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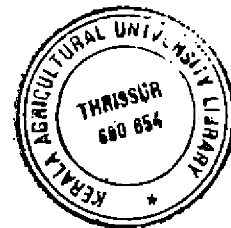
# MOLECULAR EVALUATION OF GENOMIC STABILITY OF BANANA PLANTS DEVELOPED BY *IN VITRO* CLONAL PROPAGATION

By
**ASHA. S. NAYAR**

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**2001**

# DECLARATION

I hereby declare that this thesis entitled "**Molecular evaluation of genomic stability of banana plants developed by *in vitro* clonal propagation**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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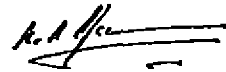
# CERTIFICATE

Certified that this thesis entitled "**Molecular evaluation of genomic stability of banana plants developed by *in vitro* clonal propagation**" is a record of research work done independently by Ms. Asha. S. Nayar (98-12-01) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.



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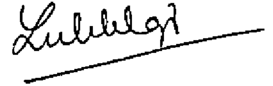
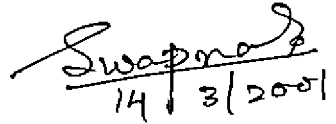
Dr. (Mrs.) G. R. SULEKHA 

Mrs. SWAPNA ALEX 

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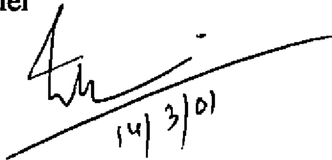

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| µl | microlitre |
| µM | micromolar |
| AFLP | amplified fragment length polymorphic DNA |
| BA | benzyl adenine |
| CTAB | cetyltrimethylammonium bromide |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxynucleotides |
| EDTA | ethylenediaminotetraacetic acid disodium salt |
| IAA | indole acetic acid |
| IBA | indole butyric acid |
| LS | Linsmaier and Skoog |
| mM | millimolar |
| MS | Murashige and Skoog |
| NAA | naphthalene acetic acid |
| ng | nanogram |
| ODAP | β-N oxalyl L-X, diaminopropionic acid |
| PCR | polymerase chain reaction |
| pM | picomolar |
| PVP | polyvinylpyrrollidone |
| RAPD | random amplified polymorphic DNA |
| RFLP | restriction Fragment Length Polymorphism |
| SCAR | sequence characterised amplified region |
| SDS | sodium dodecyl sulphate |
| SSR | simple sequence repeats |
| STMS | sequence tagged macrosatellite sites |
| Tris- HCl | tris (hydroxymethyl) aminomethane hydrochloride |
| VNTR | variable number of tandem repeats |

# INTRODUCTION

# 1. INTRODUCTION

Banana (*Musa* sp.) is the most important fruit crop of Kerala. The area under banana cultivation in Kerala is 72,000 ha (Aravindakshan, 1999). The annual production accounts to 5,70,000 t. However, the productivity (7.9 t/ ha) is not very appreciable. Incidence of serious pests and diseases, inefficient management practices and dearth of quality planting materials are among the major factors for low productivity.

Banana is propagated through suckers. Three to four month old suckers are selected for planting. The age difference in suckers leads to prolonged harvesting phase. In addition, propagation through suckers facilitates transmission of various diseases. Transportation and handling of the bulky suckers are cumbersome and expensive. The annual requirement of suckers for planting in Kerala has been estimated to be 18 crores (Aravindakshan, 1999). There is often scarcity of quality planting materials, especially due to the low rate of multiplication in banana.

*In vitro* propagation is an efficient method for rapid mass multiplication of elite plants as well as for ensuring the availability of pathogen-free planting materials. In banana, *in vitro* propagation is an excellent alternative over conventional propagation, with many advantages. In Karnataka, Tamil Nadu and Maharashtra tissue culture plants of banana are extensively used for commercial planting. However, somaclonal variation has been observed to be a problem,

assuming serious dimensions at times, while utilising tissue culture plants for large scale commercial propagation. Somaclonal variation has been reported in many species propagated *via in vitro* techniques (Munthali *et al.*, 1996 and Hashmi *et al.*, 1997). Various factors are responsible for somaclonal variations, including the number of sub-culture cycles adopted in *in vitro* propagation. Yang *et al.* (1999) reported that somaclonal variation increased with the age of the culture.

This necessitates the identification of the optimum number of subcultures, in commercial *in vitro* propagation of banana. Molecular markers like RAPD and RFLP have been reported to assess the clonal fidelity in several crops. The present study was undertaken to standardise RAPD analysis in banana and to assess the sub-culture associated genetic variation, if any.

# REVIEW OF LITERATURE

# 2. REVIEW OF LITERATURE

Micropropagation is an efficient method for rapid mass propagation of elite plants as well as pathogen–free planting materials (Ho *et al.*, 1995). In banana, *in vitro* multiplication is an excellent alternative over conventional propagation with many advantages (Vuylsteke and Ortiz, 1996). Banana is one of the most intensely micropropagated crops (Cote *et al.*, 1992).

## 2.1 *In vitro* propagation of banana

The most commonly used route of *in vitro* propagation in banana is *via* adventitious shoot formation, without intervening callus (Swamy and Sahijram, 1993). Shoot apices are commonly employed as explants for the rapid multiplication of elite varieties. With respect to multiplication rate, the potential of shoot apex culture is very high as compared to that of conventional clonal propagation. Therefore shoot apex culture is adopted as an effective technique for rapid propagation of elite types / cultivars.

There are several reports on the *in vitro* propagation of banana (Ma and Shii, 1972; Berg and Bustamante, 1974; Krikorian and Cronauer, 1984; Jarret *et al.*, 1985). At present, large scale commercial clonal propagation of banana *via* tissue culture is becoming popular. However, variation in field performance of *in vitro* raised banana plants is reported (Smith and Drew, 1990). In this chapter, a review of literature available on the *in vitro* propagation of banana, somaclonal

variations and application of molecular markers to assess the genetic fidelity of micropropagated plants is presented.

**2. 1. 1. Explant**

Various explants were tried for the micropropagation of banana plants. Shoot apices of lateral buds, eyes, peepers, suckers and parental psuedostems were used as sources of explant (Vuylsteke and de Langhe, 1985). According to Escalant *et al.* (1994), male flowers of triploid banana and plantain cultivars were used for somatic embryogenesis. *In vitro* propagation *via* adventitious shoot formation makes use of excised shoot apices as explants (Ganapathi *et al.*, 1995).

**2. 1. 2. Media**

Cronauer and Krikorian (1984) reported on the use of modified Murashige and Skoog (MS) medium containing inositol 100 mg $l^{-1}$, sucrose 40 g $l^{-1}$ and thiamine HCl 1.0 mg $l^{-1}$ for the establishment of shoot apices of banana clones (Philippine Lacatan and Grande Naine) and plantain clones (Saba and Pelipita). Murashige and Skoog medium containing macro and modified micro elements and supplemented with sucrose, glycine, thiamine HCl, nicotinic acid and pyridoxine, together with plant growth substances was used for culturing shoot apices (Banerjee and de Langhe, 1985). Wong (1986) used an initiation medium containing MS basal salts and supplemented with benzyl adenine (BA) 5.0 mg $l^{-1}$ and indole butyric acid (IBA) 0.1 mg $l^{-1}$. Linsmaier and Skoog (LS)

medium supplemented with indole acetic acid ( IAA ) 0.1mg $l^{-1}$, BA 5.0 mg $l^{-1}$ and malt extract 250 mg $l^{-1}$ was used for excised shoot apices of Karpura Chakkarakelli (Reddy, 1993). Murashige and Skoog medium along with BA 4.0 mg $l^{-1}$, inositol 100 mg $l^{-1}$ and gibberillin 1.0 mg $l^{-1}$ was used for the culture of shoot apices of Yenubontha. Murashige and Skoog medium supplemented with BA 2.0 mg $l^{-1}$ and sucrose 3.0 per cent was used to culture shoot apices of *Musa sapientum* cv.Basrai. Knop's medium was also found applicable (Ganapathi *et al.*, 1995).

Murashige and Skoog medium supplemented with Morel's vitamins, picloram 2.2 μM and sucrose 0.87 M with a pH of 5.8 was used for culturing the male flowers from *Musa sapientum* and *Musa paradisiaca* to obtain somatic embryogenesis (Escalant *et al.*, 1994).

Plantlets were obtained by placing the shoots into a rooting medium containing naphthalene acetic acid (NAA), IBA or IAA 1.0 mg $l^{-1}$ and with low levels of charcoal (Cronauer and Krikorian, 1984). Shoots cultured for one month with no plant growth substance also was reported to initiate roots (Damasco and Barba, 1985). Root induction was obtained with half strength MS containing IBA 1 μM (Banerjee *et al.*, 1986). Also rooted plants were produced by transferring the shoots to a medium containing auxin 1.0 mg $l^{-1}$. Rooting was initiated on White's medium supplemented with IBA 2.0 mg $l^{-1}$ (Reddy, 1993).

One month old plantlets were potted into soil, sand and farm yard manure (FYM) in the ratio of 2:1:1 in a mist chamber for two weeks before being planted outside (Balakrishnamurthy and Sreerangaswamy, 1988). According to Ganapathi *et al.* (1995), fully developed banana plantlets were transferred to soil in the greenhouse and then established in the field.

### 2. 1. 3. Culture conditions

Cultures were successfully stored at $15^0$ C with a light intensity of 1000 lux (Banerjee and de Langhe, 1985). For shoot apex cultures of banana, incubation was performed at $25^0$ C with a 16 hour photoperiod (Wong, 1986). Also it was reported that the cultures gave optimum results when incubated at $24^0$ C to $28^0$ C under 16 hours light and 8 hours dark photoperiod (Marroquin *et al.*, 1993; Okole and Schulz, 1996). The shoots were usually incubated at $23^0$ C to $27^0$ C, 1000 lux and 50-60 per cent relative humidity (Ganapathi *et al.*, 1995).

### 2. 2. Somalconal variations

Somaclonal variation is the general term given for the plant variation observed in cell or tissue culture (Larkin and Scowcroft, 1981). Cell or tissue cultures undergo frequent genetic changes like polyploidy, aneuploidy, chromosomal breakage, deletion, inversion, duplication, translocation and gene amplifications. These can lead to variations including agriculturally important traits.

According to Novak (1991) and Chai *et al.* (1995), somaclonal variation is the occasional phenotypic variation generated during *in vitro* culture. This may be due to genetic changes already present in the tissue of the explant *viz. in-situ* variation or variation induced by the mutagenic action of the tissue culture media used or variation as a result of the stress induced by the condition of the tissue culture environment. The first two factors are responsible for heritable changes whereas tissue culture environment induces epigenetic changes. Frequency of somaclonal variants differs widely, depending upon the genotypes and the culture conditions.

According to Reuveni *et al.* (1993), the rate of variants obtained was not affected by medium composition or by the rate of multiplication. The length of time in culture was not a mutation inducing factor.

The first detailed cytological evaluation of the somaclonal variants was carried out on regenerated sugarcane plants (Heinz and Mee, 1969). Genetic variability was found among the subcultures tested. Such differences were frequent in sugarcane due to its genetic mosaic nature (Flores *et al.*, 1984). The somaclonal variants were field tested. Stable variants (Krishnamurthi, 1982) and unstable variants (Larkin and Scowcroft, 1983) were observed. Stable variants included lines resistant to Fiji disease and downy mildew.

In potato, somaclones were recovered that contained variation for morphological characters such as growth habit, tuber colour, tuber production and tuber uniformity. Variability for disease resistance among the plants regenerated from protoplasts of potato was reported (Shepard *et al.*, 1980). Somaclones with field resistance to early blight (*Alternaria solani*) and late blight *(Phytophthora infestans)* were identified.

Chromosomal variations, single gene changes and cytoplasmic genetic variations were detected among the somaclones (Razdan, 1993). The genetic basis of somaclonal variants in tomato was described. Somaclonal variants of tomato bearing fruits with enhanced taste, better texture, colour and high (20 per cent) dry matter content were developed (Marty, 1988).

Somaclonal variation was observed in many of the micropropagated rice plant types. Rice somaclones with increased yield were identified by Evans (1989). The rice genome exhibited changes during plant differentiation or *in-vitro* culture (Bao *et al.*, 1995). These variations were found to increase with culture age (Yang *et al.*, 1999).

Among garlic regenerants, somaclonal variation was reported (Al-Zahim *et al.*, 1999). Novak (1980), reported variation in a range of phenotypic characters including plant height, leaf number, bulb weight and shape and number of cloves within a bulb. Cytological variation was also observed with almost half of the regenerants being tetraploid, aneuploid or mixoploid.

Vidal *et al.* (1993) reported a garlic somaclone which possessed a consistently higher bulb weight than the parental clone.

Somaclonal variants were reported in pigeon pea by Prasannalatha *et al.* (1999). Variations were found in micropropagated *Lathyrus sativus* in relation to leaf width, leaf length, internodal length and leaf area (Ali *et al.*, 2000). Somaclones were derived from root and internode explants. All the somaclones during development, had lower ODAP (B-N oxalyl L-X, B-diaminopropionic acid) content in their leaves compared to the parent P 24. Amongst the somaclones, Bio R 202 had the maximum leaf area, biomass as well as photosynthetic rate.

One of the concerns of using micropropagated banana plants is the detection of somaclonal variation. Somaclonal variants were observed for plant stature (mainly dwarf), leaf variegation and thickness and fruit bunches. Dwarfism was the most common variant. This accounted for 75 per cent of the total observed somaclonal variation (Stover, 1987). The second most common group of variants was characterized by different degrees of thick and rubbery narrow leaves with variable pale green mottling. Fruit bunches produced by the dwarf off-types were of inferior commercial value, causing serious economic losses to the growers.

A qualitative description on the somaclonal variants of seven banana cultivars of the Cavendish sub group obtained by *in vitro* propagation was given

by Israeli *et al.* (1991). In Cavendish banana, somaclonal variation was reported to range from 2.4 per cent to 25 per cent (Stover, 1987; Hwang and Ko, 1987; Smith and Drew, 1990; Novak, 1992). The somaclonal variation rate in micropropagation of *Musa* on a stationary liquid medium was observed to be below 5.0 per cent (Harris, 1992). For Pisang Berangan (Intan cultivar), the observed somaclonal variation ranged between 2.9 to 5.4 per cent.

In micropropagated banana the initial explant was the main factor determining the occurrence of somaclonal variants in forming stable and nonstable families (Reuveni *et al.*, 1993). With the increased use of shoot apex culture for *Musa* micropropagation, somaclonal variation was more among the regenerated plantlets (Roux, 1997).

**2. 3. Molecular markers**

Molecular markers are direct manifestations of genetic content. They serve as reliable indices of genetic variation. In the past decade, molecular markers have very rapidly complemented the classical strategies (Weising *et al.*,1995).

Molecular markers are genotypic markers. They are used to study the differences among strains at molecular level. They constitute biochemical constituents (secondary metabolites) and macromolecules (protein, DNA).

The genetic markers are used for clonal identification, linkage mapping, population diversity, taxonomy, evolutionary studies, determining the genetic fidelity during micropropagation, germplasm conservation etc. (Bretting and Widrlechner, 1995).

### 2. 3. 1. Isozymes

Isozymes and other proteins were applied to plant germplasm management. Isozymes were utilized for several crops including bananas (Jarret and Litz, 1986; Bhat *et al.*, 1992). The enzyme encoding loci do not constitute a random sample of genes and they are not randomly dispersed throughout the genome. Electrophoresis will detect only portion of the actual variability present in aminoacid sequences (Hillis and Moritz, 1990). Some isozyme variants are not selectively neutral (De Michele *et al.*, 1991). Therefore now the isozymes have been replaced by DNA- based markers (Anolles and Trigiano, 1997).

### 2. 3. 2. DNA markers

The DNA markers offer significant advantages with respect to increased number of loci detectable and mapped, overall phenotypic neutrality and the ability to score the plant at any developmental stage .

DNA markers are generally classified as hybridisation based markers and polymerase chain reaction (PCR) based markers (Swati *et al.*, 1999).

### 2. 3. 2. 1. Hybridisation based DNA marker technique

The hybridisation based DNA marker techniques are those that use labelled nucleic acid molecules as hybridisation probes (Anolles *et al.*, 1991). Probe molecules range from synthetic oligonucleiotides to cloned DNA.

Some of the important hybridisation based DNA techniques are restriction fragment length polymorphism (RFLP), hyper variable sequences and variable number of tandem repeats (VNTRs).

#### 2. 3. 2. 1. 1. Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism analysis involves digesting the subject genome with restriction enzymes, separating the fragments electrophoretically and then preferentially visualising fragments containing particular homologous sequences by hybridising them to a specific DNA probe. (Deverna and Alpert, 1990; Doowling *et al.*, 1990; Walton, 1990).

Genetic diversity in *Musa* was documented using RFLPs. Examination of 20 *Musa* species and subspecies using total DNA was done *via* RFLP. The result shows that there are two clear groupings among these species, one containing

species from sections *Musa* and *Rhodoclamys* and the other containing species from sections *Australimusa* and *Callimusa* (Gawel *et al.*, 1992).

