methylation (methylation of both strands) of either cytosine residue at the recognition site and *Msp*I is sensitive only to methylation at the external cytosine, a band detected in the *Eco*RI + *Msp*I digest but not in the *Eco*RI + *Hpa*II was referred as resulting from an internal methylation. Conversely, a band

detected in *Eco*RI + *Hpa*II digest but not in the *Eco*RI + *Msp*I digest was referred to be the result of a hemi methylation. The number of internal and hemi methylation sites were counted in each subculture, and expressed in per centage against the total number of bands detected (Joel and Zhang, 2001). The methylation per centage was calculated as follows.

$$\text{Per cent hemi-methylation (\% HM)} = \frac{\text{Number of hemimethylated sites}}{\text{Total number of scorable bands}} \times 100$$

$$\text{Per cent Internal full methylation (\% IM)} = \frac{\text{No. of internalmethylated sites}}{\text{Total number of scorable bands}} \times 100$$

Per cent total methylation (% TM) = % HM + % IM.

Results

4. RESULTS

The result of the investigations conducted on the "Morphological and molecular analysis of genetic stability in micropropagated banana var. Nendran" undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara are presented in this chapter under different sub headings.

4.1 *In vitro* multiplication

The method followed was good enough for multiplication of banana var. Nendran through *in vitro* organogenesis.

4.1.1 Micropropagation from male bud explant

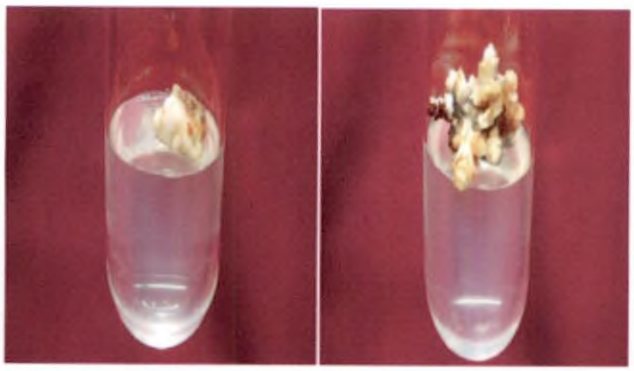
Inner core of the male bud was used as explant and the media tried gave good multiplication without formation of callus and adventitious shoots were produced. The culture establishment rate observed for male bud explant was 12 per cent. However only 16 per cent cultures gave multiple shoots. Multiplication was observed from the fifth subculture onwards and the rate of multiplication recorded was between one and six. The details are provided in Table 4 and Plate 1. Cent per cent rooting was observed in the media tried and the survival rate during hardening was 76 per cent.

4.1.2 Micropropagation from sucker explant

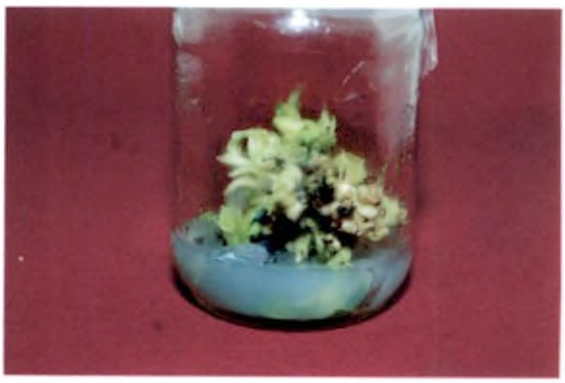
Apical bud from suckers used as explant and the media tried gave good multiplication through formation of adventitious shoots without any intervening callus. The culture establishment rate observed for sucker explants was 70 per cent 92 per cent of established cultures gave multiple shoots. Multiplication was observed from the second subculture onwards and the rate of multiplication recorded was between three and six. The details are provided in Table 4 and Plate 2. Cent per cent

a- Explant source

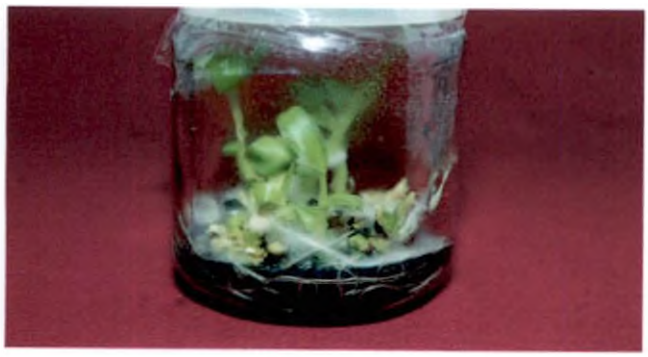
b- Explant



c- Culture initiation



d- Culture multiplication



e- Rooting

f- Hardening

g- Field planting

h- Fruit bunches

Plate 1: Different stages of tissue culture for male bud derived plants

a- Explant source

b- Explant

c- Culture initiation

d- Culture multiplication

e- Rooting

f- Hardening

g- Field planting

Plate 2: Different stages of tissue culture for sucker derived plants

Table 4: Details of *in vitro* organogenesis from different explants of banana var. Nendran

Explant used		Inner core of male bud	Apical bud from suckers
Culture establishment rate (%)		12	70
Mode of regeneration		Organogenesis from protocorm like structure	Direct adventitious shoot formation
Time taken for multiple shoot induction		15	6 weeks
% of cultures giving multiple shoots		16	92
Multiplication rate at different subcultures	1^{st} to 3^{rd}	1.5	3
	4^{th} to 10^{th}	6	6
	11^{th} to 16^{th}	2	3.5
Rooting percentage		100	100
Hardening success (%)		76	98

rooting was observed in the media tried and the survival rate during hardening was 98 per cent.

4.2 Morphological characterization

The *in vitro* derived plants obtained from different subculture intervals were hardened and planted in the field for morphological characterization. The morphological data recorded for the plants derived from different subcultures at different stages of growth are provided in Table 5, 6 and 7.

4.2.1 Morphological characterization of male bud derived tissue culture plants at different stages of growth

Male bud derived plants from different subcultures were planted out, hardened and further evaluated in the field. The plants derived from upto 10^{th} subculture attained full maturity in the field and the bunches were harvested; rest of the plants (12^{th}, 14^{th} and 16^{th} subculture) were at the vegetative phase and hence the fruit characters were not recorded. The morphological characters recorded at different stages of growth were analysed and described here under.

Stage 1 – Two months after plantout

The data recorded for different parameters are provided in Table 5. The tissue culture derived plants were planted in the field two months after plantout from the culture vessels. Hence this stage relates to the preplanting stage.

The height recorded at two months stage for the plants derived from different subcultures ranged between 11.5 and 16.4 cm. The plant height varied significantly with respect to different subculture intervals. Those that derived from upto 6^{th} subcultures were significantly superior to the rest while those derived from after 12^{th} subculture were significantly inferior to others.

Table.5: Growth parameters of male bud derived tissue culture plants of banana var. Nendran at different stages of growth.

Stage 1: Two months after plantout

Subculture number	Plant height (cm)	Number of leaves	Leaf length (cm)	Leaf breadth (cm)
Sc3	16.13[a]	4.9[a]	16.00[a]	4.30[a]
SC6	16.36[a]	5.0[a]	16.88[a]	4.38[a]
SC8	13.62[b]	4.4[ab]	15.72[a]	4.34[a]
Sc10	13.96[b]	4.4[ab]	15.98[a]	3.74[ab]
Sc12	11.78[c]	4.0[ab]	12.58[b]	3.14[b]
Sc14	11.60[c]	4.0[ab]	12.52[b]	2.26[c]
Sc16	11.52[c]	3.4[b]	12.34[b]	1.92[c]

Stage 2: Four months after field planting

Subculture number	Plant height (cm)	Number of leaves	Leaf length (cm)	Leaf leaf breath (cm)	Pseudostem girth (cm)
Sc3	173.6[a]	7.4[ab]	153.6[a]	58.8[a]	40.2[a]
SC6	173.6[a]	7.4[ab]	153.6[a]	58.8[a]	40.2[a]
SC8	133.0[b]	6.4[b]	138.8[ab]	55.8[a]	37.0[ab]
Sc10	131.6[b]	7.2[ab]	124.4[b]	52.8[a]	30.4[b]
Sc12	130.8[b]	7.6[a]	134.2[ab]	55.4[a]	34.0[ab]
Sc14	131.6[b]	7.0[ab]	134.0[ab]	56.6[a]	30.28[b]
Sc16	130.2[b]	6.4[b]	129.0[b]	48.0[a]	29.80[b]

Stage 3: Eight months after field planting

Subculture number	Plant height (cm)	Number of leaves	Leaf length (cm)	Leaf breadth (cm)	Pseudostem girth (cm)
Sc3	288.6[a]	9.8[a]	186.6[a]	57.8[ab]	64.8[a]
SC6	288.0[a]	10.2[a]	185.6[a]	60.0[ab]	60.16[a]
SC8	288.6[a]	9.6[a]	177.4[a]	62.6[a]	60.9[a]
Sc10	289.1[a]	9.4[a]	177.2[a]	56.6[b]	61.16[a]
Sc12	286.6[a]	9.6[a]	179.2[a]	62.4[a]	60.06[a]
Sc14	280.4[b]	9.4[a]	180.4[a]	62.7[a]	59.58[a]
Sc16	267.2[c]	9.6[a]	179.2[a]	60.0[b]	57.16[a]

The number of leaves at stage 1 varied from 3.4 to 5. There was a gradual reduction in the number of leaves produced while the subculture advanced and it was significantly low at 16th subculture (3.4), when compared to those upto 6th subculture (5).

The leaf length and breadth recorded at stage 1 also indicated similar trend. The leaf length of the plants was almost the same upto the 10th subculture while it was significantly low from 12th subculture onwards. The leaf breadth was significantly low from 14th subculture onwards.

Stage 2- Four months after field planting

The data recorded for different parameters at stage 2 are provided in Table 5.

The tissue culture derived plants were planted in the field after two months of hardening.

The height recorded at stage 2 for the plants derived from different subcultures ranged between 130.2 and 173.6 cm. The plant height varied significantly with respect to subculture intervals. Those that derived from upto 6th subculture (173.6 cm) were significantly superior to the rests while the plant height was inferior at 16th subculture (130.2 cm).

The number of leaves at stage 2 varied from 6.4 to 7.6. Plants derived from 12th subculture (7.6) were superior to rest. While plants derived from 16th subculture (6.4) were significantly inferior to others.

The leaf length recorded at stage 2 ranged between 124 and 153.6 cm. Those that derived from upto 6th subculture (153.6 cm) were significantly superior to the rests. While those derived from 8th to 16th subculture were significantly inferior to others. The leaf breadth at stage 2 varied from 48 to 58.8 cm. There was no significant variation observed for the plants derived from all the subcultures.

The pseudostem girth at stage 2 varied from 29.8 to 40.2 cm. The plants derived from upto 6th subculture (58.8 cm) were significantly superior to others while it was significantly inferior at 14th and 16th subculture.

Stage 3 – Eight months after field planting

The data recorded for different parameters at stage 3 are provided in Table 5.

The height recorded eight months after field planting for the plants derived from different subcultures was ranged between 267.2 and 289.1 cm. The height of the plant was almost similar upto 12th subculture while it was significantly less in further subcultures.

The number of leaves at stage 3 varied from 9.4 to 10.2. The number of leaves were almost similar for the plants derived from all the 16th subcultures without any significant variation.

The leaf length at stage 3 also indicated similar trend. The leaf length was almost the same for all the subcultures; ranged between 179.2 and 186.6 cm. While the leaf breadth ranged between 56.6 and 62.7 cm. Plants derived from 12th and 14th subculture (62 cm) were significantly superior to others. While those derived from subculture 10th and 16th were significantly inferior to others.

The pseudostem girth at stage 3 varied from 57.16 to 64.8 cm. Considering the pseudostem girth, there was no significant difference observed for the plants derived from all the subcultures.

4.2.1.1 Fruit characters

The fruit characters were recorded (Table 6) for those plants from which the bunches were obtained (upto subculture 10th). Plants derived from subculture 6rd recorded significantly superior values for all the bunch characters than the plants derived from later subcultures. The maximum yield was obtained in 6th and 8th

Table.6. Fruit characters recorded for male bud derived plants

Subculture number	Total bunch wt (Kg)	Number of hands	Number of fruits per hand	Weight of hand (Kg)	single fruit wt (Kg)	Length (single fruit) (cm)	Circumference (single fruit) (cm)	TSS °Bx
Mp	12.50	6.0	10	1.816	0.195	20.6	13.7	30.4
Sc3	8.36^{c}	5.8^{a}	9.8^{a}	1.324^{c}	0.128^{b}	18.9^{b}	11.8^{c}	27.7^{a}
Sc6	11.02^{a}	6.0^{a}	10.2^{a}	1.733^{a}	0.164^{a}	19.6^{a}	13.3^{a}	29.9^{a}
Sc8	10.50^{a}	6.0^{a}	9.8^{a}	1.639^{ab}	0.152^{ab}	19.1^{b}	12.9^{b}	29.8^{c}
Sc10	9.58^{b}	5.6^{a}	8.6^{b}	1.578^{b}	0.177^{a}	19.7^{a}	12.9^{b}	28.5^{b}

subculture derived plants which was 11 and 10.5 Kg. There was no significant difference recorded for number of hands throught all subcultures (approximately 6). However for all the plants, bunch characters were comparable to the mother plant. Total Soluble Solids recorded for mother plant was 30.4° Bx while in different subcultures it varied from 27.7 to 29.9 °Bx.

4.2.1.2 Abnormalities observed

Some of the abnormalities were also observed during the tissue culture of male bud derived plants. The occurrences of these off types were not on large scale and regular.

Three different kinds of abnormalities were observed (Plate 3). These are elongated petiole, wavery leaf lamina and unequal leaf base. But these abnormalities were not observed in later stages of their vegetative growth.

4.2.2 Morphological characterization of sucker derived tissue culture plants at different stages of growth.

Sucker derived plants from different subcultures were planted out, hardened and further evaluated in the field. The plants derived from only upto 10^{th} subculture had reached the stage 2 (four months after field planting); rest of the plants (12th, 14th and 16th subculture) were at stage less than four months after field planting and hence the morphological parameters were not recorded for those plants. Fruit characters also not recorded for any of the subculture since all the plants were at vegetative phase. The morphological characters recorded (Table 7) at different stages of growth were analysed and described here under.

Stage 1 – Two months after plantout

The data recorded for different parameters at stage 1 are provided in Table 7.

a - Elongated petiole

b - Wavery leaf lamina

Table.7: Growth parameters of sucker derived tissue culture plants of banana *var.* Nendran at different stages of growth.

Stage 1: Two months after plantout

Subculture number	Plant height (cm)	Number of leaves	Leaf length (cm)	Leaf breadth (cm)
Sc3	11.92^{bc}	5.4^{a}	14.6^{a}	3.8^{abc}
SC6	13.64^{ab}	5.0^{ab}	15.0^{a}	4.4^{a}
SC8	13.84^{a}	4.4^{abc}	14.78^{a}	3.9^{ab}
Sc10	11.82^{bc}	4.0^{bc}	13.04^{b}	3.62^{bcd}
Sc12	12.18^{abc}	4.0^{bc}	12.96^{b}	3.9^{ab}
Sc14	11.88^{bc}	3.4^{c}	12.26^{b}	3.22^{cd}
Sc16	11.72^{c}	3.4^{c}	12.56^{b}	2.98^{d}

Stage 2: Four months after field planting

Subculture number	Plant height (cm)	Number of leaves	Leaf length (cm)	Leaf breadth (cm)	Pseudostem girth (cm)
Sc3	145.56^{ab}	7.8^{a}	143.4^{a}	48.6^{a}	38.4^{a}
SC6	155.4^{a}	7.2^{a}	143.8^{a}	50.6^{a}	34.6^{a}
SC8	136.0^{ab}	7.2^{a}	138.8^{a}	54.6^{a}	33.2^{a}
Sc10	126.0^{b}	7.2^{a}	128.0^{a}	53.2^{a}	35.4^{a}

The height recorded at two months stage for the plants derived from different subcultures ranged between 11.72 and 13.84 cm. The plant height varied significantly with respect to different subculture intervals. Those that derived from 8^{th} subculture (13.8 cm) were significantly superior to rest while those derived from 16^{th} subculture (11.72 cm) were significantly inferior to others.

The number of leaves at stage 1 varied from 3.4 to 5.4. There was a gradual reduction in the number of leaves produced while the subculture advanced and it was significantly inferior at 16^{th} subculture (3.4 cm). When compared to those upto 6^{th} subculture (5 cm).

