MOLECULAR CHARACTERIZATION OF BANANA *VAR.* NANJANAGUDU RASABALE AND IDENTIFICATION OF FRUIT RIPENING SPECIFIC PRODUCTS

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by

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Dedicated To my family,

Guide & friends

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DECLARATION

I, **L. VENKATACHALAM**, certify that this thesis is the result of research work done by me under the supervision of **Dr. Bhagyalakshmi Neelwarne** at Plant Cell Biotechnology Department of Central Food Technological Research Institute, Mysore- 570 020, India during the period 2003-2008. I am submitting this thesis for possible award of the **DOCTOR OF PHILOSOPHY (Ph.D.)** degree in **BIOTECHNOLOGY** of the University of Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree / diploma of this or any other University.

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This is to certify that the thesis entitled "**MOLECULAR CHARACTERIZATION OF BANANA *VAR.* NANJANAGUDU RASABALE AND IDENTIFICATION OF FRUIT RIPENING SPECIFIC PRODUCTS"** submitted by **Mr. L. Venkatachalam,** to the **University of Mysore** for the award of the degree of **Doctor of Philosophy** in **Biotechnology** is the result of research work carried out by him under my guidance in **Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore** during the period October 2003 to October 2008.

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L. Venkatachalam

Abstract

Banana is a major fruit crop and India is the place of origin and diversity for banana. The cultivar under consideration for the present study is an endangered one namely "Nanjanagudu rasabale" (NR) having genotype AAB. The present study has established genetic relationship of NR, among other 21 commercially important banana cultivars of South India, by using genetic markers. Analyses using 50 Randomly Amplified Polymorphic DNA (RAPD) and 12 Inter Simple Sequence Repeats (ISSR) primers resulted in the amplification of totally 641 bands of 200 – 3100 bp, of which 382 bands were polymorphic. Based on these data, a genetic similarity matrix was established and a dendrogram for each set of primers was developed by UPGMA. The Genetic similarity coefficients in RAPD analysis ranged from 0.3177 to 0.7818 and in ISSR analysis from 0.1800 to 0.8462. The presence of a specific RAPD (OPC 5_{800}) band was observed for an endemic cultivar - NR. A group of 8 cultivars was identified having highly diverse relationship from one another and this information would be useful for generating 2X and 4X breeding populations for further application in breeding secondary hybrids.

Since edible banana plants are sterile hybrids, obtaining variants through biotechnological methods would greatly assist in the generation of genetically diverse and agronomically improved clones for which *in vitro* regeneration is a pre-requisite. Thus aiming, the leaf explants were cultured first on medium with a high level (22.4 µM) of benzyladenine (BA), second on indole-3-butyric acid (IBA) supplemented medium, and third on reduced BA medium under incubation in the dark. The highest adventitious shoot regeneration in 24% of the explants, with the number of shoots ranging from 2-3 per explant, occurred in the explants incubated at the first step in medium with 24.6 µM and 0.198 µM of IBA. Addition of various levels of (10-50 µM) spermine, spermidine, and putrescine to cultures with secondary embryogenesis showed that about 50% of embryogenic calli rapidly produced secondary embryos only in the presence 40 µM spermine but not in other treatments. The shoot cultures were checked for their performance on solid medium (SM) and partial immersion system (PIS). The rate of shoot multiplication was higher in PIS than in SM. A micropropagation protocol was also developed where high levels of Benzylamino purine (BAP) up to 53.28 µM and Kinetin (Kn) up to 55.80 µM showed direct correlation between the two resulting in a highest number of 80 shoot buds per segment in BAP (31.08 µM) treatment. The plantlets were analysed for their genetic stability using 50 RAPD and 12 ISSR genetic markers that resulted in 625 distinct

bands showing homogeneous patterns. The absence of any genetic variation indicates that the micropropagation protocol developed in this study as well as for developing stable regenerants of NR for rapid *in vitro* multiplication is appropriate and applicable for clonal propagation.

Fruit ripening and softening involve depolymerization of complex cell wall components. More than one class of enzymes and other proteins are known involved in this process. In the present study the regulation of fruit softening studied at molecular level and demonstrated the simultaneous activities and gene expressions of expansins (the cell wall loosening proteins) and other cell wall hydrolyzing enzymes. Two types of expansin genes, *MaEXPA-1* and *MaEXPA-2*, were found to be banana fruit-ripening-specific and their expressions significantly and differentially altered by ethylene inducers/inhibitors. Activities of pectin methyl esterase (PME), polygalacturonase (PG) and pectate lyase (PEL) in banana cv. NR fruit were measured over a period of 10 days after ripening was initiated with ethylene. Ethylene-stimulated activities of the above three enzymes was differentially suppressed by GA, MH, SA and IAA whereas ABA, ethrel and smoking stimulated the activities of all hydrolases, except polygalacturonase. These treatments appear to play a major role in up- / down-regulation of the activities of various cell wall hydrolases.

To gain a better insight on the molecular regulation of banana fruit ripening, the mRNA Differential Display technique coupled with silver-staining was used and specific transcripts differentially expressed during ripening were first identified. Using 71 primer combinations and four populations of mRNA (Pre-climacteric; Climacteric 1; Climacteric II and post climacteric), a total of 120 transcripts were cloned into T/A cloning vector and sequenced. DNA sequence analyses revealed significant homology to transport protein, sucrose phosphate synthase, heat shock protein, transcriptional regulator, senescence protein. These proteins can be associated to biological processes like primary, secondary and RNA metabolism, signal transduction and stress responses or defense. The RNA dot analysis showed the expression of most of the up- and down-regulated genes are specific to fruit and ripening. Since banana lacks information about the molecular regulation of ripening, the results of the present finding provide better insight for the characterization of the changes in gene expression that accompanies the ripening process. The enormous data developed through these studies form the basis for developing chemical formulations for controlled ripening of banana fruit, probably with extended shelf life.

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List of Abbreviations

AA	:	Amino acids
BLAST	:	Basic Local Alignment Search Tool
bp	:	base pairs
BSA	:	Bovine Serum Albumin
ºC	:	Degree Centigrade
cDNA	:	Complementary Deoxyribonucleic Acid
cm	:	Centimeter
cRACE	:	Circular Rapid Amplification of cDNA ends
CTAB	:	Hexadecyltrimethylammonium bromide
DDRT-PCR	:	Differential Display Reverse Transcription- PCR
DEPC	:	Diethyl-pyrocarbonate
DIG	:	Digoxigenin
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxynucleotide triphosphate
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
GA	:	Giberellic acid
GUS	:	β-glucuronidase
HRMM	:	High Rate Multiplication Medium
IPTG	:	Isopropyl-β–D–thiogalactopyranoside
kb	:	Kilobase
kDa	:	Kilodalton
kn	:	Kinetin
3’	:	Hydroxyl- terminus of DNA molecule
5’	:	Phosphate-terminus of DNA molecule
d	:	day
DNase I	:	Deoxyribnuclease I
dsDNA	:	Double strand Deoxyribonucleic Acid
EST	:	Expressed Sequence Tag
FC	:	Folin and Ciocalteul
FW	:	Fresh Weight
g	:	gram
h	:	hour
ISSR	:	Inter Simple Sequence Repeats
LB	:	Luria- Bertani
L	:	Litre
M	:	Molar
mg	:	Milli gram
min	:	minute
MH	:	Maleic Hydrazide
ml	:	Millilitre
mM	:	Millimolar
MOPS	:	4-Morpholinepropanesulfonic acid
mRNA	:	messenger RNA
N	:	Newton
nm	:	nanomolar

ng	:	nanogram
nt	:	nucleotide
NCBI	:	National Centre for Biotechnology Information
NBT	:	Nitroblue tetrazolium
NR	:	Nanjanagudu Rasabale
OD	:	Optical density
PCR	:	Polymerase Chain Reaction
PEG	:	Polyethylene glycol
PEL	:	Pectate Lyase
PG	:	Polygalacturonase
PGR	:	Plant Growth Regulators
pmol	:	Picomol
PME	:	Pectin Methyl Esterase
RAPD	:	Rapid Amplification of Polymorphic DNA
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rRNA	:	ribosomal RNA
rpm	:	revolution per minute
RT	:	Reverse transcription
RT-PCR	:	Reverse Transcriptase Polymerase Chain Reaction
SA	:	Salicylic acid
SD	:	Standard Deviation
SDS	:	Sodium dodecyl sulphate
sec	:	Second
SSC	:	Saline sodium citrate
TAE	:	Tris-acetate-EDTA
Taq	:	*Thermus aquaticus*
TBE	:	Tris-Borate-EDTA
TE	:	Tris-EDTA buffer
Tris	:	Tris (hydroxymethyl) amino methane
X-GAL	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Glc	:	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
U	:	Unit enzyme
UV	:	Ultra Violet
V	:	Volt
v/v	:	Volume per volume
wk	:	week
W	:	Weight
w/v	:	Weight per volume
α	:	Alpha
β	:	Beta
µg	:	Micro gram
µM	:	Micro molar
µl	:	Micro litre
%	:	Percent

General Introduction and Review of Literature

G1.1. Introduction

The word "banana" is a general term embracing a number of species or hybrids in the genus *Musa* of the family *Musaceae* known simply as the banana family. Two *Musa* species namely *Musa acuminata* and *Musa balbisiana*, out of the 28 described, were first noted in 1865 by Kurz (as reported in Stover and Simmonds 1987) as being the origin of most of the commercial clones. Cultivated clones are parthenocarpic and often quite sterile with seedless starchy fruits that develop without fertilization. Indeed, wild types are very fertile; their fruits are full of seeds with little starch. To the consumer, "banana" seems a simple name for the yellow fruits so abundantly marketed for consumption as fresh fruit, and "plantain" for the larger more angular fruits intended for cooking but also edible in raw form when fully ripe. However, the distinction is not precise and the terms are sometimes reversed.

G1.2. Origin and history of cultivation

Edible *Musa spp.* originated in south-eastern Asia, distributed from eastern part of India to northern Australia. Early Filipinos probably spread the banana eastward to the pacific islands, including Hawaii, prior to recorded history. Westward, banana likely followed the major trade routes that transported other fruits, and it is known to have arrived in east Africa around 500 AD. Bananas were not carried to Europe until the 10^{th} century, and Portuguese traders obtained it from West Africa, during the age of discovery. Plants were taken from West Africa to the Canary Islands and South America in the 16^{th} century, and spread throughout the Caribbean with settlement of the area in the 16^{th}-17^{th} centuries. Bananas are now grown pantropically in more countries than any other fruit crop in the world (Morton 1987).

G1.3. Socioeconomic importance of bananas

- Bananas are the developing world's fourth most important food crop (after rice, wheat and maize). The crop is grown in more than 100 countries throughout the tropics and sub-tropics with annual world production of 88 million tons (Table 1.1)
- As a crop bananas represent consist of a wide range of varieties, including both cooking and dessert types
- Around 87% of all the bananas grown worldwide are produced by small scale farmers for home consumption or for sale in local and regional markets

- Bananas provide a staple food for millions of people, particularly in Africa, an area where the green revolution has had little influence
- Bananas are an important food security crop, providing a cheap and easily produced source of energy. In addition, they are rich in vitamins A, C, B_6 and β-carotene
- Bananas are also rich in mood elevators, tryptophan, serotonin and melatonin
- Growing urbanization in many developing countries means that the crop is becoming more and more important as a source of revenue, sometimes providing the main source of income for rural communities. Bananas thus play an important role in poverty alleviation
- Bananas grow year-round in a range of environments, thus providing a source of energy during the 'hungry-period' between the harvests of other crops
- Bananas are particularly suited to intercropping systems and to mixed farming with livestock. Due to their suitability for production in backyard systems, bananas are also an important component of peri-urban agriculture
- When grown in perennial production systems, bananas maintain cover throughout the year, thus protecting the soil from erosion due to rain and wind
- Approximately 13% of worldwide banana production is destined to the export market. For many countries, especially in Latin America and Caribbean, bananas provide an essential source of foreign exchange and the crop plays a vital role in the social and political fabrics of the islands, including the French departments of Guadeloupe and Martinique
- They provide an important source of fibre (for example Abaca/Manila hemp in the Philippines), and among other uses, raw pulp can be fermented to produce alcohol
- Bananas have been considered as a useful tool to deliver edible vaccines. Since the fruits are eaten uncooked, it is sterile before peeling and it is often the first solid food eaten by babies
- Millions of small-scale farmers in Africa, south Asia and northern Latin America grow the fruit for household consumption and / or local markets and the highest consumption of bananas per person is in Uganda, estimated as about to 1 kg per person per day (Edmeades et al. 2006). The two major banana producing countries in the world are India and Brazil but neither of these exports significant quantities

Table G1. Major dessert banana producing countries

Country	Production in 2006 (× 1000 tons)	% of bananas exported
India	11,710.30	0.1
Brazil	7,088.02	3
Philippines	6,794.56	29
China	6,7083.00	0.6
Ecuador	6,118.42	67

Data compiled from FAO 2007

Table G2. Major dessert banana exporting countries

Country	Export (× 1000 tons)
Ecuador	4,085.35
Philippines	1,964.39
Costa Rica	1,597.08
Colombia	1,381.25
Guatemala	1,125.60

Data compiled from FAO 2007

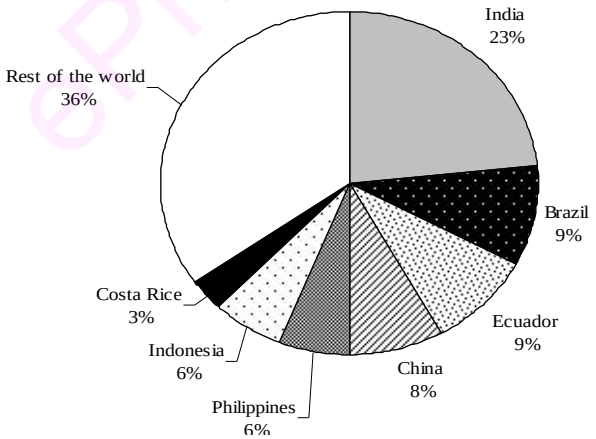

Figure G1. Global distribution of banana production. (Average on the 2001-2006 period)