Chloroplast-DNA RFLPs were used to study cytoplasmic genetic diversity in various *Musa* species and subspecies (Gawel and Jarret, 1991). Bhat *et al.* (1994) examined nuclear and chloroplast DNA RFLP variability within 57 *Musa* germplasm collections in order to evaluate the ability of RFLPs to identify and classify the Indian bananas.

**2. 3. 2. 1. 2. Hypervariable sequences and Variable number of tandem repeats (VNTRs)**

Jeffreys *et al.* (1985) were the first to report on the presence of minisatellite hypervariable sequences in the human genome that could be used for DNA fingerprinting. The presence of hypervariable sequences was confirmed in plants and animals by Gupta *et al.* (1996). Kaemmer *et al.* (1993) used oligonucleotide probes to differentiate *Musa* cultivars in various genomic groups. Bhat *et al.* (1995) found that DNA fingerprinting using oligonucleotide probes was useful for cultivar identification and for overall genome analysis to establish relatedness among the various accessions of *Musa* germplasm.

### 2. 3. 2. 2. Polymerase chain reaction (PCR) based DNA marker technique

These are fingerprinting techniques that use an *in vitro* enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules (Anolles and Trigiano, 1997; Michelli and Bova, 1996).

Among the PCR based DNA marker techniques the important ones are amplified fragment length polymorphism (AFLP), microsatellites, sequence characterised amplified region (SCAR), random amplified polymorphic DNA (RAPD) etc.

### 2. 3. 2. 2.1. Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism is based on PCR amplification of restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of few nucleotide bases (Vos *et al.*, 1995). Lin and Kuo (1995) have compared three different DNA mapping techniques RFLP, RAPD and AFLP in their efficiency in detecting polymorphisms in soybean. They found that AFLP is the most efficient technique in detecting polymorphism in soybean. This technique is yet to be applied in banana for the detection of polymorphism. This technique was found to be very useful in saturation mapping and for determination between varieties in oilpalm (Singh *et al.*, 1998).

### 2. 3. 2. 2. 2. Microsatellites

Microsatellites consist of tandemly arrayed di-tri-tetra nucleotide repeats, which are hypervariable and ubiquitously distributed throughout eukaryotic genomes. Micro satellite DNA markers, which can be directly amplified by PCR, have been developed using the unique sequences that flank microsatellites (Litt and Luty, 1989; Tantz, 1989; Weber and May, 1989). In several crop plants including soybean (Akkaya *et al.*, 1992), maize (Senior and Huem, 1993), brassica (La - gercrantz *et al.*, 1993), barley (Becher and Heun, 1995), wheat (Rodder *et al.*,1995) and rice (Mc Couch *et al.*, 1997), specific amplification of microsatellite loci indicated that microsatellite DNA markers are more variable than RFLPs .

Two size selected genomic libraries from banana were screened for the presence of simple sequence repeats (SSR) by Kaemmer *et al.* (1997). They demonstrated that SSR are readily applicable to the study of *Musa* genetics. Comprehensive analysis of a significant number of banana sequence tagged macrosatellite sites (STMS) would add to the knowledge on the structure and phylogeny of genomes of the *Musa* species and suggested the use of microsatellites as anchor markers for a banana genetic core map.

### 2. 3. 2. 2. 3. Sequence characterised amplified region (SCAR)

Sequence characterised amplified region DNA analysis was developed to produce reliable PCR- based results. Parent and Page (1998) used this technique to identify raspberry cultivars. Damasco *et al.* (1998) used markers based on SCAR to detect dwarf off-types of *in vitro* grown Cavendish bananas.

### 2. 3. 2. 2 .4. Random amplified polymorphic DNA (RAPD)

Polymerase chain reaction in conjunction with random primers, was used for fingerprinting genomes (Welsh and Mc Clelland, 1990), for population biology studies (Astley, 1992), for identification of genome-specific markers and other uses (Williams *et al.*,1990 and Erlich *et al.*,1991).

Several authors have applied the RAPD technique to investigate genetic variability and found the technique very efficient and reliable (Brown *et al.*, 1993; Munthali *et al.*, 1996).

Analysis of RAPDs offers several advantages when compared to RFLPs. The advantage of RAPD over RFLP is that RAPD does not require a labour intensive procedure. It is not necessary to construct or maintain a genomic library. RAPD requires far smaller quantities of genomic DNA than RFLP analysis. Also it is less costlier when compared to RFLP.

Random amplified polymorphic DNA markers offer a nearly unlimited supply of molecular traits and have been demonstrated as a useful tool for characterising cultivars of various crop species including *Brassica* spp. (Kresovich *et al.*, 1992), rice (Mackill, 1995) and mango (Schnell *et al.*, 1995).

Random amplified polymorphic DNA markers have been used successfully to detect genetic variation among low land and upland rice cultivars, the genetic characterisation and classification of *japonica* cultivars into temperate and tropical groups and foı analysis of genetic variability in rice populations (Yu and Nguyen, 1994). In general, a higher level of polymorphism was found between *japonica* and *indica* subspecies while fewer polymorphisms were found between upland and lowland cultivars within the indica subspecies. Thus among rice cultivars, genetic variation was easily detected using RAPDs. According to Verma *et al.* (1999), RAPD analysis allows the identification and discrimination of the individual genotypes of Basmati rice including the identification of the duplicates in genetic resource collections. Random amplified polymorphic DNA technique was used to detect the genetic variation at the level of DNA among aromatic and non-aromatic cultivars by Baishya *et al.* (2000).

To establish polymorphisms among local sugarcane varieties, RAPD markers were used (Iqbal *et al.*, 1995). The amplification profiles of the varieties L118, L116, BL 4, BF 162, Co 144 and Co 154 were compared and polymorphisms were detected.

According to Hu and Quiros (1992), RAPD markers provided a quick and reliable alternative to identify broccoli and cauliflower cultivars. Random amplified polymorphic DNA markers generated by four arbitrary 10-mer primers, discriminated 14 broccoli (*Brassica oleracaea italica*) and 12 cauliflower (*B . oleraceae botrytis*) cultivars by banding profiles.

The trait associated with the seed oil content in Indian mustard was identified with three RAPD markers *viz.* OPH 11, OPJ 06 and OPL 15 (Sharma *et al.*, 1999). It was revealed that there is significant association of oil content with these markers.

Randomly amplified polymorphic DNA markers have been used to characterise cocoa clones representing the three main cultivated subpopulations *viz.* Criollo, Forestro and Trinitario (Wilde *et al.*, 1992). The use of single primers of arbitrary nucleotide sequence resulted in the selective amplification of DNA fragments which were unique to the individual cocoa clones studied.

Mulcahy *et al.* (1993) characterised twenty five accessions of apple, representing eight cultivars (Golden Delicious, Delicious, Gala, Jonathan, Jonagold, Florina, Fior di Cassia and Imperate Dallago) with RAPD. Using two separate ten base pair primers, it was possible to obtain a distinctive fingerprint for each of the cultivars. Thus RAPD provided a simple and reliable method for cultivar identification in apple.

Using the technique of RAPD, Howell *et al.* (1994) identified 116 amplification products in *Musa* germplasm using nine primers. This enabled them to identify RAPD markers that are specific to each of nine genotypes of *Musa* representing AA, AAA, AAB, ABB and BB genomes. Fifty seven accessions of *Musa* including cultivated clones of six genomic groups (AA, AB, AAA, AAB, ABB, ABBB), *Musa balbisiana* Colla (BB), *Musa acuminata* Colla spp. *banksii* F. Mucll. (AA), *Musa acuminata* Colla ssp. *malaccensis* Ridl.(AA) and *Musa velutina* Wendl. and Drude were examined by Bhat and Jarret (1995) for RAPD genetic markers using PCR with sixty 10-mer random primers, which generated 605 polymorphic amplification products.

Randomly amplified polymorphic DNA studies were conducted by Martin *et al.* (1998), to assess genetic variability among three wild species of *Musa* and cultivars of dessert and cooking bananas. The polymorphism observed in the study allowed them for identification of different species and genomic groups. However, no polymorphism was detected within Cavendish sub-group. They also employed the technique for identification of height variants originated from *in vitro* cultures of Grande Naine and Petite Naine cultivars.

Randomly amplified polymorphic DNA using Operon primers was used to evaluate the genetic variability of 66 *Musa* species accessions in the germplasm collections of the National Banana Corporation of Costa Rica (CORBANA) and the Agronomic Centre for Research and Teaching (CATIE) also in Costa Rica (Cabrera *et al.*, 1998). High variability among the AA clones

and a low variability among the AAA, AAB, ABB triploids were observed. Randomly amplified polymorphic DNA analysis was performed on several clones of the variety Williams by Iqbal *et al.* (1995).

Randomly amplified polymorphic DNA markers were used for fingerprinting genotypes within and between *Annona* species (Renning *et al.*, 1995). The use of RAPD analysis för *Mangifera* germplasm classification and clonal identification was reported by Schnell *et al.* (1995). Graham and Mc Nicol (1995) generated RAPD markers from different *Rubus* species in order to assess the degree of similarity between species. Inter and intra specific / varietal variations were observed in the RAPD analysis of 42 accessions of *Vitis*, representing 13 species (Wang *et al.*, 1999). In addition, RAPD was used to detect genetic diversity within grape cultivars. These markers were also generated for identifying grape vine root stock (Wolf *et al.*, 1999). According to Lanham and Brennen (1999), RAPD markers were used to fingerprint and to examine genetic diversity among twelve genotypes of gooseberry. Six hazelnut (*Corylus avellana*) cultivars were identified using RAPD markers (Galderisi *et al.*, 1999). The analysed cultivars were distinguished by their RAPD fingerprints using the DNA primers U2, U3, U4, U11 and U14.

Early identification of sex in dioecious plants like papaya (Somri, 1998) and nutmeg (Shibu *et al.*, 2000) was possible with the help of RAPD markers .

Randomly amplified polymorphic DNA analysis was done to determine intra-specific variability in *Andrographis paniculata*. The results indicated that RAPD could be effectively used for genetic diversity analysis in wild species of prospective value as it is reliable, rapid and superior to those based on pedigree information (Padmesh *et al.*, 1999). Similarly in *Angelica* species, which is a natural medicine, RAPD analysis was carried out to identify the varietal difference (Watanabe *et al.*, 1998). Also RAPD markers were used to reveal genetic homogenity in the endangered Himalayan species *Meconopsis paniculata* and *Meconopsis simplicifolia* (Sulaiman and Hasnain, 1996). Four elite genotypes of *Vetiveria zizanioides viz.* BDP 1, BMH 1, MBR 5 and KS 1 were analysed by RAPD profiling to develop unique pattern of these genotypes as well as to assess the extent of diversity for placing them in a phylogenetic dendrogram (Shasamy *et al.*, 1998).

Randomly amplified polymorphic DNA markers were extensively used for the molecular characterisation in various crop species. Efforts were done in *Piper longum* to find out the genetic difference among the varieties using RAPD analysis by Philip *et al.* (2000). Genotypic and morphogenetic differences among three female varieties of *Piper longum*, one variety each from Assam, Calicut and one variety Viswam released from Kerala, were investigated using RAPD analysis and it was revealed that these varieties were genetically different . In *Piper longum*, RAPD technique was used to investigate the molecular basis of genotypic differentiation between the male and female plants (Banerjee *et al.*,

1999). As a result, male sex associated RAPD markers were identified for the first time in *Piper longum*.

Randomly amplified polymorphic DNA markers were found to be useful for confirmation of genetic fidelity in micropropagated plants (Gupta *et al.*, 1999). There are reports which document the occurrence of somaclonal variation among plants derived through somatic embryogenesis, organogenesis and axillary branching cultures. Molecular analysis carried out during various cultural passages, would help detect genetic variation, if any.

Four different types of somaclonal variants were identified and characterised in banana plants generated by meristem culture (Walther *et al.*,1997). Tissue cultured off-types did not display any visual differences during *in vitro* culture. But after six weeks of hardening in a commercial nursery, the field established plants showed significant phenotypic difference. Randomly amplified polymorphic DNA analysis of somaclonal variants revealed the presence of polymorphic bands with at least one set of primers. This enabled the early detection of mutants *in vitro.*

Randomly amplified polymorphic DNA analysis was used to detect genetic variation in micropropagated Cavendish bananas (Damasco *et al.*, 1996). The use of RAPD marker at the *in vitro* stage provides a reliable means for early detection of somaclonal variants. This allows the elimination of off-types before

planting of micropropagated plants in the field.

In pigeon pea, somaclonal variants were distinguished at the molecular level by RAPD analysis using specific arbitrary primers (Prasannalatha *et al.*, 1999). A higher level of polymorphism was evident with the primer OPA-20 where as low level was observed with the primer OPA-07. These served as molecular markers for specific somaclonal variants, thereby providing a method for selecting somaclones with better agronomic performance.

According to Lu *et al.* (1996), RAPDs are useful for establishing a genetic basis for somaclonal variation in rice. The results of RAPD analysis in cultured rice showed that somaclonal variation might have occurred in transfer RNA, ribosomal protein and other genes during cell culture. Also somaclonal variation was found to increase with culture age (Yang *et al.*, 1999).

The genetic variations in somaclones of Phalaenopsis True Lady B79-19 derived from tissue culture were evaluated (Chen *et al.*, 1998). The RAPD data indicated that normal and variant somaclones were not genetically identical. The results of this study suggested that considerable somaclonal variations in flower morphology, including colour and shape occurred in Phalaenopsis regenerants derived from tissue culture and that it should be possible to discern some of the somaclonal variants using genetically characterised RAPD markers.

Somaclonal variations were reported in *Triticum aestivum* (Brown *et al.*, 1993), populus (Rani *et al.*, 1995), beet (Munthali *et al.*, 1996) peach (Hashmi *et al.*, 1997), tomato (Hong *et al.*, 1999) and grapes (Verdisson *et al.*, 1999) using RAPDs. Rani *et al.* (1995) reported variation to the extent of 2.0 per cent in micropropagated *Populus deltoides* plants.

Using the RAPD technique, various investigators have reported the absence of genetic variation in micropropagated *Picea mariana* (Isabel *et al.*, 1993), *Festuca patiens* (Valles *et al.*, 1993), *Hordeum* (Devaux *et al.*, 1993) and norway spruce (Fourre *et al.*, 1997).

Random amplified polymorphic DNA markers were used in characterisation of micropropagated clones of four tree species, namely *Populus deltoides*, *Eucalyptus terticornis*, *Eucalyptus camaldulensis* and *Coffea arabica* (Rani and Raina, 1998).

Plants regenerated by somatic embryogenesis from long term callus cultures derived from five garlic cultivars were subjected to RAPD analysis (Al- Zahim *et al.*, 1999). Certain band changes were found in the regenerants of different cultivars, suggesting the existence of somaclonal variants.

According to Goto *et al.* (1998), RAPD markers were used to determine genetic stability of long term propagated shoots of Japanese black pine (*Pinus thunbergii* Parl.). The results showed that regenerants from plant

micropropagation system are genetically stable.

Randomly amplified polymorphic DNA primers were used to study genetic variation at the DNA level among the peach somaclonal variants (Hammerschlag *et al.*, 1996; Hashmi *et al.*, 1993). Sixty RAPD primers (10-mer) were screened and ten were found useful as markers to detect polymorphisms, thus establishing a genetic basis for somaclonal variation.

Randomly amplified polymorphic DNA technology was applied to monitor the genetic fidelity of micropropagated, meadow fescue *viz. Festuca pretensis* (Valles, 1993), norway spruce (Heinze and Schmidt, 1995) and strawberries (Kumar *et al.*, 1995).

# MATERIALS AND METHODS

# 3. MATERIALS AND METHODS

Investigations were carried out at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani during January 1999 to November 2000 for evaluating the genomic stability of *in vitro* propagated Red banana plantlets at molecular level. The materials and methods tried for the research work are described in this chapter.

## 3. 1. *In vitro* propagation

### 3. 1. 1. Preparation of the culture medium

The basal medium used for culturing banana was Murashige and Skoog (Murashige and Skoog, 1962). Analytical grade chemicals from British Drug House (Mumbai), Sisco Research Laboratory (Mumbai) and Merck (Mumbai) were used for the preparation of the media. Standard procedures were followed for the preparation. Stock solutions of major and minor nutrients, were prepared by dissolving the required quantities of chemicals in specific volume of double glass distilled water. They were stored under refrigerated condition (4.0 $^{0}$ C).

The culture vessels (Borosil brand 250 ml Erlenmeyer flasks) used were washed with 1000 times diluted 'Labolene' and tap water, rinsed with double glass distilled water and kept overnight in a hot air oven ($160^0$ C) for drying and pre-sterilisation.

All items of glassware (Borosil brand) used for the preparation of culture media were washed thoroughly in 1000 times diluted 'Labolene' and tap water and rinsed with double glass distilled water. Specific quantities of stock solutions were pipetted into a beaker. The required quantities of sucrose, inositol and glucose were freshly added. Benzyl adenine (2.0 mg $l^{-1}$ or 5.0 mg $l^{-1}$) was also added. The pH of the medium was adjusted to 5.6 to 5.8 using 0.1 N NaOH or 0.1 N HCl with the aid of an electronic pH meter (Philips PP – 9046). The final volume was made up to the desired quantity using double glass distilled water. The medium was then poured to pre-sterilised culture vessels at the rate of 100 ml per Erlenmeyer flask. The flasks were tightly plugged with non-absorbent cotton, covered with aluminium foil or paper and autoclaved (NAT Steel Equipment, Mumbai) at $121^0$ C and 1.06 kg $cm^{-2}$ pressure for 20 minutes.