The leaf length recorded at stage 1 also indicated similar trend. The leaf length of the plants was almost same upto the 8^{th} subculture (14 cm) while it was significantly less from 10^{th} subculture (12 cm) onwards. The leaf breadth at two months stage varied from 2.9 to 4.4 cm. The leaf breadth was superior for the plants derived from 6^{th} subcultures (4.4 cm) while it was significantly inferior to those derived from 16^{th} subculture (2.98 cm).

Stage 2- Four months after field planting

The data recorded for different parameters at stage 2 are provided in Table 7.

The plant height recorded at stage 2 ranged between 126 and 155 cm. Plants derived from 6^{th} subculture (155 cm) were significantly superior to others while those derived from subculture 10^{th} (126 cm) was significantly inferior to others.

The number of leaves recorded four months after field planting varied from 7.2 to 7.8. There was no significant variation recorded for the plants derived from upto 10^{th} subculture.

Leaf length, leaf breadth and pseudostem girth recorded at stage 2 also indicated similar trends; observed almost similar values for each parameter for all the plants derived from upto 10^{th} subculture.

4.3 Molecular analysis

4.3.1 Isolation, purification and quantification of DNA

Genomic DNA isolated through the CTAB method reported by Roger and Benedich (1994) was pure with slight RNA contamination (Plate.4). RNase treatment given after the DNA isolation was found effective than RNase treatment given during the DNA isolation. The agarose gel electrophoresis indicated clear discrete band without contamination and spectrophotometric analysis gave ratio of UV absorbance ($A_{260}/_{280}$) between 1.8 and 2.0.

4.3.2 ISSR analysis

ISSR analysis was carried out for the two sets of plants regenerated from male bud and sucker explants.

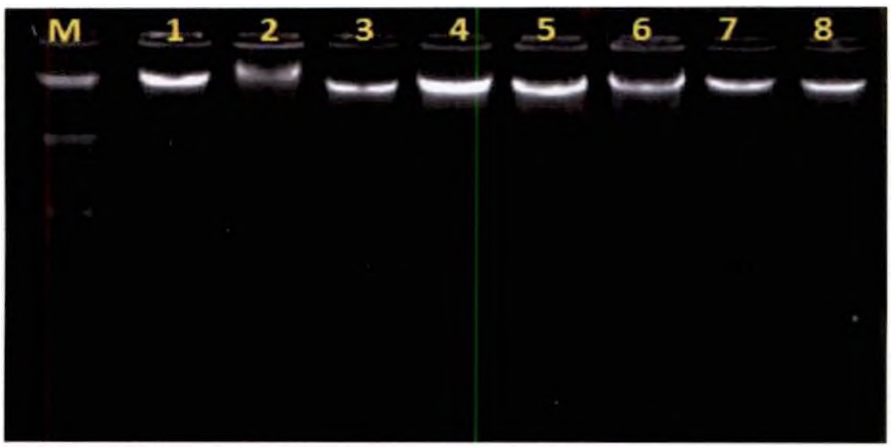
Fourteen ISSR primers reported earlier (Rakeshkumar, 2011) were attempted. Out of which ten were selected for detailed analysis. The sequence data of the primer selected for analysis is already provided in Table 2 under section 3.6.1

4.3.2.1 Regenerants from male bud explant

The amplification pattern obtained for each of the primer with respect to plants derived from male bud is provided in Plates (5 to 9). Details are as follows.

UBC 835:

A total of four amplicons were obtained after DNA amplification with the primer UBC 835. The pattern of amplification is shown in Plate 5a. All amplicons



M- Marker (λDNA/*Eco*RI + *Hind*III)

Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th

Plate 4. Intact DNA isolated through CTAB method from regenerants of specific subcultures

obtained with this primer were monomorphic for the subculture selected. The molecular weight of the bands varied from 0.720 to 1.21 kb.

UBC 843

Amplification with this primer generated six amplicons of which only one was polymorphic and the remaining were monomorphic. The pattern of amplification is shown in Plate 5b. Two polymorphic bands of molecular weight 1.012 kb were present in 10^{th} and 14^{th} subculture regenerants.

UBC 820

A total of six amplicons were obtained after DNA amplification with the primer UBC 820. The pattern of amplification is shown in Plate 6a. Regenerants from subculture 14^{th} and 16^{th} showed two polymorphic amplicons in each. The molecular weight of the bands varied from 0.280 to 1.156 Kb.

UBC 890

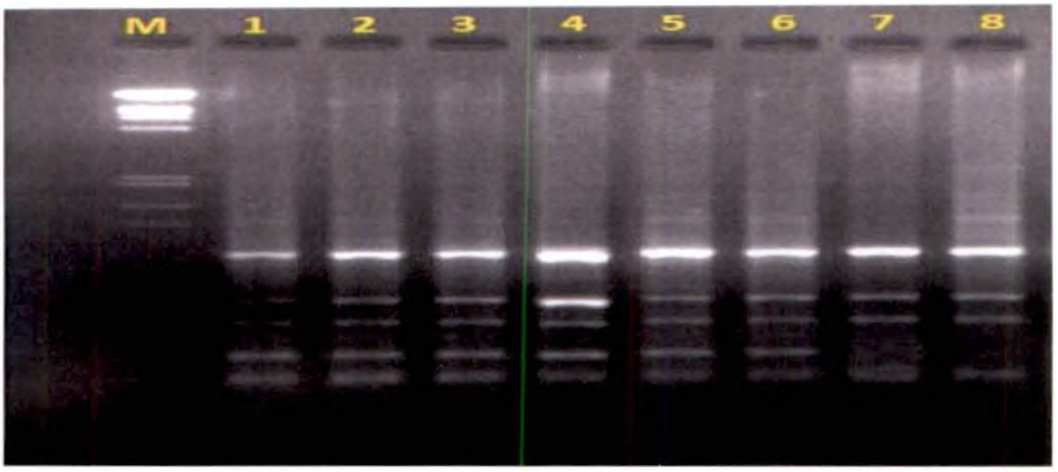
A total of three amplicons were obtained after DNA amplification with the primer UBC 890. The pattern of amplification is shown in Plate 6b. The amplicons obtained with this primer were monomorphic. The molecular weight of the bands varied from 0.660 to 0. 990 kb.

UBC 836

Five clear amplicons were observed on the agarose gel for the DNA amplified with the primer UBC 836 (Plate 7a). Only one band was polymorphic in subculture 10^{th} and 14^{th}; the rest were monomorphic. The molecular weights of the products ranged between 0.564 and 1.584 Kb

UBC 868

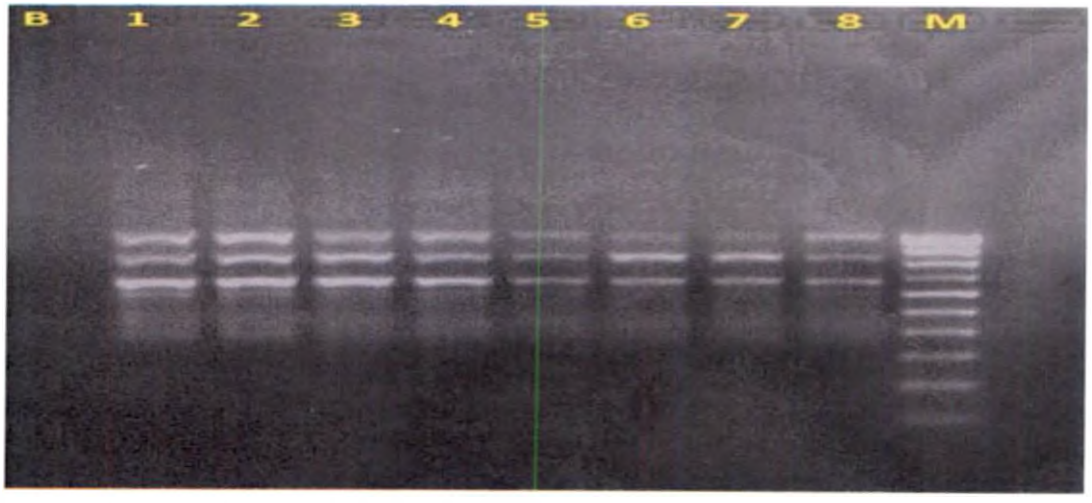
Amplification with this primer generated four amplicons. The amplicons obtained with this primer were almost monomorphic with a single band being



M- Marker (λDNA/*Eco*RI + *Hind*III), B: Control

a - Amplification with UBC 820

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.

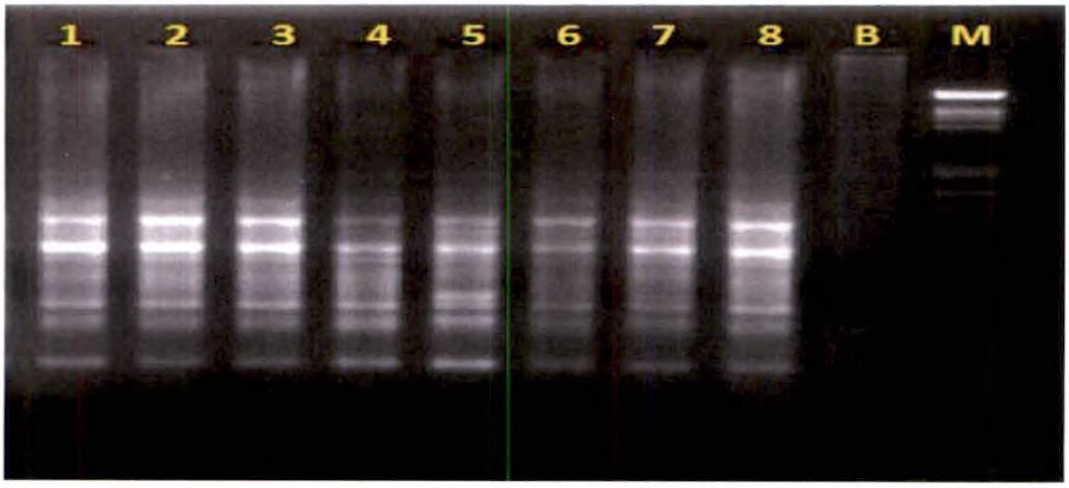


M: 100bp ladder/Marker, B: Control

b - Amplification with UBC 890

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.

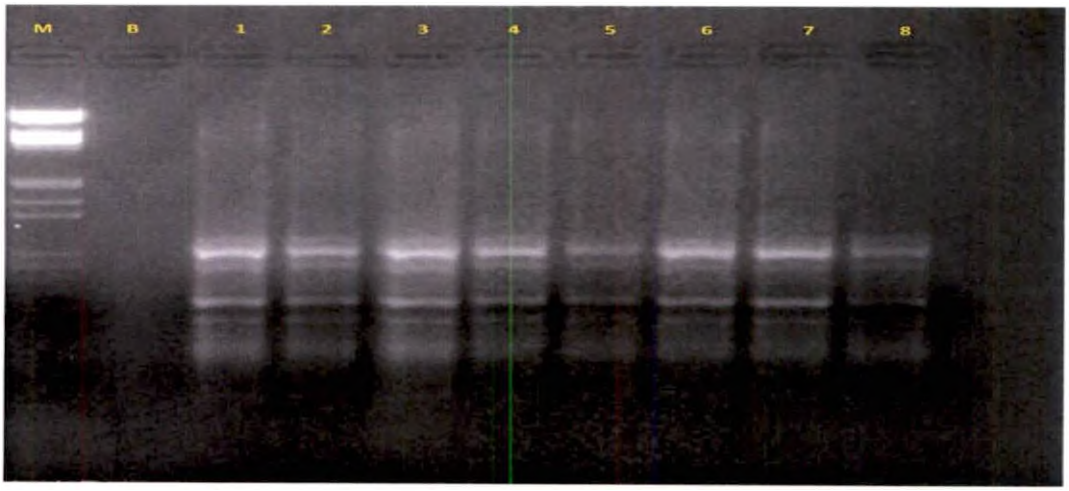
Plate 6: ISSR amplification pattern of micropropagated banana plants (MBD) with primers UBC 820 and UBC 890



M: 100bp ladder/Marker, B: Control

a - Amplification with ISSR primer UBC 836

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.



M- Marker (λDNA/*Eco*RI + *Hind*III), B: Control

b - Amplification with UBC 868

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.

Plate 7: ISSR amplification pattern of micropropagated banana plants (MBD) with primers UBC 836 and UBC 868

polymorphic in last subculture and the rest were monomorphic. The pattern of amplification is shown in Plate 7b. The molecular weight of the products ranged between 0.564 to 0.994 Kb

ISSR 3

Amplification with this primer generated six clear amplicons of which two were polymorphic and rest were monomorphic. The pattern of amplification is shown in Plate 8a. The molecular weight of the amplicons ranged between 0.564 Kb to 1.05.

UBC 848

A total of six amplicons were obtained after DNA amplification with the primer UBC 848. The pattern of amplification is shown in Plate 8b. Three amplicons obtained with this primer were polymorphic. The molecular weight of the bands varied from 0.350 to 1.1 kb.

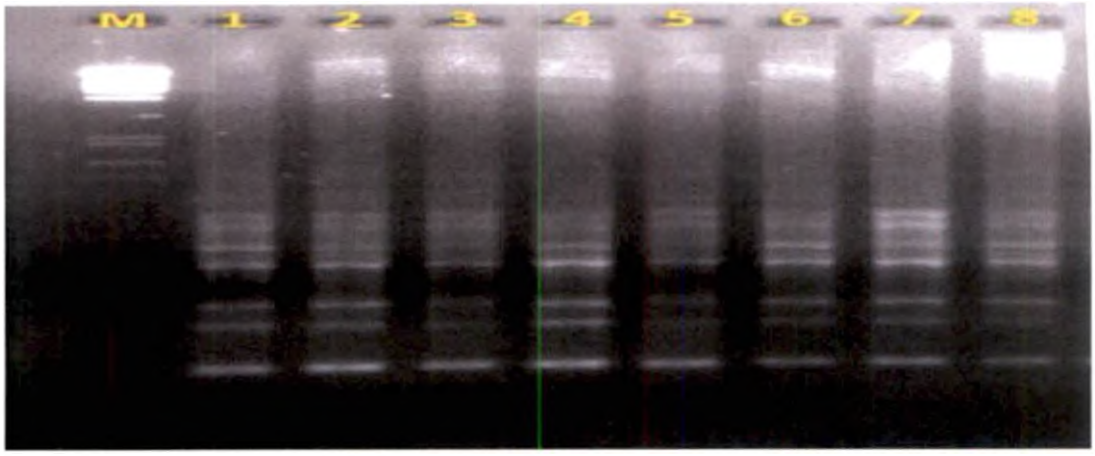
UBC 845

Amplification with this primer generated seven clear amplicons of which two were polymorphic and rest were monomorphic. The pattern of amplification is shown in Plate 9a. The molecular weight of the amplicons ranged between 0.220 to 1.064 Kb.

UBC 814

A total of five amplicons were obtained after DNA amplification with the primer UBC 814. The pattern of amplification is shown in Plate 9b. The amplicons obtained with this primer were monomorphic. The molecular weight of the bands varied from 0.647 to 1.464 Kb.

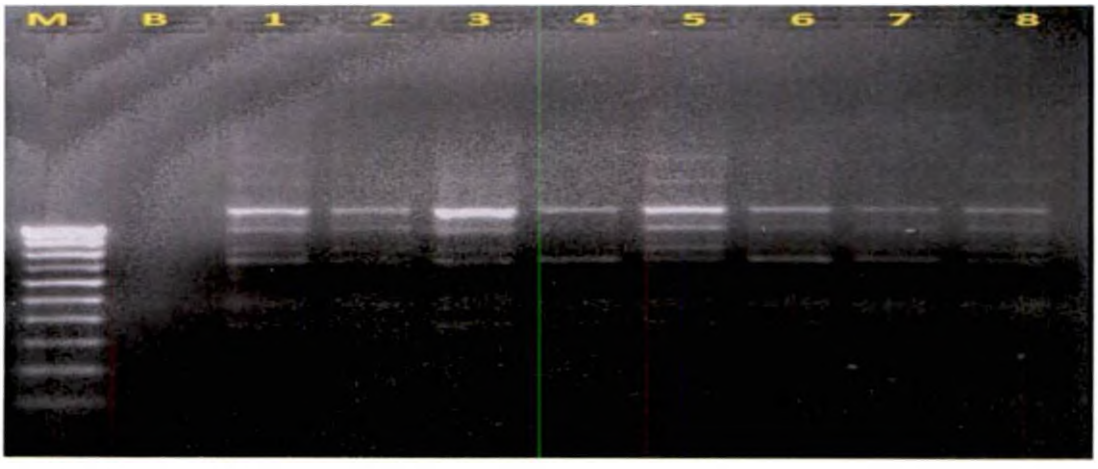
The extent of amplification and polymorphism observed is tabulated and provided in Table 8. Three to seven amplicons were observed with respect to each primer and the polymorphism observed ranged between 0 to 33 per cent.