Table G3. Nutritional composition of banana (per 100 g of edible portion)*

	Banana (Cavendish)				**Plantain**		
	Ripe	**Green**	**Dried**	**Flour (green)**	**Ripe**	**Green**	**Dried (green)**
Calories	65.5-111	108	298	340	110.7-156.3	90.5-145.9	359
Moisture (%)	68.6-78.1	72.4	19.5-27.7	11.2-13.5	52.9-77.6	58.7-74.1	9.00
Protein (g)	1.1-1.87	1.1	2.8-3.5	3.8-4.1	0.8-1.6	1.16-1.47	3.30
Fat (g)	0.016-0.4	0.3	0.8-1.1	0.9-1.0	0.1-0.78	0.10-0.12	1.40
Carbohydrates (g)	19.33-25.8	25.3	69.9	79.6	25.50-36.81	23.4-37.61	83.90
Fiber (g)	0.33-1.07	1.0	2.1-3.0	3.2-4.5	0.30-0.42	0.40-0.48	1.00
Ash (g)	0.60-1.48	0.9	2.1-2.8	3.1	0.63-1.40	0.63-0.83	2.40
Calcium (mg)	3.2-13.8	11.0	NA	30-39	5.0-14.2	10.01-12.2	50.00
Phosphorus (mg)	16.3-50.4	28.0	NA	93-94	21.0-51.4	32.5-43.2	65.00
Iron (mg)	0.4-1.50	0.9	NA	2.6-2.7	0.40-0.11	0.56-0.87	1.10
β-Carotene (μg)	6.0-151.0				110.0-1320	60.0-1380.0	45000.0
Thiamine (mg)	0.04-0.54				0.04-0.11	0.06-0.09	0.10
Riboflavin (mg)	0.05-0.07				0.04-0.05	0.04-0.05	0.16
Niacin (mg)	0.60-1.05				0.48-0.70	0.32-0.55	1.90
Ascorbic Acid (mg)	5.60-36.4				18-31.2	22.2-33.8	1.00
Tryptophan (mg)	17-19				8-15	7-10	14.0
Methionine (mg)	7-10				4-8	3-8	
Lysine (mg)	58-76				34-60	37-56	

*Source: INIBAP; NA: Data not available

G1.4. Taxonomy

The genus name *Musa* is thought to be derived from the Arabic name for the plant (mouz) which, in turn, may have been applied in honour of Antonius Musa (63-14 BC), physician to Octavius Augustus, first emperor of Rome (Hyam and Panhurst 1995). The name 'banana' is derived from the Arabic banan = finger (Boning 2006). While many revisions of taxonomy of banana had taken place, a taxonomic advisory group for *Musa* was formed by the International Network for banana and plantain in 2006, four sections are compiled below (Table G4; Ploetz et al. 2007).

Table G4. Indicative listing of the possible species in *Musa*

Chromosome No.	Section	'Minor' section	Species
2n=2x=14	*Ingentimusa*		*M. ingens*
2n=2x=20	*Callimusa*	*Callimusa*	*M. angcorensis; M. bauensis* *M. bomeensis; M. campestris* *M. coccinea; M. exotica* *M. flavida; M. gracilis* *M. lawitiensis; M. paracoccinea* *M. pigmaea; M. salaccensis* *M. suratii; M. violascens*
		Australimusa	*M. alinsanaya; M. angustigemma* *M. beccarii; M. boman* *M. bukensis; M. fitzalanii* *M. hirta; M. insularimontana* *M. johnsii; M. lolodensis* *M. maclayi; M. monticola* *M. muluensis; M. peekelii* *M. textiles; M. tuberculata*
2n=2x=22	*Musa*	*Musa*	*M. acuminata; M. balbisiana* *M. basjoo; M. cheesmanii* *M. flaviflora; M. halabanensis* *M. itinerans; M. nagensium* *M. ochracea; M. schizocarpa* *M. sikkimensis; M. griersonii*
		Rhodochlamys	*M.aurantiaca; M. laterita* *M. mannii; M. ornate* *M. rosea; M. rubra* *M. sanguinea; M. thomsonii* *M. velutina*

Most of the cultivated sweet bananas and plantains belong to the section *Musa* and are triploid varieties that evolved from two wild diploid species, *M. acuminata*, given the genome designation 'AA' and *M. balbisiana*, given the genome designation 'BB'

(Simmonds and Shepherd 1955). The formation of heterogenomic triploid (2n=3x) hybrids with the 'AAA' genotype occurred within *M. acuminata* leading to the development of cultivars that comprises the sweet bananas (Daniells et al. 2001). Crosses of the diploid and triploid types of *M. acuminata* with *M. balbisiana* led to the formation of heterogenomic triploid hybrids that are mostly plantains (AAB) and other cooking bananas (ABB).

Table G5. Examples of genome nomenclature for some common cultivars (with emphasis on cultivars grown in India)

Genome group	Sub-group	Common cultivars
AA	Sucrier	Chingan, Matti, Kadali, Anai koomban
AB	Ney poovan	Ney poovan, Safed velchi, Soneri, Devabale, Puttabale, Elakki bale
AAA	Dwarf Cavendish	Basrai, Loton, Kabuli, Vamankeli, Pachavazhai, Pachabale, Kuzhivazhai, Mauritus, Bhusaval
	Giant Cavendish	Harichal, Bombay green, Pedda pacha arati, Bongali jahaji
	Red & Green red	Chenkadali, Lalkel, Venkadali, Sevvazhai, Anupan, Red banana
	Mysore	Poovan, Lal velchi, Champa, Karpura chakkarakeli
AAB	Silk	Rasathali, Mutheli, Morthoman, Marlaban, Malbhog, Sabari, Sonkel, Rasabale, Amrithapani, Nanjangudu rasabale
	French Plantain	Nendran, Rajeli, Ethakai, Myndoli
	Pome	Virupakshi, Sirumalai, Vannan, Malavazhai, Dacca martaban
ABB	Bluggoe	Monthan, Bankel, Khasadia, Kanchkala, Madhurangabale, Peyan
	Pisangawak	Kostha bontha, Pey kunnan, Manuva kola
AABB		CO-1 (A new hybrid)

Tetraploid (2n=4x) and other diploid combinations also exist (Pillay et al. 2004). Hybrids of *M. acuminata* and *M. balbisiana* can be referred to as *Musa paradisiaca*. *M. paradisiaca* was the name first given to the 'type banana' by Linnaeus.

G1.5. Banana diversity

Malaysia, Indonesia, the Philippines and New Guinea, were the primary centres and India is a secondary centre for banana diversity (Simmonds and Shepherd 1955). Edible diploids of *M. balbisiana* underwent a parallel natural evolution in drier parts of Asia and hybrids of the seeded types were produced. The end result of the parallel evolution and subsequent hybridization of the two species, *M. acuminata* and *M. balbisiana* resulted in the occurrence of the range of genotypes *i.e.,* homo-genomic and hetero-genomic diploids, triploids and tetraploids. The genomes of the two species contributed different traits, with *M. acuminata* largely contributing for fruit sweetness, parthenocarpy and sterility (Simmonds and Shepherd 1955) and *M. balbisiana* contributing hardiness, drought tolerance, disease resistance and fruit starchiness (Pillay et al. 2002).

Africa, both east and west represents the other main secondary centre for *Musa* diversity, with unique 60 highland cultivars of AAA genotypes (Karamura 1998). Another secondary centre for diversity is Polynesia, to where the AAB hybrids were carried from Philippines more than 4,000 years ago (De Langhe' 1995). The modern day bananas are a mix of wild and cultivated species, hybrids of *M. acuminata* and *M. balbisiana*. *M. acuminata* is the most widespread of the species in section *Musa* (Daniells et al. 2001) and the centre of origin is thought to be Malaysia (Simmonds 1962) or Indonesia (Horry et al. 1997). Clones of the diploids were cultivated in wetter parts of Southeast Asia (Valmayor et al. 2000) and the development of vigorous seedless triploid cultivars was the result of chromosome restitution (Raboin et al. 2005) and / or crosses between edible diploids and with *M. acuminata* (Daniells et al. 2001). A brief history of the domestication of banana is given by De Langhe (1995). It is claimed that there was written reference to bananas, Sanskrit as early as 500 BC. It is thought that traders from Arabia, Persia, India and Indonesia distributed banana suckers around coastal regions of the Indian Ocean between the 5^{th} and 15^{th} centuries. Today the cultivation of bananas occurs throughout the tropics and sub-tropics of Asia, America, Africa and Australia.

The most widely distributed banana cultivar is Dwarf Cavendish (Ploetz et al. 2007). It is derived by mutation from tall members of the Cavendish subgroup (Constantine and Rossel 2001). It is thought that the original plants were first obtained by Charles Telfair of China during 1826 and taken to Mauritius (Marin et al. 1998). Then it spread to Samoa (1838), Tonga and Fiji in 1840s and Australia, India during 1850s.

G1.6. Diversity analysis

Biotechnological applications must not only respond to the challenges of improving food security and fostering socio-economic development but also, in doing so, promote the conservation, diversification and sustainable use of plant genetic resources for food and agriculture. Nowadays the biotechnology toolbox available to plant breeders offers several new possibilities for increasing productivity, crop diversification and production, while developing a more sustainable agriculture. Molecular markers have already played a major role in the genetic characterization and improvement of many crop species. They have also contributed to and greatly expanded our abilities to assess biodiversity, reconstruct accurate phylogenetic relationships, and understand the structure, evolution and interaction of plant and microbial populations. Molecular markers are now routinely used for characterization of genetic diversity, DNA fingerprinting, genome mapping, genome evolution, ecology, taxonomy, and plant breeding.

DNA-based markers are abundant, highly polymorphic and independent of environment or tissue type. Most DNA-based markers can be classified into three categories based on the technique used: Hybridization-based DNA markers, arbitrarily primed Polymerase Chain Reaction (PCR)-based markers, and sequence targeted and single locus DNA markers. Restriction fragment length polymorphism (RFLP) markers are based on hybridization in which DNA polymorphism is detected by digesting DNA with restriction enzymes followed by DNA blotting and hybridizations with probes. Arbitrarily primed PCR-based markers are employed in organisms for which no genome sequence is available. Following table lists the most commonly used genetic markers.

Table G6. Some widely used markers and their applications

Markers	Applications
PCR Based markers	
AFLP: Amplified Fragment Length Polymorphism (D)	Fingerprinting, mapping, F1, Varietal identification, Gene tagging, Marker-assisted selection, Map-based gene cloning
CAPS: Cleaved Amplified Polymorphic Sequences (CD) **SCAR:** Sequence Characterized Amplified Region (CD)	Framework mapping, Can be converted to allele-specific probes, F1 identification, Gene tagging, Bulk segregant analysis, Diversity studies, Marker-assisted selection, Map-based cloning
EST: Expressed Sequence Tag (D/CD) **STS:** Sequence Tagged Site (D/CD)	Fingerprinting, Varietal identification, Genetic maps, F1 identification, Gene tagging and identification, Bulk segregant analysis, Diversity studies, Marker-assisted selection, Novel allele detection, High-resolution mapping, Map-based cloning
IPCR: Inverse Polymerase Chain Reaction (CD) **IRAP:** Inter-Retrotransposon Amplified Polymorphism (CD) **REMAP:** Retrotransposon-Microsatellite Amplified Polymorphism (CD)	Fingerprinting, Varietal identification F1 identification, Gene tagging Bulk segregant analysis, Diversity studies Marker-assisted selection, High-resolution mapping, Seed testing
ISSR: Inter-Simple Sequence Repeat amplification (D)	Fingerprinting, Varietal/line identification, Genetic maps, F1 identification, Gene tagging Breeding Bulk segregant analysis, Diversity studies Marker-assisted selection, High-resolution mapping, Seed testing
RAPD: Random Amplified Polymorphic DNA (D)	Genetic maps, F1 identification, Varietal/line identification, Breeding, Bulk segregant analysis, Diversity studies, Marker-assisted selection, Seed testing, Map-based gene cloning
SNP: Single Nucleotide Polymorphism (CD)	Genetic maps, F1 identification, Breeding, Gene tagging, Alien gene introduction, Bulk segregant analysis, Diversity studies, Novel allele detections, Marker-assisted selection, High resolution mapping
SSR: Simple Sequence Repeat (CD)	Fingerprinting, Varietal/line identification, Framework/region specific mapping, Genetic maps, F1 identification, Comparative mapping, Breeding,

	Bulk segregant analysis, Diversity studies Novel allele detections, Marker-assisted selection, High-resolution mapping, Seed testing, Map-based gene cloning
Non PCR-based markers	
RFLP: Restriction Fragment Length Polymorphism (CD)	Comparative maps, Framework maps, Genetic maps, Breeding, Varietal/line identification, Marker-assisted selection, F1 identification, Diversity studies, Novel allele detections, Gene tagging, Bulk segregant analysis, Map-based gene cloning

D: Dominant, CD: Co-Dominant

G1.6.1. Characteristics of ideal genetic markers

- Should not have detrimental effect on phenotype
- Co-dominant in expression
- Single copy
- Economic to use
- Highly polymorphic
- Easily assayed
- Multi-functional
- Easily available / synthesizable
- Genome-specific in nature (especially with reference to polyploids)
- Can be multiplexed
- Ability to automate

In the present study, we used RAPD (Williams et al. 1990) and ISSR markers to fingerprint the micropropagated banana plantlets as well as south Indian banana cultivars to determine genetic variation induced during tissue culture process.

G1.6.2. Diversity study in banana

The diversity of bananas spreads across southern states of India (Simmonds 1962; Singh and Uma 2000; Uma et al. 2008). The earliest attempt made to evaluate the clonal situation in south India resulted in a monograph published by Jacob in 1952 (cited by Uma et al. 2006). Since then, attempts have been made in the states of Tamil Nadu and Kerala to evaluate accessions, resolve synonymy, identify mutants and classify cultivars based on cytological and morpho-taxonomic characters. While the presence of genetic

diversity is most important in any crop improvement programme, prospection, collection and conservation of clones need attention to save the erosion of gene pool and ensure its utilization in breeding programme. Such an effort has resulted in the collection of a total of 525 accessions from all over India, including the major field gene-banks, and the clones have been pooled at the National Research Centre for Banana (NRCB), Tamil Nadu. While some clones have been evaluated partially for their reaction to Sigatoka, *Fusarium* wilt and nematodes, the others have been evaluated for better yield and fruit qualities. Because most of the cultivated banana varieties are the result of natural mutation and selection, it is necessary to locate promising clones through intensive evaluation. *In vitro* propagation of such selected clones holds a great promise for future genetic improvement programmes on banana.