### 3.1.2. Explant

A high yielding Red banana plant along with its suckers were selected for the study. The suckers were removed out of the soil. Leaves and pseudostem

were cut about 30 cm above soil level. The suckers were then cleaned by removing the adhering soil and roots were cut off to expose the corm. The outer sheathing leaf bases of the pseudostem were peeled off one at a time until they became too small to be removed by hand. Then they were transferred to 1000 times diluted 'Labolene' solution, allowed to remain in the same for 30 minutes while swirling occasionally. Soap solution was then decanted and the rhizomes were washed in distilled water.

**3.1. 3. Surface sterilisation**

Surface sterilisation of the shoot apices was carried out inside the laminar air flow chamber just before inoculation. The shoot apices were treated with freshly prepared 0.08 per cent mercuric chloride for 10 minutes. The solution was drained and the shoot apices were washed four to five times with sterile double glas distilled water. The last remaining leaves were removed and the shoot apex was excised. Sterile blades of commercial sizes 11 and 21 were used.

**3.1. 4. Inoculation and incubation**

All inoculation operations were carried out inside a laminar air flow chamber. The vessels and tools (beakers, petriplates, scalpels, blades, forceps

etc.) required for inoculation were washed thoroughly, rinsed with double glass distilled water, oven dried, covered air-tight with aluminium foil and autoclaved at $121^0$ C and 1.06 kg $cm^{-2}$ pressure for 45 minutes. They were further flame sterilised just before inoculation using a spirit lamp inside the laminar air flow chamber. Cotton plugs of the culture vessels were removed and the mouth was flamed. The excised shoot apices were inoculated into a MS medium supplemented with BA 2.0 mg $l^{-1}$. The mouth of the culture vessels were flamed again and cotton plugs were replaced. Cultures were transferred to the culture rooms at a temperature of $26+2^0$ C. Light (3000 lux, 16 hours photoperiod) was provided using cool white flourescent tubes.

### 3. 1. 5. Stimulation of Multiple Shoot Formation

Three or four weeks after inoculation of the shoot apex, it was taken out and cut longitudinally. Leaves were removed. Also the lower part of the explant was trimmed to remove darkened or necrotic tissue. The halves were transferred into proliferation medium (MS supplemented with BA 5.0 mg $l^{-1}$). The proliferating shoots were repeatedly subcultured on fresh medium at four weeks interval.

### 3. 2. Isolation of genomic DNA

For the standardisation of DNA isolation methods, the tissues from the emerging leaves of the mother plant of the Red banana before they fully unfurl were used. The isolation was done with the help of one of the four methods described below. Tissues from young leaves belonging to the first three subcultures were used for DNA isolation with the help of the selected methods.

#### 3. 2. 1. Method –1 (Aljanabi and Martinez, 1997)

About 50-100 mg of emerging leaves of field grown Red banana before they fully unfurl were used for DNA extraction. The fresh tissue was homogenised in 400μl of sterile salt homogenising buffer using a mortar and pestle for 10 to 15 seconds. The homogenising buffer is made of 0.4 M sodium chloride (NaCl), 10mM tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), pH 8.0 and 2 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0. Then 40 μl of 20 per cent sodium dodecyl sulphate (SDS-2.0 per cent final concentration) and 8.0 μl of 20 mg $l^{-1}$ proteinase k (400 μg $ml^{-1}$ final concentration) were added and mixed well. The samples were incubated at 55-65$^0$ C for at least one hour or overnight, after which 300 μl of 6.0 M NaCl was added to each sample. Samples were vortexed for 30 seconds at maximum speed

and tubes spun down for three minutes at 10,000 *g*. The supernatant was transferred to fresh tubes. An equal volume of absolute ethanol was added to each sample, mixed well and samples were incubated at $-20^0$ C for one hour. Samples were then centrifuged for 20 minutes, $4.0^0$ C at 10,000 *g*. The pellet was washed with 70 per cent ethanol, dried and finally resuspended in 300 to 500 μl sterile distilled water. This was stored at $4.0^0$ C.

**3. 2. 2. Method – 2 (Walbot, 1988)**

The emerging leaves of high yielding Red banana plant before they fully unfurl (1.0 g) were homogenised in a cold mortar by rapid grinding till a fine paste was formed. Volume of grinding buffer (15 per cent (w/v) sucrose, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA and 250 mM NaCl) added was 0.5 ml. The fluid was transferred to a microfuge tube kept on ice. The mortar was rinsed twice with 0.5 ml of the same solution to transfer residue. The tubes were spun at low speed (5000 rpm) for 5.0 minutes in a microfuge at $4.0^0$ C . The supernatant was discarded. The pellet contained nuclei. It was then suspended in 0.3 ml of suspension buffer (20 mM Tris–HCl, pH 8.0, 10 mM EDTA). A volume of 20 μl of 20 per cent SDS was added to the resuspended nuclei and mixed by inverting the tube rapidly several times. The tubes were then incubated at $70^0$ C for 15 minutes in a water bath. To this 150 μl of 7.5 M ammonium acetate was added and mixed thoroughly by inverting the tube several times. The tubes were

cooled on ice for 30 minutes. Again they were spun at high speed (15,000 *g*) for 5.0 minutes to sediment ammonium dodecyl sulphate. The supernatant was transferred to a new microfuge tube, 0.7 ml of ethanol was added and allowed to remain as such for 15 minutes. DNA in the string form was pelleted in a microfuge tube when centrifuged for five minutes at 14,000 rpm at $4^0$ C. The supernatant was discarded and the pellet was dried. The pellet in each tube was dissolved in 25µl of Tris-HCl and EDTA (TE -10 mM Tris HCl, pH 8.0 and 1 mM EDTA) buffer.

**3. 2. 2. 1. Purification of DNA**

Genomic DNA was purified by phenol : chloroform : isoamyl alcohol (25 : 24 : 1) extraction. To the DNA sample, equal volume of the phenol : chloroform : isoamyl alcohol mixture was added and mixed by repeated inversions. The mixture was centrifuged at 14000 rpm for 10 minutes at room temperature. The aqueous phase was transferred carefully to a fresh 1.5 ml microfuge tube. The above step was repeated until the interphase disappeared. To the aqueous phase, equal volume of chloroform : isoamyl alcohol (24 : 1) was added and mixed by repeated inversions. The mixture was centrifuged at 14,000 rpm for 10 minutes and the aqueous phase was transferred to another tube.

To the aqueous phase 1/10$^{th}$ volume of 3.0 M sodium acetate (pH 8.0) and 2.0 volumes of absolute ethanol were added and thoroughly mixed by inversions and kept overnight. DNA was pelleted by centrifugation at 10,000 rpm for 5.0 minutes the next day. The supernatant was discarded, the pellet was dried and dissolved in 100 or 200 μl of TE. The tissue samples were stored at 4.0$^{0}$ C.

**3. 2. 3. Method – 3**

This is a modified method given by Walbot (1988). About 10 g of emerging leaves of Red banana before they fully unfurl was used for DNA extraction. The leaves were placed in porcelain container and liquid nitrogen was poured over the leaf material. The leaves were ground well to a fine powder and transferred to the extraction buffer, (168 g urea, 28ml 5 M NaCl, 20 ml 1 M Tris–HCl, pH 8.0, 16 ml 0.5 M EDTA, 20 ml phenol, made upto 400 ml with sterile water) placed in the water bath at a temperature of 55 $^{0}$ C. A volume of 2.5 ml of 20 per cent SDS and a pinch of polyvinylpyrrollidone (PVP) were added and mixed gently. The temperature of the water bath was set at 50 $^{0}$ C and the lysis buffer was allowed to equilibrate to that temperature. The solution was kept at 50 $^{0}$ C for 10 minutes. The lysate was then squeezed through four layers of sterile muslin cloth. Then 25 ml of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) solution was added to the above solution and kept at room temperature for 15 minutes. The same was then centrifuged at 10,000 rpm for 10

minutes at $4.0^0$ C. After collecting the upper phase, the phenol : chloroform : isoamylalcohol (25 : 24 : 1) extraction was repeated. This step was repeated until the interphase disappeared. After that, to the aqueous phase collected, equal volume of chloroform : isoamylalcohol (24 : 1) solution was added and the two phases were mixed gently. Centrifugation was done at 10,000 rpm for 10 minutes at $4.0^0$ C. To the upper phase collected $1/10^{th}$ volume of 3.0 M sodium acetate and two volumes of cold absolute ethanol were added. It was mixed gently and DNA strands were spooled out with a glass rod. After that 70 per cent ethanol wash was given to the DNA strands. The pellet was allowed to dry and then dissolved in 100 μl to 200 μl TE. DNA samples were stored at $4.0^0$ C.

**3. 2. 4. Method - 4 ( Rogers and Bendich, 1994 )**

The emerging unfurled leaves of Red banana were ground with liquid nitrogen in a mortar and pestle. The ground tissue was transferred to a centrifuge tube or a microfuge tube. When the liquid nitrogen sublimed away, hot ($65^0$ C) 2x cetyltrimethylammonium bromide (CTAB) buffer (2.0 per cent CTAB w/v, 100 mM Tris – HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 1.0 per cent PVP) and 1.5 per cent β-mercaptoethanol were added. The same was allowed to remain in water bath at $56^0$ C – $58^0$ C for 30 minutes. To this one volume of chloroform : isoamyl alcohol (24 : 1) solution was added and mixed thoroughly to form an emulsion. Centrifugation was done at 10,000 rpm for 10 minutes. The

upper aqueous phase was transferred to a new microfuge tube. Then 1/5$^{th}$ volume of the 10 per cent CTAB solution (10 per cent CTAB w/v, 0.7 M NaCl) was added and mixed. Again chloroform: isoamyl alcohol (24 : 1) solution extraction was done as mentioned above. To the aqueous phase collected, equal volume of CTAB precipitation buffer (1 per cent CTAB, 50mM Tris p$^{H}$ 8.0, 10mM EDTA p$^{H}$ 8.0) was added and mixed gently while keeping it on ice for 5.0 to 30 minutes. Centrifugation was done at 10000 rpm for 5.0 minutes. The supernatant was discarded and the pellet was rehydrated in high salt TE (10 mM Tris – HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1 M NaCl) buffer. To that two volumes of cold ethanol was added and mixed gently and centrifuged. The pellet was subjected to 80 per cent ethanol wash, dried, rehydrated in 0.1 x TE (1.0 m M Tris – HCl pH 8.0, 0.1 m M EDTA pH 8.0) and stored at 4.0$^{0}$ C.

**3. 3. Quantification of DNA**

Reliable quantification of DNA concentration is important for many applications in molecular biology including amplification of target DNA polymerase chain reaction. DNA quantification was carried out with the help of UV – Vis spectrophotometer (Spectronic Genesys 5).

The buffer in which the DNA was already dissolved was taken in a cuvette and used for the calibration of the spectrophotometer at 260 nm as well

as 280 nm wavelength. The optical density (OD) of the DNA sample dissolved in the buffer was recorded at both 260 nm and 280 nm. Then the DNA concentration was estimated by employing the following formula :

Amount of DNA (μg / μl) = $A_{260}$ x 50 x dilution factor/1000, where,

$A_{260}$ - absorbance at 260 nm

The quality of the DNA could be judged from the ratio of the OD values recorded at 260 nm and 280 nm. The $A_{260}$ /$A_{280}$ ratio between 1.8 and 2.0 indicates best quality of DNA.

$A_{280}$ - absorbance at 280 nm

### 3. 4. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit supplied by the Bangalore Genei. Required amount of agarose was weighed out (0.9 per cent for visualising the genomic DNA and 1.4 per cent for visualising the amplified products) and melted in 1x TAE buffer (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0). Agarose was dissolved by boiling. After cooling to about $50^0$ C ethidium bromide was added to a final concentration of 0.5 mg / ml. The mixture was poured immediately to a pre-set template with appropriate comb. After solidification, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank. To the

DNA sample, required volume of sample buffer (6.0x loading dye *viz.* 40 per cent sucrose, 0.25 per cent bromophenol blue) was added. Each well was loaded with 20 µl of sample. One of the wells was loaded with 5.0 µl of PCR molecular weight marker along with required volume of the sample buffer. Electrophoresis was performed at 50 volts until the loading dye reached $3/4^{th}$ of the length of the gel. The gel was visualised using a ultraviolet-visible (UV-Vis) transilluminator.

### 3. 5. Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA analyses were performed following the method recommended by Bhat and Jarret (1995) with required modifications. Forty arbitrarily designed decamer primers supplied by Operon Inc., CA, USA, were used.

Genomic DNA (20 ng) was amplified *in vitro* in a 2.5 µl 10x buffer (10 mM Tris HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01 per cent gelatin). 10 pM primer, 250 µM each of deoxynucleotides (dNTPs) and 0.6 units of Taq DNA polymerase were the optimum quantities of the reagents required for the amplification. Amplifications were carried out in a Programmable Thermal Controller (MJ Research, Inc.) set for the following programme: An initial denaturation at $95^0$ C for 3.0 minutes, followed by 45 cycles of denaturation at $95^0$ C for 1.0 minute, annealing at $36^0$ C for 1.0 minute 30 seconds and extension at $72^0$ C for 2.0 minutes. The synthesis step of the final cycle was extended

further by 6.0 minutes. Finally the products of amplification were cooled to $4.0^{0}$ C until attended. A negative control, containing water instead of template was included in each reaction set.

Amplified products along with PCR molecular weight marker supplied by U. S. Biochemicals were separated by 1.4 per cent agarose gel electrophoresis, stained with ethidium bromide and photographed with the help of gel doc.

# RESULTS

# 4. RESULTS

The results of the investigations carried out for evaluating the genomic stability of *in vitro* propagated Red banana plantlets at molecular level are presented in this chapter.

## 4.1. *In vitro* propagation of Red banana

Twenty per cent of cultures survived in Murashige and Skoog (MS) medium supplemented with benzyl adenine (BA) 2.0 mg $l^{-1}$. Swelling was observed from the explants *viz.* shoot apices, ten to fourteen days after inoculation. Two to three weeks after inoculation of the shoot apex, the cleaned tissue was subcultured to MS medium supplemented with BA 5 mg $l^{-1}$, after removing the outermost leaves and blackened shoot bases. This produced multiple shoots.

An average of 15.0 shoots were produced with 3.0 leaves per shoot in the first subculture. Second and third subculture produced an average of 14.0 and 15.0 shoots, respectively. The number of leaves produced were 2.0 in both second and third subculture.

## 4. 2. DNA isolation

### 4. 2. 1. Methods of Isolation

Of the four methods used for DNA isolation, purity of DNA was the highest in the fourth method (see appendix-2) described by Rogers and Bendich (1994). Purity of the DNA was the least in the second method described by Walbot (1988). The optical density ($A_{260}$/ $A_{280}$) ratio was 1.81 in the Rogers and Bendich's method, whereas it was only 0.55 in the Walbot's method. Aljanabi and Martinez's method and modified Walbot's method (see appendix-1) gave the $A_{260}$/ $A_{280}$ ratio of 0.93 and 1.44, respectively (Table 1).

The quantity of DNA obtained was the highest in the Walbot's method, and was the lowest in the Aljanabi and Martinez's method. DNA yield was 3000 ng/µl and 760 ng/µl in Walbot's method and Aljanabi and Martinez's method, respectively. The quantity of DNA obtained was 2825 ng/µl in modified Walbot's method and it was 1185 ng/µl in Rogers and Bendich's method.

Table – 1 **Effect of methods of isolation on the quantity and quality of the DNA of Red banana mother plant**

| Sl.No. | Method | Source material of plant tissue | $A_{260}$ | $A_{280}$ | DNA yield ng/ µl | $A_{260}$/ $A_{280}$ |
|---|---|---|---|---|---|---|
| 1. | Aljanabi and Martinez's method, 1997 | Tissue from emerging leaves before they fully unfurl. | 0.152 | 0.163 | 760 | 0.93 |
| 2. | Walbot's method, 1988 | Tissue from emerging leaves before they fully unfurl. | 0.600 | 1.095 | 3000 | 0.55 |
| 3. | Modified Walbot's method, 1988 | Tissue from emerging leaves before they fully unfurl. | 0.565 | 0.393 | 2825 | 1.44 |
| 4. | Rogers and Bendich's method, 1994 | Tissue from emerging leaves before they fully unfurl. | 0.237 | 0.131 | 1185 | 1.81 |

$A_{260}$ - absorbance at 260 nm.

$A_{280}$ - absorbance at 280 nm.