M- Marker (λDNA/*Eco*RI + *Hind*III), B: Control

a - Amplification with ISSR 3

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.



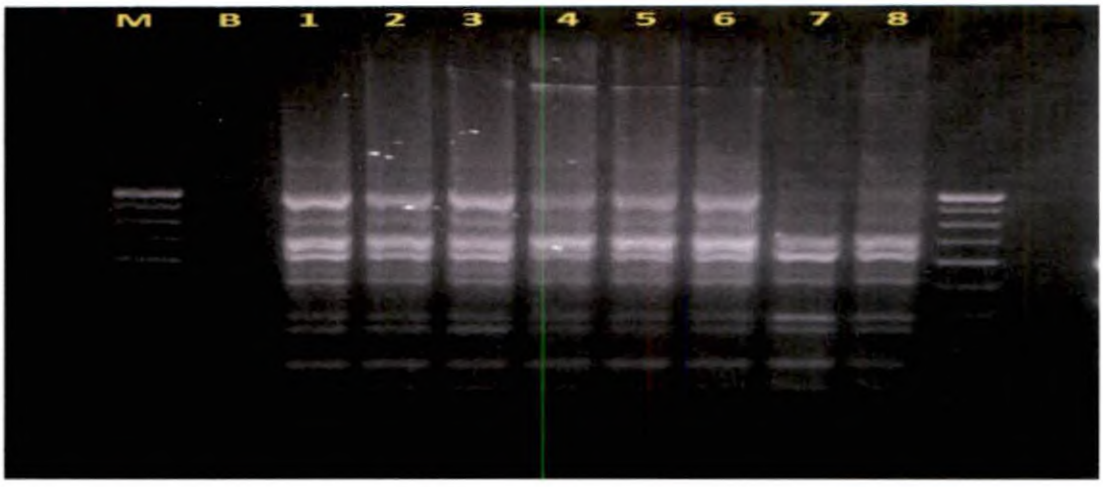
M: 100bp ladder/Marker, B: Control

b - Amplification with UBC 848

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.

2-

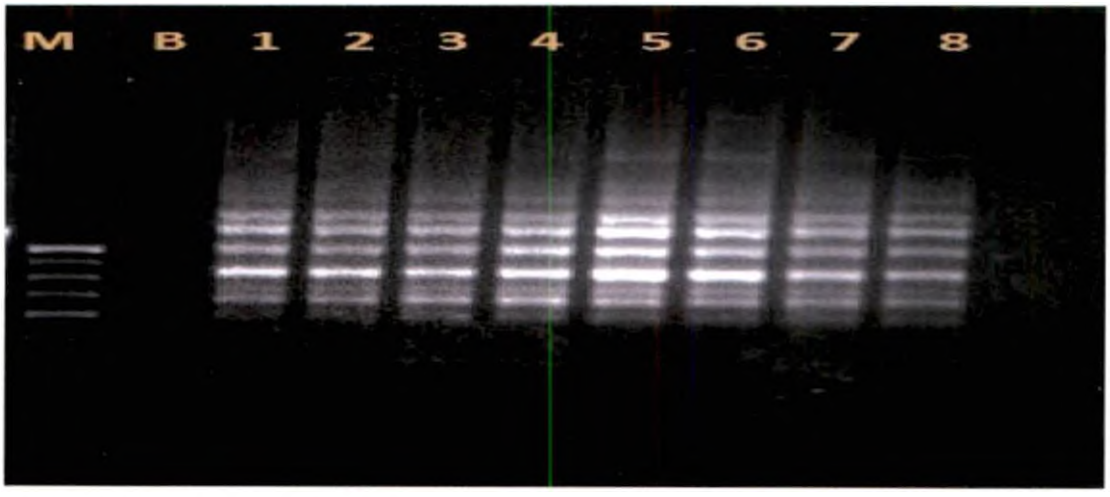
Plate 8: ISSR amplification pattern of micropropagated banana plants (MBD) with primers ISSR 3 and UBC 848



M: 100bp ladder/Marker, B: Control

a - Amplification with UBC 845

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.



M: 100bp ladder/Marker, B: Control

b - Amplification with UBC 814

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.

Plate 9: ISSR amplification pattern of micropropagated banana plants (MBD) with primers UBC 845 and UBC 814

Table. 8: Amplification pattern observed in ISSR assay of micropropagated banana regenerated from male bud

Primer	MP	Sc3		Sc6		Sc8		Sc10		Sc12		Sc 14		Sc16	
	T	P	%	P	%	P	%	P	%	P	%	P	%	P	%
UBC 835	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UBC 843	6	0	0	0	0	0	0	1	16.6	0	0	1	16.6	0	0
UBC 820	6	0	0	0	0	0	0	0	0	0	0	2	33	2	33
UBC 890	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UBC 836	5	0	0	0	0	0	0	1	20	0	0	1	20	0	0
UBC 868	4	0	0	0	0	0	0	0	0	0	0	0	0	1	25
ISSR 3	6	0	0	0	0	0	0	0	0	0	0	0	0	2	33
UBC 848	6	0	0	0	0	0	0	1	16.6	1	16.6	0	0	1	16.6
UBC 845	7	0	0	0	0	0	0	0	0	0	0	2	28.5	2	28.5
UBC 814	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0

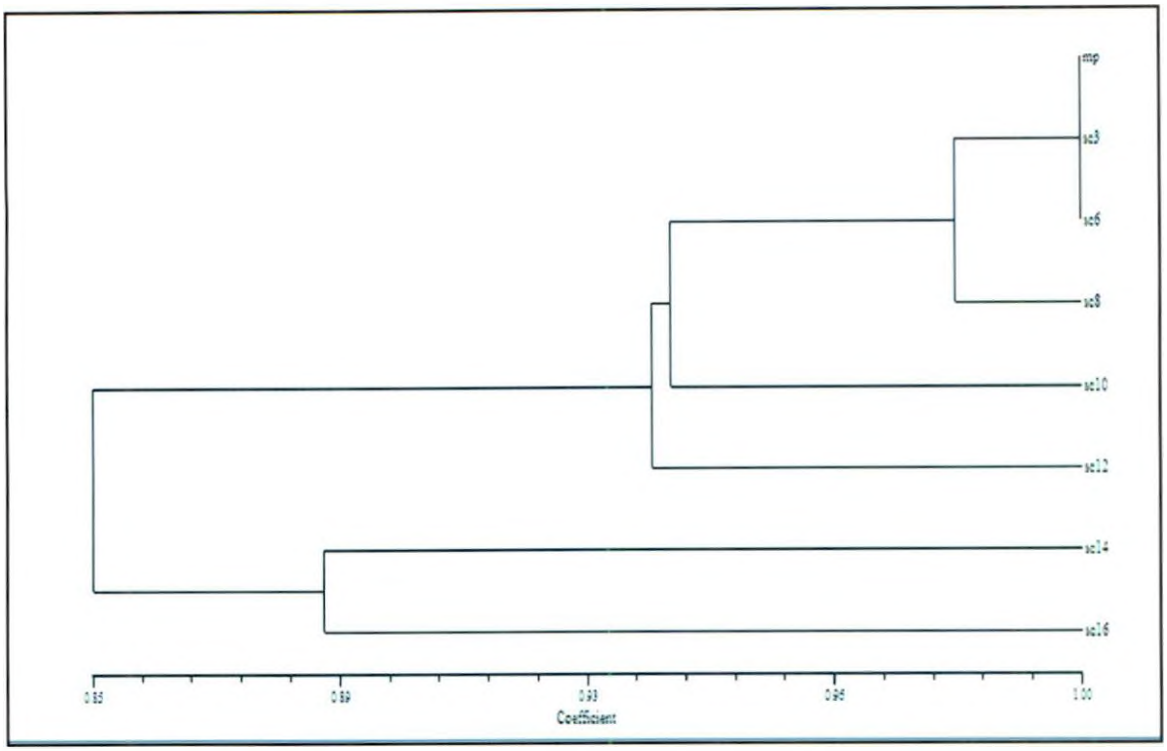
MP- Mother Plant,

Sc3.....Sc16 - subculture 3 to 16

T- Total number of amplicons,

P- Number of polymorphic amplicons (in relation to mother plant),

% - Percentage polymorphism



MP- Mother Plant,

Sc3……Sc16 - subculture 3 to 16

Plate 10: Dendrogram developed out of ISSR analysis of male bud derived plants

The dendrogram developed out of the ISSR data is provided in Plate 10. The variation observed among the regenerants ranged between 1 to 15 per cent. The plantlets regenerated through different subcultures were grouped differently. Variability was less (6 per cent) in the regenerants derived in the initial six subcultures.

4.3.2.2 Regenerants from sucker explant

The amplification pattern obtained for each of the primer with respect to plants derived from sucker is provided in Plates 11 to 15. Details are as follows.

UBC 835

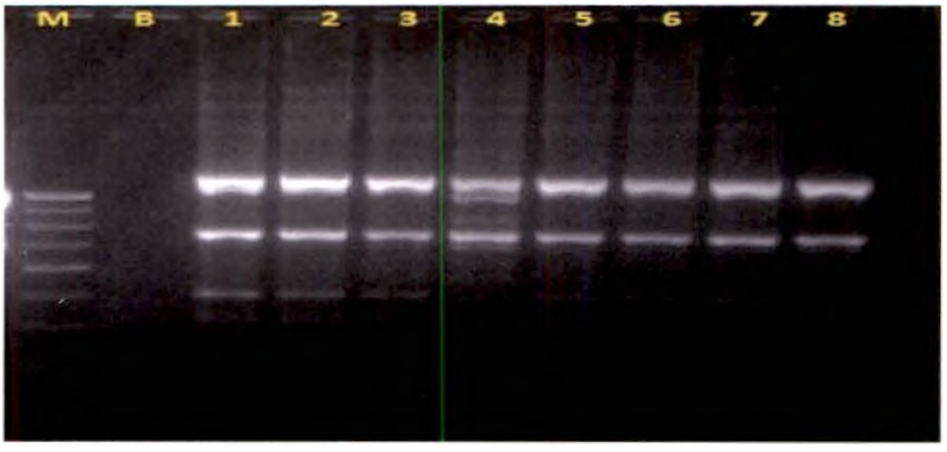
Amplification with this primer generated four amplicons of which one was polymorphic and the rest were monomorphic. The pattern of amplification is shown in Plate 11a. The molecular weight of the amplicons ranged between 0.400 and 1.1 Kb. Polymorphic bands was present in 16^{th} subculture.

UBC 843

A total of six amplicons were obtained after DNA amplification with the primer UBC 843. The pattern of amplification is shown in Plate 11b. All amplicons obtained with this primer were monomorphic. The molecular weight of the bands varied from 0.250 to 1.4 Kb.

UBC 820

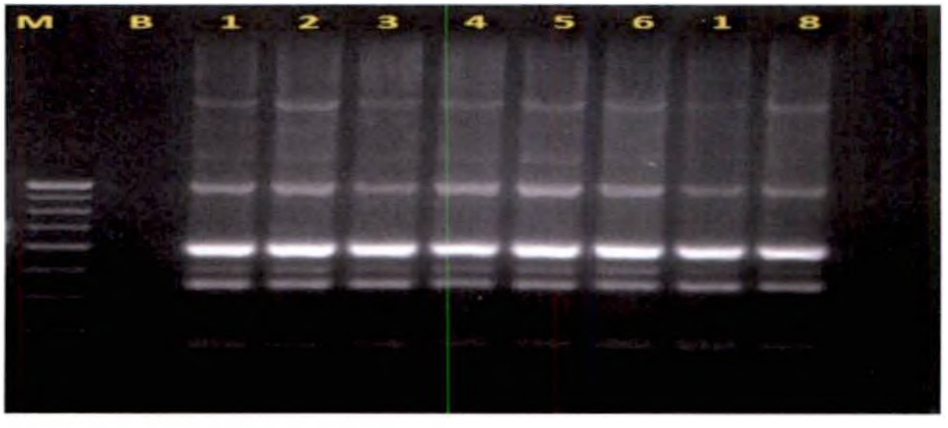
Amplification with this primer generated seven amplicons of which four were polymorphic and rests were monomorphic. The pattern of amplification is shown in Plate 12a. The polymorphism was observed from 6^{th} subculture onwards. The molecular weight of the amplicons ranged between 0.564 to 1.4 kb.



M: 100bp ladder/Marker, B: Control

a - **Amplification with UBC 835**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.

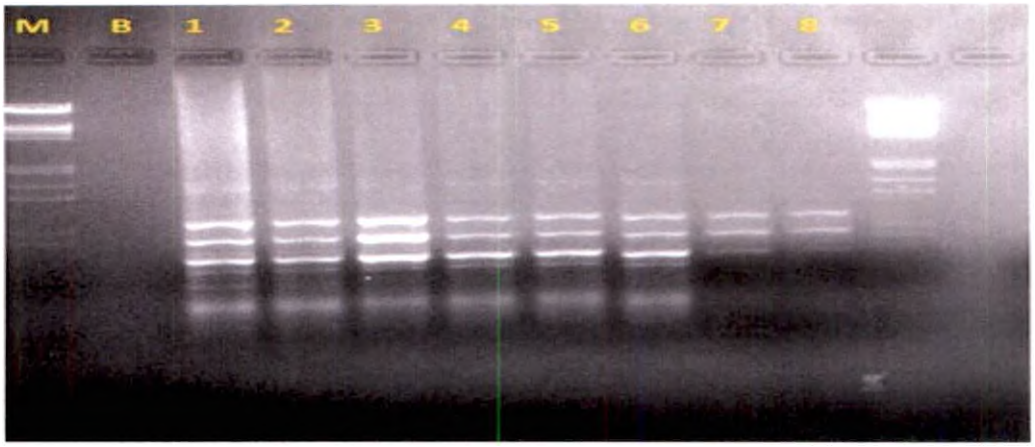


M: 100bp ladder/Marker, B: Control

b - **Amplification with UBC 843**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.

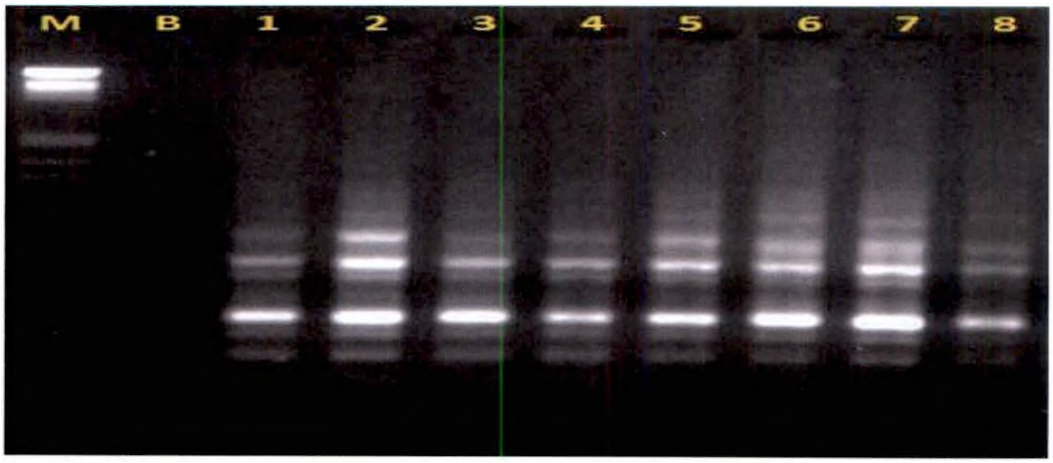
Plate 11: ISSR amplification pattern of micropropagated banana plants (SD) with primers UBC 835 and UBC 843



M- Marker (λDNA/*Eco*RI + *Hind*III), B: Control

a - **Amplification with UBC 820**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6- subculture 12th, 7- subculture 14th, 8- subculture 16th.



M: 100bp ladder/Marker, B: Control

b - **Amplification with UBC 890**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6- subculture 12th, 7- subculture 14th, 8- subculture 16th.