Several methods have been used to investigate the genetic variability present in *Musa* germplasm. Morpho-taxonomic characters were first developed and optimized for bananas and 119 descriptors were defined to characterize *Musa* germplasm (Horry et al. 1997). Edible bananas are generally the hybrids between *Musa acuminata* (represented as 'A' genome) and *Musa balbisiana* (represented as 'B' genome). Thus the various commercial clones used in the present study (Table 2.1) are the hybrids between the two, some being sterile diploids (AB), or triploids (AAB, ABB) or auto-polyploids (AAA). The genome-type is decided on the basis of morphological descriptors as well as preliminary cytological studies. Therefore, genome with more of '*balbisiana'* and less '*acuminata'* characteristics are named as ABB and so on. In addition, genetic changes may be expected because many vegetative characteristics are influenced by the environmental variations and a high degree of plasticity of the genome. The development and application of technologies based upon molecular markers provide the powerful tools that are adequate enough to reveal polymorphism at the DNA sequence level, and are often robust to detect genetic variability within the populations and between individuals (Kresovich et al. 1995; Simmons et al. 2007). Therefore, these markers have also been routinely applied for genetic analysis for formulating taxonomic, phylogenic and heterotic groupings, which may be of considerable value for defining core collections and developing efficient breeding schemes. DNA analysis through markers has been found extremely useful for predicting the breeding value of parental genotypes (Crouch et al.

1997; 1998a; Creste et al. 2004). One of the most important marker-based characteristics identified is that for parthenocarpy (Crouch et al. 1998b), which has tremendously benefited the breeders. Genetic diversity in plants can be estimated in several ways. The RAPD (DNA, Williams et al. 1990) and ISSR technique have been used extensively for this because of the ease and rapidity with which data can be generated in a less laborious manner than other methods such as RFLP and AFLP.

G1.7. Micropropagation

The inherent vegetative multiplication has made banana an ideal material for micro-propagation research. Therefore, propagation of banana through *in vitro* techniques has been reported by several researchers using different explants such as shoot buds, meristems and floral buds (Novak et al. 1989; Bhagyalakshmi and Singh 1995). Micropropagation has played a key role in plantain and banana improvement programs worldwide (Rowe and Rosales 1996; Smith and Drew 1990; Vuylsteke et al. 1997; Vuylsteke 1998). Apart from its potential to provide genetically uniform, pest- and disease-free planting materials, micropropagation-derived plants establish faster, grow more vigorously, are taller, have a shorter and more uniform production cycle, and yield higher than conventional propagules (Drew and Smith 1990; Robinson et al. 1993; Vuylsteke and Ortiz 1996). Maximum yield gains from *in vitro* derived plants range from 20% in bananas to 70% in plantains. However, this superior field performance does not appear to be consistent and requires optimal crop husbandry (Vuylsteke 1998).

Different micropropagation protocols have been reported for various banana varieties by several researchers (Cronauer and Krikorian 1983; 1988; Banerjee et al. 1986; Escalant and Teisson 1989; Bhagyalakshmi and Singh 1995; Dhed'a et al. 1991; Navarro et al. 1997). Though a very few exogenous growth regulators have been reported useful for the micropropagation of banana (Escalant and Teisson 1989; Bhagyalakshmi and Singh 1995; Dhed'a et al. 1991; Albany et al. 2005), the sub- and supra-optimal levels of growth substances, especially the synthetic ones, were found to cause somaclonal variation (Peres et al. 1999; Smith 1988; Martins et al. 2004; Martin et al. 2006). Even in cases where immediate variations were not noticed, the long-term usage of high levels of cytokinins resulted in somaclonal or epigenetic variations in some of the mericlones (Israeli et al. 1996; Damasco et al. 1996) questioning the very fidelity

of their clonal nature. That apart, banana shoots *in vitro* are known to synthesize and accumulate a natural cytokinin, 2-ip (2-isopentenyl adenine), at the basal portions of shoot clusters resulting in *de-novo* bud formation, especially under the exogenous supply of Benzylaminopurine (BAP) (Zaffari et al. 1998; Zaffari et al. 2000; Venkatachalam et al. 2006).

G1.8. Regeneration studies

In banana, two pathways of plantlet regeneration have been observed, the most common being the somatic embryogenesis and rarely observed is the direct organogenesis. Reports on the formation of non-organogenic callus (Escalant and Tiesson 1987) and root forming callus (Escalant et al. 1994; Shii et al. 1992) were the initial success stories on developing protocols for regeneration. Subsequently, somatic embryogenesis was achieved using floral explants (Panis and Swennen 1993; Navarro et al. 1997), as were cell suspensions of diploid and triploid cultivars with genomes AA, AAA and ABB (Novak et al. 1989) and AAB (Ganapathi et al. 2001). In plantains (ABB), regeneration of somatic embryos from the callus, which sloughed off from shoot cultures in liquid medium, was observed (Cronauer and Krikorian 1988). However, further culture of such somatic embryos resulted in the growth of roots only. Similarly, in 'Cavendish' (AAA), spherical masses that developing from leaf base explants which also resulted in root formation (Novak et al. 1989). Regeneration via somatic embryogenesis was reported in diploid and triploid *M. acuminata* (AA and AAA) using reproductive explants (Jarret et al. 1985; Cote et al. 1996; 2000; Navarro et al. 1997 and Becker et al. 2000; Chung et al. 2006), haploids from anther culture (Assani et al. 2003) and rhizome tissue (Bakry and Rossingnol 1985; Novak et al. 1989). Organogenesis has been reported from callus cells (Dhed'a et al. 1991), protoplasts (Panis and Swennen 1993; Assani et al. 2006) and leaf-base micro-sections (Okole and Schulz 1996) for certain *Musa* genotypes. Pro-embryogenic callus around the periphery of cultured corm tissue of *M. acuminata* were observed that had the potential to regenerate into plantlets (May et al. 1995). Shii et al (1992) reported the production of embryogenic cell suspensions of several Cavendish cultivars, using immature male flowers as the explant.

G1.9. Genetic transformation

There have been a number of reports on the genetic transformation of banana (Sagi et al. 1995; May et al. 1995). However, there is only one report on the production of transformed Cavendish plants. May et al (1995) produced transgenic Cavendish cv 'Grand Nain' by co-cultivating wounded meristems with *Agrobacterium tumefaciens*. Although this technique is appealing because of its relatively short regeneration time, it may be of limited value because of the potential generation of chimeric plants (May et al. 1995). Transformation of totipotent single cells would overcome this limitation. Shii et al. (1992) reported the production of embryogenic cell suspensions of several Cavendish cultivars using immature male flowers as the explant. Escalant et al (1994) and Cote et al. (1996) further refined this system and applied it to *cv* 'Grand Nain'. The refined system had a very high regeneration capacity, and it was considered that somatic embryos were most likely unicellular in origin. Such tissue is potentially an ideal target for genetic transformation.

G1.10. Ripening of banana fruit

The ripening of fleshy fruits is characterized by a series of biochemical, physiological and structural changes which make the fruit attractive to the consumer. Although these processes vary from one type of fruit to the next, fruits can be divided into two broad groups, namely climacteric and non-climacteric. Climacteric fruits, such as banana, are characterized by a surge in ethylene production at the onset of ripening and a peak in respiration. Their ripening is accelerated and their shelf life reduced by ethylene (Jiang et al. 1999). An increase in the respiration rate during the climacteric peak is accompanied by an increase in the rate of ripening and *vice versa*. In general, fruit with a higher rate of respiration ripens faster and has a shorter shelf life than the one with a lower respiration rate.

Banana ripening is a synchronized event, which is characterized by a series of biochemical, physiological and structural changes. It is characterized by a surge in ethylene biosynthesis (Burg and Burg 1965; Dominguez and Vendrell 1993) and a climacteric rise in the respiration (Salisbury and Ross 1985; Kotecha and Desai 1998). Several other physiological changes also occur concomitantly during ripening, inducing pulp softening (Marriot 1980; Civello et al. 1999; Zhou et al. 2001), sugar production

(Mendoza 1968; Marriot 1980), increased moisture loss (Marin et al. 1998; Kotecha and Desai 1998), and colour change from green to yellow (Salisbury and Ross 1985; Peacock 1966; Marriot 1980). The climacteric pattern gives an indication of the physiological status of the fruit. The basic respiration pattern of the fruit can be used to predict the shelf life and general physiological status of the product. Any deviation from this standard pattern is a clear indication that the fruit experienced some kind of stress

G1.10.1. Change in firmness of the peel and pulp during ripening

Tissue softening accompanies the ripening of many fruit, and initiates the processes of irreversible deterioration (Marriott 1980). It is suggested that ethylene is essential for promoting the proper sequence of cell wall hydrolysis required for normal fruit softening (Zhou et al. 2001). The storage life of plant produce is normally synonymous with the pre-climacteric period after harvest, since once the climacteric stage commences; the fruit softens rapidly and becomes much more susceptible to mechanical damage and fungal infection, causing increased wastage (Marriott 1980). This marked decrease in the firmness of the pulp can be ascribed to the breakdown of starch, cellulose and hemicellulose (Kotecha and Desai 1998). Civello et al (1999) investigated the role of expansins (plant cell wall proteins) in the disruption of hydrogen bonds within the cell wall polymer matrix. They found that expansins are a common component of ripening and that non-climacteric signals other than auxin, may coordinate the onset of ripening and the softening of the fruit. In view of Lyons's (1973) statements concerning the effect of chilling injury on the firmness of the pulp and peel, it stands to reason that a decrease in cell wall integrity would be enhanced by stress conditions.

G1.10.2. Change in ethylene production during ripening

Ethylene has been recognized as a gaseous plant hormone since 1901 (Sisler and Serek 1999) and the production of ethylene by bananas and its significance to the ripening process have been the subject of great interest to many (McCarthy and Palmer 1962; Mapson and Robinson 1966).

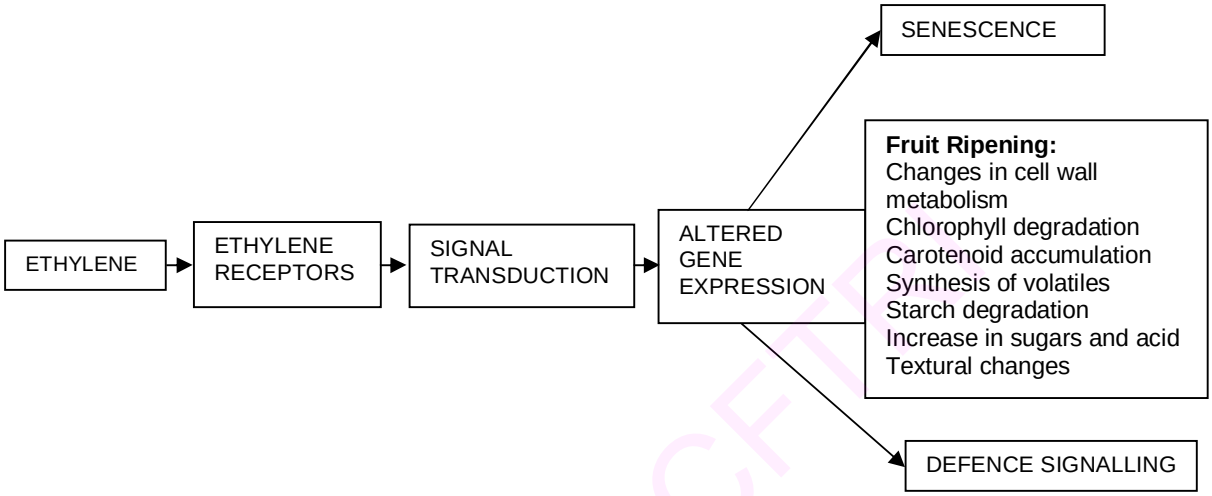

Figure G2. Schematic representation of the role that ethylene plays during banana fruit ripening

As little as one µl l^{-1} of ethylene in the air will hasten the onset of the climacteric rise in respiration (Mendoza 1968). The effect of ethylene in accelerating ripening, however, has only been shown in pre-climacteric fruits. The onset of natural ripening in bananas is indicated by a sharp increase in ethylene production followed within a few hours by the climacteric rise in respiration (Vendrell and McGlasson 1970). The identification of the genes encoding the key ethylene biosynthetic enzymes has allowed the generation of transgenic fruit with reduced ethylene production (Lelièvre et al. 1997). Climacteric fruit show a sharp increase in climacteric ethylene production at the onset of ripening that is considered to contribute to controlling the initiation of changes in colour, aroma, texture, flavour and other biochemical and physiological attributes. This accelerated ethylene production rate may be required to raise the internal content of ethylene to a stimulatory level, or it may be an autocatalytic response initiated when tissue becomes sensitive to the low level of ethylene present throughout the early pre-climacteric period (Burg and Burg 1965; Dominguez and Vendrell 1993).