$A_{260}$/ $A_{280}$ - optical density (O.D.) ratio

**4. 2. 2. Plant tissue**

Various sources of plant tissue were tried for DNA isolation from Red banana (Table 2). Leaves from *in vitro* culture, mature leaves from the peeper sucker, emerging leaves of the mother plant before they fully unfurl and mature leaves from the mother plant were used for DNA isolation. The emerging leaves of the mother plant before they fully unfurl were found to be the best explant with respect to DNA yield (2825 ng/µl). The $A_{260}/A_{280}$ ratio of the same was 1.44. The highest $A_{260}/A_{280}$ ratio of 1.76 was obtained when leaves from *in vitro* culture were used. But DNA yield from the leaves from *in vitro* culture was only 220 ng/µl. The least $A_{260}/A_{280}$ ratio was 0.82 when mature leaves of peeper sucker were used for DNA isolation. Thus the purity of DNA was the highest in *in vitro* grown leaves, whereas it was the least in mature leaves of the peeper sucker. The lowest DNA yield (185 ng/µl) was obtained when the mature leaves of the peeper sucker was used. Mature leaves of the mother plant when used gave the $A_{260}/A_{280}$ ratio of 1.24 with a DNA yield of 1315 ng/µl.

Table – 2 **Effect of source of the plant tissue on the quantity and the quality of the DNA of Red banana**

| Sl.No | Source of plant tissue | $A_{260}$ | $A_{280}$ | $A_{260}/A_{280}$ | Yield of DNA ng/µl |
|---|---|---|---|---|---|
| 1. | Immature leaves from *in vitro* cultures | 0.044 | 0.025 | 1.76 | 220 |
| 2. | Emerging leaves before they fully unfurl | 0.565 | 0.393 | 1.44 | 2825 |
| 3. | Mature leaves of mother plant | 0.263 | 0.212 | 1.24 | 1315 |
| 4. | Mature leaves of peeper sucker | 0.037 | 0.045 | 0.82 | 185 |

$A_{260}$ - absorbance at 260 nm.

$A_{280}$ - absorbance at 280 nm.

$A_{260}/A_{280}$ - optical density (O.D.) ratio

**4. 2. 3. Proteinase k**

Proteinase k when added to the DNA, dissolved in Tris HCl and EDTA (10: 1) buffer, and kept overnight, the purity of DNA increased (Table 3). Optical density (OD) ratio of DNA obtained from emerging leaves of mother plant before they fully unfurl was 1.33 . Their OD ratio was changed to 1.46 by addition of proteinase k. But the DNA yield was reduced by the addition of proteinase k from 2326.7 ng/μl to 955 ng/μl.

**4. 2. 4. Purification of DNA**

Additional purification step with phenol: chloroform: isoamylalcohol (25:24:1) treatment followed by chloroform: isoamyl alcohol (24:1) treatment after the DNA pellet was obtained, increased the purity of DNA. While the initial OD ratio was 0.93, after the additional purification step, the ratio was found to increase to 1.18.

The DNA sample obtained was brown in colour when extraction was done following the modified Walbot's method. Polyvinyl pyrrollidone (0.1 per cent) when included in the extraction buffer, in addition to the other reagents, reduced the browning of the DNA sample.

Table – 3 **Effect of proteinase k on the quantity and quality of the genomic DNA of the Red banana plant**

| Sl. No | $A_{260}$ | | $A_{280}$ | | $A_{260}/A_{280}$ | | Yield of DNA ng/μl | |
|---|---|---|---|---|---|---|---|---|
| | Without proteinase k | With proteinase k | Without proteinase k | With proteinase k | Without proteinase k | With proteinase k | Without proteinase k | With proteinase k |
| 1. | 0.43 | 0.21 | 0.35 | 0.14 | 1.24 | 1.42 | 2145 | 1065 |
| 2. | 0.40 | 0.22 | 0.31 | 0.15 | 1.30 | 1.47 | 2010 | 1120 |
| 3. | 0.57 | 0.14 | 0.39 | 0.10 | 1.44 | 1.48 | 2825 | 680 |
| Mean | 0.46 | 0.19 | 0.35 | 0.13 | 1.33 | 1.46 | 2326.7 | 955 |

$A_{260}$ - absorbance at 260 nm.

$A_{280}$ - absorbance at 280 nm.

$A_{260}/A_{280}$ - optical density (O.D.) ratio

In the fourth method (Rogers and Bendich, 1994), 1.5 per cent β-mercaptoethanol was added in addition to the other reagents in the extraction buffer to avoid the browning of the tissue. This was the most efficient method that completely avoided the browning of tissue. So the inclusion of 0.1 per cent PVP and 1.5 per cent β-mercaptoethanol was ideal to obtain transparent DNA pellet.

**4. 3. Gel electrophoresis**

Various concentrations of agarose were tried to prepare the gel for visualising the genomic DNA and the RAPD banding pattern. Agarose at 0.7 per cent, 0.8 per cent, 0.9 per cent and 1.0 per cent concentrations were tried for the genomic DNA. Of these, 0.9 per cent was found to be the best. Agarose at 1.2 per cent, 1.3 per cent, 1.4 per cent and 1.5 per cent concentrations were tried for the RAPD analysis. Of these, 1.4 per cent was the most reliable for the RAPD analysis.

**4. 4. Polymerase chain reaction (PCR)**

Polymerase chain reaction conditions were standardised for the amplification of the DNA from the Red banana plant. Quantity of the reagents required for the PCR reaction as well as the specific conditions in which the amplification of the DNA of the Red banana plants were optimised. The

quantity of DNA used was 20 ng, 250 µM each of dNTPs, 0.6 units Taq DNA polymerease and 10 pM primer in presence of the 10x assay buffer gave the best amplification results. The optimum programme consisted of an initial denaturation at $95^0$ C for 3.0 minutes, followed by 45 cycles of denaturation at $95^0$ C for 1.0 minute, annealing at $36^0$ C for 1.0 minute 30 seconds, and extension at $72^0$ C for 2.0 minutes. The synthesis step of the final cycle was extended further by 6.0 minutes. The amplification products were cooled to $4.0^0$ C after the reaction.

The PCR amplification was carried out using forty decamer primers (Operon Inc., CA, USA) of kit A and kit B (Table 4). All of them yielded amplification products when amplified with the DNA from the Red banana mother plant, except OPA-06, OPB-10 and OPB-14. The amplified products were separated on 1.4 per cent agarose gel and stained with ethidium bromide (0.01 per cent), visualised on a UV transilluminator and photographed (Plate-I)

A total of 134 RAPDs (average of 3.4 bands per primer) were generated, of which 97.0 per cent (130 bands) were polymorphic. This accounts for an average of 3.2 polymorphic bands per primer. Primers varied in their ability to yield banding patterns with the template DNA.

Monomorphic bands were produced by the primers OPA-08, OPB-02, OPB-07 and OPB-16.

Plate 1 **Amplification profiles of the DNA of Red banana mother plant using 40 primers (belonging to kit A and kit B) supplied by the Operon Inc., CA, USA.**( The primers used for the amplification is given against the respective lanes)

**1**

Lane – 1 : OPA –01
Lane – 2 : OPA –02
Lane – 3 : OPA –03
Lane – 4 : OPA –04
Lane – 5 : OPA –05
Lane – 6 : OPA –06
Lane – 7 : OPA –07
Lane – 8 : PCR molecular weight marker supplied by U.S. Biochemicals

**2**

Lane – 1: OPA - 08
Lane – 2: OPA - 09
Lane – 3: OPA - 10
Lane – 4: OPA - 11
Lane – 5: OPA - 12
Lane – 6: OPA - 13
Lane – 7: OPA - 14
Lane – 8: OPA - 15
Lane – 9: OPA - 16
Lane–10: OPA - 17
Lane – 11:OPA-18
Lane – 12:OPA-19
Lane – 13:OPA-20
Lane – 14:OPB-01
Lane – 15:OPB-02
Lane – 16:OPB-03
Lane – 17:OPB-04
Lane – 18:PCR molecular weight marker by U. S.B

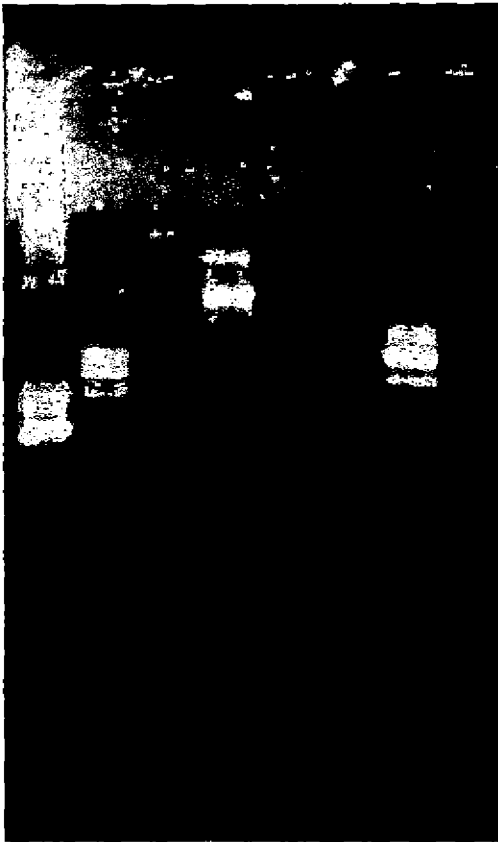
**3**

Lane – 1: OPB-05
Lane – 2: OPB-06
Lane – 3: OPB-07
Lane – 4: OPB-08
Lane – 5: OPB-09
Lane – 6: OPB-10
Lane – 7: OPB-11
Lane – 8: OPB-12

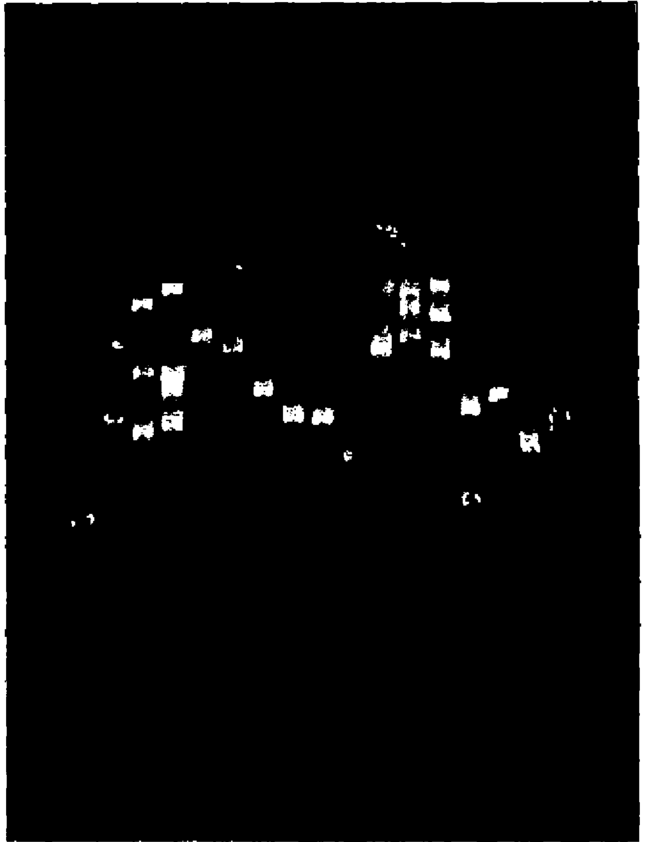
**4**

Lane – 1: OPB-13
Lane – 2: OPB-14
Lane – 3: OPB- 15
Lane – 4: OPB- 16
Lane – 5: OPB- 17
Lane – 6: OPB -18
Lane – 7: OPB -19
Lane- 8 : OPB- 20

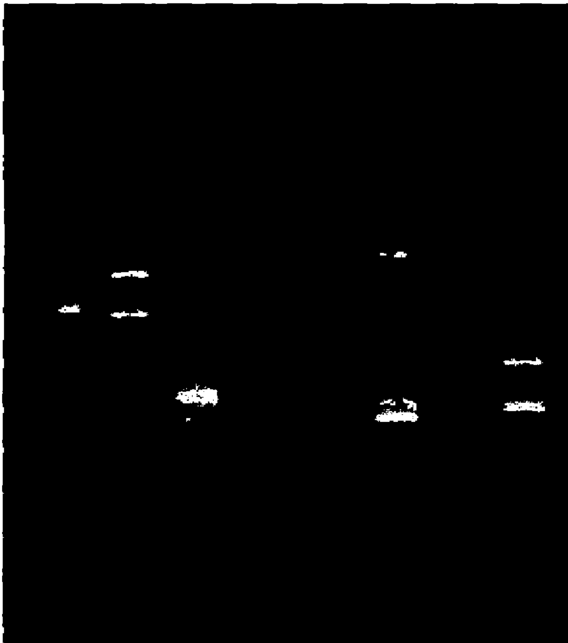
PLATE - 1



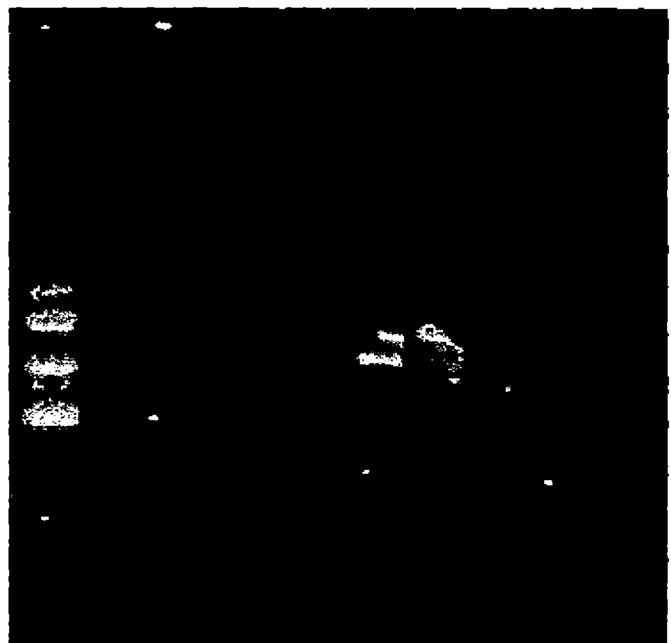

1




2




3




4

OPA-20 produced a total of 8.0 bands. Intense bands were 5.0 in number, where as faint bands were 3.0. This was the primer selected for further comparison studies.

Maximum number of RAPDs were produced by OPB-08. Out of the total 9.0 bands, 8.0 were faint and 1.0 was intense.

OPA-13 and OPB-06 produced 7.0 bands each. OPA-13 produced 3.0 intense and 4.0 faint bands, whereas, OPB-06 produced 2.0 intense and 5.0 faint bands in the amplification reaction.

OPA-04, OPA-07 and OPA-19 when tried for RAPD analysis, produced 6.0 bands each. Both OPA-07 and OPA-19 produced the highest number of intense bands (3.0). It was OPA-04 that produced the lowest number of intense bands (1.0). The highest number of faint bands was produced by the primer OPA-04. OPA-07 and OPA-19 produced the lowest number of faint bands (3.0) (Table-4).

Five bands each were produced when OPA-02, OPA-10, OPA-18, OPB-11 and OPB-12 were used for amplification reactions. Both OPA-10 and OPB-12 gave the highest number of intense bands (3.0). It was OPA-18 that produced the lowest number of intense bands (1.0). The highest number of faint bands were 4.0, produced by OPA-18, where as, the lowest number of the same (2.0) were given by OPA-10 and OPB-12 (Table-4).

Table – 4 **Primer-associated banding patterns with the DNA of Red banana mother plant using 40 primers (belonging to kit A and kit B ) supplied by the Operon Inc., CA, USA.**

| Sl.No. | Primers | Total no. of bands | Intense bands | Faint bands |
|---|---|---|---|---|
| 1. | OPA-01 | 2 | 1 | 1 |
| 2. | OPA-02 | 5 | 2 | 3 |
| 3. | OPA-03 | 4 | 0 | 4 |
| 4. | OPA-04 | 6 | 1 | 5 |
| 5. | OPA-05 | 4 | 0 | 4 |
| 6. | OPA-06 | 0 | 0 | 0 |
| 7. | OPA-07 | 6 | 3 | 3 |
| 8. | OPA-08 | 1 | 1 | 0 |
| 9. | OPA-09 | 2 | 2 | 0 |
| 10. | OPA-10 | 5 | 3 | 2 |
| 11. | OPA-11 | 3 | 3 | 0 |
| 12. | OPA-12 | 3 | 1 | 2 |
| 13. | OPA-13 | 7 | 3 | 4 |
| 14. | OPA-14 | 2 | 1 | 1 |
| 15. | OPA-15 | 2 | 1 | 1 |

| Sl.No. | Primers | Total no. of bands | Intense bands | Faint bands |
|---|---|---|---|---|
| 16. | OPA-16 | 2 | 1 | 1 |
| 17. | OPA-17 | 3 | 0 | 3 |
| 18. | OPA-18 | 5 | 1 | 4 |
| 19. | OPA-19 | 6 | 3 | 3 |
| 20. | OPA-20 | 8 | 5 | 3 |
| 21. | OPB-01 | 4 | 3 | 1 |
| 22. | OPB-02 | 1 | 1 | 0 |
| 23. | OPB-03 | 2 | 1 | 1 |
| 24. | OPB-04 | 2 | 0 | 2 |
| 25. | OPB-05 | 2 | 2 | 0 |
| 26. | OPB-06 | 7 | 2 | 5 |
| 27. | OPB-07 | 1 | 0 | 1 |
| 28. | OPB-08 | 9 | 1 | 8 |
| 29. | OPB-09 | 3 | 0 | 3 |
| 30. | OPB-10 | 0 | 0 | 0 |
| 31. | OPB-11 | 5 | 2 | 3 |
| 32. | OPB-12 | 5 | 3 | 2 |
| 33. | OPB-13 | 3 | 0 | 3 |
| 34. | OPB-14 | 0 | 0 | 0 |
| 35. | OPB-15 | 4 | 0 | 4 |
| 36. | OPB-16 | 1 | 0 | 1 |

| Sl.No. | Primers | Total no. of bands | Intense bands | Faint bands |
|---|---|---|---|---|
| 37. | OPB-17 | 2 | 2 | 0 |
| 38. | OPB-18 | 3 | 0 | 3 |
| 39. | OPB-19 | 2 | 0 | 2 |
| 40. | OPB-20 | 2 | 0 | 2 |

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OPA-03, OPA-05, OPB-01 and OPB-15 gave 4.0 bands each with the DNA in the amplification reactions. OPA-03, OPA-05 and OPB-15 produced the maximum number of faint bands (4.0). The lowest number of faint bands (1.0) was given by the primer OPB-01. The highest number of intense bands (3.0) were produced by the primer OPB-01. OPA-03, OPA-05 and OPB-15 produced no intense bands (Table-4).