Plate 12 : ISSR amplification pattern of micropropagated banana plants (SD) with primers UBC 820 and UBC 890

UBC 890

A total of three amplicons were obtained after DNA amplification with the primer UBC 890. The pattern of amplification is shown in Plate 12b. All amplicons obtained with this primer were monomorphic. The molecular weight of the bands varied from 0.250 to 1.4 Kb.

UBC 836

Amplification with this primer generated seven amplicons of which three were polymorphic in 16th subculture and the rests were monomorphic. The pattern of amplification is shown in Plate 13a. The molecular weight of the amplicons ranged between 0.320 to 1.92 Kb.

UBC 868

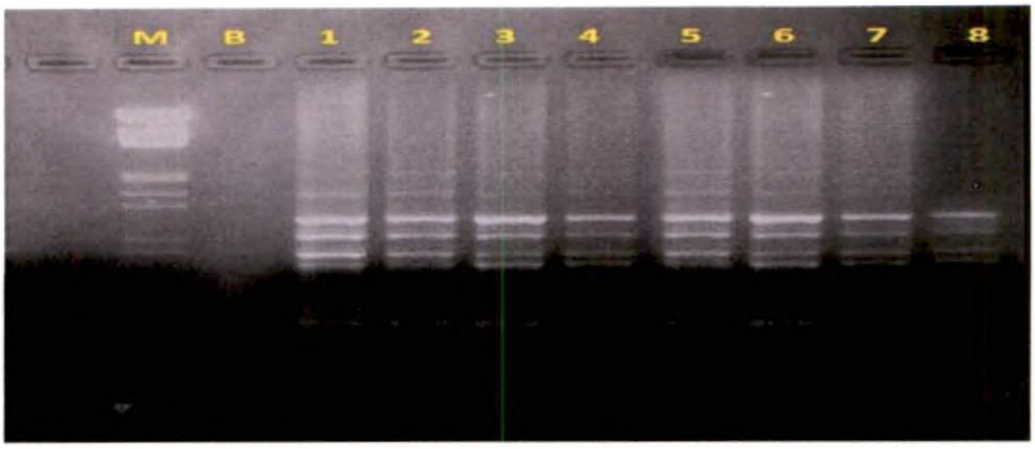
A total of five amplicons were obtained after DNA amplification with the primer UBC 868. The pattern of amplification is shown in Plate 13b. Two amplicons obtained with this primer was polymorphic and rests were monomorphic. The molecular weight of the bands varied from 0.277 to 1.164 Kb.

ISSR 3

Amplification with this primer generated five amplicons and all were monomorphic. The pattern of amplification is shown in Plate 14a. The molecular weight of the amplicons ranged between 0.420 to 1.064 Kb.

UBC 848

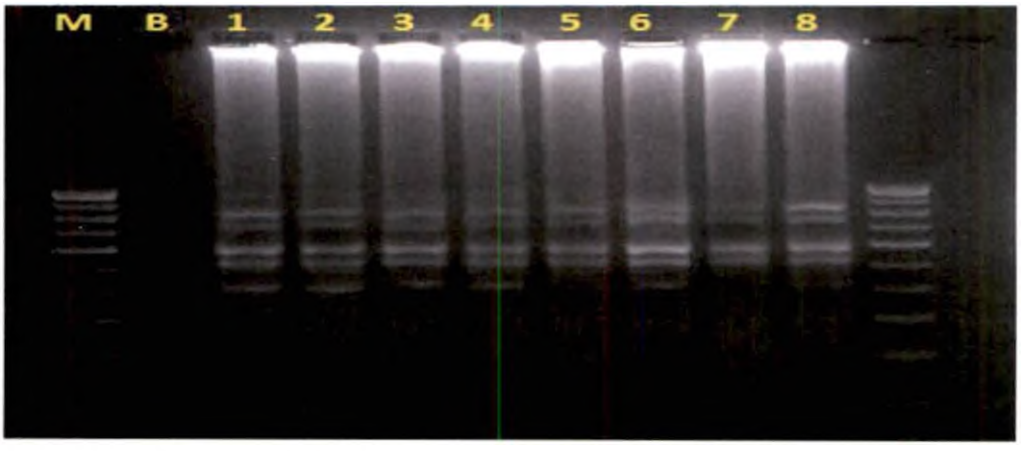
Six amplicons were observed on the agarose gel for the DNA amplified with the primer UBC 848 (Plate 14b). One band was polymorphic in 8th subculture and the rest were monomorphic. The molecular weights of the products ranged between 0.284 to 0.990 Kb.



M- Marker (λDNA/*Eco*RI + *Hind*III), B: Control

a - **Amplification with UBC 836**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6- subculture 12th, 7- subculture 14th, 8- subculture 16th.

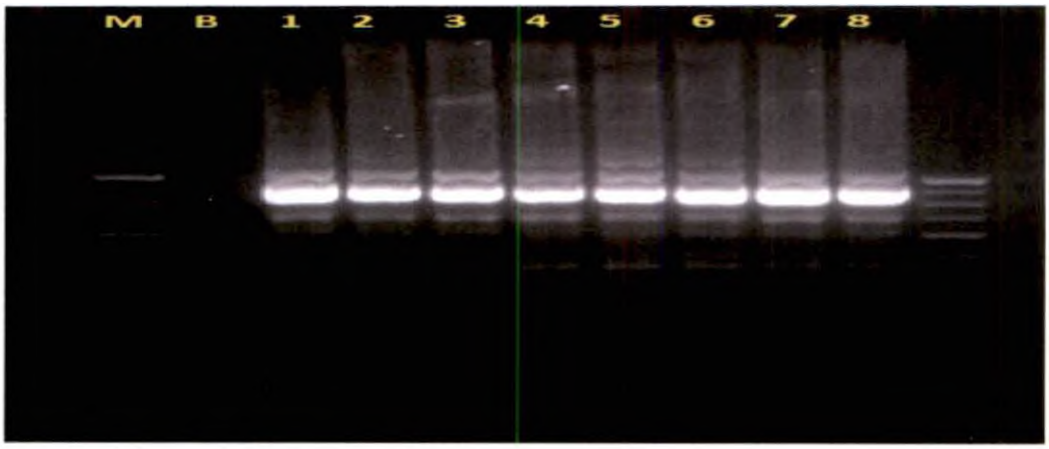


M: 100bp ladder/Marker, B: Control

b - **Amplification with UBC 868**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6- subculture 12th, 7- subculture 14th, 8- subculture 16th.

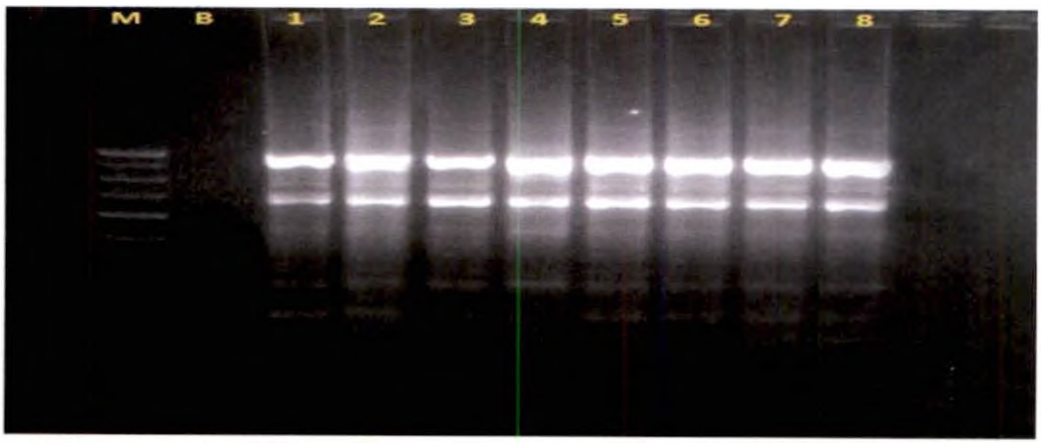
Plate.13 : ISSR amplification pattern of micropropagated banana plants (SD) with primers UBC 836 and UBC 868



M: 100bp ladder/Marker, B: Control

a - **Amplification with UBC ISSR 3**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6- subculture 12th, 7- subculture 14th, 8- subculture 16th.



M: 100bp ladder/Marker, B: Control

b - Amplification with UBC 848

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6- subculture 12th, 7- subculture 14th, 8- subculture 16th.

Plate 14 : ISSR amplification pattern of micropropagated banana plants (SD) with primers ISSR 3 and UBC 848

UBC 845

Amplification with this primer generated seven amplicons of which two were polymorphic and the rest were monomorphic. The pattern of amplification is shown in Plate 15a. The molecular weight of the amplicons ranged between 0.448 to 1.464 Kb.

UBC 814

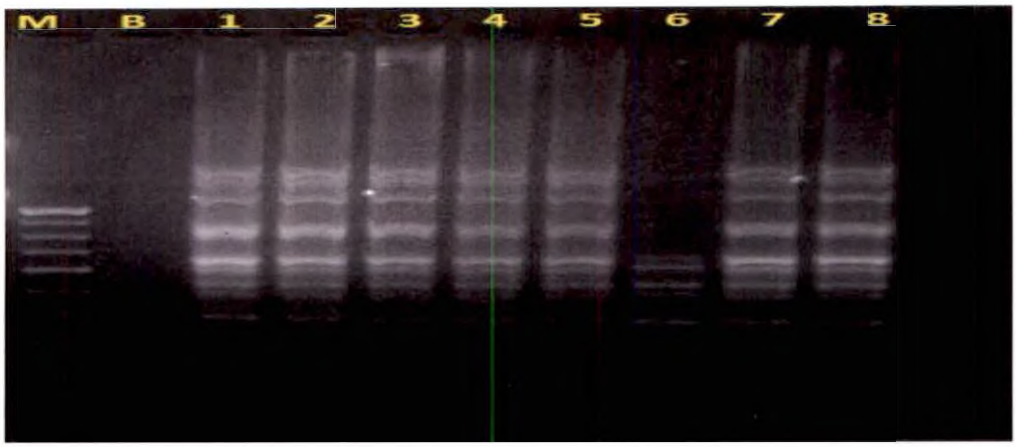
A total of four amplicons were obtained after DNA amplification with the primer UBC 814. The pattern of amplification is shown in Plate 15b. All amplicons obtained with this primer were monomorphic. The molecular weight of the bands varied from 0.250 to 1.23 Kb.

The extent of amplification and polymorphism observed is tabulated and provided in Table 9. Three to seven amplicons were observed with respect to each primer and the polymorphism observed ranged between 0 to 57.1 per cent.

The dendrogram developed out of the ISSR data is provided in Plate 16. The variation observed among the regenerants ranged between 1 to15 per cent. The plantlets regenerated through different subcultures were grouped differently. Variability was less (8 per cent) in the regenerants derived in the initial six subcultures.

4.3.3 MSAP analysis

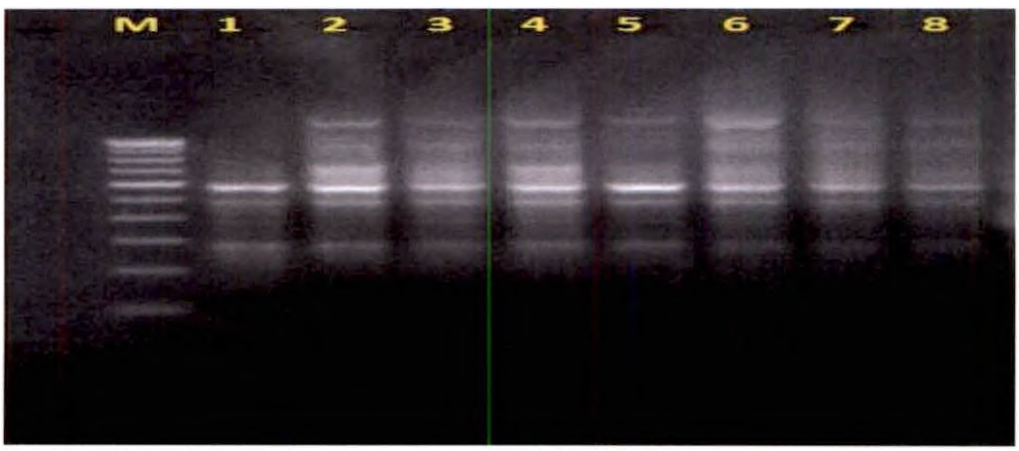
The plants derived from different subcultures through *in vitro* organogenesis were used for molecular analysis to study the levels and patterns of DNA methylation. The data were analysed for per cent hemi methylation, internal methylation and total methylation. The results obtained are presented in this section.



M: 100bp ladder/Marker, B: Control

a - **Amplification with UBC 845**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th.



M: 100bp ladder/Marker, B: Control

b - **Amplification with ISSR primer UBC 814**

1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5-subculture 10th, 6-subculture 12th, 7- subculture 14th, 8- subculture 16th

Plate 15: ISSR amplification pattern of micropropagated banana plants (SD) with primers UBC 845 and UBC 814

Table 9: Amplification pattern observed in ISSR assay of micropropagated banana regenerated from sucker

Primer	MP	Sc3		Sc6		Sc8		Sc10		Sc12		Sc 14		Sc16	
	T	P	%	P	%	P	%	P	%	P	%	P	%	P	%
UBC 835	4	0	0	0	0	1	20	0	0	0	0	0	0	1	20
UBC 843	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UBC 820	7	0	0	0	0	2	28.57	2	28.57	2	28.57	4	57.14	4	57.14
UBC 890	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UBC 836	7	0	0	0	0	0	0	0	0	0	0	0	0	3	42.85
UBC 868	5	0	0	0	0	0	0	1	20	0	0	1	20	0	0
ISSR 3	6	0	0	0	0	1	16.6	1	16.6	1	16.6	1	16.6	1	16.6
UBC 848	6	0	0	0	0	2	33.3	0	0	0	0	0	0	0	0
UBC 845	7	0	0	0	0	0	0	0	0	0	0	1	14.28	1	14.28
UBC 814	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0

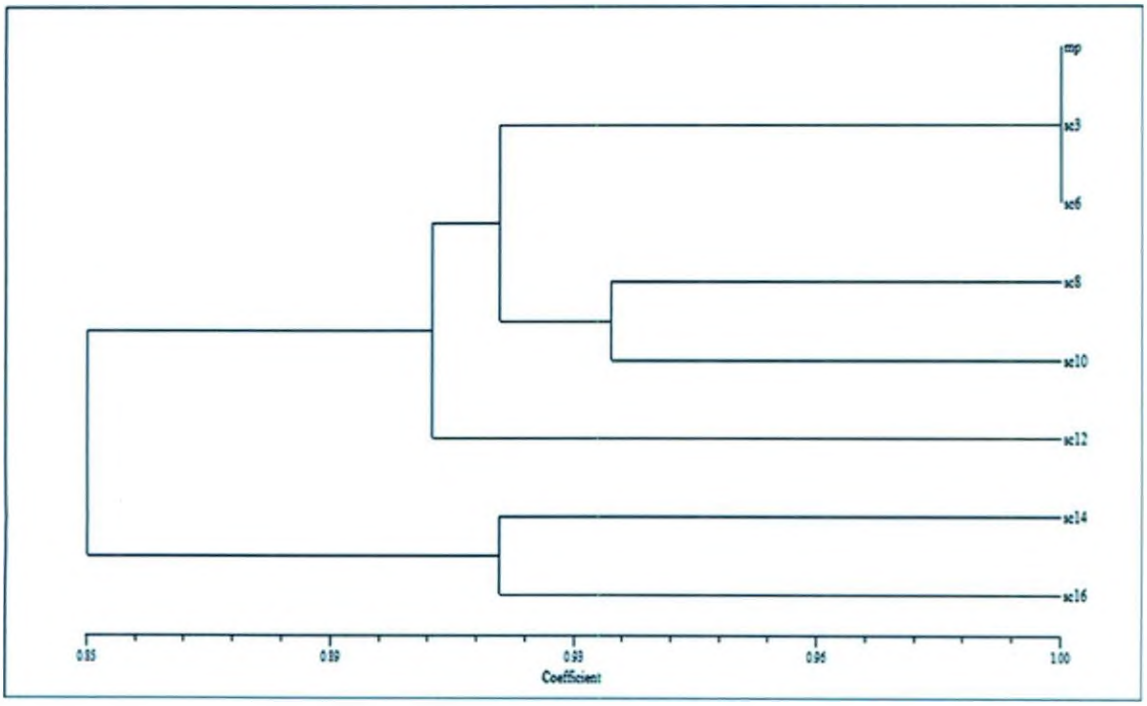
MP- Mother Plant,

Sc3.....Sc16 - subculture 3 to 16

T- Total number of amplicons

P- Number of polymorphic amplicons (in relation to mother plant)

% - Percentage polymorphism



MP- Mother Plant,

Sc3……Sc16 - subculture 3 to 16

Plate 16: Dendrogram developed out of ISSR analysis of sucker derived plants

4.3.3.1 MSAP analysis of male bud derived plants regenerated in different subcultures

An attempt was made to detect the cytosine methylation at 5'CCGG 3' in different subcultures of sucker derived banana plants using 4 primer combinations. The pattern of amplification is provided in Plate 17 and the compiled data for all the four primers is provided in Table 10.