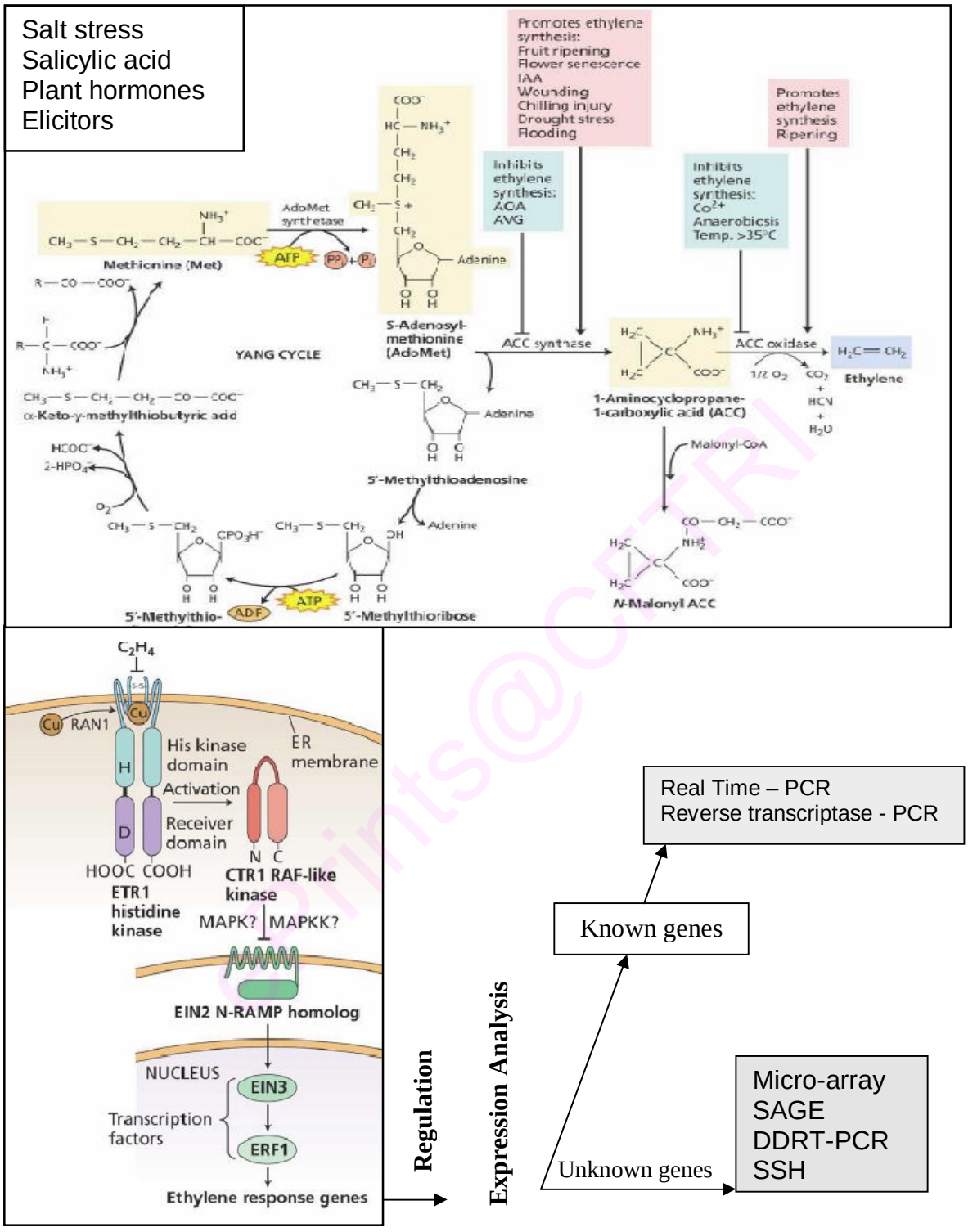

Figure G3. A diagrammatic representation of ethylene response pathway for the regulation of ripening genes expressions. Modified and redrawn from Guo and Ecker (2004)

Treatment with ethylene does not affect those fruits already in the climacteric phase due to endogenous production of the ripening gas. In climacteric fruit, the transition to autocatalytic ethylene production appears to result from a series of events where developmentally regulated ACC (1-aminocyclopropane-1-carboxylic acid) oxidase (ACO) and ACC synthase (ACS) gene expression initiates a rise in ethylene production, setting in motion the activation of autocatalytic ethylene production. Besides the differences in their rates and patterns of respiration, climacteric and non-climacteric fruit also differ in their internal ethylene concentration during the growth and ripening phases.

Environmental factors to which fruit are exposed during transportation, storage and postharvest ripening treatments have the potential to influence the level of ethylene biosynthesis (Lelièvre et al. 1997). Temperatures outside a fruit's acclimatized range can also accelerate ethylene synthesis and induce premature ripening in temperate fruit such as pears. Chilling treatment also modifies the sensitivity to ethylene. For example, bananas stored at lower temperature (13-15°C) expressed lower ethylene resulting in delayed ripening. It has also been found that the rate of ethylene production of bananas under water stress increase by a factor of two (Marriott 1980). This is significant since bananas experience an increase in moisture loss during temperatures stress (if the relative humidity is not preserved). This increase in moisture loss can then stimulate ethylene production, which in turn can aggravate the symptoms of temperature stress.

G1.10.3. Regulation of ripening by controlling the ethylene metabolism

Many effective synthetic chemical agents are available for blocking/enhancing the ethylene receptors in the membranes. This gaseous compound blocks the action of exogenous and endogenous ethylene (Feng et al. 2000), and thus provides new ways of controlling ethylene synthesis, binding, ripening, senescence and other ethylene related responses.

G1.11. Identification of genes differentially expressed during ripening of banana

The major biological process of living organisms is driven by changes in the levels and patterns of gene expression. Thus the co-ordinated regulation of gene expression is fundamental to the control of normal physiological phenomena within a cell, specifying cellular characteristic and responses to intracellular, extracellular and

external cues. In order to reveal active processes at the molecular level and to dissect key components of molecular pathways, differential gene expression studies provides a foundation for the elucidation of dynamic molecular mechanisms. Furthermore, the study of differential gene expression is particularly useful when an experimental hypothesis has not been established, as many occur in an uncharacterized biological model.

Various approaches to investigate differential gene expression have been utilized including differential hybridization (Sambrook et al. 1989), subtractive hybridization (Lee et al. 1991; Byers et al. 2000), and differential display (Liang and Pardee 1992), predecessors to the more advanced technologies of SAGE (Serial Analysis of Gene Expression), TOGA (Total Gene Expression Analysis), and GeneCalling, collectively referred to as 'open' architecture systems, that do not need any pre-existing biological or sequence information (Green et al. 2001). This is in contrast to the so called 'closed' systems such as quantitative PCR and oligonucleotide or cDNA miroarray technologies (Schena et al. 1995) that requires knowledge about a given genome (Green et al. 2001)

G1.11.1. Differential Display

Differential display (DD) (Liang and Pardee 1992) and RNA arbitrary primed polymerase chain reaction (RAP-PCR) (McClelland et al. 1993) were simultaneously introduced in 1992 as a means to identify and compare genes expressed as mRNA in various cell populations under specified conditions (Liang and Pardee 1995). The DDRT-PCR technique only requires small amounts of RNA and it allows the comparison of several RNA populations simultaneously (Bosch and Lohmann 1997; Jorgensen et al. 1997). Nonetheless differential display has advantages over other methods which include: the use of small amounts of total RNA, the identification of mRNA species independent of prevalence (Wan et al. 1996), and the detection of rare and abundant transcripts of both known and novel genes (Matz and Lukyanov 1998). Though effective, the technique does have some drawbacks. The major limitation is the high incidence of false positive. However the single greatest contributor to false positives is arbitrarily primed PCR using short oligos (Matz and Lukyanov 1998), which can be solved with the use of longer arbitrary primers (Zhao et al. 1995). Classical differential display employs the use of a degenerate single base or two-base- anchored oligo-dT primer [$d(T)_{11}$ NN

Differential Display RT- PCR

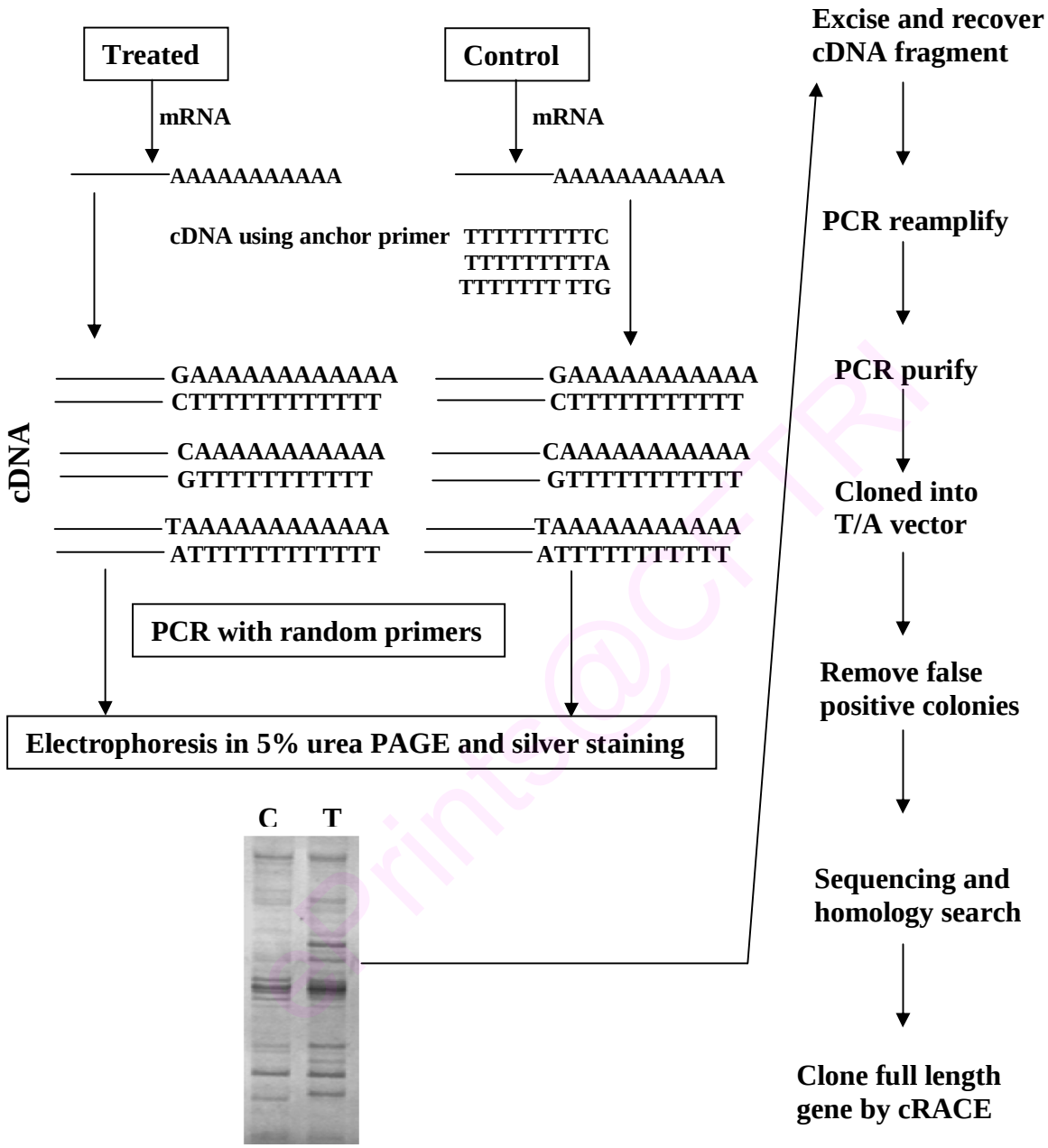

Figure G4. Schematic diagram showing the general method used for mRNA differential display. Transcripts produced in both control and treated are displayed simultaneously as PCR products when identical primer combinations are used. The presence of a PCR product from only one sample strongly suggests differential transcriptional activity of the corresponding gene

where N= G, A, T, C] to reverse transcribe a subset of the total mRNA population extracted from cell lines or tissues 'under various conditions (Liang and Pardee 1992). Amplification of the cDNA by PCR using the 3' anchored oligo dT primer and a 5' arbitrary decamer which anneals randomly along the length of the cDNA results in the amplification of a subset of the total cDNA population, which is resolved by denaturing polyacrylamide get electrophoresis (PAGE). In order to increase the cDNA amplified, it may be necessary to perform multiple amplifications with different primer combinations, although redundancy in cDNA amplification is likely as degenerative decamer may anneal upstream of a cDNA already represented in an amplified subpopulation of another primer set (Matz and Lukyanov 1998). Given that twenty different arbitrary decamers (priming as 6- to 7- mers) combined with twelve different combinations of oligo $d(T)_{11}$ MN primers are estimated to be required to display the approximately fifteen thousand individual mRNA species expressed in an average eukaryotic cell (Liang and Pardee 1992; 1995), which likely represents an underestimate if system redundancy is taken into account, many amplifications and gels would need to be run to display the whole set, a disadvantage of differential display.

Differentially expressed genes are represented by bands that are present on the gel in one sample but not in another, or at different intensities indicating varying levels of abundance between samples (Matz and Lukyanov 1998). Thus specific advantages of differential display include the simultaneous detection of both up- regulated and down-regulated genes and the comparison of more than two RNA populations; in fact limitations to the number of comparisons that can be done are only imposed by the size of the gel.

Approaches like subtractive hybridization and differential library screening have been employed in climacteric fruit like kiwifruit (Ledger and Gardner 1994), banana (Clendennen and May 1997; Medina-Suarez et al. 1997) and melons (Hadfield et al. 2000) and in non-climacteric fruit like strawberry (Nam et al. 1999) and grapes (Davies and Robinson 2000), while mRNA differential display has been used to study ripening in tomato (Zegzouti et al. 1999) and raspberry (Jones et al. 2000). Recently, transcriptome analyses in apricot (Grimplet et al. 2005) and tomato (Alba et al. 2005) have also been

performed in an effort to get an insight into the vast array of genes that may be involved in ripening.

G1.11.2. Advantages of Differential Display

1. A minimal amount of starting material is required. (Differential Display can be performed with as little as 200 μg of total RNA per sample)
2. Multiple samples can be analyzed simultaneously. (Alternative methods are limited to comparing two samples, whereas Differential Display is limited only by the number of lanes available on the gel apparatus)
3. Sensitivity (As Differential Display is a PCR- based procedure, transcripts present at very low copy numbers are included in the analysis)
4. Speed

Table G7. Identification of ripening related genes; A few earlier studies have tried the identification of ripening–related genes in banana and other fruits

Source	Conditions	Methodology	Authors
Banana	Differential expression of genes during banana fruit development, ripening and 1-MCP treatment	DDRTPCR	Gupta et al. 2006
Banana	Identification of genes differentially expressed during ripening of banana	SSH	Trujillo et al. 2007
Banana	Low temperature induce differential expression genes in banana fruits	DDRTPCR	Velázquez et al. 2007
Banana	Differentially expressed cDNAs at the early stage of banana ripening	Micro array/SSH	Xu et al. 2007
Banana	Identify genes differentially expressed during early banana fruit development undergoing changes in ethylene responsiveness	SSH	Mbéguie'-A-Mbéguie' et al. 2007
Banana	Ethylene-induced ripening in banana evokes expression of defense and stress related genes in fruit tissue	SSH	Kesari et al. 2007
Pear	Monitoring gene expression along pear fruit development, ripening and senescence	cDNA microarrays	Fonseca et al. 2004
Melon	Characterization of ripening-regulated cDNAs and their expression in ethylene-suppressed charentais melon fruit	DDRTPCR	Hadfield et al. 2000

Apple	Molecular identification of novel differentially expressed mRNAs up-regulated during ripening of apples	DDRTPCR	Goulao and Oliveira 2007
Strawberry	Cloning and characterization of cDNAs from genes differentially expressed during ripening	SSH	Medina-Escobar et al. 1998
Wild strawberry	Isolation and characterization of mRNAs differentially expressed during ripening	SSH	Nam et al. 1999
Grape berry	Cloning and characterization of cDNAs encoding putative cell wall and stress response proteins	SSH	Davies and Robinson 2000
Strawberry	Identification of mRNAs with enhanced expression in ripening	DDRTPCR	Wilkinson et al. 1995
Strawberry	Isolation of a set of ripening-related genes from: their identification and possible relationship to fruit quality traits	DDRTPCR	Manning 1998
Tomato	Ethylene-regulated gene expression in fruit: characterization of novel ethylene-responsive and ripening-related genes	DDRTPCR	Zegzouti et al. 1999
Raspberry	Profiling of changes in gene expression during fruit ripening	DDRTPCR	Jones et al. 2000
Grapes	Molecular analysis of fruit ripening: the identification of differentially expressed sequences	cDNA-AFLP	Venter et al. 2001

With this background knowledge, the objectives of the present research work were laid as follows

- **To develop the biochemical and genetic markers to differentiate NR**
- **To develop the regeneration and genetic transformation protocol for banana**
- **To identify the genes expressed to the maximum during ripening**

The subsequent chapters deals with the molecular fingerprinting of NR, regeneration, micro-propagation and transformation protocol for NR and differential expression of ripening related cDNAs by RT-PCR. Finally the cloning of ripening specific-gene from ripening banana fruit using PCR based mRNA Differential Display approach and preliminary characterization of the genes by nucleic acid sequencing and analyses. The information thus generated could be of great use in regulating the ripening related genes by chemical agents for improving the shelf life of banana.