Three bands each were obtained when OPA-11, OPA-12, OPA-17, OPB-09, OPB-13 and OPB-18 were used for the amplification reaction. The maximum number of intense bands (1.0) was obtained when OPA-12 was used, where as, no intense bands were obtained with the primers OPA-11, OPA-17, OPB-09 and OPB-13. The highest number of faint bands (3.0) were obtained with the primers OPA-11, OPA-17, OPB-09 and OPB-13. OPA-12 gave the lowest number of faint bands (Table-4).

Only 2.0 bands each were produced in a reaction when OPA-01, OPA-09, OPA-14, OPA-15, OPA-16, OPB-03, OPB-04, OPB-05, OPB-17, OPB-19 and OPB-20 were used (Table-4). The highest number of intense bands was obtained with the primers OPA-09, OPB-05 and OPB-17. The lowest number of intense bands was obtained when OPA-01, OPA-14, OPA-15, OPA-16 and OPB-03 were used for amplification. OPB-04 and OPB-19 produced the maximum number of faint bands(2.0). OPA- 01, OPA-14, OPA-15, OPA-16 and OPB-03 gave the lowest number of faint bands (1.0).

Plate – 2 **RAPD analysis of the mother plant and three subcultures of the DNA of Red banana using OPA-20 supplied by Operon Inc., CA, USA**

Lane – 1 : PCR molecular weight marker (U. S. Biochemicals)

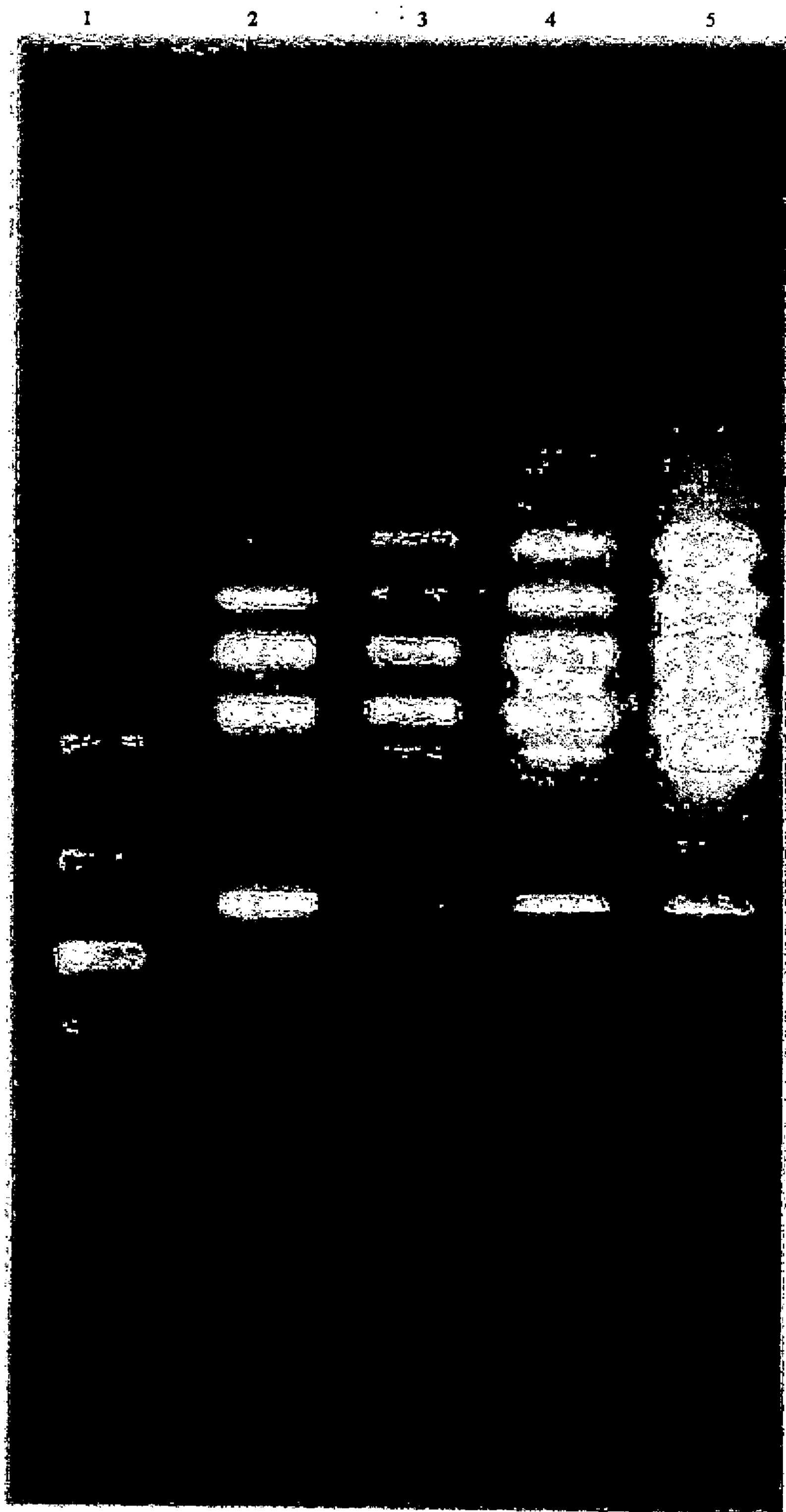
Lane – 2 : Amplification of the DNA (Red banana) from first subculture using OPA-20

Lane – 3 : Amplification of the DNA (Red banana) from second subculture using OPA-20

Lane – 4 : Amplification of the DNA (Red banana) from third subculture using OPA-20

Lane – 5 : Amplification of the DNA (Red banana) from the mother plant using OPA-20

**PLATE - II**



The DNA concentration in the reaction mixture was important for successful PCR amplifications in banana. The DNA concentration influenced the number and intensity of products amplified. DNA concentration between 20 ng and 40 ng resulted in good amplification and the products were easy to score. Amplifications were even better at 20 ng of DNA concentration, which was found to be optimum.

Amplification reaction when done using OPA-20 in the DNA of the mother plant, 5.0 bands were intense and 3.0 were faint. When the same amplification reaction was done using the DNAs of the three subcultures, no difference was found in RAPD banding pattern from that of the mother plant (Plate-II). Of the 5.0 intense bands one was of 1100 kbp and the other four were greater than 1100 kbp. Of the 3.0 intense bands, one was between 1100 kbp and 700 kbp, other one was between 700 kbp and 500 kbp and the third one was between 400 kbp and 300 kbp.

# DISCUSSION

# 5. DISCUSSION

Banana (*Musa* sp.) is the most important fruit crop of Kerala with respect to area and production. However, the low productivity of banana can be attributed to deficiencies in crop management, incidence of serious pests and diseases and dearth of quality planting materials. The production as well as productivity of banana has to be increased to meet the internal demand.

*In vitro* propagation is an excellent alternative over conventional propagation of banana, with many advantages. With respect to the rate of multiplication, potential for shoot apex culture is enormous. It was found that thousands of plantlets would be derived from a single explant within a short span of time. In Karnataka, Tamil Nadu and Maharashtra tissue culture plants of banana are extensively used for commercial planting.

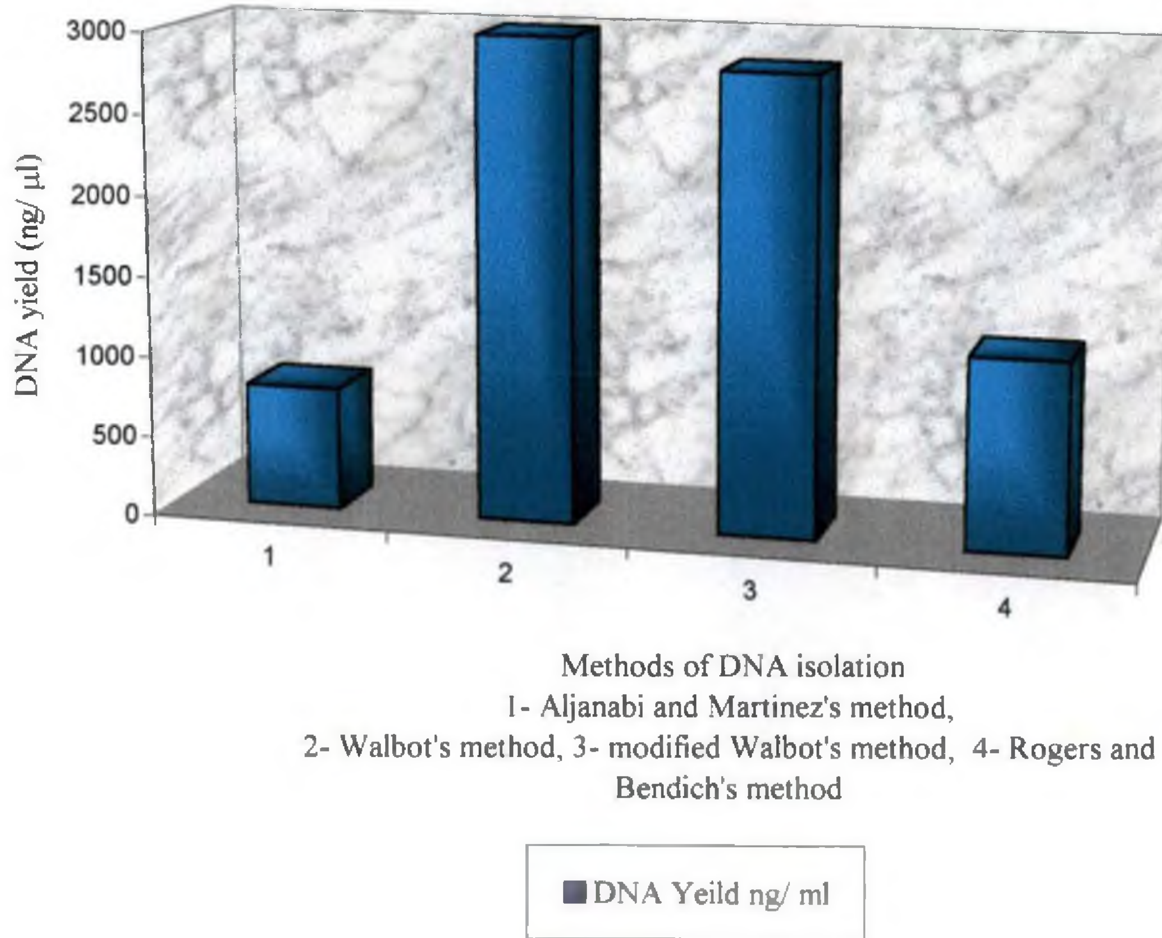
The present study was undertaken to standardise the PCR conditions required for RAPD analysis in Red banana and to assess the sub-culture associated variations, if any. The results obtained are discussed in detail in this chapter.

**DNA isolation**

Standardisation of isolating DNA of reasonable purity is the pre-requisite for any RAPD work. DNA isolation from phenol rich plant encounters a number of problems (Pich and Schubert, 1993). Banana contains large amount of phenols, tannins, pigments and polysaccharides that impair the quantity and purity of isolated DNA. This can inhibit the activity of most DNA synthesising and modifying enzymes which may lead to difficulties during the RAPD analysis. Efficient DNA isolation methods (Fig-1 and Fig-2) were standardised which could be used for the preparation of large number of samples (DNA yield : 1180ng/µl - 3000 ng/µl and optical density ratio : 1.5-1.8) for RAPD analysis.

Various sources of plant tissue were tried for the DNA isolation from Red banana. These included leaves from *in vitro* culture, mature leaves of the peeper sucker, emerging leaves (before they fully unfurl) and mature leaves of the mother plant (Fig. 3 and Fig. 4). The emerging leaves of the mother plant before they fully unfurl were found to yield the highest amount of DNA (2825 ng/ µl). A critical factor in the effective isolation of the plant DNA is the efficient disruption of the plant cell wall. The tender leaves gave better DNA yield probably because of the easy cell disruption during the isolation steps as described by Mondal *et al.* (2000). Mature leaves of the peeper sucker gave the

Fig-1 **Effect of methods of isolation on the quantity of the DNA of Red banana**