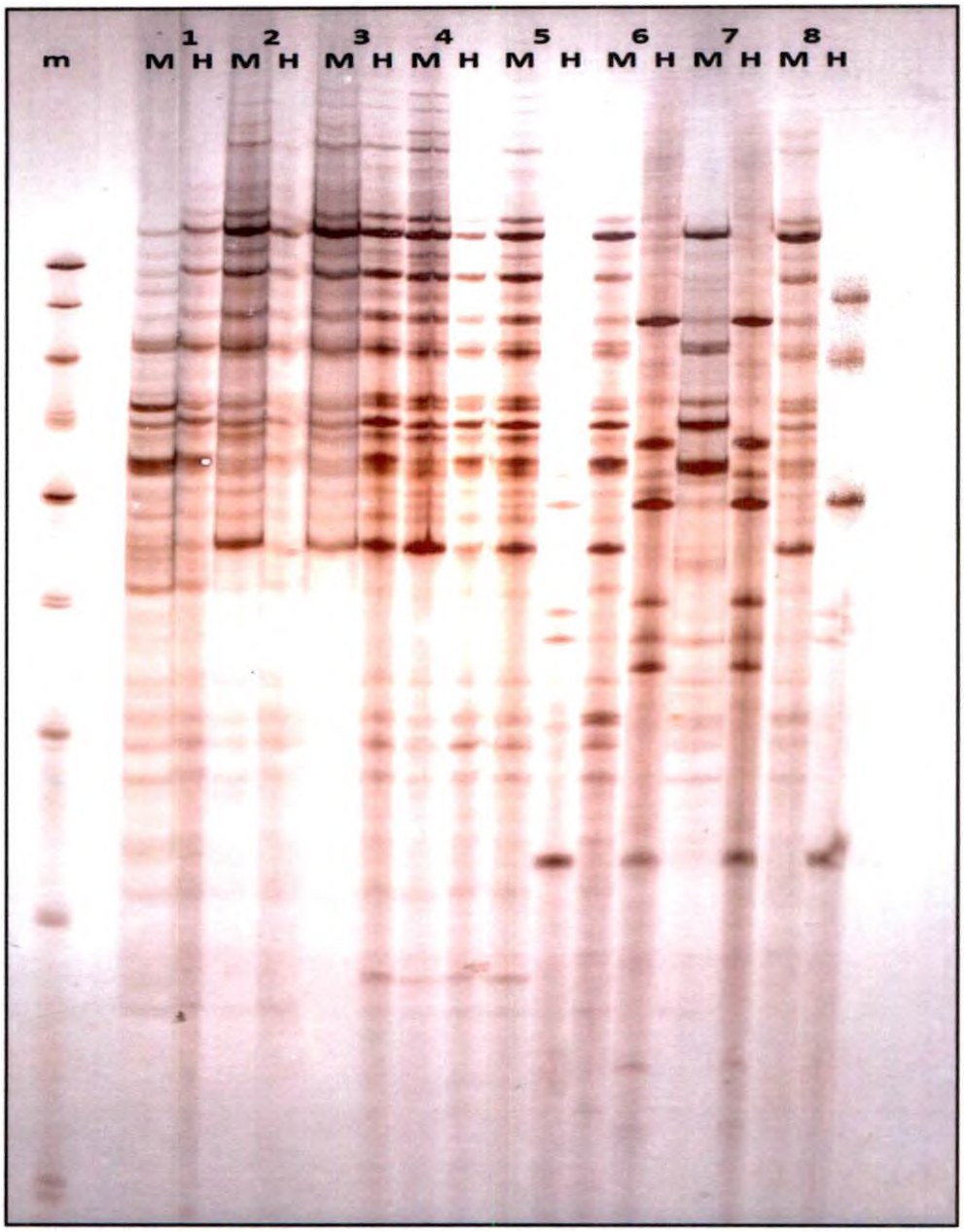
The total number of bands varied from 17 to 32 for different primers. The number of methylated sites varied from 1 to 11. The plants derived from later subcultures (10^{th} onwards) scored maximum methylated sites compare to those derived from initial subcultures. The extent of cytosine methylation is provided in Table 11.

A total of 8 (3.0%) hemimethylated sites and 13 (6.3%) internal methylatied sites were observed in the mother plant. Thus a total methylation of 9.3 per cent was observed in mother plant.

The subculture 3^{rd} showed slight increase in hemimethylaed sites to 10 (3.2%) and the internal methylation sites to 16 (9.20%). A total methylation observed was 12.4 per cent in plants derived from 3^{rd} subculture.

A total hemimethylated sites in subculture 6^{th} were 8 (3.20%) and internal methylation sites were 14 (6.30%). While the total methylation was 9.15 per cent in plants derived from 6^{th} subculture.

The plants derived from subculture 8^{th} and 10^{th} showed 5 (3.42%) and 9 (10.68%) hemimethylated sites respectively. While the internal methylated sites were 5 (9.3%) and 11 (14.8%) in 8^{th} and 10^{th} subculture respectively. A total methylation percentage for 8^{th} subculture was 12.78 while for 10^{th} subculture it was 25.52.



m- Marker DNA (100 bp), 1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5- subculture 10th, 6- subculture 12th, 7- subculture 14th, 8- subculture 16th

Plate 17: MSAP amplification profile for male bud derived plants with the primer E+AG- MH+ ATG

Table 10. Variation in number of methylated sites in MSAP assay with respect to different primers for male bud derived plants

Primer name	Mp	Sc3	Sc6	Sc8	Sc10	Sc12	Sc14	Sc16
E+AG-MH+ATG	2	5	1	1	4	8	9	11
E+AT-MH+CA	8	9	7	5	5	6	6	5
E+AC-MH+ATG	9	10	11	4	4	8	9	11
E+AG-MH+GT	2	2	2	0	7	7	10	11
Total	21	26	22	10	20	29	34	38

Table 11. Extent of cytosine methylation in male bud derived banana plants regenerated in different subcultures.

Subculture no.	Hemi methylation (%)	Internal full-methylation (%)	Total methylation (%)
Mp	3.00	6.30	9.3
Sc3	3.20	9.20	12.4
Sc6	3.20	6.15	9.15
Sc8	3.42	9.36	12.78
Sc10	10.68	14.84	25.52
Sc12	16.89	26.79	43.68
Sc14	18.12	28.33	46.45
Sc16	18.27	30.43	48.7

Plants derived from subculture 10th onwards showed more methylation per centage to those derived in initial subcultures. A total of 13 (16.89%) hemimethylated and 16 (26.69%) internal methylated sites were observed. The total methylation was 43.68 per cent; showed approximately 10 per cent increase compared to initial subcultures.

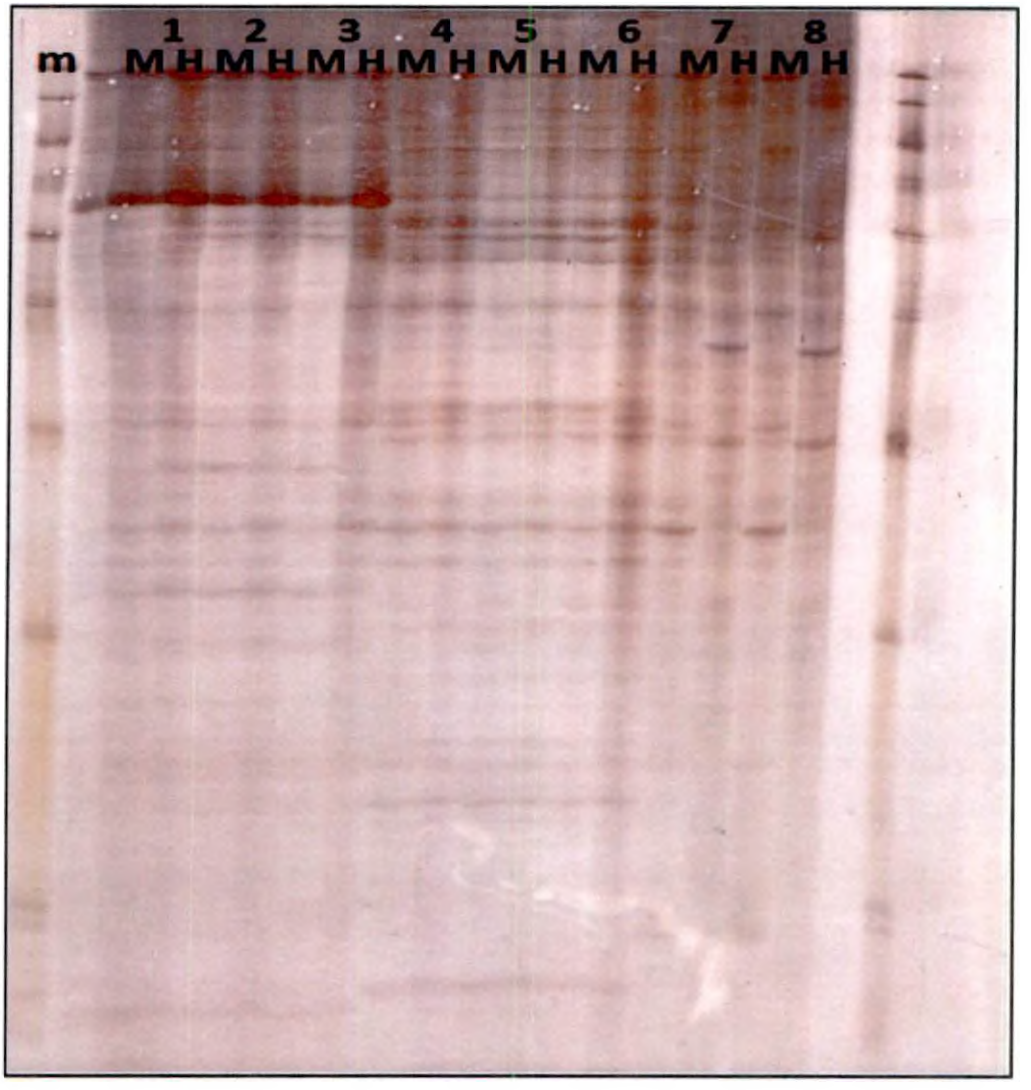
A total of 15 (18.12%) and 16 (18.27) hemimethylated sites were observed in 14th and 16th subculture derived plants respectively. While the total internal methylation sites were 19 (28.33%) in 14th subculture and 22 (30.43%) in 16th subculture derived plants. A total methylation was 46.45 and 48.7 per cent respectively in 14th and 16th subculture derived plants.

The differential sensitivity of isoschizomers *Hpa*II and *Msp*I revealed the abundance of internal methylated and hemimethylated sites in all the subcultures. The level of internal full methylation was higher than hemi-methylation for all the subcultures.

4.3.3.2 MSAP analysis of sucker derived plants regenerated in different subcultures

An attempt was made to detect the cytosine methylation at 5'CCGG 3' in different subcultures of sucker derived banana plants using 4 primer combinations. The pattern of amplification is provided in Plate 18 and the compiled data for all the four primers is provided in Table 12.

The total number of bands varied from 16 to 28 for different primers. The number of methylated sites varied from 2 to 11. The plants derived from later subcultures (10th onwards) scored maximum methylated sites compare to those derived from initial subcultures. The extent of cytosine methylation is provided in Table 13.



m- Marker DNA (100 bp), 1- Mother plant, 2- Subculture 3rd, 3- Subculture 6th, 4- subculture 8th, 5- subculture 10th, 6- subculture 12th, 7- subculture 14th, 8- subculture 16th

Plate 18 : MSAP amplification profile for sucker derived plants with the primer E+AG- MH+ ATG

Table 12. Variation in number of methylated sites in MSAP assay with respect to different primers for sucker derived plants

Primer name	Mp	Sc3	Sc6	Sc8	Sc10	Sc12	Sc14	Sc16
E+AG-MH+ATG	2	2	2	4	4	5	10	11
E+AT-MH+CA	7	6	6	6	6	6	8	8
E+AC-MH+ATG	10	10	9	9	6	9	10	10
E+AG-MH+GT	2	2	2	2	6	11	11	11
Total	21	20	19	22	22	31	39	40

Table 13. Extent of cytosine methylation in sucker derived banana plants regenerated in different subcultures

Subculture no.	Hemi methylation (%)	Internal full-methylation (%)	Total methylation (%)
Mp	10.25	16.45	26.7
Sc3	6.32	18.98	25.3
Sc6	6.75	18.91	25.66
Sc8	9.45	20.27	29.72
Sc10	12.98	15.58	28.56
Sc12	13.41	24.39	37.8
Sc14	20.23	26.19	46.42
Sc16	16.25	33.75	50

A total of 8 (10.25%) hemimethylated sites and 13 (16.45%) internal methylatied sites were observed in the mother plant. Thus a total methylation of 26.7 per cent was observed in mother plant.

The subculture 3rd showed decrease in hemimethylaed sites to 5 (6.32%) but the internal methylation sites increased to 15 (18.98%). A total methylation observed was 25.3 per cent in plants derived from 3rd subculture.

A total hemimethylated sites in subculture 6th were 5 (6.75%) and internal methylation sites were 14 (18.71%). While the total methylation was 25.6 per cent in plants derived from 6th subculture.

The plants derived from subculture 8th and 10th showed 7 (9.45%) and 10 (12.98%) hemimethylated sites respectively. While the internal methylated sites were 15 (20.27%) and 12 (15.58%) in 8th and 10th subculture respectively. A total methylation percentage for 8th subculture was 29.7 while for 10th subculture it was 28.56.

Plants derived from subculture 12th onwards showed more methylation per centage to those derived in initial subcultures. A total of 11 (13.41%) hemimethylated and 20 (24.39%) internal methylated sites were observed. The total methylation was 37.8 per cent; showed approximately 10 per cent increase compared to initial subcultures.

A total of 17 (20.23%) and 13 (16.25) hemimethylated sites were observed in 14th and 16th subculture derived plants respectively. While the total internal methylation sites were 22 (26.19%) in 14th subculture and 27 (33.75%) in 16th subculture derived plants. A total methylation was 46.4 and 50 per cent respectively in 14th and 16th subculture derived plants.

The differential sensitivity of isoschizomers *Hpa*II and *Msp*I revealed the abundance of internal methylated and hemimethylated sites in all the subcultures. The

level of internal full methylation was higher than hemi-methylation for all the subcultures.

Discussion

5. DISCUSSION

In vitro regeneration in banana var. Nendran was attempted from two different explant sources namely male bud and suckers. Plantlets regenerated at different subcultures were hardened and evaluated for morphological and genetic stability. The results obtained in different aspects of the study are discussed here under.

5.1 *In vitro* regeneration

Plants could be derived *in vitro* from both male bud and sucker derived explants. Since the male buds are already differentiated for a reproductive pathway, the natural programme had to be reversed to bring them back to vegetative multiplication in tissue culture cycle, unlike the sucker derived explants. Specific media components were required for altering the male bud explants from reproductive to vegetative pathway. Half MS media with reduced salt concentration supplemented with biotin and auxins (2, 4-D, NAA and IAA) induced bulging of male bud explants which developed as protocorms. These protocorms when subcultured in the shoot multiplication media (Full MS, BA and adenine sulphate) gave large number of multiple shoots. However the culture establishment rate, the time taken for multiple shoot induction and frequency of multiplication were low in male bud derived cultures. The sucker derived cultures gave good multiplication from the 2^{nd} subculture onwards. Adventitious shoots were observed all around the explant and multiplication rate was reasonably high (Table 4).

Earlier workers have also reported variation in *in vitro* regeneration of banana from different explants. However very few such reports are there in var. Nendran. Culture establishment and multiplication media having varying combinations of adenine sulphate, cytokinin (BA) and auxine (2, 4-D, IAA, IBA and NAA) have been reported earlier for *in vitro* regeneration from male bud explants in banana (Dore-Swami *et al.*, 1989).