CHAPTER - I

Molecular Fingerprinting of NR

SUMMARY

The cultivar under consideration for the present study is "Nanjanagudu rasabale", which is local variety and hence its genetic relationship is unknown. Therefore, its genetic relationship and variations were established using 21 commercially important banana cultivars of South India and 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. A strong linear relationship was observed between the Resolving power (Rp) of primers and their ability to distinguish genotypes. Based on these data, a genetic similarity matrix was established and a dendrogram for each set of primers was developed by UPGMA. The Genetic similarity coefficients in RAPD analysis ranged from 0.3177 to 0.7818 and in ISSR analysis from 0.1800 to 0.8462. A fingerprinting key was generated where the presence / absence of specific RAPD/ISSR bands were recorded for each cultivar. The presence of a specific RAPD (OPC 5_{800}) band was observed for an endemic cultivar - Nanjanagudu Rasabale (NR). The study resulted in the identification and molecular classification of south Indian banana cultivars of which Robusta and Williams are global and others have either limited geographical distribution or purely endemic to South India. A group of 8 cultivars was identified having highly diverse relationship from one another. The members of this group may be useful for generating 2X and 4X breeding populations for further use in breeding secondary triploid hybrids.

1.1. INTRODUCTION

India is recognized as one of the major centres of origin and diversity of *Musa* at a global level, along with South East Asian countries. The diversity of bananas spreads across southern states of India (Simmonds 1962; Singh and Uma 2000). The earliest attempt made to evaluate the clonal situation in south India resulted in a monograph published by Jacob in 1952 (cited by Uma et al. 2006). Since then, attempts have been made in the states of Tamil Nadu and Kerala to evaluate accessions, resolve synonymy, identify mutants and classify cultivars based on cytological and morpho-taxonomic characters. While the presence of genetic diversity is most important in any crop improvement programme, prospection, collection and conservation of clones need attention to save the erosion of gene pool and ensure their utilization in breeding programmes. Such an effort has resulted in the collection of a total of 525 accessions from all over India, including the major field gene-banks, and the clones have been pooled at the National Research Centre for Banana (NRCB), Tamil Nadu. While some clones have been evaluated partially for their reaction to Sigatoka, *Fusarium* wilt and nematodes, the others have been evaluated for better yield and fruit qualities. Because most of the cultivated banana varieties are the result of natural mutation and selection, it is necessary to locate promising clones through intensive evaluation. *In vitro* propagation of such selected clones holds a great promise for future genetic improvement programmes on banana.

Several methods have been used to investigate the genetic variability present in *Musa* germplasm. Morpho-taxonomic characters were first developed and optimized for bananas and 119 descriptors were defined to characterize *Musa* germplasm (Horry et al.1997). Edible bananas are generally the hybrids between *Musa acuminata* (represented as 'A' genome) and *Musa balbisiana* (represented as 'B' genome). Thus the various commercial clones used in the present study (Table 1.1) are the hybrids between the two, some being sterile diploids (AB), or triploids (AAB, ABB) or auto-polyploids (AAA). The genome-type is decided on the basis of morphological descriptors as well as preliminary cytological studies. Therefore, more of "*balbisiana*" and lesser "*acuminata*" characteristics are named as ABB and so on. The hybrids are scientifically represented as *Musa sapientum.* In addition, genetic changes may be expected because many vegetative

characteristics are influenced by the environmental variations and a high degree of plasticity of the genome. The development and application of technologies based upon molecular markers provide the powerful tools that are adequate enough to reveal polymorphism at the DNA sequence level, and are often robust to detect genetic variability within the populations and between individuals (Kresovich et al. 1995; Simmons et al. 2007). Therefore, these markers have also been routinely applied for genetic analysis for formulating taxonomic, phylogenic and heterotic groupings, which may be of considerable value for defining core collections and developing efficient breeding schemes. DNA analysis through markers has been found extremely useful for predicting the breeding value of parental genotypes (Crouch et al. 1997; 1998a; Creste et al. 2004). One of the most important marker-based characteristics identified is that for parthenocarpy (Crouch et al. 1998b), which has tremendously benefited the breeders.

Genetic diversity in plants can be estimated in several ways, of which the use of genetic markers such as RAPD, RFLP, SSH, STR and ISSR are very popular. Among these, the RAPD (Random Amplified Polymorphic DNA, Williams et al. 1990) technique has been used extensively for this because of the ease and rapidity with which data can be generated in a less laborious manner than other methods such as RFLP (Restriction Fragment Length Polymorphism) and AFLP (Amplified Fragment Length Polymorphism).

1.1.1. RAPD Technique

This technique uses any DNA segment that is amplified using short oligo-deoxy-nucleotide primers of arbitrary nucleotide sequence (amplifiers) and polymerase chain reaction procedures (PCR). Random amplified polymorphic DNAs (RAPDs) are produced by PCR using genomic DNA and arbitrary primers. PCR is typically carried out using two random oligonucleotide primers that flank the DNA fragment to be amplified. These primers hybridise to complementary strands of the target sequence and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers. The result is an exponential accumulation of the specific target fragment by the de *novo* synthesis of the region of DNA flanked by the two primers (Figure 1.1).

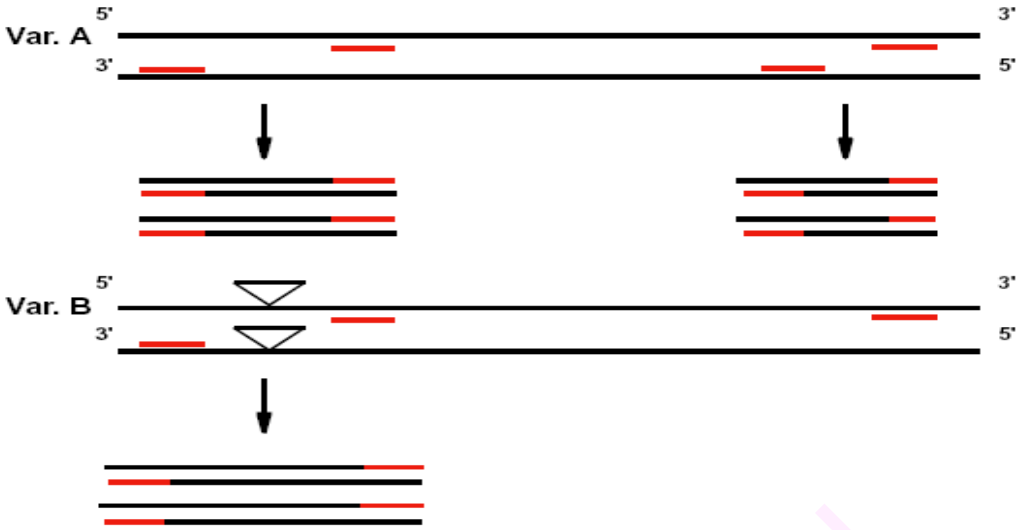

Figure 1.1. Schematic diagram showing the general principle of RAPD

1.1.2. ISSR Technique

A variant of the PCR that uses simple sequence repeat primers (e.g. $[AC]_n$) to amplify regions between their target sequences (Figure 1.2). The technique exploits the abundant and random distribution of SSRs in plant genomes by amplifying DNA sequences between closely linked SSRs. More complex banding patterns can be achieved using 5'-anchored primers that incorporate the SSR regions in their amplification.

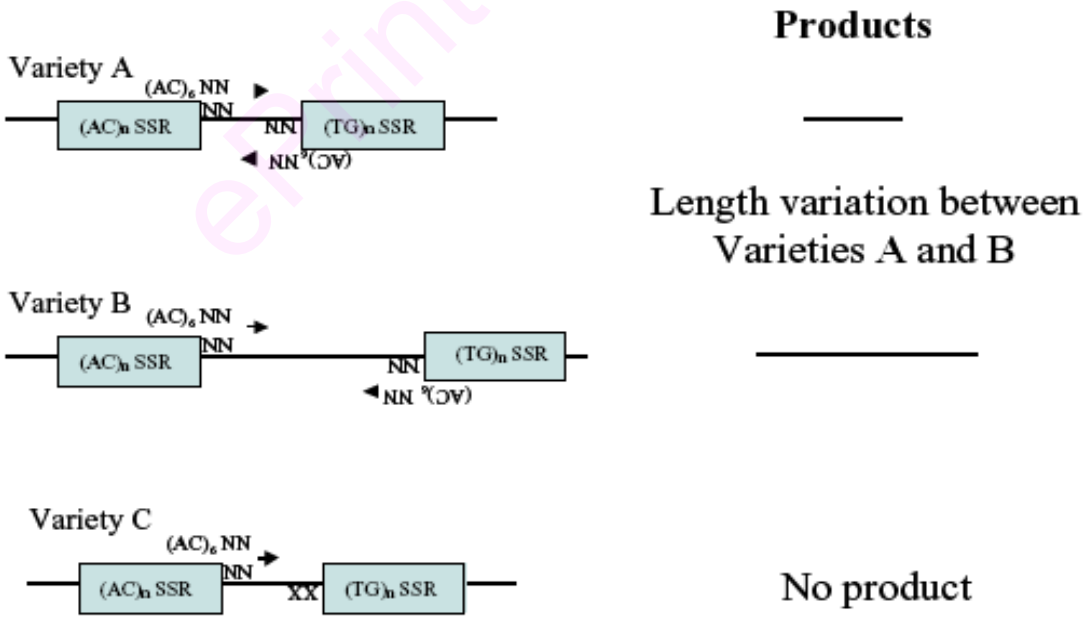

Figure 1.2. Schematic diagram showing the general principle of ISSR

1.1.3. RAPD and ISSR analyses in banana

RAPD technique has been used to assess genetic relationships in many plants and banana itself for distinguishing *Musa* germplasm (Howell et al. 1994), African bananas (Ude et al. 2003; Pillay et al. 2001; Onguso et al. 2004), landraces of AAB group of *Musa* (Crouch et al. 2000), and in BB *Musa* (Uma et al. 2006). Several reasons have been given to justify the use of these dominant markers for phylogenetic analysis rather than sampling sequences or morphological characters. The dominant markers, RAPD and ISSR, sample multiple loci from across the different genomes, in contrast to single gene analysis (Albertson et al. 1999; El-Rabey et al. 2002). Therefore, these PCR-based analytical tools are robust to address problems of introgression, lineage and unrecognized paralogy (Simmons and Webb 2006). Therefore, the successful use of dominant markers to resolve phylogenetic relationships has lead to wide-spread application of ISSRs (Joshi et al. 2000; Wolfe and Randle 2001) and RAPDs (Bowditch et al. 1993; Spooner et al. 1996).

A dominant marker is also expected to result in high band informativeness helpful in distinguishing among the genotypes as well as between primers. This functional property, commonly known as Resolving power (Rp) of a primer, has been found to correlate strongly with its ability to distinguish between genotypes. This function is well suited for comparing primers, primer-enzyme combinations or probe-enzyme combinations generated by RAPD, ISSR, AFLP and RFLP analyses. Essentially, Rp provides quantitative data allowing comparisons between primers (or probe-enzymes etc.), including those that are able to distinguish all genotypes examined in a study. Rp can also be used to predict the performance of group of primers (Prevost and Wilkinson 1999).

The information on genetic relationships is essential and decisive for developing an efficient breeding programme. Advances in *Musa* breeding have established that crossing of divergent genotypes and subsequent selection of improved hybrids are the essential steps for the production of new banana cultivars (Ortiz and Vuylsteke 1996). However, the information on genetic diversity and phylogenetic relationships within the south Indian banana cultivars is scarce. The present study was aimed at using RAPD and ISSR markers to assess the levels of genetic diversity in popular cultivars of South India

and to construct a dendrogram demonstrating the genetic relationships amongst them. Since the present evaluation involves commercially important diploid, triploid and tetraploid cultivars, the information generated is expected to be of great help in finding genetic relationships and planning breeding programmes.

1.2. MATERIALS AND METHODS

1.2.1. Plant material

The banana suckers of the most popular cultivars of southern part of the India were collected from germplasm maintained at Tamil Nadu Agricultural University, Tamil Nadu, India. The suckers were planted and maintained at Plant Cell Biotechnology Department of Central Food Technological Research Institute, Mysore, India for conservation and research purposes. Twenty one cultivars were used for the present study (Table 1.1). The young unfurled leaves were collected from five different plants of each cultivar and used for DNA extraction.