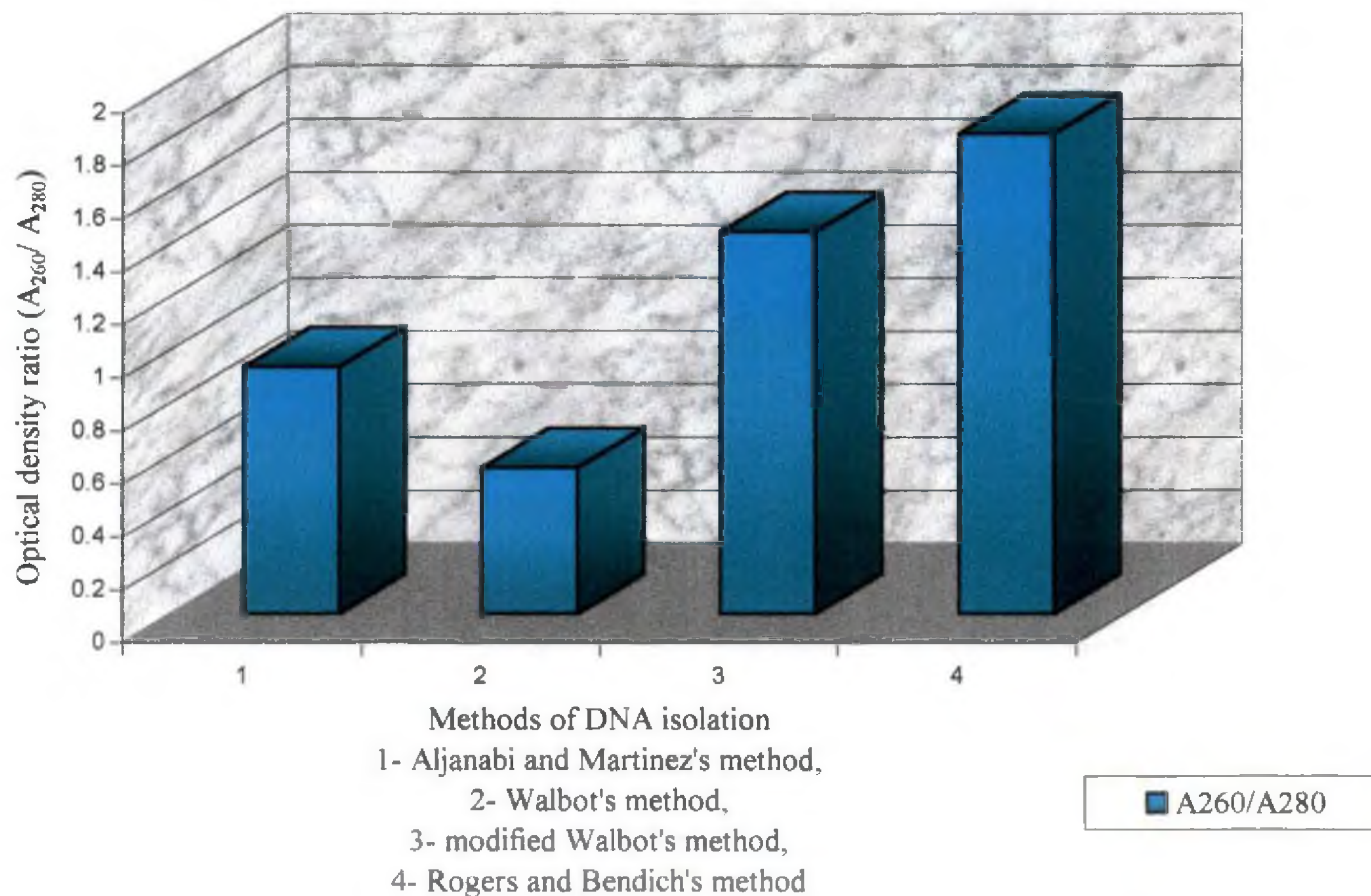
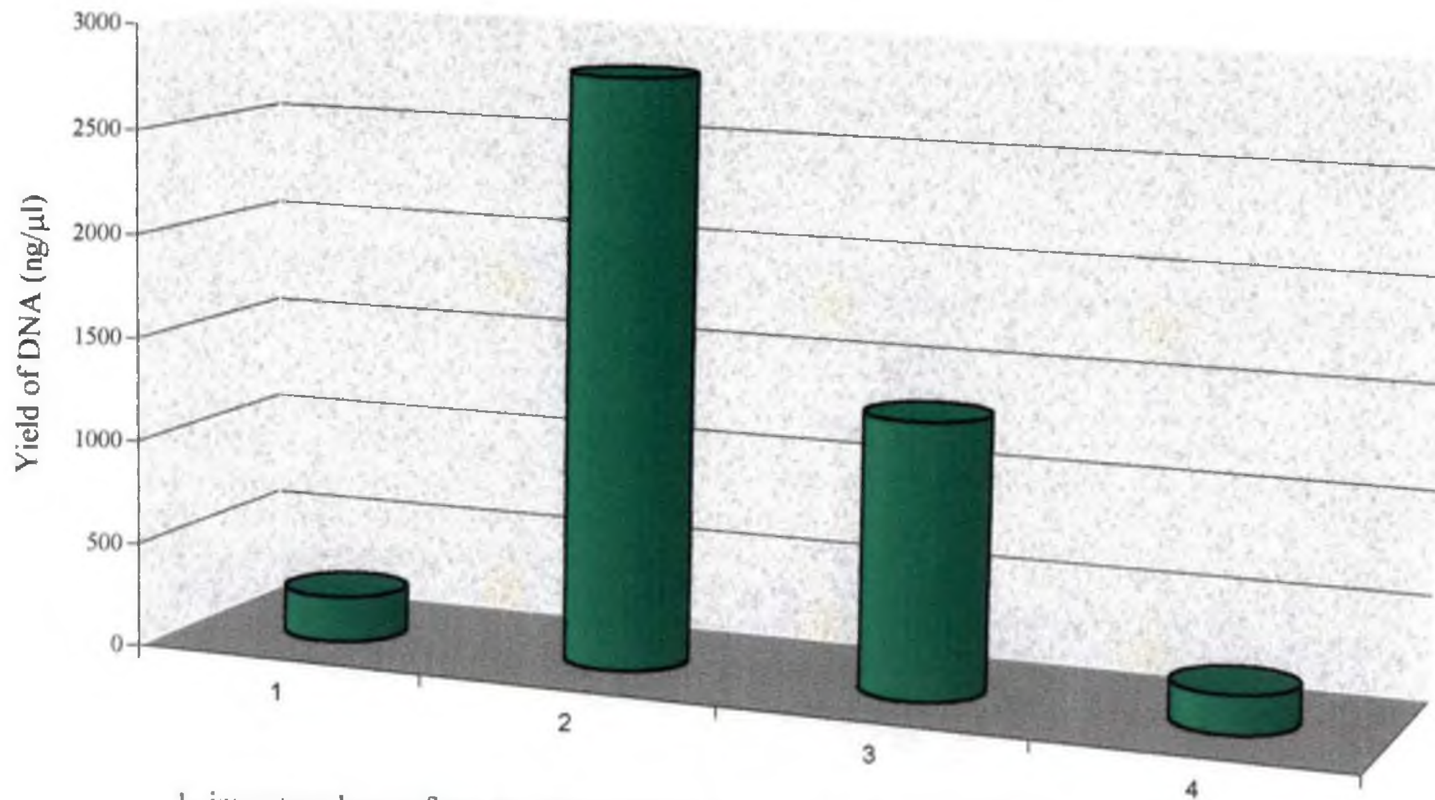


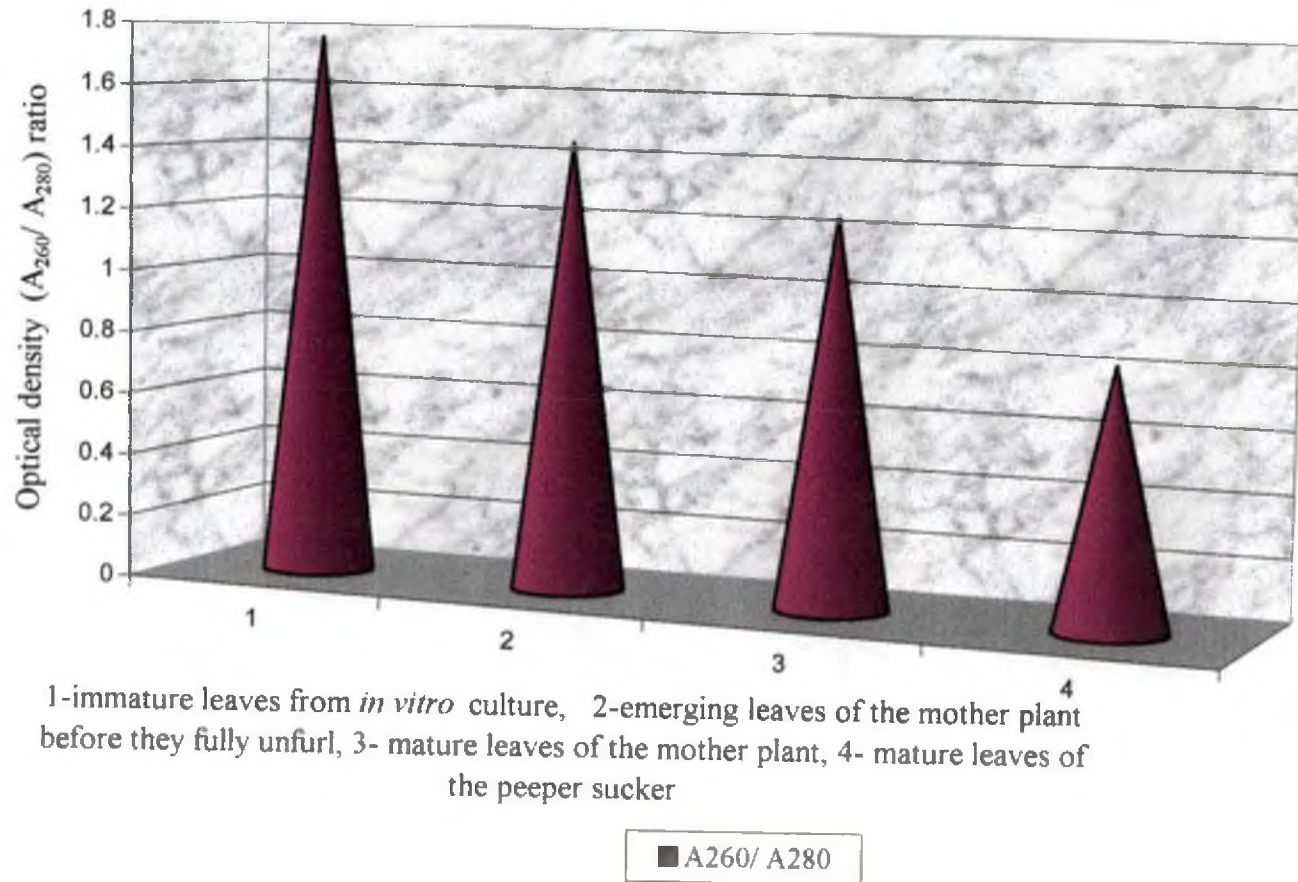

Fig-2 **Effect of methods of isolation on the optical density ratio ($A_{260}/A_{280}$) of the DNA of Red banana**

Fig-3 **Effect of source of plant tissue on the quantity of the DNA of Red banana**




1- immature leaves from *in vitro* culture, 2- emerging leaves of the mother plant before they fully unfurl, 3- mature leaves of the mother plant, 4- mature leaves of the peeper sucker

Fig-4 **Effect of source of plant tissue on the optical density ($A_{260}/A_{280}$) ratio of the DNA of Red banana**




1-immature leaves from *in vitro* culture, 2-emerging leaves of the mother plant before they fully unfurl, 3- mature leaves of the mother plant, 4- mature leaves of the peeper sucker

lowest DNA yield (185 ng/ μl). The cell disruption might not have been efficient in mature leaves of the peeper sucker.

The DNA sample obtained was brown coloured when the isolation was done according to modified Walbot's method. When 0.1 per cent PVP was included in the extraction buffer in addition to the other reagents in the modified Walbot's method, browning of DNA was found reduced. According to John (1992), the browning occurred due to the binding of tannin like material to DNA which leads to degradation of DNA even after cell lysis. Weising *et al.* (1995) reported that high phenolic oxidation in coffee tissues to brown coloured quinonic compounds damaged DNA and proteins. This could be effectively counteracted by the use of certain ingredients in the extraction medium which could prevent or reverse the action of polyphenol oxidase. Similar observation was made by Mondal *et al.* (2000) who used 0.2 per cent PVP in the extraction buffer for the isolation of chloroplast DNA from tea. The PVP acted as an antioxidant, which bound to the phenolic compounds and co- precipitated in subsequent centrifugation.

In the present study, when 1.5 per cent β - mercaptoethanol was included in the extraction buffer in addition to the other reagents, in Rogers and Bendich's method, browning was completely avoided. Inclusion of a variety of reducing agents inhibit browning (Mondal *et al.*, 2000). The use of reducing agents was an imperfect substitute for actual inhibition of phenol oxidation. Thus the use of polymer, PVP or the antioxidant β - mercaptoethanol alone could not prevent

browning of the extract. In the presence of both β - mercaptoethanol and PVP, the extraction efficiency was improved.

Reliable methods are required for isolating the DNA from the cells. CTAB method was found to be useful in several species like banana (Howell *et al.*, 1994) and Phalaenopsis (Chen *et al.*, 1998). In the present study, it was found that for routine extraction of high molecular weight DNA, a modified Rogers and Bendich's (1994) method was ideal.

Additional purification step with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) treatment, followed by chloroform : isoamyl alcohol (24 : 1) treatment after the DNA pellet was obtained, increased the optical density (O. D.) ratio from 0.93 to 1.18. The phenol : chloroform : isoamyl alcohol (25 : 24 : 1) treatment removed proteins from nucleic acid solutions (Walbot,1988). This procedure had the advantage that de-proteinisation was more efficient as two different organic solvents were used instead of one. The subsequent extraction with chloroform : isoamyl alcohol (24 : 1) treatment removed the lingering traces of phenol from the nucleic acid preparation.

The optical density (O. D.) ratio of DNA was increased from 1.33 to 1.46 when proteinase k was added and kept overnight. The reduction in protein content increased the O. D. ratio. However the yield of DNA was reduced after the proteinase k treatment, from 2326.7 ng/μl to 955 ng/μl (Fig-5 and Fig-6).

Fig:-5 **Effect of proteinase k on the quantity of the DNA of Red banana mother plant**

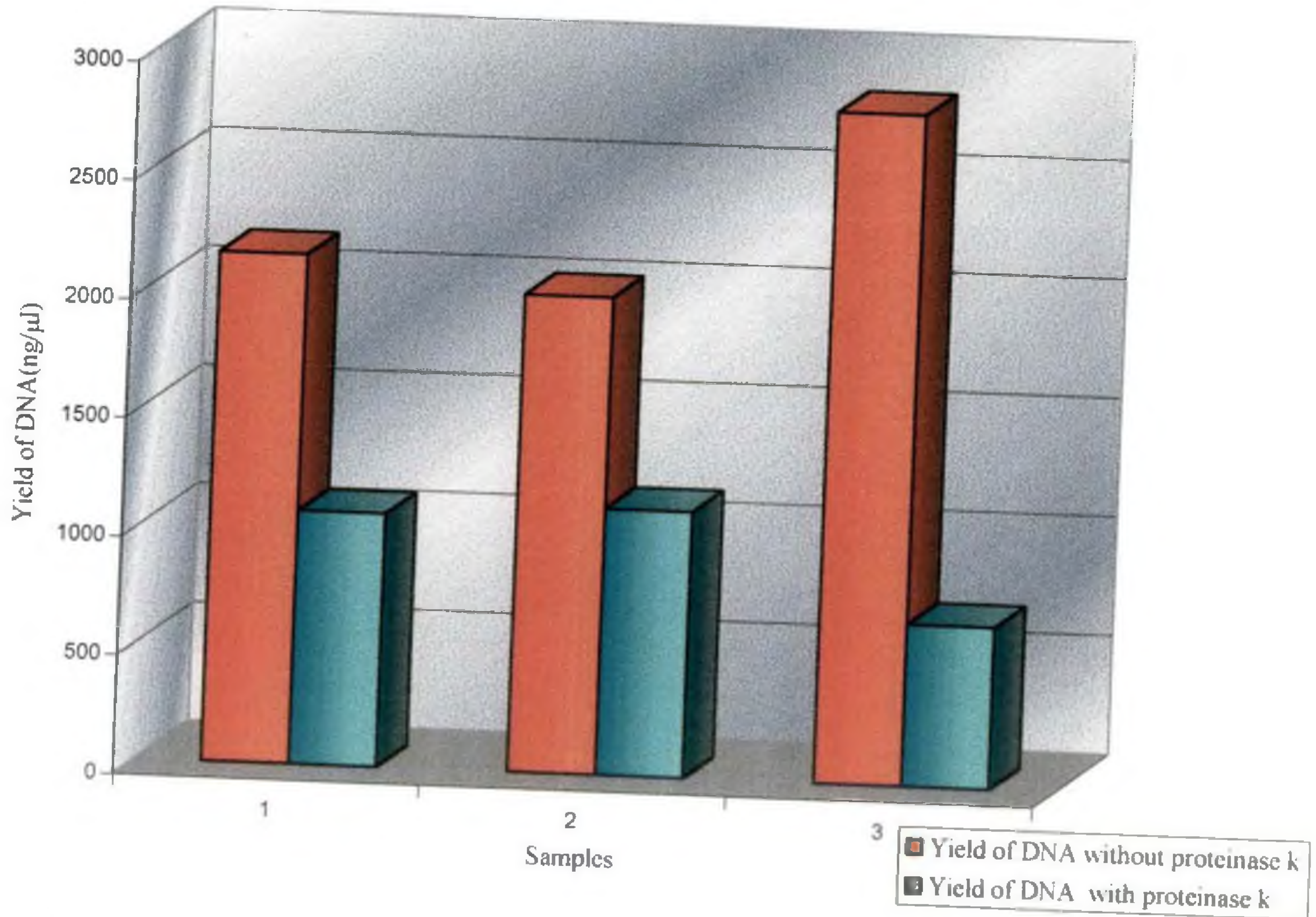
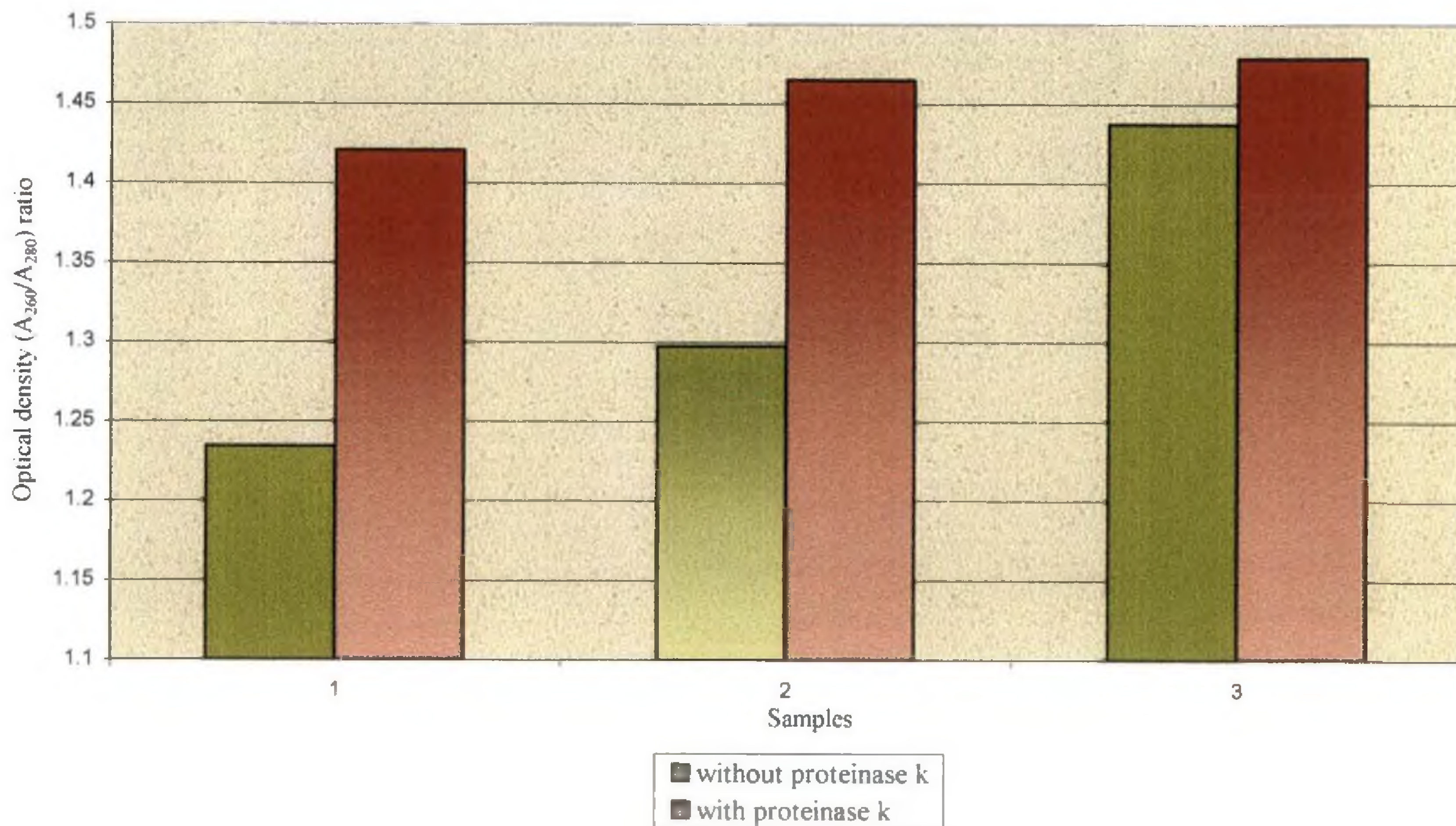


Fig:-6 **Effect of proteinase k on the optical density (A260/A280) ratio of the DNA of Red banana mother plant**



While using modified Walbot's method the speed of centrifugation was changed from 4,000 rpm to 10,000 rpm. It was observed that more extensive centrifugation caused the pellet of nucleic acid to adhere tightly to the centrifuge tube.