Matsumoto and Yamaguchi (1989) reported protocorm like bodies during the *in vitro* regeneration of banana cv. Nanicavo (AAA group). Protocorm like bodies in tissue culture of several orchids were reported earlier (Murdad *et al.*, 2006 and Luo *et al.*, 2008).

Silayoi (2001) reported more multiplication rate in cultures from sucker explants compared to cultures from male bud explant.

There are various reports of formation of callus from male bud explants using the identical media combinations in banana (Ganapathi *et al.*, 1999; Meenakshi *et al.*, 2011). However there are different factors reported which affects and defines the formation of callus from male bud explant, some of which are explant type, growth regulators, physiological conditions, genetics of explants, *etc* (Gaj, 2004). Absence of callus phase in the present study may be due to above factors reported earlier.

Sidha *et al.* (2006) obtained callus from immature male flowers of different banana cultivars (Ardhapuri, Basrai, Grande Naine, Lalkela, Mutheli and Shrimanti) on MS medium containing 2, 4-D, IAA and NAA and the callus induction response varied with respect to different cultivars. These findings differ with the results of the present study. This may be due to the difference in cultivar used and the media combinations tried.

5.2 Morphological characterization

5.2.1 Male bud derived plants

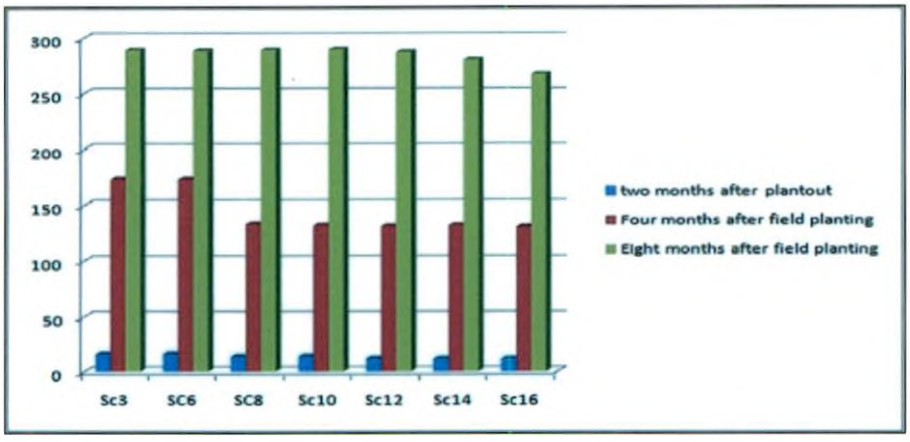
The morphological characters recorded (Table 5) at different stages of growth for the tissue culture plants derived from different subcultures recorded much variation.

The plant height varied significantly with respect to different subcultures. At two months after plantout, the plants derived from initial subculture were superior to those derived from later subcultures and the similar trend was observed at four months after field planting also. However the plants did not show much difference for plant height at eight months after field planting. At two months after plantout, the maximum difference recorded for plant height was 28 per cent and at four months after field planting it was 21.38 per cent. At eight months after planting, the lowest height was recorded for the plants from last (16^{th}) subculture and the variation was found reduced by 20.1 per cent.

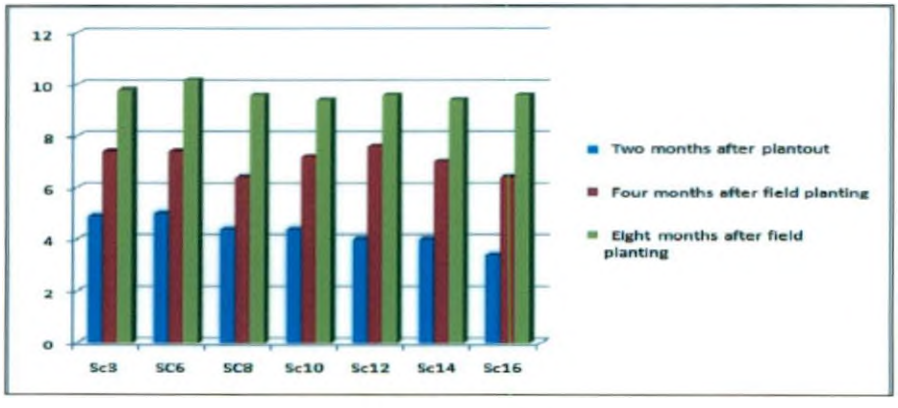
Similar trend was also observed for the number of leaves and leaf length. The maximum difference recorded for number of leaves was 32 and 16.2 per cent at two and four months of planting respectively, while for leaf length it was 26.6 and 15.6 per cent at two months of plantout and four months of field planting respectively. At two months after plantout, the maximum difference recorded for leaf breadth and pseudostem girth was 55.8 and 25.8 per cent respectively. The observations recorded are depicted in figure 1 and 2.

It was quite obvious to observe that at early stages of growth, the plants from initial subculture performed better. However, such a variation was not observed at later stages (8 months after planting), indicating the epigenetic nature of such variations. Further the climatic conditions that provided at planting time were entirely different for the plants derived from different subcultures since the planting was a continuous process in the study. This would have also contributed to the phenotypic variation observed.

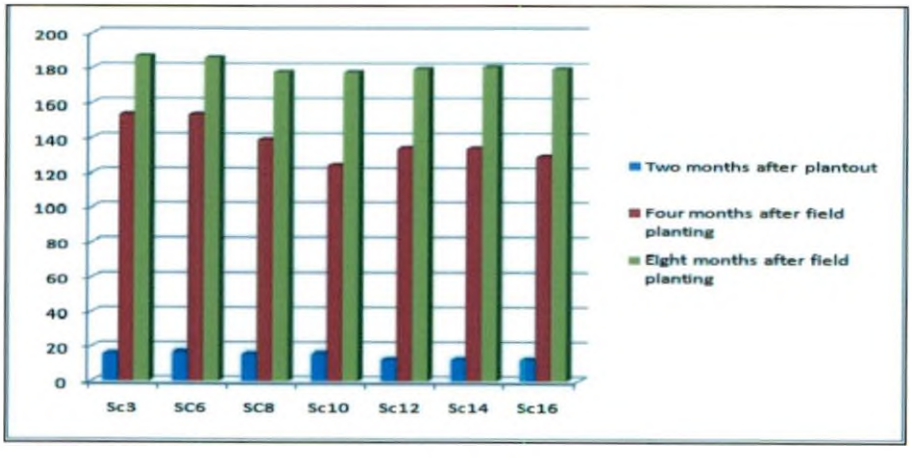
Vishwas *et al.* (2010) indicated similar results and trend during the evaluation of variation in micropropagated banana plants. In their study it is reported that the growth and yield characteristics were inferior in plants derived from later subcultures than those derived from initial subcultures.



a – Plant height

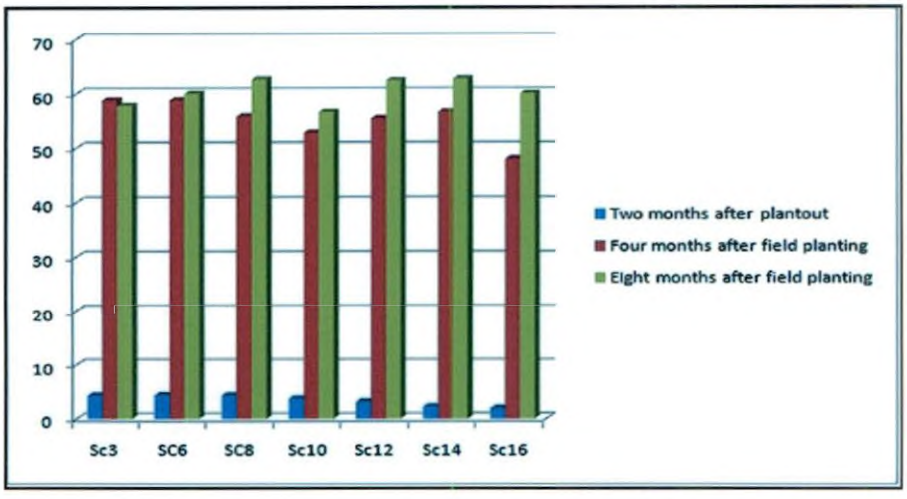


b – Number of leaves

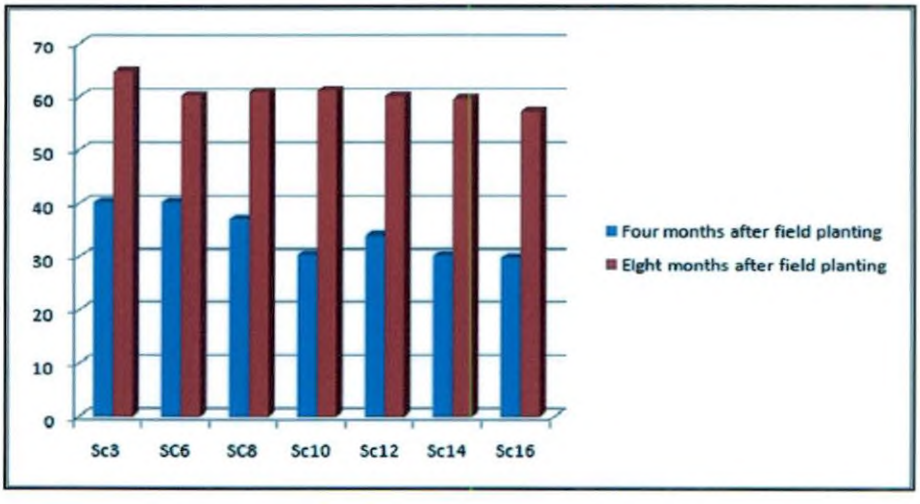


c – Leaf length

Fig. 1. Variation in plant height, number of leaves and leaf length recorded at different stages for MBD plants



a – Leaf breadth



b – Pseudostem girth

Fig. 2. Variation in leaf breadth and pseudostem girth recorded at different stages for MBD plants

5.2.1.1 Fruit characters

As explained earlier (section 4.2.2.1), the bunch characters were recorded only upto 10th subculture derived plants as the others are still at vegetative growth phase. The average bunch weight observed for different subculture, were comparable to the mother plant though the bunch weight was less in plants derived from 3rd subculture. This may be due to instability in the reversion process from reproductive to vegetative phase during regeneration of male bud derived explants. Apart from mother plant, the maximum bunch weight was recorded for 6th and 8th subculture derived plants but for 10th subculture derived plants it was significantly less. Vishwas *et al.* (2010) reported 24.9 per cent decrease in yield of 15th subculture plants compared to initial subcultures.

5.2.1.2 Abnormalities observed

Some of the abnormalities were observed during the tissue culture of the male bud derived plants. The occurrence of the abnormalities was not on large scale and regular. Three different kinds of abnormalities, wavery leaf lamina, elongated petiole and abnormal leaf base were observed during early stages of growth (Plate 3).

Apparently, it was neither the effect of a microbial infection (as they were grown under hygienic conditions), nor an effect of nematode infection (being grown on synthetic sterilized growth medium). Since all plants (normal and abnormal) were grown under identical nethouse conditions, variation could not be attributed to the physiological factors at early stages of growth. Therefore it appears to be an expression of tissue culture induced variation

Various off types of banana were identified during the laboratory evaluation and hardening of tissue cultured banana var. *Grand naine* (Vasane *et al.*, 2009). Silayoi (1995) reported great variation and abnormalities like dwarf plants and

irregular leaf shape in banana plants derived from male bud and also mentioned that the variation rectifying in later stages of growth.

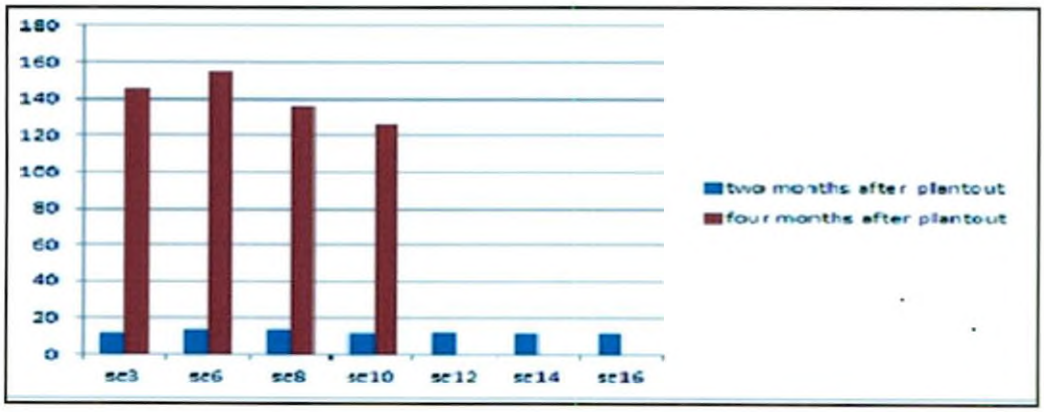
5.2.2 Morphological characterization of sucker derived plants

The different morphological characters recorded (Table 7) at different stages of growth for the tissue culture plants derived from different subcultures recorded much variation.

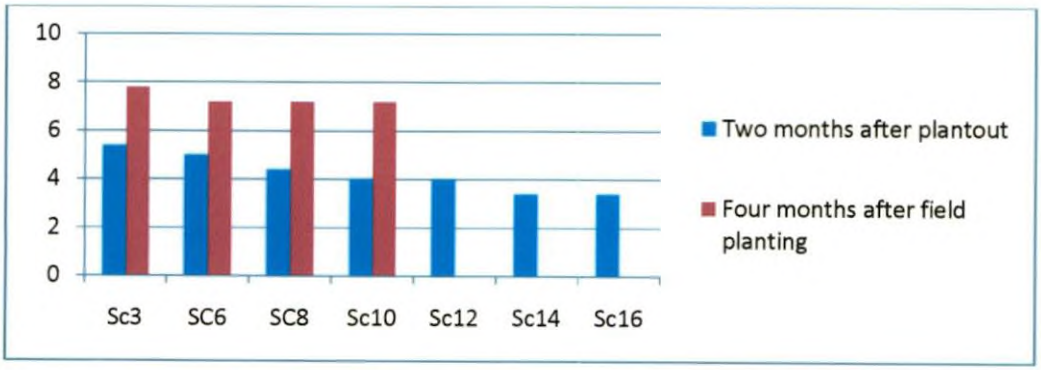
The plant height varied significantly with respect to different subcultures. At two months after plantout, the plants derived from initial subcultures were superior to those derived from later subcultures and the similar trend was observed at four months after field planting also. At two months after plantout, the maximum difference recorded for plant height was 15.17 per cent and at four months after field planting it was 15.66 per cent.

Similar trend was also observed for number of leaves and leaf length at two months after plantout only. At two months after plantout, the maximum difference recorded for number of leaves and leaf length was 37.1 and 18 per cent respectively. Superior leaf breadth was recorded in initial subcultures only at two months after plantout; no significant difference observed at four months after field planting. There was no significant difference observed for pseudostem girth. The observations recorded are depicted in Figure 3 and 4.