Table 1.1. List of cultivars, type of utilization, ploidy and genome composition of banana plants used in the present genetic diversity study

Sl.No.	Cultivars	Type	Ploidy	Genome composition
1.	Monthan	Cooking	3 X	ABB
2.	Elakki bale	Dessert	2 X	AB
3.	NR	Dessert	3 X	AAB
4.	Ney poovan	Dessert	2 X	AB
5.	CO1	Both	4 X	AABB
6.	Nendran	Both	3 X	AAB
7.	Rasthali	Dessert	3 X	AAB
8.	Red banana	Dessert	3 X	AAA
9.	Poovan	Dessert	3 X	AAB
10.	Peyan	Cooking	3 X	ABB
11.	Karpooravalli	Dessert	3 X	ABB
12.	Virupakshi	Dessert	3 X	AAB
13.	Kunnan	Cooking	3 X	ABB
14.	Kadali	Dessert	2 X	AA
15.	Robusta	Dessert	3 X	AAA
16.	Williams	Dessert	3 X	AAA
17.	Motta poovan	Both	3 X	AAB
18.	Matti	Dessert	2 X	AA
19.	Laden	Both	3 X	AAB
20.	Anai koomban	Both	2 X	AA
21	Sirumalai	Dessert	3 X	AAB

1.2.2. Genomic DNA extraction

Each cultivar was reduced to a pool of 5 plants, and 100 mg of young leaves from each plant were used to create the pool. These leaves were frozen in liquid nitrogen and

stored at -80 °C. DNA was extracted according to the following protocol. Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to a concentration of 25 ng μl^{-1} before use.

Reagents Required

CTAB – extraction buffer (500 ml)

Tris-HCl (pH 8.0)	:	100.00 mM
Sodium chloride	:	1.40 M
EDTA (pH 8.0)	:	20.00 mM
CTAB	:	2.0 % w/v
2-mercaptoethanol	:	0.1% v/v

(The extraction buffer must be autoclaved without 2-mercaptoethanol. 2-mercaptoethanol should be added only prior to use)

RNase solution	:	10 mg/ml
Chloroform:isoamylacohol	:	24:1

TE buffer

Tris HCl (pH 8.0)	:	10.00mM
EDTA (pH 8.0)	:	1.00 mM
Sodium chloride	:	3.00 M
Sodium acetate	:	3.00 M

1.2.2.1. Protocol: Approximately, 2 g of leaf tissue/cells/organs was ground in liquid nitrogen using a mortar and pestle and transfer the ground material to a 50 ml centrifuge tube. To the ground material 15 ml CTAB extraction buffer pre-heated to 60°C was added and suspended thoroughly. Incubate the suspension at 65°C for 30 minutes with occasional mixing, the mixture were cooled to room temperature. Then, equal volume of chloroform:isoamyl alcohol mixture added and mix well to get an emulsion by inverting the tubes several times for 15 minutes. The mixture was centrifuged at 12000 rpm for 5 minutes and the aqueous layer was collected. The chromosomal DNA was precipitated by adding 0.7 volume of isopropanol and kept at a room temperature for 15 minutes. The DNA was transferred to a clean tube and air dried for 15 minutes. The DNA was dissolved in minimal volume of TE buffer (up to 5 ml) and incubated at 65°C for 10 minutes will accelerates the dissolution of DNA and inactivate residual DNase I. 1/100

volume of 10 mg/ml RNase A solution was added. The mixture was thoroughly mixed by inversion and incubated at 37°C for 30 minutes. To this an equal volume of chloroform isoamyl alcohol was added and centrifuged at 12000 rpm for 10 minutes. The aqueous layer was collected and to which (2 volumes of absolute alcohol and 1/10 the volume 3 M sodium acetate was added and spun for 5 minutes at 10000 rpm. The supernatant was discarded and the precipitate was washed with 70% ethanol. Then the pellet was air dried and dissolved in 100 µl of TE buffer.

1.2.3. Selection of primers and band profile reproducibility

Initially, 75 RAPD and 25 ISSR primers were screened against genomic DNA of two banana cultivars for their ability to amplify DNA fragments. Three replicate DNA extractions (pool of five plants) from leaves of two different banana cultivars, Nanjanagudu Rasabale (NR) and Robusta, were used to assess the consistency of the band profiles. Of all the primers, 50 RAPD and 12 ISSR primers produced robust amplification patterns and no band was detected in any negative control amplification. The reproducibility of the RAPD and ISSR amplifications were assessed using selected primers (OPA 14, OPC 05, OPC 09, OPM 20, UBC 811, UBC 826 and UBC 890) with different DNA samples isolated independently from same cultivars (NR and Robusta) and amplified at different times.

1.2.4. RAPD and ISSR analyses

The RAPD primers were decamer oligonucleotides (Operon Technologies, Alameda, CA, USA) and for ISSR analyses, twelve primers based on di-nucleotide or tri-nucleotide repeats were utilized (Tables 1.2 and 1.3) following the previous criterion (Venkatachalam et al. 2007a; 2007b; 2007c). These oligonucleotides were obtained from UBC primer set 100/9 (University of British Columbia)

1.2.5. PCR amplification

The protocol for RAPD analysis was adapted from Williams et al. (1990) and for ISSRs, the protocol of Zietkiewicz et al. (1994) was adapted. Initially, optimum PCR conditions for both RAPD and ISSR were standardized with various quantities of template DNA (12.5, 25 and 50 ng), dNTPs (100, 200 and 300 µM) and $MgCl_2$ (0, 1, 2

and 3 mM). The PCR reaction was carried out by combining the following reaction components in 25µl reaction volume.

Components	**Volume (µl)**	**Final concentration**
Nuclease-free water	17.67	…..
10 X Reaction Buffer	2.50	1 X
dNTP mix (10 mM)	0.50	0.2 mM
Taq polymerase (3U/ µl)	0.33	0.04U/µl
Primers	2.0	0.2 µM
Template (~25 ng)	2.0	2 ng/µl

Later, RAPD amplifications were performed routinely using PCR mixture (25 µl) which contained genomic DNA as template, 1X PCR buffer (Fermentas GmbH, Germany), dNTPs (Fermentas GmbH, Germany), 1 unit (U) of Taq DNA polymerase (Fermentas GmbH, Germany) and primer (Table 2.2 and 2.3) and the contents of the tube were mixed by a brief spin in a micro centrifuge. PCR was performed using a thermal cycler (Eppendorf, Germany) and the reaction parameters were as follows:

a. Initial Denaturation : 94°C for 4 min
b. Denaturation : 94°C for 60 sec
c. Annealing : 36°C for 45 sec } 35 cycles
d. Extension : 72°C for 2min
e. Final extension : 72°C for 10 min

In case of ISSR primers, optimal annealing temperature was found to vary according to the base composition of the primers. PCR mixture (25 µl) contained 25 ng of genomic DNA as template, 1X PCR buffer (Fermentas GmbH, Germany), 200 µM dNTPs (Fermentas GmbH, Germany), 1 U of *Taq* DNA polymerase (Fermentas GmbH, Germany). PCR was performed at initial denaturation temperature of 94^{o}C for 4 min followed by 40 cycles of 1 min denaturation at 94^{o}C, 1 min at 2^{o}C lower than specified annealing temperature for each primer and 2 min extension at 72^{o}C with a final extension at 72^{o}C for 10 min using a thermal cycler (Eppendorf, Germany).

1.2.6. Analysis of PCR product by agarose gel electrophoresis

The PCR product was analyzed by agarose gel (2.0%) electrophoresis as described below.

Materials and solutions

1. Agarose (SRL, Mumbai, India).
2. TAE 50 X buffer:

Tris base	:	24.2 g
Glacial acetic acid	:	5.71 ml
0.5M EDTA (pH 8.0)	:	10 ml
Distilled water	:	vol to 80 ml

The pH was adjusted to 7.2 and the final volume was brought to 100 ml with distilled water. The buffer was sterilized by autoclaving and stored at room temperature.

3. 3kb DNA marker (Fermentas GmbH, Germany)
4. Gel casting boat
5. Midi gel apparatus and power supply (Bangalore Genie, India).
6. Ethidium bromide stock solution (10 mg ml^{-1}):

   10 mg of ethidium bromide (Sigma, USA) was dissolved in 1 ml of distilled water. The solution was stored in a micro centrifuge tube wrapped in aluminium foil at 4°C.

1.2.6.1. Methodology: The boat was sealed with an adhesive tape and the comb was placed for the wells. 1.2 g of agarose was added to 100 ml of 1 X TAE buffer. The mixture was heated on a hot plate to allow the agarose to dissolve. The solution was cooled to 50°C and poured into the sealed boat. The gel was allowed to polymerize. The comb and the adhesive tape were removed and the gel was placed in the electrophoresis tank with sufficient volume of 1 X TAE buffer to cover the surface of the gel. The PCR reaction sample and the standard DNA size marker were loaded in the wells. Electrophoresis was carried out at 50 volts till the dye reached 3/4th of the gel. The gel was removed from the tank and stained by soaking in a solution of 0.5 μg ml^{-1} ethidium bromide for 30 min at room temperature. The gel was de-stained in distilled water for 10 min, examined on a UV transilluminator and documented using Gel Documentation system (Herolab Laborgerate, Germany). The size of the amplification products was estimated from 100 bp DNA ladder (Fermentas GmbH, Germany). The band intensity

histogram of each gel was checked using EasyWin 32 software (HeroLab Laborgerate, Germany).

1.2.7. Resolving power (Rp)

Resolving power (Rp) for each primer was calculated following the method of Prevost and Wilkinson's (1999) for selecting primers that can distinguish a maximal number of accessions. Resolving power (Rp) of a primer is = ∑Ib where Ib (band informativeness) takes the value of: Ib = 1 - [2 x | 0.5 – p |], p being the proportion of the 21 genotypes (banana cultivars analyzed) containing the bands.

1.2.8. Data analysis

For both RAPD and ISSR profiles, the well-resolved and consistently reproducible fragments ranging from 200-3100 bp were scored as present (1) or absent (0) for each analysis. Bands with the same migration distance were considered homologous. A pair-wise similarity matrix was computed and analyzed with NTSYS (Rohlf 1998) version 2.02 using the simple matching coefficient (Sokal and Michener 1958). The similarity matrix was used to construct a dendrogram by the un-weighed pair-group method with arithmetical averages (UPGMA).

1.3. RESULTS

1.3.1. Genomic DNA Extraction

The quality of DNA was inspected by agarose gel electrophoresis (Figure 1.3). All samples showed bands typical of un-degraded DNA and free from RNA contamination. DNA obtained from banana leaf of 21 cultivars shown in the figure 1.3.

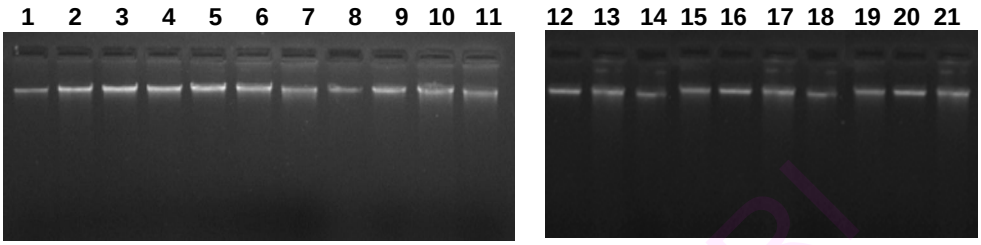

Figure 1.3. Agarose gel electrophoresis of genomic DNA extracted from twenty one cultivars Lanes 1: Monthan, 2: Elakki bale, 3: NR, 4: Ney poovan, 5: CO1, 6: Nendran, 7: Rasthali, 8: Red banana, 9: Poovan, 10: Peyan, 11: Karpooravalli, 12: Virupakshi, 13: Kunnan, 14: Kadali, 15: Robusta, 16: Williams, 17: Motta poovan, 18: Matti, 19: Laden, 20: Anai koomban and 21: Sirumalai.

The issue of DNA quality was assessed by calculating the A260/A280 ratio to determine RNA and protein contamination. The closest ratio to 2.0 (indicative of pure DNA) was obtained for all the samples.

1.3.2. Selection of Primers and Reproducibility

To begin with, only two cultivars of banana, i.e., Robusta and NR (the first one is fairly studied and the second one is never studied), were subjected for genetic analysis to screen the primers for their ability to amplify DNA fragments. The bands were scored by eye in two independently prepared sets and were un-affected by DNA extraction method, parts of the plant used and PCR replication (Figure 1.4). Based on the results of their ability to produce good number of distinct bands, 50 RAPD and 12 ISSR primers were selected. DNA samples from each of the 21 cultivars were amplified using the decamers listed in Tables 1.2 and 1.3, where all the primers produced distinctly robust bands varying in numbers. For any of the primers used, band scores did not differ between repeat assessments or between gels.

The majority of band positions varied between cultivars. Each of the primers produced large numbers of polymorphic bands. The data on the total number of bands

(TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (%PB), Resolving power (Rp), amplified fragment size (AFS) obtained for each primer are shown in Tables 1.2 and 1.3.

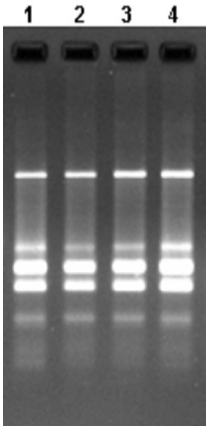

Figure 1.4. Agarose gel electrophoresis of PCR amplified products using genomic DNA extracted from different parts leaf, stem, leaf sheath and fruit of the cultivars NR using the OPC 05

1.3.3. RAPD analysis

The total amplified products of 50 RAPD primers was 537 (average of 10.74 bands per primer) ranging from 200 to 3100 bp, of which 323 were polymorphic (60.15%). The number of bands for each RAPD primer varied from 5 (OPJ 04 and OPJ 14) to 22 (OPM 20). The resolving power (Rp) of the 50 RAPD primers ranged from 0.66 for primer OPD 04 to 8.19 for primer OPM 20 (Table 1.2). Samples of RAPD analysis are shown in Figure 1.5.