Ribonucleic acid (RNA) was observed as a thick band below the genomic DNA in gel electrophoresis. But the presence of RNA did not interfere in amplification. Mondal *et al.*, (2000), reported that RNA will not interfere in RAPD analysis.

The technique of gel electrophoressis is important, as it represents the way by which the nucleic acid fragments may be visualised directly. Here the DNA fragments are separated according to their size. Agarose concentration has an important role in the separation of nucleic acid fragments. Lesser the pore size, more the movement of DNA molecules in the gel. Thus in the present study, agarose concentration of 0.9 per cent and 1.4 per cent was optimum in gel electrophoresis for genomic DNA and RAPD analysis, respectively. Mulcahy *et al.* (1993) and Yu and Nguyen (1994) used 0.9 per cent agarose for visualising the genomic DNA. Prasannalatha *et al.* (1999), reported the separation of amplified products through 1.4 per cent agarose gel.

The PCR amplification (as per the procedure mentioned in **3.5**, materials and methods) was carried out using forty decamer primers (Operon Inc., CA, USA) of kit A and kit B with the DNA of Red banana mother plant. All the 37 primers out of the 40 primers yielded amplification products. Among the 37 primers there were variations in the banding patterns. The total number of bands ranged from 1.0 to 9.0. OPA - 06, OPB - 10 and OPB – 14 did not yield any bands. This indicates that there is no sequence complementary to the sequence of the primers OPA - 06, OPB - 10 and OPB – 14 in the mother plant DNA.

The concentration of DNA is an important factor in PCR amplification. Damasco *et al.* (1996) reported that the DNA concentration between 25 to 75 ng produced better amplification in Cavendish banana.In the present study, the DNA concentration between 20ng and 40 ng resulted in good amplification. Use of concentrations above 80 ng was not beneficial.

A total of 134 RAPDs (average of 3.4 bands) were generated with the mother plant DNA, of which 97.0 per cent (130 bands) were polymorphic (Table-4). This accounts to an average of 3.2 polymorphic bands per primer. High level of polymorphism was evident with all the primers of kit A and kit B except OPA – 06, OPB - 10, OPB – 14, OPA – 08, OPB – 02, OPB – 07 and OPB –16. This high level of polymorphism with certain primers could be explained by the capability of individual primers to amplify the less conserved and highly repeated regions of the DNA (Prasannalatha *et al.*, 1999). There is a

Fig-7 **Amplification profiles (total bands) of the DNA of Red banana mother plant using 20 primers (belonging to kit A) supplied by Operon Inc., CA, USA.**

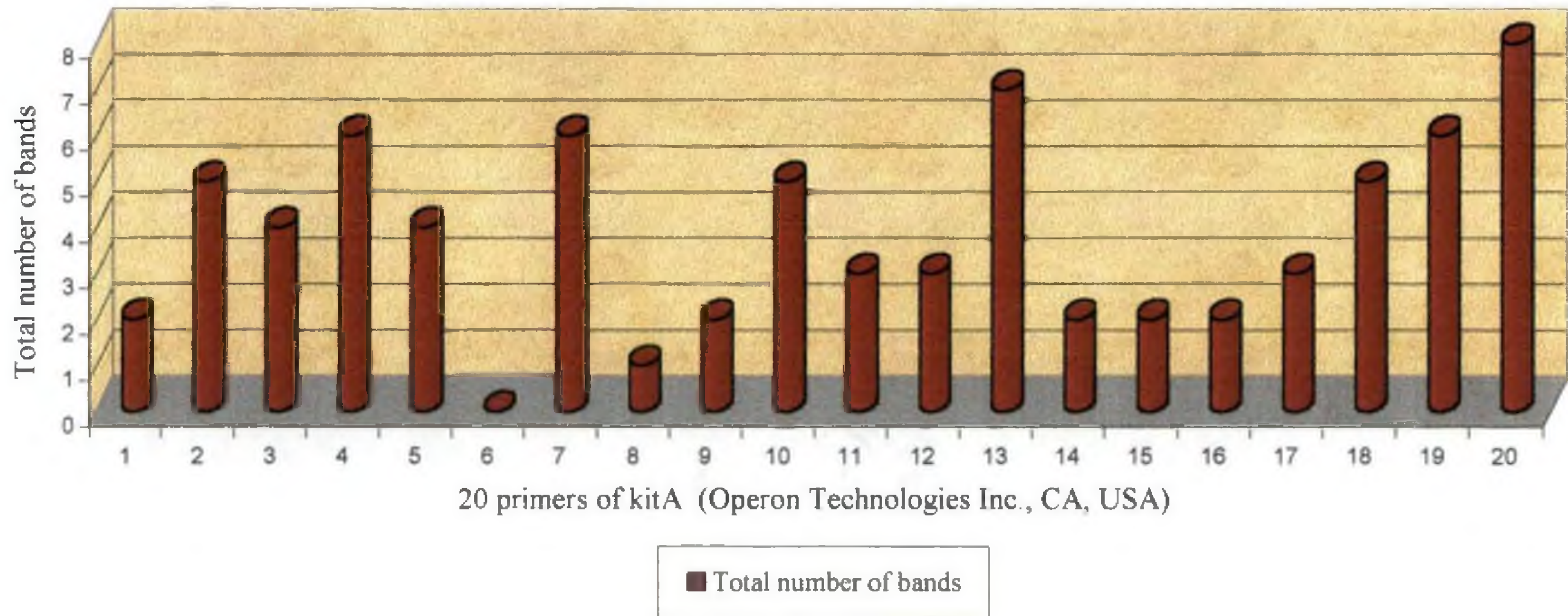


Fig-8 **Amplification profiles (Total bands) of the DNA of Red banana mother plant using 20 primers (belonging to kit B) supplied by Operon Inc., CA, USA**

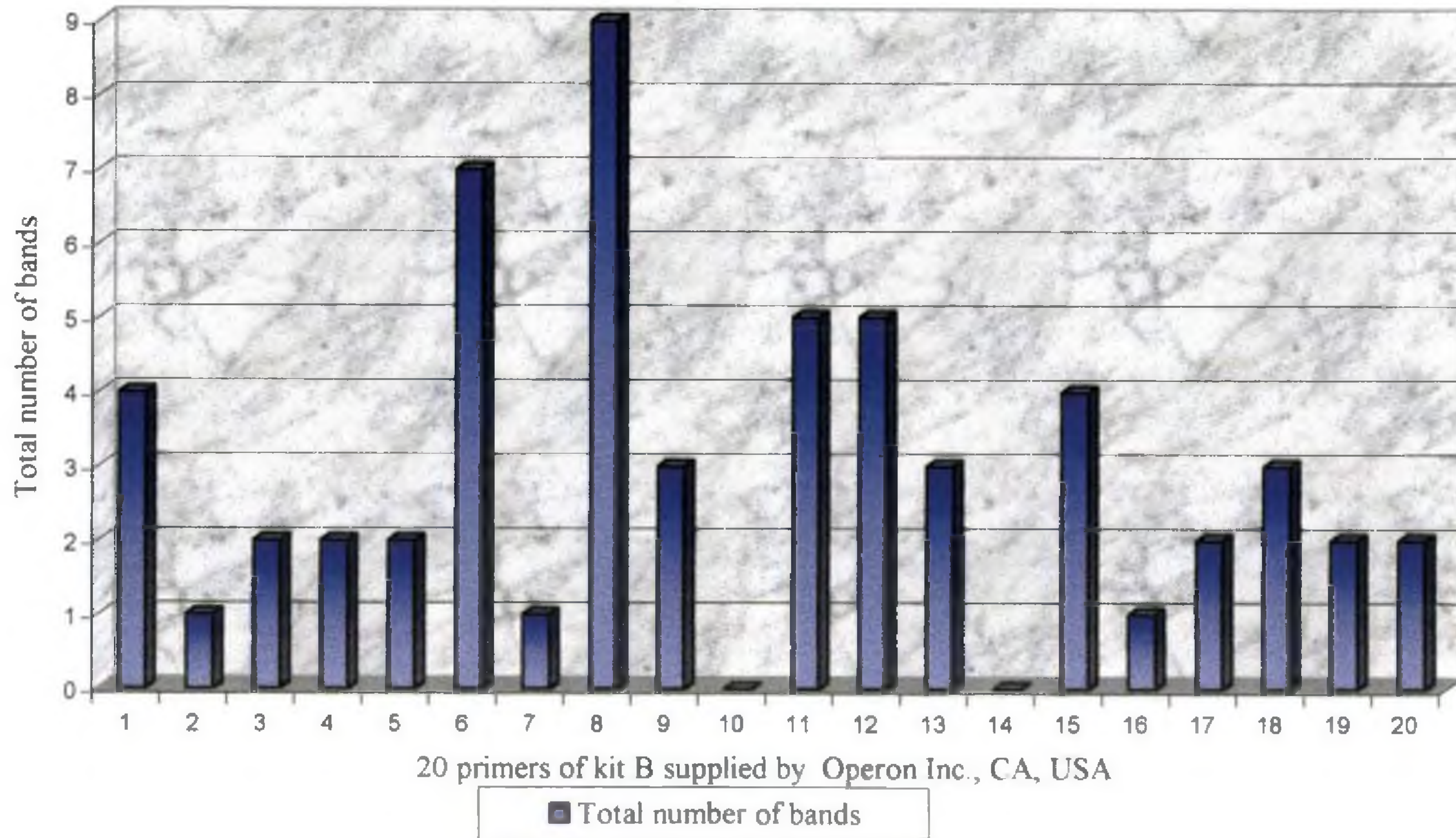


Fig-9 **Amplification profiles (Intense and faint bands) of the DNA of the Red banana mother plant using the primers of kit A supplied by the Operon Inc., CA, USA**

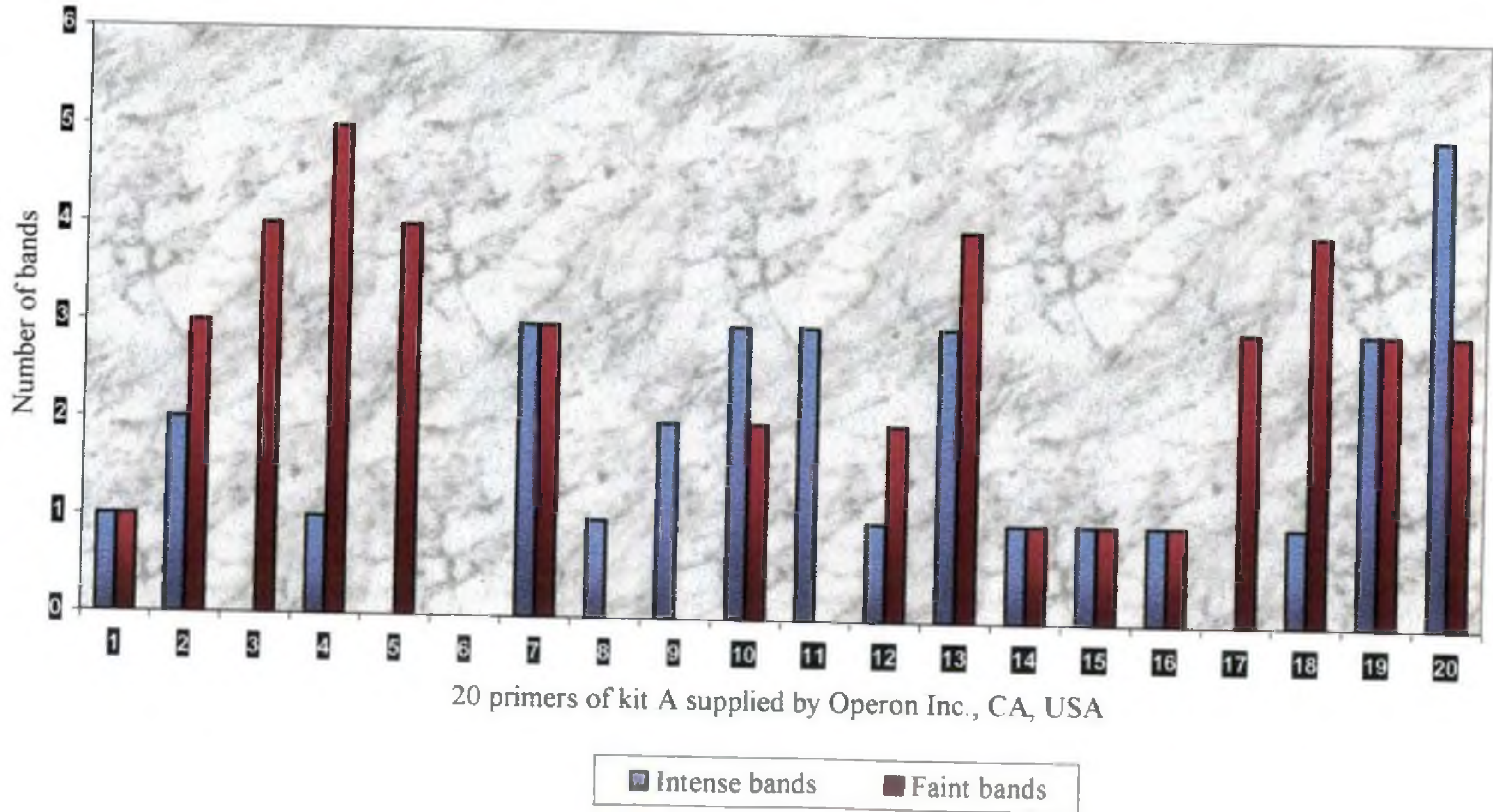
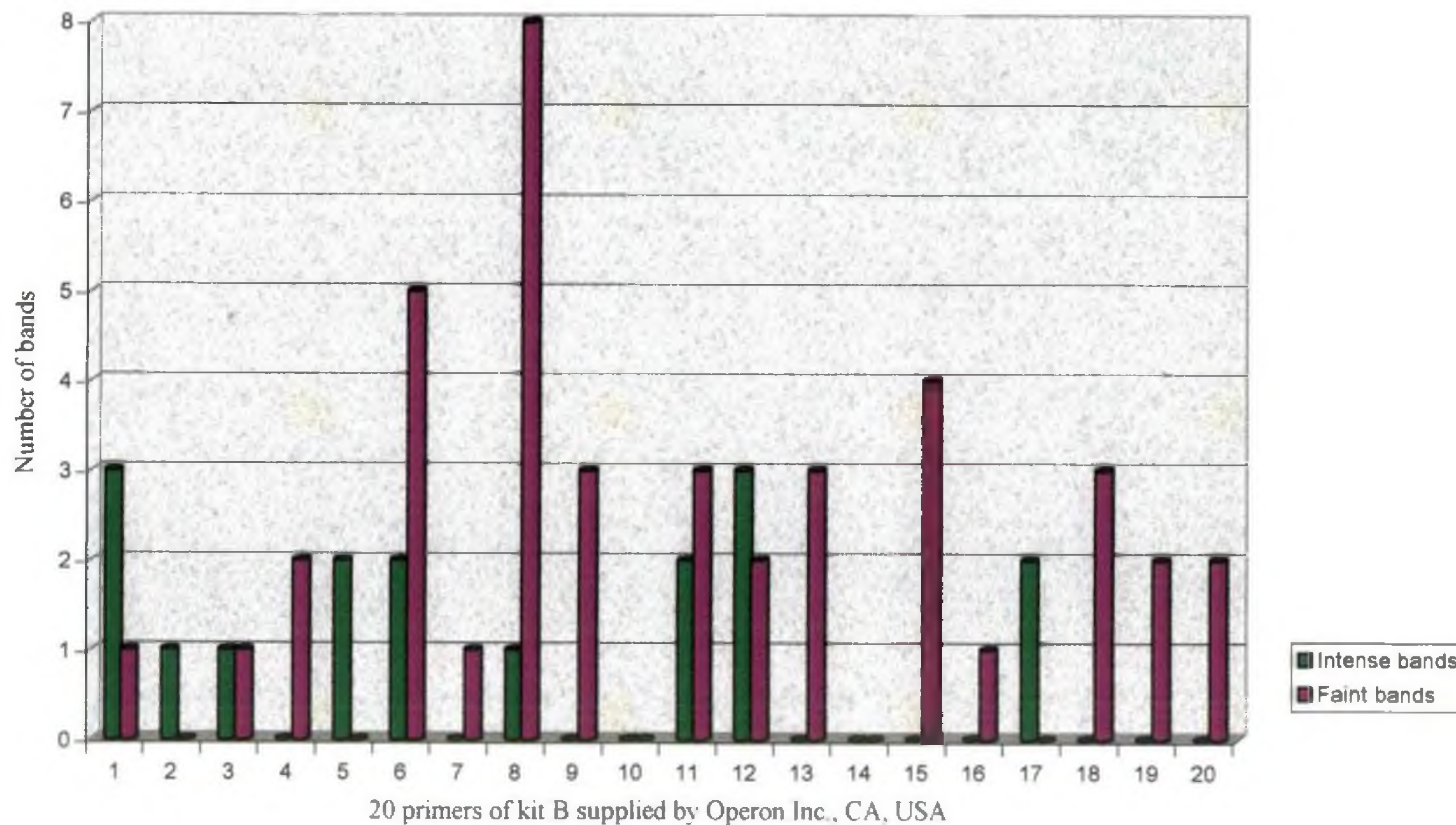


Fig-10 **Amplification profiles (Intense and faint bands) of the DNA of Red banana mother plant using the primers of kit B supplied by the Operon Inc., CA, USA**



high probability for the amplified fragments to contain repeated sequences (Fig-7 to Fig-10).