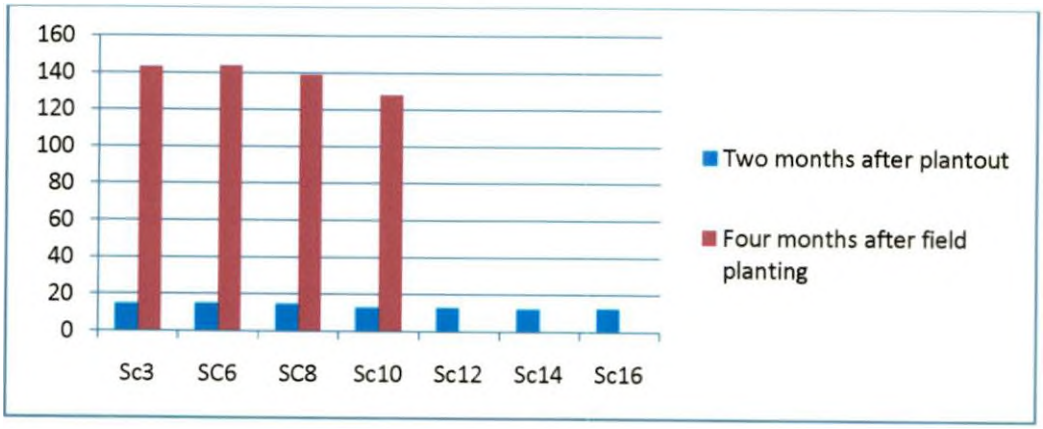
There have been other reports of morphological and vigour variation from field populations. In micropropagated bananas, somaclonal variation is detectable at the level of phenotype. Daniells and Smith (1993) reported as high as 91 per cent variants in tissue cultured plants in banana. The range of phenotypic variation has been reported to vary between 1 and 50 per cent (Israeli *et al.,* 1995). Gomez and Garcia (1997) also observed that the percentage of phenotypic variants differed in between different Cavendish banana genotypes. Jambhale *et al.* (2000) had suggested



a – Plant height

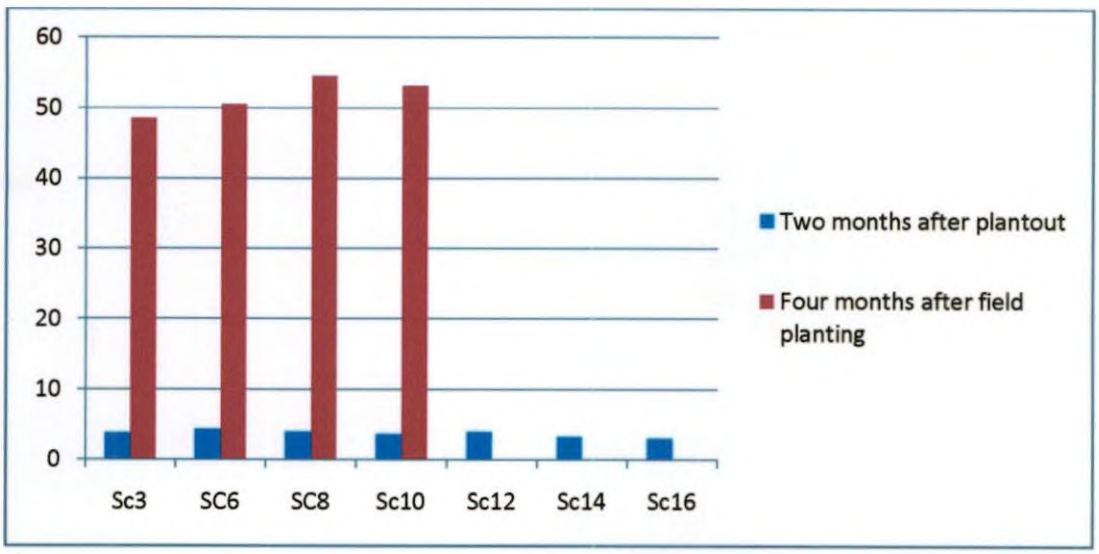


b – Number of leaves

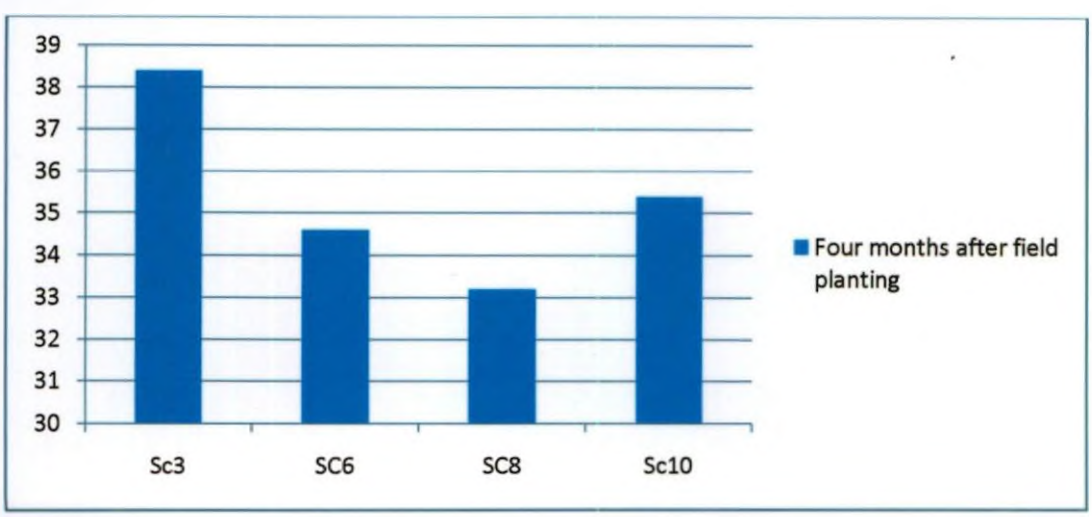


c – Leaf length

Fig. 3. Variation in plant height, number of leaves and leaf length recorded at different stages for SD plants



a – Leaf breadth



b – Pseudostem girth

Fig. 4. Variation in leaf breadth and pseudostem girth recorded at different stages for SD plants

that the number of subcultures in micropropagation of banana should be restricted to eight. They observed that some plantlets were conspicuously distinct from the parental clones in the populations of hardened plants after the 8th subculture. Morphological variations were observed in the 10th, 12th and 14th subculture of all the clones except Safed Velachi. Three other cultivars studied exhibited moderate frequency of variation i.e. Basrai (1.0–5.5%), Nendran (15.87–36.49%) and Lal Kela (3.0–7.2%). In their study, Nendran exhibited maximum variants in the 10th (15.87%), 12th (26.585%) and 14th (36.49%) subcultures.

5.2.2.1 Growth, yield and quality of banana influenced by planting time

In the present study, the plants derived from different subcultures were planted in the field throughout the year. The planting time was not uniform for all the subculture derived plants since they were planted out from different subcultures of one parental line. So the duration of subculturing has reflected on planting time.

There is possibility of influence of different planting time on the variation recorded for vegetative and bunch characters. For exact evaluation of morphological variation in *in vitro* regenerated plants the planting need to be identical for all subculture derived plants.

There are several reports which suggest the influence of planting time on growth and other parameters of banana.

Negative influence of different planting time on banana production was reported earlier (world meteorological report, 1988). Deshmukh *et al.* (2003) reported the variation in days for flowering and harvesting influenced by planting time in banana. Ara *et al.* (2011) also reported the great significant variation in vegetative, bunch characters and quality of banana at six different planting times throught the year suggesting the influence of planting time on yield of banana.

5.3 Molecular characterization

There are very few reports on characterization of tissue culture derived banana by using both morphological and molecular markers. Molecular markers combined with morphological markers will be the ideal method to evaluate the variation in tissue culture derived banana plants.

5.3.1 DNA isolation

The DNA was isolated from young leaves of banana plants of different subcultures. The protocol suggested by Rogers and Bendich (1994) modified with 4x CTAB extraction buffer yielded good quality DNA. The electrophoresed DNA showed distinct bands without shearing.

The homogenisation, pulverisation and uniformity of grinding of plant tissue were essential during DNA extraction. Excess liquid nitrogen was used for the homogenisation of the leaf tissue. Liquid nitrogen helps in maintaining the frozen tissue, preventing nucleic acid degradation and effect of secondary metabolites and a better mechanical disruption of tissue (Hernandez and Oyarzum, 2006). The problem of polyphenols was overcome by the addition of ß- mercaptoethanol and Poly Vinyl Pyrrolidone (PVP) along with the extraction buffer. ß- mercaptoethanol disrupts the protein disulphide bond and is thereby capable of initiating protein degradation. Poly Vinyl Pyrrolidone (PVP) removes polyphenols and inhibits co-precipitation of polysaccharides which resulted in good quality DNA. This was confirmed by De la Cruze *et al.* (1997) and Matasyoh *et al.* (2008).

The detergent present in the extraction buffer, CTAB, helps in the release of nucleic acids into the buffer after disruption of the cell membrane. The released DNA is protected from the action of DNase enzyme by EDTA present in the extraction buffer. It is a chelating agent, which efficiently blocks Mg^{2+}, the major cofactor of DNase enzyme. The DNA isolated by CTAB method was freed from chlorophyll by

using the Chloroform: Isoamyl alcohol which aids 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 is dissolved and stored. RNase treatment was given in order to remove RNA contamination from the isolated DNA samples as was reported by Sambrook *et al.* (1989).

The yield of DNA and its purity varied with clones. The yield ranged from 531.14 ng/µl to 3538 ng/µl. The purity (A260/ A280) ranged from 1.84 to 2.06. This could be due to the interference of various compounds in the plant tissue during the procedure.

5.3.2 Molecular marker analysis

In the present study, two PCR-based techniques, ISSR and MSAP were adopted for evaluation of genetic stability in banana plantlets. Palombi and Damiano (2002) also suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation while working on micropropagated plants of kiwi fruit. A better analysis of genetic stability of plantlets can be made by using a combination of two types of markers that amplify different regions of the genome (Martins *et al.*, 2004).

5.3.3 ISSR analysis

The DNA isolated from plants of different subcultures were bulked together and subjected for ISSR analysis.

5.3.3.1 Male bud derived regenerants

Out of the ten primers selected for ISSR assay of male bud derived banana plants only one primer UBC 890 gave monomorphic amplicons while others were polymorphic. The polymorphism was observed only in those plants which were derived after 8th subculture. A total of 53 amplicons were observed in the mother

plant and in those derived from upto 8th subculture. While the number of amplicons varied from 8th subculture onwards with respect to the mother plant. Maximum number of polymorphic amplicons were observed in 14th and 16th subculture derived plants. The primer UBC 848 was good enough to detect the polymorphism in plants derived from later subculture (10th onwards) and thus could be utilized as a marker for genetic stability analysis in micropropagated banana.

The amplification pattern was scored and analysed for quantifying the variation among the plants derived from different subcultures using male bud as explant. The computer package NTSYS-pc was used for cluster analysis. Plants derived from different subcultures were grouped into two distinct clusters. Plants derived from upto 12th subculture were found grouped in first cluster (<10 % variation) and those derived from 12th subculture onwards were found grouped in second cluster. The maximum genetic variation detected through ISSR assay was 15 per cent for male bud derived plants and the variation was relatively more in the plants derived in later subcultures (12th onwards).

5.3.3.2 Sucker derived regenerants

Out of the ten primers selected for ISSR assay of sucker derived banana plants three primers UBC 843, UBC 890 and UBC 814 gave monomorphic amplicons while others were polymorphic. The polymorphism was observed only in those plants which were derived after 6th subculture. A total of 55 amplicons were observed in the mother plant and those derived from upto 6th subculture. Maximum number of polymorphic amplicons were observed in 14th and 16th subculture derived plants. The primer ISSR 3 and UBC 820 gave the maximum number of polymorphic amplicons in the plants derived after 8th subculture onwards.

The amplification pattern was scored and analysed for quantifying the variation among the plants derived from different subcultures using sucker as explant. The computer package NTSYS-pc was used for cluster analysis. Plants derived from

different subcultures were grouped into two distinct clusters. Plants derived from upto 12th subculture were found grouped in first cluster (<10 % variation) and those derived from 12th subculture onwards were found grouped in second cluster. The maximum genetic variation detected through ISSR assay was 15 per cent for sucker derived plants and the variation was relatively more in the plants derived in later subcultures (12th onwards).

The polymorphism in ISSR amplification products could result from changes in either the sequences of the primer binding sites or from changes that could have altered the sizes of the DNA fragments (of template). Variation could also result due to the prevention of the successful amplification of a target DNA fragment (e.g., insertions, deletions, and inversions) suggested by Venkatachalam *et al.* (2007).

Earlier there have been reports of genetic stability analysis of micropropagated banana cultivars using molecular markers (Martin *et al.*, 2004; Lakshmanan *et al.*, 2007). On the other side, there are some reports which detected no or very less variation on ISSR analysis in micropropagated banana. Ray *et al.* (2006) reported 5.08% polymorphism between the mother and few micropropagated plants of Robusta cultivar. Venkatachalam *et al.* (2007) observed homogeneous RAPD and ISSR patterns on genetic analysis of micropropagated plants of banana. Rout *et al.* (2009) concluded in vitro multiplication as the safest mode for multiplying true-to-type plants. On ISSR analysis of micropropagated plantlets they observed that most of ISSR markers showed monomorphic banding pattern. Very few plants showed variation at the DNA level, but morphologically they were similar.

Usually plantlets from commercial firms show high morphological variation in field. They go for more subcultures in excess to reduce their economic cost as with fresh explant usage there are more chances of contamination associated losses. Recycling of sucker explants from previously micropropagated banana transplanted to field back to lab, further accumulates variation. Number of subculture, explant

source and hormonal concentration should be strictly followed to improve the genetic stability of micropropagated banana (Borse *et al.*, 2011).

5.3.4 DNA Methylation Analysis

In the present study, MSAP technique was used to detect cytosine methylation in the micropropagated banana genome. The results showed that this technique is highly efficient for large scale detection of cytosine methylation as suggested by Xiong *et al.* (1999). A distinct advantage of this approach compared to other methods is direct identification of the methylated sequences in the genome.

Previous analysis of the methylation status of in vitro-cultured plants have most frequently utilized restriction fragment length polymorphism (RFLP). However, this technique has several disadvantages such as the high number of probes required, the lower amount of information produced and the necessity for prior sequence information. MSAP is based on the AFLP technology (Vos *et al.*, 1995) and therefore there is no requirement for prior genome information other than the approximate genome size. Other advantages are the high number of methylation events detected using a relatively small number of primer combinations and the additional ability to clone and characterize novel methylated sequences. Thus MSAP technique could be considered as a suitable technique to evaluate epigenetic changes at the level of DNA methylation which may be associated with the phenomenon of somaclonal variation.

It should be pointed out that the technique also has three major constraints associated with resolving power. It relies on the template DNA quality. It can only detect a limited spectrum of bands (50 to 1,500 bp) on the 4 to 6 per cent acrylamide gel, shorter or longer DNA fragments cannot be detected (Xu *et al.*, 2000). Also, the technique can only investigate cytosines in the CCGG sites, restricted to the recognition site of the isoschizomers used, but cannot detect other cytosine methylation in CAG and CTG sites.

In the past, nucleotides modified by methylation were not considered to be part of primary nucleotide sequence of an individual. However, since DNA methylation occurs at defined target sequences, it represents a potentially important form of polymorphism. In this way, epigenetic information systems, like DNA methylation, could generate epigenetic variation that had never been considered as the cause of phenotypic variation. Tsaftaris and Polidoros (2000) speculated that the presence of epigenetic variation could be of particular importance in creation of variation in plants, single plant heritability, hybrid vigour, etc. Although it remains to be determined whether epigenetic variations generate phenotypic variation, they might be potentially associated with cryptic changes of phenotype like naturally occurring floral variants such as those determined by *clark kent* allele of Arabidopsis *SUPERMAN* locus (Jacobson and Meyerowitz, 1997). Remarkably, the first characterized natural mutant of flower symmetry in *Linaria vulgaris* was caused by DNA hyper methylation of the underlying gene *Lcyc* (Cubas *et al.*, 1999).

This is the first report of the use of the MSAP technique to evaluate DNA methylation changes in plants derived from tissue culture of Indian banana cultivar.

Results of the present study indicated that methylation pattern varied in each subculture (Fig. 5) for both male bud and sucker derived banana plants. The total methylation percentage varied from 9.3 to 48.7 per cent in different subcultures of male bud derived banana plants, while it was between 25.3 and 50 per cent in different subcultures of sucker derived plants. So for male bud derived plants the net methylation percentage was 39.4 per cent and for sucker derived plants it was 23.3 per cent. The methylation percentage observed was more in later subcultures compared to initial ones in both male bud and sucker derived plants.