Table 1.2. List of RAPD primers selected from Operon. Total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (%PB), resolving power (Rp) and amplified fragment size (AFS) obtained per RAPD primer

Sl. No.	Primer	Primer (5' – 3')	TNB	NPB	% PB	Rp	AFS
1.	OPA-03	AGTCAGCCAC	8	7	87.50	2.48	500-2800
2.	OPA-04	AATCGGGCTG	12	11	91.66	6.19	300-3000
3.	OPA-06	GGTCCCTGAC	10	5	50.00	3.33	300-1031

4.	OPA-09	GGGTAACGCC	8	4	50.00	2.48	400-1800
5.	OPA-11	CAATCGCCGT	18	12	66.66	4.57	300-3100
6.	OPA-14	CTCGTGCTGG	13	6	46.15	2.10	200-3000
7.	OPA-20	GTTGCGATCC	14	4	28.57	0.95	200-2600
8.	OPC-01	TTCGAGCCAG	12	5	41.66	3.72	280-1800
9.	OPC-02	GTGAGGCGTC	12	5	41.66	2.10	300-2800
10.	OPC-04	CCGCATCTAC	15	9	60.00	3.33	220-2000
11.	OPC-05	GATGACCGCC	14	13	92.86	6.10	320-1900
12.	OPC-07	GTCCCGACGA	16	10	62.50	4.48	300-2600
13.	OPC-08	TGGACCGGTG	16	15	93.75	5.33	290-3100
14.	OPC-09	CTCACCGTCC	14	12	85.71	5.91	480-1800
15.	OPC-11	AAAGCTGCGG	6	2	33.33	1.14	200-2000
16.	OPC-13	AAGCCTCGTC	8	3	37.50	1.71	480-1800
17.	OPC-20	ACTTCGCCAC	7	3	42.86	1.14	300-2000
18.	OPD-03	GGTCTACACC	6	3	50.00	1.16	290-1800
19.	OPD-04	TCTGGTGAGG	10	3	30.00	0.66	300-2000
20.	OPD-07	TTGGCACGGG	13	5	38.46	3.14	380-2500
21.	OPD-08	GTGTGCCCCA	7	5	71.43	2.19	290-2400
22.	OPD-16	AGGGCGTAAG	14	13	92.86	7.53	400-2000
23.	OPF-12	ACGGTACCAG	6	3	50.00	1.62	400-1031
24.	OPF-15	CCAGTACTCC	7	4	57.14	2.00	200-2400
25.	OPJ-01	CCCGGCATAA	8	6	75.00	2.48	200-3000
26.	OPJ-02	CCCGTTGGGA	9	8	88.88	3.81	380-2400
27.	OPJ-03	TCTCCGCTTG	8	6	75.00	3.05	500-2400
28.	OPJ-04	CCGAACACGG	5	3	60.00	1.43	500-1031

29.	OPJ-06	TCGTTCCGCA	10	4	40.00	2.95	220-2800
30.	OPJ-07	CCTCTCGACA	11	5	45.45	3.14	300-2000
31.	OPJ-08	CATACCGTGG	6	3	50.00	0.76	400-3000
32.	OPJ-09	TGAGCCTCAC	8	5	62.50	2.48	400-2000
33.	OPJ-10	AAGCCCGAGG	11	5	45.45	2.95	220-1800
34.	OPJ-11	ACTCCTGCGA	8	3	37.50	1.14	400-2200
35.	OPJ-14	CACCCGGATG	5	1	20.00	0.86	500-1031
36.	OPJ-15	TGTAGCAGGG	9	5	55.55	3.62	220-1800
37.	OPJ-16	CTGCTTAGGG	8	2	25.00	1.05	220-2000
38.	OPJ-17	ACGCCAGTTC	6	3	50.00	1.24	500-2200
39.	OPJ-20	AAGCGGCCTC	7	4	57.14	2.38	800-2400
40.	OPL-14	TCGTGCGGGT	8	7	87.50	3.81	1031-2400
41.	OPM-12	CACAGACACC	12	9	75.00	2.48	300-2600
42.	OPM-16	GTAACCAGCC	18	13	72.22	4.29	400-2000
43.	OPM-20	AGGTCTTGGG	22	16	72.22	8.19	320-2000
44.	OPM-18	CACCATCCGT	7	5	71.42	2.10	400-2000
45.	OPN-03	GGTACTCCCC	18	12	66.66	4.57	300-2600
46.	OPN-04	GACCGACCCA	12	6	50.00	3.71	400-2800
47.	OPN-06	GAGACGCACA	11	9	81.81	4.95	340-2000
48.	OPN-09	TGCCGGCTTG	18	13	72.22	5.33	240-3000
49.	OPN-10	ACAACTGGGG	19	5	26.32	1.91	240-3000
50.	OPN-12	CACAGACACC	7	3	42.86	1.72	240-2400
Total			537	323	60.15	151.76	

Key to symbols: R = A+G, Y = C+T, H = A+T+C

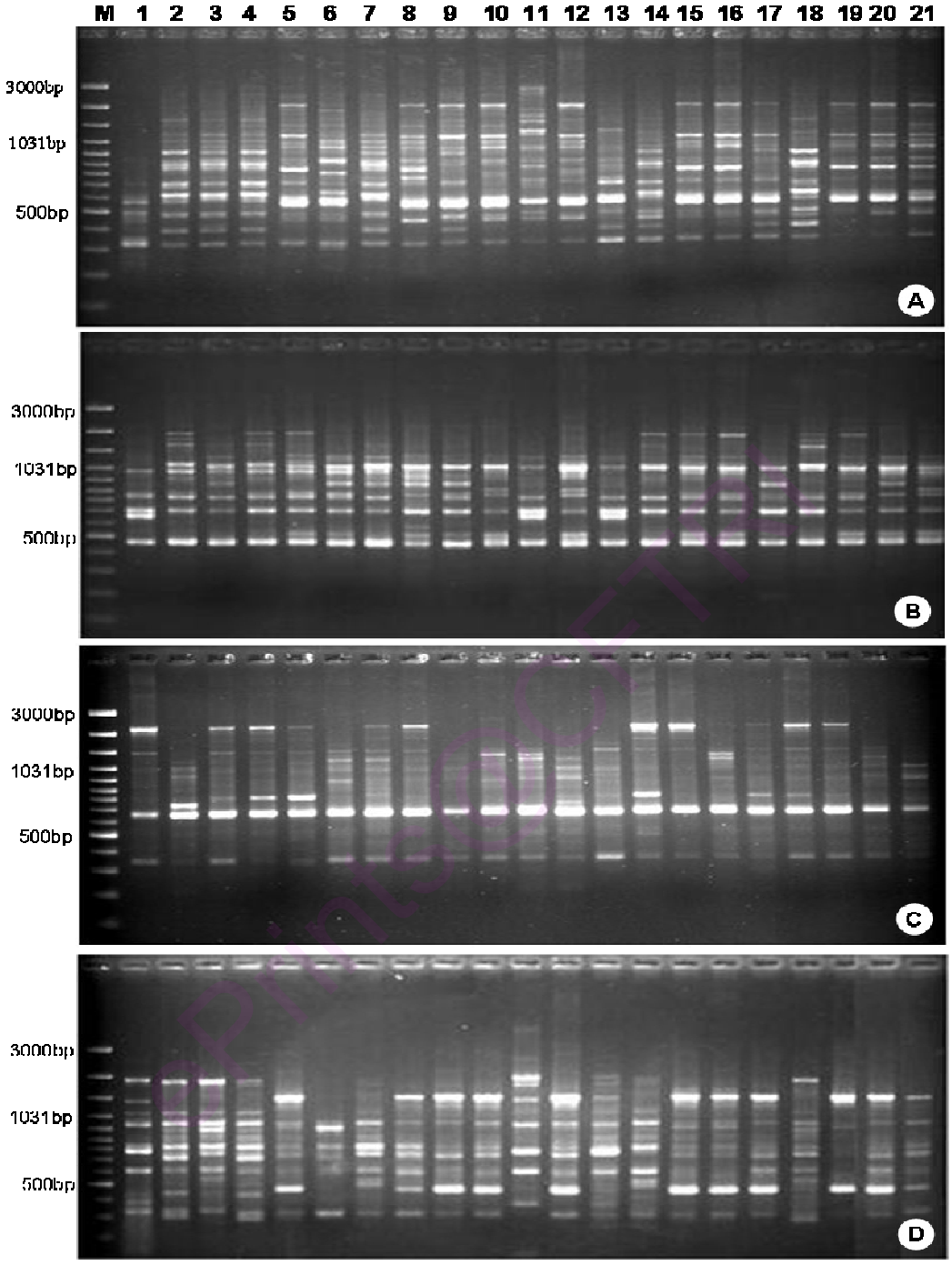

Figure 1.5. RAPD profile of DNA from 21 cultivars of South Indian banana cultivars using primers OPA 14 (A), OPN 06 (B), OPM 20 (C) and OPC 05 (D). Lane M: 100 bp GeneRuler DNA ladder

Lanes 1: Monthan, 2: Elakki bale, 3: NR, 4: Ney poovan, 5: CO1, 6: Nendran, 7: Rasthali, 8: Red banana, 9: Poovan, 10: Peyan, 11: Karpooravalli, 12: Virupakshi, 13: Kunnan, 14: Kadali, 15: Robusta, 16: Williams, 17: Motta poovan, 18: Matti, 19: Laden, 20: Anai koomban and 21: Sirumalai

1.3.4. ISSR analysis

For ISSR analyses, after screening 25 primers, 12 were selected for the PCR amplifications of DNA samples from 21 cultivars resulting in highly reproducible amplification products. TNB, NPB, %PB, Rp and AFS obtained for each primer are shown in Table 1.3. The total amplified products of 12 ISSR primers was 104 (average of 8.67 bands per primer) ranging from 200 to 3000 bp, 59 of them polymorphic (56.73%). Number of bands for each primer varied from 4 (UBC-863) to 15 (UBC-890). The Rp of the 12 ISSR primers ranged from 1.24 for primer UBC-820 to 3.33 for primers UBC-836 and UBC-890. A sample of ISSR results is shown in Figure 1.6.

Table 1.3. List of ISSR primers selected from UBC. Total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (%PB), resolving power (Rp) and amplified fragment size (AFS) obtained per ISSR primer

Sl. No.	Primer	Primer sequence (5' – 3')	TNB	NPB	% PB	Rp	AFS
1.	UBC-811	$(GA)_8C$	7	6	85	3.24	250-2500
2.	UBC-813	$(CT)_8$ T	5	4	80	1.90	200-1031
3.	UBC-817	$(CA)_8A$	8	6	75	3.05	400-2800
4.	UBC-820	$(GT)_8T$	11	4	36.36	1.24	200 -2000
5.	UBC-826	$(AC)_8C$	8	5	62.50	2.48	350-2000
6.	UBC-834	$(AG)_8YT$	13	5	38.46	2.67	700-3000
7.	UBC-836	$(GA)_8YA$	11	6	54.54	3.33	200-1000
8.	UBC-840	$(GA)_8YT$	11	3	27.27	1.33	200-1000
9.	UBC-845	$(CT)_8RG$	6	6	100	3.24	300-1400
10.	UBC-848	$(CA)_8RG$	5	4	80	2.48	200-1400
11.	UBC-863	(GAA)6	4	3	75	2.48	500-1800
12.	UBC-890	$VHV(GT)_7$	15	7	46.66	3.33	450-1800
Total			104	59	56.73	30.76	

Key to symbols: R = A+G, Y = C+T, H = A+T+C

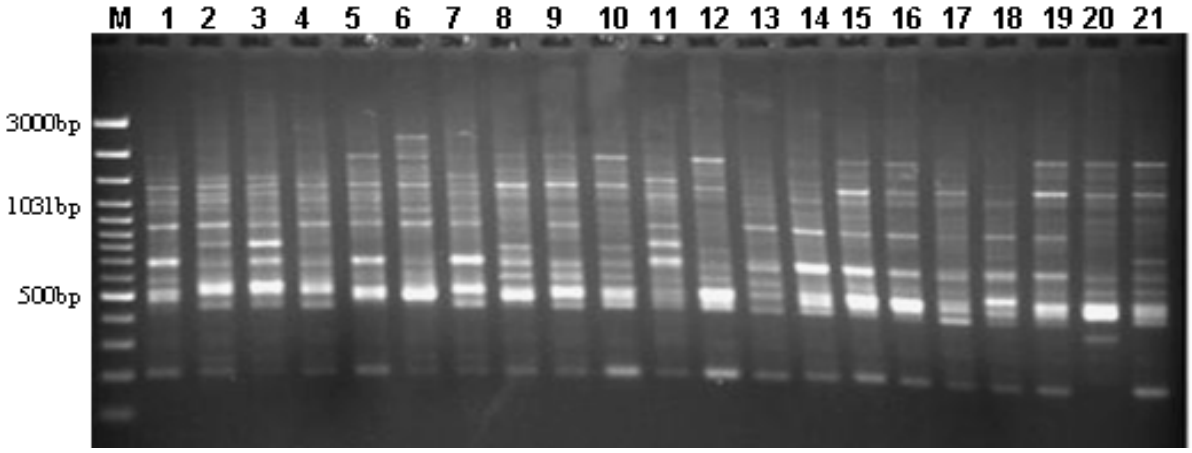

Figure 1.6. ISSR pofile of DNA from 21 cultivars of South Indian banana cultivars using primer UBC 810. Lane Marker represents 100 bp GeneRuler DNA ladder.

Lanes 1: Monthan, 2: Elakki bale, 3: NR, 4: Ney poovan, 5: CO1, 6: Nendran, 7: Rasthali, 8: Red banana, 9: Poovan, 10: Peyan, 11: Karpooravalli, 12: Virupakshi, 13: Kunnan, 14: Kadali, 15: Robusta, 16: Williams, 17: Motta poovan, 18: Matti, 19: Laden, 20: Anai koomban and 21: Sirumalai.