In the present study four promising primers for the RAPD analysis was identified. They were OPA-20, OPB-08, OPA-13 and OPB-06. OPA-20 was recommended for further PCR programmes. OPA–20 produced a total of 8.0 bands. Intense bands were 5.0 in number, where as faint bands were 3.0. This was the primer selected for further comparison studies. The PCR amplification was carried out according to the following procedure using OPA-20 as the primer. An initial denaturation at $95^0$ C for 3.0 minutes, followed by 45 cycles of denaturation at $95^0$ C for 1.0 minute, annealing at $36^0$ C for 1.0 minute 30 seconds, and extension at $72^0$ C for 2.0 minutes. The synthesis step of the final cycle was extended further by 6.0 minutes. Finally the products of amplification were cooled down to $4.0^0$ C until attended. Emphasis was given to the definition of PCR conditions, which allow the distinct, prominent and consistent amplification band from a range of DNA. Numerous adjustments were initially examined in order to optimise the RAPD assay. Among the parameters annealing temperature was found to be the most critical (Mondal *et al.*, 2000). Below $36^0$ C (annealing tempeature) produced non – specific bands where as above $36^0$ C failed to amplify.

When the same amplification reaction was done using the DNAs of the three subcultures, no difference was found in RAPD banding pattern from that of the mother plant. This indicates the absence of somaclonal variations. Using the

RAPD technique, various investigators had reported the absence of genetic variation in Norway Spruce (Fourre *et al.*, 1997), *Picea mariana* (Isabel *et al.*, 1993), *Festuca patensis* (Valles *et al.*, 1993) and *Hordeum* (Devaux *et al.*, 1993). In contrast, somaclonal variations were reported in *Triticum aestivum* (Brown *et al.*, 1993), poplar (Rani *et al.*, 1995), beet (Munthali *et al.*, 1996) and peach (Hashmi *et al.*, 1997) using RAPDs.

According to Goto *et al.* (1998), the presence or absence of variation depends on the source of the explant and method of regeneration or on the source of the regenerants (callus, protoplast and cell). Shoot apex culture has been described as a comparatively low risk method for *in vitro* propagation (Pierik, 1991), as organised structures are more resistant to genetic changes than unorganised callus. However, based on the leads of the present work, it is necessary to asses the safe number of subcultures that can be adopted for large scale commercial *in vitro* clonal multiplication of banana.

# SUMMARY

# 6.SUMMARY

Attempts were made for evaluating the genomic stability of *in vitro* propagated Red banana plantlets at molecular level, during January 1999 to November 2000 at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani. The salient features of the studies are summarised below:

1. Twenty per cent of cultures survived in Murashige and Skoog (MS) medium supplemented with benzyl adenine (BA) 2.0 mg $l^{-1}$.
2. The emerging leaves before they fully unfurl gave the highest DNA yield of 2825 ng/ µl as compared to *in vitro* leaves, mature leaves of the peeper sucker and mature leaves of the mother plant. The $A_{260}/A_{280}$ ratio obtained was the highest in *in vitro* leaves (1.76) as compared to the emerging leaves before they fully unfurl, mature leaves of the peeper sucker and mature leaves of the mother plant.
3. Out of the four methods used for DNA isolation, the purity of DNA was the highest (1.81) in the fourth method given by Scott *et al.* (1994) where as, the quantity of the DNA was the highest (3000 ng/ µl) in the second method given by Walbot (1988).
4. The optical density (OD) ratio of the pelleted DNA was increased from 1.33 to 1.46 on addition of proteinase k. Additional purification step with phenol: chloroform: isoamyl alcohol (25: 24: 1) followed by chloroform: isoamyl alcohol (24: 1), until the interphase disappeared, increased the $A_{260}/A_{280}$ ratio from 0.93 to 1.18. Colourless transparent DNA pellet was obtained on

the addition of 1.0 per cent polyvinyl pyrrolidone (PVP) and 1.5 per cent β-mercaptoehanol to the modified method of Walbot (1988) and Scott *et al.* (1994) method respectively.

5. Agarose concentration of 0.9 per cent was the best for the genomic DNA, as compared to 0.7 per cent, 0.8 per cent and 1.0 per cent. Similarly 1.4 per cent agarose concentration was the best for the RAPD pattern than 1.2 per cent, 1.3 per cent and 1.5 per cent.

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6. 20 ng of the DNA, 250 µM each of dNTPs, 0.6 units Taq DNA polymerase and 10 pM primer in presence of the assay buffer gave the best amplification results. The optimum programme was : An initial denaturation at $95^0$ C for 3.0 minutes, followed by 45 cycles of denaturation at $95^0$ C for 1.0 minute, annealing at $36^0$ C for 1.0 minute 30 seconds, and extension at $72^0$ C for 2.0 minutes. The synthesis step of the final cycle was extended further by 6.0 minutes. The products of amplification were kept at $4.0^0$ C until attended.
7. A total of 134 RAPDs were generated, when PCR amplification was carried out using forty decamer primers (Operon Inc., CA, USA) of kit A and kit B. 130 bands were polymorphic which accounted to an average of 3.2 polymorphic bands per primer. OPA- 06, OPB-10, OPB- 14 produced no amplification. OPA- 20 produced five intense bands and three faint bands when subjected to amplification reaction with the genomic DNA of the mother plant.
8. No marked difference was found in RAPD banding pattern (using OPA- 20 as primer) between the three subcultures and the mother plant of Red banana.

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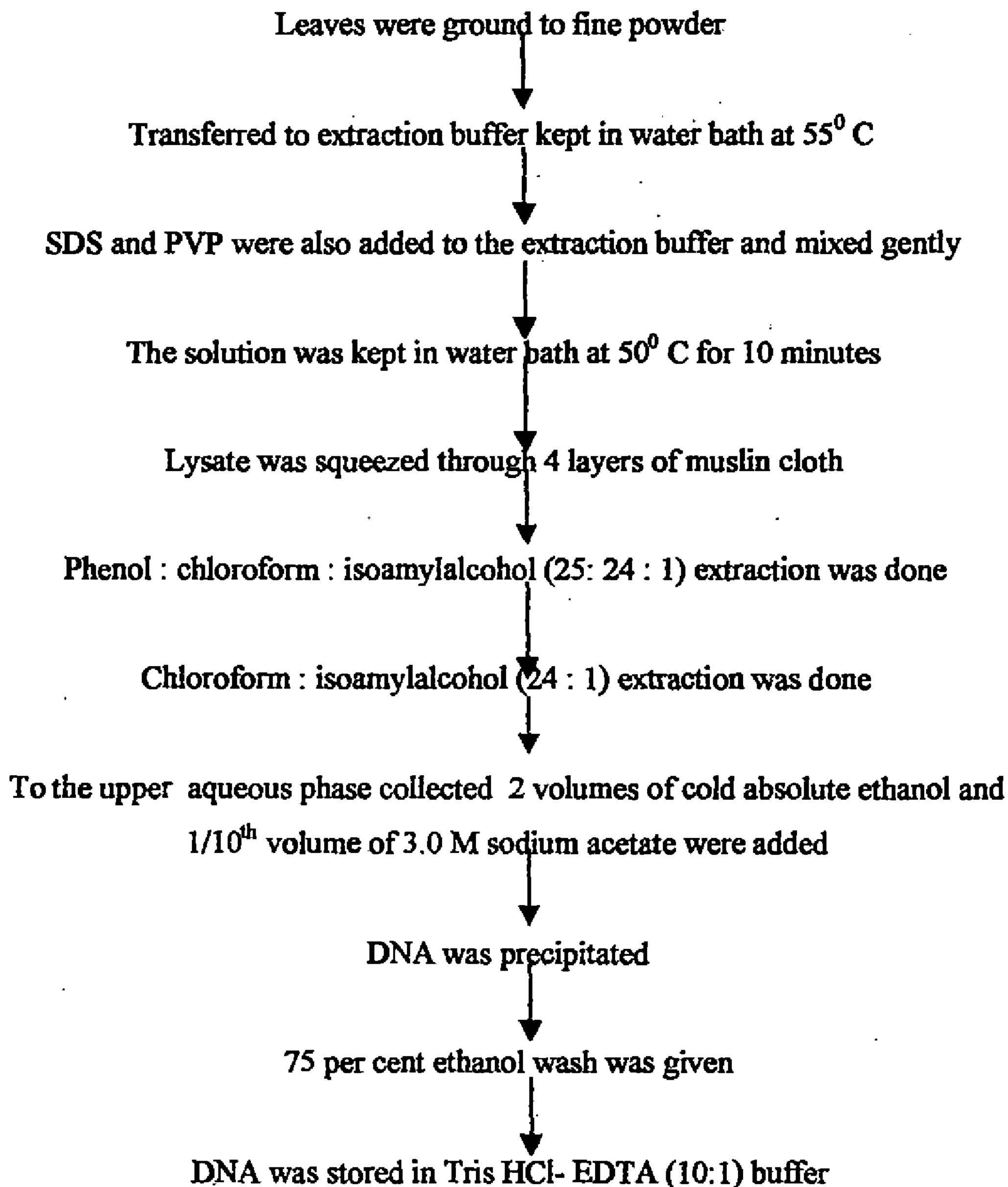
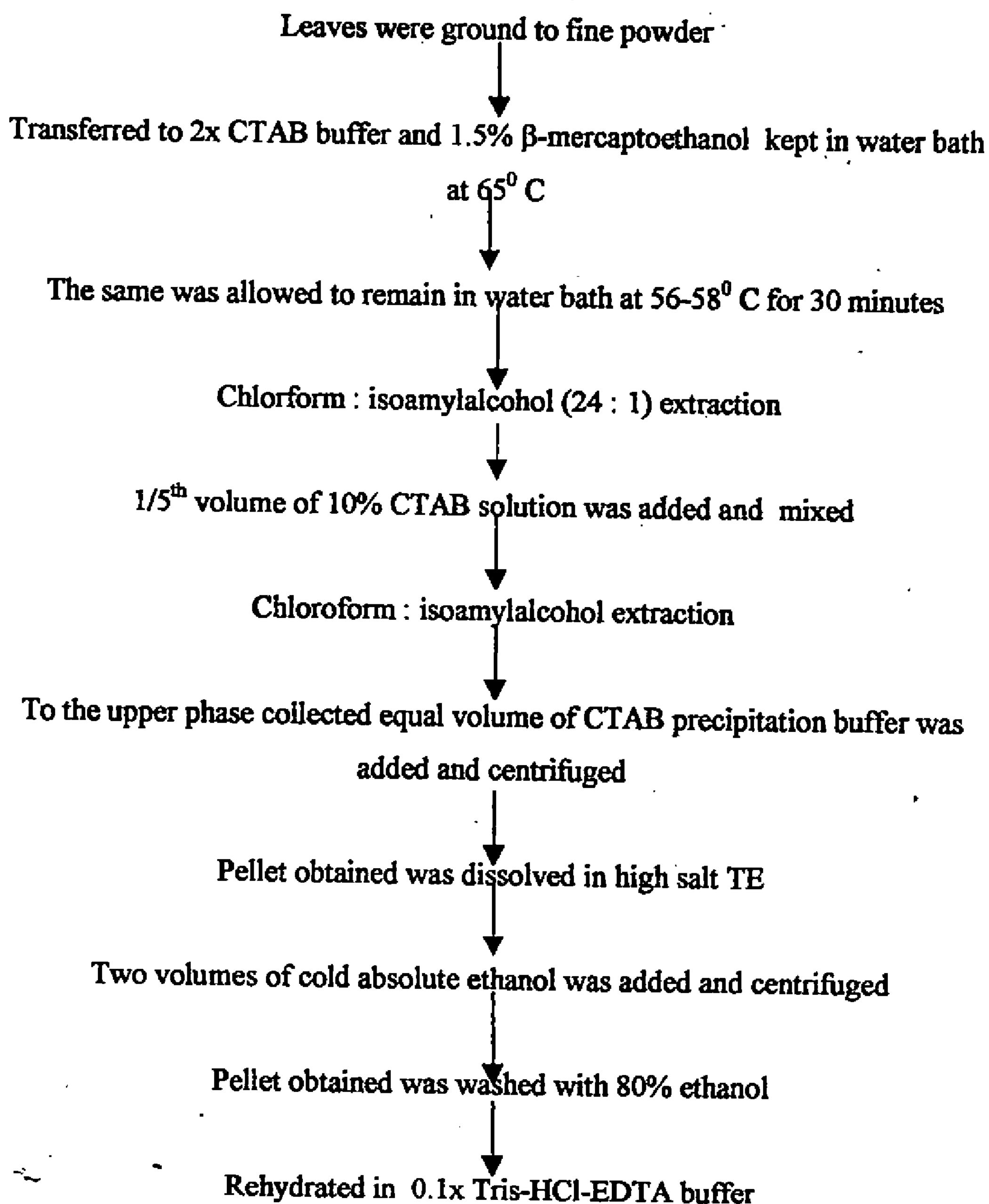
## Appendix-1 **MODIFIED WALBOT'S METHOD**



## Appendix-2 **ROGERS AND BENDICH'S METHOD**



# MOLECULAR EVALUATION OF GENOMIC STABILITY OF BANANA PLANTS DEVELOPED BY *IN VITRO* CLONAL PROPAGATION

By
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ABSTRACT OF THESIS
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# ABSTRACT


Attempts were made for evaluating the genomic stability of *in vitro* propagated Red banana plantlets at molecular level, during 1999- 2000, at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani. Efforts were made to standardise the DNA isolation method and PCR amplification conditions, to identify the primer producing reproducible polymorphic bands and to compare the banding patterns characteristic to the subcultures and the mother plant. The emerging leaves of the Red banana plants before they fully unfurl, gave the highest DNA yield of 2825 ng/ µl whereas, the *in vitro* leaves gave the highest optical density (OD) ratio of 1.76. The purity of DNA was the highest (O. D. ratio 1.81) while CTAB method (Scott et al., 1994) was adopted. The DNA quantity was the highest in the Walbot's method, *viz.* 3000 ng/ µl. The OD ratio increased from 1.33 to 1.46 on addition of proteinase k. Additional purification step increased the OD ratio from 0.93 to 1.18. One per cent polyvinyl pyrrolidone (PVP) and 1.5 per cent β-mercaptoethanol, when added in the extraction buffer produced transparent DNA pellet. 0.9 per cent and 1.4 per cent of agarose concentration were found to be the best for the genomic DNA and RAPD banding patterns respectively. The optimum PCR programme was an initial denaturation at $95^0$ C for 3.0 minutes, followed by 45 cycles of denaturation at $95^0$ C for 1.0 minute, annealing at $36^0$ C for 1.0 minute and 30 seconds, and extension at $72^0$ C for 2.0 minutes. The synthesis step was extended further by 6.0 minutes. A total of 134 RAPDs were generated when PCR amplification was done of which 130 were polymorphic. OPA- 06, OPB-10 and

OPB- 14 produced no amplification. No difference was found in the banding pattern of the three subcultures and the mother plant of Red banana, when amplification reaction was carried out using OPA-20. A total of five intense bands and three faint bands were obtained with OPA-20.