Peraza–Echeverria (2001) also found the 23 per cent total methylation in micropropagated banana. Similar proportions of methylated CCGG sites were found

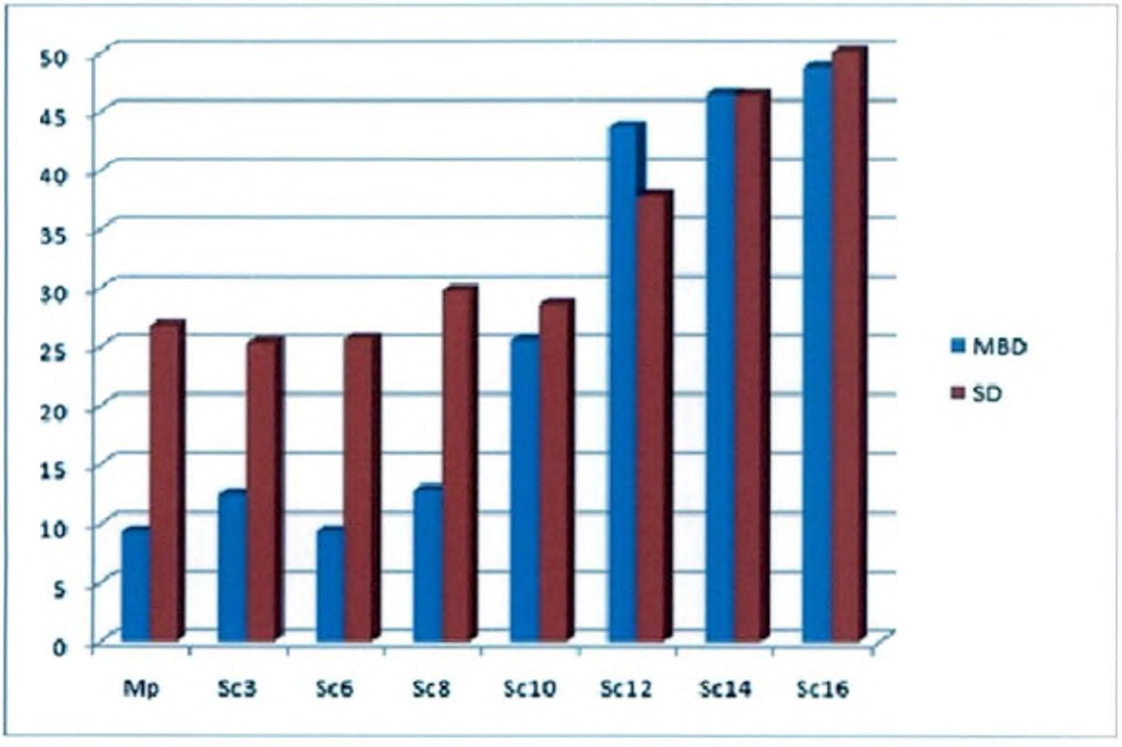


Fig. 5. Total methylation percentage in male bud (MBD) and sucker derived (SD) plants

in maize inbreed lines (21.88 %) reported by Yanli (2008). Sakthivel (2006) reported 33.39 per cent methylation in rice genome.

Another observation from this study is that the internal full methylation occurs more often than hemi-methylation in both male bud and sucker derived plants. In male bud derived plants the percentage of hemimethylated sites varied between 3 and 18.27 per cent and for sucker derived plants; ranged between 6.32 and 20.23 per cent. The percentage of internal full methylation ranged between 6.15 and 30.43 for male bud derived plants. For sucker derived plants; ranged between 15.58 and 33.75 per cent.

The high level of internal full methylation than hemimethylation may be due to the fact that hemi-methylated sites are unstable with respect to methylation status and by the process of maintenance methylation, the hemi methylated cytosine residues are converted to homo-methylated, i.e full or both strands methylated, as suggested by Otto and Walbot (1990). Similar findings were also observed by Xiong *et al.* (1999) and Joel and Zhang (2001) in their studies on rice.

Another important observation is the existence of polymorphism for both internal and hemi-methylation in plants derived from different subcultures; which reveal the presence of variability for methylation level in banana. Such a variation beyond DNA sequence variation is referred as 'epigenetic variability' in the form of DNA methylation. Wang *et al.* (2004) and Joel and Zhang (2001) reported the presence of natural epigenetic variation based on differential methylation states in rice genome.

5.3.4.1 DNA Methylation and Transposable Elements

DNA methylation is generally associated with the epigenetic variation and there are several reports of control of transposable element activity by DNA methylation level in the genome (Martienssen, 2001). More than 80 per cent of the

wheat genome is composed of transposable elements which reported to be associated with DNA methylation (Cantu *et al.,* 2010). Similarly it was also reported in *Arabodopsis* genome (Furner, 2010).

Recently, banana genome has been sequenced (CIARD, 2012); it reveals that 50 per cent of the banana genome is composed of transposable elements. So this study has great significance in detecting the hotspots in banana genome. However it must be pointed out that variation detected by using ISSR marker may be mainly due to changes in sequence and primer binding sites. But as reported earlier these types of variation influenced by transposons can be the effect of DNA methylation of the genome. Thus the DNA methylation could be the major cause of variation in tissue culture derived plants.

The present study could bring out valid results pertaining to micropropagation in banana. The variations observed in TC cycle were confirmed to be of genetic origin through ISSR and MSAP assay. Such variations were reasonably more after 12^{th} subculture. Thus it is recommended to restrict the subculture to 12^{th} cycle in the multiplication stage for micropropagation in banana in order to reduce the genetic variation (<10%). The variation in methylation pattern in advanced subcultures can be highlighted as an important factor contributing to somaclonal variation in banana.

Summary

6. SUMMARY

The study entitled "Morphological and molecular analysis of genetic stability in micropropagated banana (*Musa* spp.) var. Nendran" was carried at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara during the period 2010-2012. The objectives of the study were to characterize the variation in banana plants regenerated in different subcultures through *in vitro* organogenesis using morphological and molecular markers.

The salient features of the findings are as follows:

1. Male bud and suckers were used as explant source for in vitro organogenesis. Specific media combinations used for establishment of male bud explants induced bulging of explants which resulted in formation of protocorm like bodies.

2. Adventitious shoots were induced from both the sucker and male bud derived explants. The culture establishment rate, the time taken for multiple shoot induction and frequency of multiplication were low in male bud derived cultures. The sucker derived cultures gave good multiplication from the 3rd subculture onwards. Adventitious shoots were observed all around the explant and the multiplication rate was reasonably high. Regular rooting and plantout was followed for specified subcultures.

3. Vegetative characters were recorded at different stages of growth for male bud derived plants. The plant height, number of leaves and leaf length varied significantly with respect to different subcultures at initial stages. However, such a variation was not observed at later stages (8 months after planting).

4. The fruit characters of the male bud derived plants were found to be comparable to the mother plant though bunch weight and size were less in early and later subculture.

5. Some of the abnormalities were observed during the tissue culture of the male bud derived plants. These include wavery leaf lamina, elongated petiole and abnormal leaf base during early stages of growth.

6. Vegetative characters were recorded upto the fourth month for sucker derived plants. The TC sucker derived plants were at four months of field planting stage; later growth performances were not recorded. The plant height, number of leaves and leaf length varied significantly with respect to different subcultures at initial stages. There was no significant difference observed for pseudostem girth.

7. The protocol suggested by Rogers and Bendich (1994) was tested for extraction of genomic DNA from banana leaves. DNA was isolated from young unfurled leaves. The RNA contamination was completely removed through RNAse A treatment which resulted in DNA with no impurities and suitable for ISSR and MSAP assay.

8. ISSR assay was found good enough to detect variations in micropropagated banana plants. Out of the ten primers selected for ISSR assay of male bud derived banana plants, only one primer UBC 890 gave monomorphic amplicons while others were polymorphic.

9. The polymorphism was observed only in those plants which were derived after 8^{th} subculture. A total of 53 amplicons were observed in the mother plant and in those derived from upto 8^{th} subculture. While the number of amplicons varied from 8^{th} subculture onwards with respect to the mother plant. Maximum number of polymorphic amplicons were observed in 14^{th} and 16^{th} subculture in male bud derived plants

10. The amplification pattern was scored and analyzed using computer package NTSYS-pc for cluster analysis. Plants derived from different subcultures were grouped into two distinct clusters. Plants derived from

upto 12th subculture were found grouped in first cluster (<10 % variation) and those derived from 12th subculture onwards were found grouped in second cluster. The maximum genetic variation detected through ISSR assay was 15 per cent for male bud derived plants.

11. Out of the ten primers selected for ISSR assay of sucker derived banana plants, three primers UBC 843, UBC 890 and UBC 814 gave monomorphic amplicons while others were polymorphic. A total of 55 amplicons were observed in the mother plant and those derived from upto 6th subculture. Maximum number of polymorphic amplicons were observed in 14th and 16th subculture in sucker derived plants.

12. Plants derived from different subcultures were grouped into two distinct clusters. Plants derived from upto 12th subculture were found grouped in first cluster (<10 % variation) and those derived from 12th subculture onwards were found grouped in second cluster. The maximum genetic variation detected through ISSR assay was 15 per cent for sucker derived plants

13. Methylation in the present study was observed as an important factor which contributed to variation in micropropagated banana plants. MSAP technique was used to detect cytosine methylation using four primer combinations and it was found that MSAP assay could detect the variation in methylation pattern in micropropagated banana plants.

14. The methylation pattern obtained was highly variable in plants derived from later subcultures (10th onwards). Total methylation observed in male bud derived plants was 39.4 percent and in sucker derived plants it was 23.3 per cent. Percentage of internal methylation was relatively more than hemimethylation in both male bud and sucker derived plants.

15. The present study concluded that the culture response is low in male bud and thus suckers are the best explants for micropropagation of banana.

Micropropagated banana plants were observed to be uniform and exactly similar to the mother plant up to 6^{th} subculture. Variability observed up to 12^{th} subculture was < 10 per cent and it was 15 per cent by the 16^{th} subculture. Changes in methylation pattern and nucleotide sequence contributed to the variation.

16. Future line of work includes; further molecular characterization needed at the later stages of growth to detect reversion if any in methylation pattern. The primers identified could be further exploited for fidelity analysis in National Certification System for TC banana. Similar work to be taken up in other varieties of banana and since the banana genome is sequenced recently; the information generated could be exploited to detect the hotspots in banana genome.

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Annexures

ANNEXURE I

Details of laboratory equipment items used for the study

Refrigerated centrifuge	: Kubota 6500, Japan
Horizontal electrophoresis System	: BIO-RAD, USA
Thermal cycler	: 1. Master cycler, Eppendorf : 2. Master Cycler, Eppendorf, Gradient
Gel documentation system UVP (Inc. CA)	: BIO-RAD, USA
Nanodrop® ND-1000 Spectrophotometer	: Nanodrop® Technologies, Inc. USA
Water purification system	: Millipore, Germany
Ice flaking machine	: F 100 Compact, Ice matics
Laminar Air Flow	: HML- 104, Thermadyne

ANNEXURE II

Chemical composition of the Murashige and Skoog medium

Stock	Chemical	mg/litre	Stock concentration	Stock
I	$(NH_4)NO_3$	1,650	50 X	82.5g/l
	KNO_3	1,900		95.0g/l
	KH_2PO_4	170		8.5 g/l
	$MgSO_4.7H_2O$	370		18.5 g/l
II	$CaCl_2.2H_2O$	440	50X	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	100 X	2.23 g/l
	ZnSO4.7H2O	8.6		860 mg/l
	H_3BO_3	6.2		620 mg/l
	Kl	0.83		83.0 mg/l
	$Na_2MoO_4.2H_2O$	0.250		25.0 mg/l

	$CuSO_4.5\ H_2O$	0.025		2.5 mg/l
	$CoCl_2.6H_20$	0.025		2.5 mg/l
V	Vitamins			
	glycine	2.0	100 X	200mg/l
	Nicotinic acid	0.5		50 mg/l
	Pyridoxine acid-HCl	0.5		50 mg/l
	Thiamine- HCl	0.1		10 mg/l
	100 mg/l myo-inositol			
	30 g/l sucrose			
	6-9 g/l Agar 7.5 semi solid			
	pH 5-7-5.8			

$CuSO_4.5\ H_2O$	0.025		2.5 mg/l
$CoCl_2.6H_20$	0.025		2.5 mg/l

V Vitamins

glycine	2.0	100 X	200mg/l
Nicotinic acid	0.5		50 mg/l
Pyridoxine acid-HCl	0.5		50 mg/l
Thiamine- HCl	0.1		10 mg/l

100 mg/l myo-inositol

30 g/l sucrose

6-9 g/l Agar 7.5 semi solid

pH 5-7-5.8

ANNEXURE III

CTAB method of DNA Isolation as per Rogers and Bendich (1994)

Reagents:

(a) 2X CTAB Extraction Buffer

100 ml stock solution of each reagents were prepared separately and stored in refrigerator

Stock. I - CTAB (10%, w/v)

Stock. II - 1M Tris Buffer (pH 8)

Stock.III - 0.5M EDTA (pH 8)

Stock. IV - 4M NaCl

60 ml of 2X CTAB extraction buffer was prepared by pipetting following stocks

Stock .I - 12ml

Stock. II - 6 ml

Stock.III - 2.4ml

Stock .IV - 21ml

Distilled water- 18.6ml

Total - 60ml

(b) 10% CTAB Solution

10% CTAB (w/v)

0.7M NaCl

(c) TE Buffer

10mM Tris (pH 8)

1mM EDTA (pH 8)

ANNEXURE IV

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

242g Tris base

57.1ml glacial acetic acid

100ml 0.5 EDTA (pH 8.0)

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/l in water and was stored at room temperature in a dark bottle.

MORPHOLOGICAL AND MOLECULAR ANALYSIS OF GENETIC STABILITY IN MICROPROPAGATED BANANA (*MUSA* SPP) VAR. NENDRAN

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Banana is an important fruit crop widely grown throughout the world. With the increasing demand and vast export potential coupled with the farmers desire to grow banana on a large area, *in vitro* propagated plants are becoming increasingly important as planting material for rapid multiplication of economically important commercial varieties. *In vitro* propagation has many advantages, such as higher rates of multiplying clean (pest and disease-free) planting material and the small amount of space required to multiply large number of plants.

A major problem associated with micropropagation is the occurrence of genetic variation resulting from *in vitro* cultures, i.e., somaclonal variation amongst sub-clones of one parental line. The molecular basis of such variation is not well explained till date.

The study entitled "Morphological and molecular analysis of genetic stability in micropropagated banana (*Musa* spp) *var.* Nendran" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, during the period from 2010 to 2012 with the objective of evaluation and characterization of the variation in tissue culture derived banana plants regenerated in different subcultures through *in vitro* organogenesis.

Micropropagation of banana was carried out using male bud and sucker as explant source. Adventitious shoots were induced from both the sucker derived and male bud derived explants. The culture establishment rate and multiplication frequency were significantly low in male bud derived explants. The plantlets derived in specific subcultures (3, 6, 8, 10, 12, 14 and 16) were planted out and subjected for further evaluation. Considering vegetative characters during the field performance;

plants derived from later subcultures were found inferior to others in both male bud and sucker derived plants. The fruit characters of the male bud derived plants were comparable to the mother plant though bunch weight and size was less in later subculture (10^{th}).

The CTAB procedure reported by Rogers and Benedich (1994) for the extraction of nucleic acids was used for the isolation of genomic DNA from tissue culture derived banana plants. The young unfurled leaves from healthy plants were collected early in the morning and used for the genomic DNA isolation. The RNA contamination was completely removed through RNase treatment. Good quality DNA with UV absorbance ratio (A_{260}/A_{280}) 1.80 - 1.89 was used for further analysis.

The PCR conditions were optimized for Inter Simple Sequence Repeats (ISSR) assay. DNA isolated from each subculture were bulked and amplified using 10 selected primers. The amplification pattern was scored and analysed for quantifying the variation among the plants derived from different subcultures using male bud and sucker as explant. The computer package NTSYS-pc was used for cluster analysis. Plants derived from different subcultures were found grouped into two distinct clusters. Plants derived from upto 12^{th} subculture were grouped in first cluster (<10 % variation) and those derived from 12^{th} subculture onwards were found grouped in second cluster. The maximum genetic variation detected through ISSR assay was 15% for both male bud and sucker derived plants and the variation was relatively more in the plants derived in later subcultures (12^{th} onwards).

Changes in DNA methylation (addition of -CH3 to cytosine) has been hypothesized as an underlying mechanism of tissue culture induced variation due to the high frequency of quantitative phenotypic variation, the activation of transposable elements, heterochromatin-induced chromosome breakage events etc. An attempt was made to detect the extent of methylation pattern in micropropagated banana plants using Methylation Sensitive Amplification Polymorphism (MSAP) assay.

In the study, four MSAP primer combinations were used to amplify the fragments cleaved by restriction enzymes *Msp*I and *Hpa*II (isoschizomers). For male bud derived plants a total of 39.4 percent methylation sites were detected while for sucker derived plants it was only 23.3 percent. Variation in methylation pattern was observed more in later subcultures (10^{th} onwards) than initial. Percentage of internal methylation was relatively more compared to hemimethylation for both male bud and sucker derived plants.

From the study, it was observed that the plants derived from initial subcultures showed lesser variation (<10 %) compared to later subcultures (12^{th} onwards). The methylation pattern detected through MSAP assay was observed as an important factor inducing variation in tissue cultured banana. The ISSR and MSAP primers which detected polymorphism could be further utilized in quality control of tissue culture derived banana plants. Since the banana genome is sequenced recently, the information generated could be exploited to detect the hotspots in banana genome.