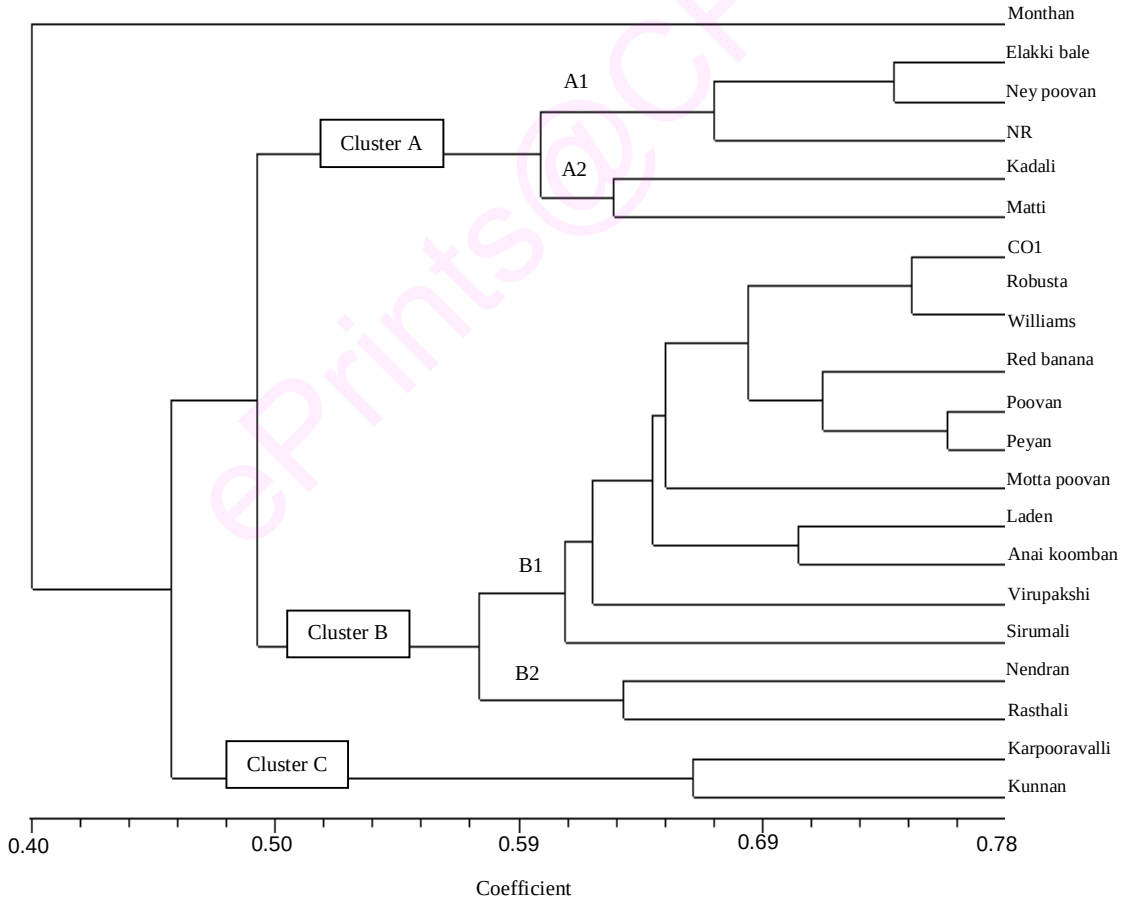

Figure 1.7. Dendrogram displaying the genetic distances among banana cultivars obtained from cluster analysis of RAPD data

Table 1.4. Genetic similarity co-efficient among the commercially important south Indian banana cultivars based on ISSR (top wedge) and RAPD (bottom wedge) analyses

Cultivars	Monthan	Elakki bale	NR	Ney poovan	CO1	Nendran	Rasthali	Red banana	Poovan	Peyan	Karpooravalli	Virupakshi	Kunnan	Kadali	Robusta	Williams	Motta poovan	Matti	Laden	Anai koomban	Sirumalai
Monthan	1	0.1800	0.1875	0.3404	0.4167	0.3913	0.3556	0.3125	0.4130	0.3913	0.4250	0.3750	0.4872	0.4390	0.4651	0.4889	0.3696	0.3404	0.4186	0.3830	0.3333
Elakki bale	0.3333	1	0.5676	0.6410	0.4082	0.3542	0.4762	0.3617	0.3750	0.3542	0.3182	0.3958	0.3721	0.4634	0.3333	0.3600	0.3617	0.4884	0.2917	0.3200	0.3830
NR	0.3536	0.6503	1	0.5500	0.3400	0.3696	0.4286	0.3478	0.3913	0.3696	0.2444	0.3265	0.3256	0.4146	0.2917	0.3469	0.4419	0.5122	0.2766	0.3617	0.3404
Ney poovan	0.4031	0.7381	0.6864	1	0.5870	0.5682	0.5714	0.5111	0.5556	0.5333	0.4091	0.4792	0.5000	0.6842	0.5111	0.5319	0.4167	0.6190	0.4348	0.4894	0.4681
CO1	0.4409	0.5052	0.4394	0.6211	1	0.6444	0.4490	0.5532	0.7045	0.7209	0.4565	0.6889	0.5111	0.4681	0.7381	0.7907	0.5532	0.5208	0.6512	0.6304	0.6444
Nendran	0.4194	0.4922	0.4712	0.5916	0.6612	1	0.5581	0.5682	0.6512	0.5909	0.3696	0.5652	0.4884	0.5116	0.5682	0.5870	0.4681	0.5333	0.5227	0.5778	0.5217
Rasthali	0.4294	0.5829	0.5882	0.6461	0.5260	0.6328	1	0.6098	0.5455	0.4565	0.2766	0.4375	0.4186	0.4762	0.5000	0.5217	0.5349	0.5714	0.4545	0.5814	0.4565
Red banana	0.3842	0.4794	0.4660	0.5538	0.6117	0.6519	0.6842	1	0.7073	0.6429	0.2653	0.6512	0.3696	0.4545	0.7000	0.6364	0.5814	0.5111	0.5714	0.7073	0.5682
Poovan	0.4402	0.5052	0.4921	0.5885	0.6944	0.6538	0.6201	0.7143	1	0.7317	0.3913	0.6977	0.4773	0.5000	0.7500	0.7209	0.6279	0.5556	0.7000	0.6744	0.5778
Peyan	0.4086	0.4896	0.4531	0.5729	0.7159	0.6464	0.5598	0.7069	0.7588	1	0.4318	0.7143	0.5238	0.5116	0.6829	0.6977	0.6429	0.5333	0.5952	0.6512	0.5556
Karpooravalli	0.4269	0.4255	0.3737	0.4639	0.4869	0.5054	0.4084	0.4162	0.4789	0.5027	1	0.3542	0.6286	0.3810	0.3778	0.3750	0.3191	0.3191	0.3333	0.2800	0.2857
Virupakshi	0.3177	0.4352	0.3568	0.4798	0.6461	0.5538	0.4479	0.6089	0.6292	0.6590	0.3795	1	0.4348	0.4255	0.7317	0.7442	0.5435	0.4792	0.6429	0.6591	0.6744
Kunnan	0.4512	0.4475	0.4011	0.5191	0.5027	0.5393	0.4693	0.4599	0.5193	0.5367	0.6601	0.4140	1	0.5128	0.5000	0.5227	0.4318	0.4000	0.4186	0.4444	0.3913
Kadali	0.4176	0.5588	0.5176	0.6529	0.4635	0.5137	0.5376	0.4603	0.4787	0.4628	0.4199	0.4074	0.5494	1	0.4884	0.4783	0.4222	0.6000	0.4091	0.4043	0.3830
Robusta	0.4358	0.4410	0.4124	0.5469	0.7168	0.5838	0.5243	0.6591	0.6897	0.6534	0.4368	0.6395	0.5085	0.4917	1	0.8462	0.5814	0.5111	0.7368	0.7073	0.5682
Williams	0.4402	0.4670	0.4242	0.5641	0.7733	0.6270	0.5676	0.6760	0.7062	0.7283	0.4789	0.6763	0.5278	0.4632	0.7818	1	0.6364	0.5652	0.7949	0.7209	0.5870
Motta poovan	0.4302	0.4737	0.4757	0.5179	0.6264	0.5450	0.5611	0.6348	0.6743	0.6763	0.4316	0.5272	0.4778	0.4385	0.6102	0.6743	1	0.6190	0.6098	0.7073	0.5333
Matti	0.3925	0.5899	0.6047	0.6798	0.5255	0.5285	0.5955	0.5155	0.5417	0.5260	0.4175	0.4416	0.4309	0.6287	0.4845	0.5337	0.5769	1	0.5349	0.5556	0.5000
Laden	0.4181	0.4249	0.4031	0.5156	0.6724	0.5769	0.5249	0.5889	0.6744	0.6286	0.4202	0.5862	0.4341	0.4270	0.6471	0.7349	0.6316	0.5243	1	0.7000	0.5952
Anai koomban	0.3474	0.4141	0.4145	0.4874	0.6354	0.5956	0.5189	0.6257	0.6278	0.6384	0.3807	0.6149	0.4536	0.4010	0.6011	0.6743	0.6416	0.4718	0.7012	1	0.6905
Sirumalai	0.3552	0.4624	0.4247	0.4845	0.6180	0.5519	0.5000	0.6264	0.6193	0.6207	0.3968	0.5780	0.4333	0.4032	0.5829	0.6102	0.5682	0.4762	0.6036	0.6727	1

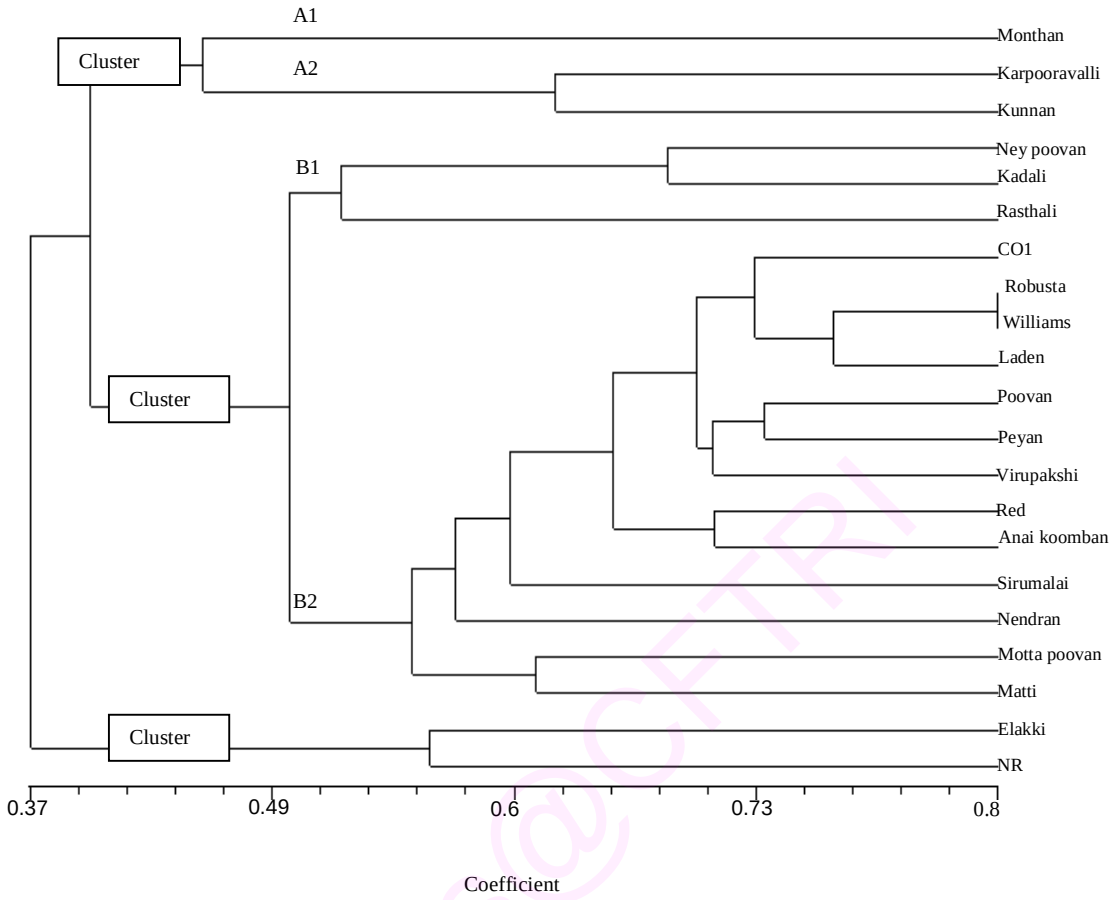

Figure 1.8. Dendrogram displaying the genetic distances among banana cultivars obtained from cluster analysis of ISSR data

1.3.5. Molecular analysis and fingerprinting of Banana cultivars

The genetic similarity (GS) coefficients for 21 cultivars obtained with RAPD markers ranged from 0.3177 (between the cultivars Monthan and Virupakshi) to 0.7818 between Robusta and Williams). The UPGMA analysis made it possible to discriminate all of the genotypes of this study. The dendrogram, based on RAPD data, showed a clear distinction into major and minor clusters (Figure 1.7). The dendrogram obtained using GS coefficient (Table 1.4) showed the presence of 3 main clusters (A, B and C) having 5, 13 and 2 cultivars in each respectively. The cultivar Monthan did not group with any other variety and hence can be considered as "isolate". The dendrogram derived on the basis of ISSR analyses using GS coefficient (Table 1.4) showed a clear distinction into 3 main clusters (A, B and C) with 3, 16 and 2 cultivars in each respectively (Figure 1.8).

The cluster A had 2 sub-clusters (A1 and A2), where the subcluster A1 showed the presence of 3 cultivars, Elakki, Ney poovan and NR; and the subcluster A2 represented 2 cultivars - Kadali and Matti. The cluster B showed the presence of two subclusters (cluster B1 and B2) where the subcluster B1 included 11 cultivars and the subcluster B2 showed 2 cultivars – Nendran and Rasthali. The cluster C showed the presence of 2 cultivars – Karpooravalli and Kunnan (Figure 1.7).

The GS coefficients for 21 cultivars obtained with ISSR markers ranged from 0.1800 (cultivar Monthan and Elakki bale) to 0.8462 (Robusta and Williams). The UPGMA analysis clearly showed phenetic discrimination for all the genotypes (21 cultivars) of this The cluster A showed 2 subclusters (A1 and A2), where subcluster A1 showed one cultivar – Monthan and subcluster A2 included the two cultivars – Karpooravalli and Kunnan. The cluster B showed 2 subclusters (B1 and B2), where subcluster B1 included 3 cultivars (Ney poovan, Rasthali and Kadali) and the subcluster B2 showed the presence of 13 cultivars. The cluster C showed two cultivars – Elakki bale and NR (Figure 1.8).

1.4. DISCUSSION

Morphological markers for identifying the cultivars of banana are limited in number and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of the traits (Smith and Smith 1989). In contrast, DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationships studies (Reddy et al. 2002). RAPD-based phylogenetic analyses have been conducted using neighbor-joining and /or UPGMA with Jaccard and raw-character-difference distances (Pharmawati et al. 2004; Simmons and Web 2006; Simmons et al. 2007). Similarly, ISSR-based phylogenetic analyses have variously been conducted in plant varieties (Iruela et al. 2002; Pharmawati et al. 2004). In our earlier studies, the genetic markers of di-, tri- and tetra-nucleotide repeat primers were used to ascertain the genetic fidelity in long-term micropropagated (Venkatachalam et al. 2007c) and regenerated banana plants (Venkatachalam et al. 2007b).

The present study was conducted to determine the extent of genetic diversity in the most popular cultivars of banana, including certain rare cultivars (such as NR) of South India that were not analysed earlier. We chose to analyse the banana germplasm, based on marker systems (RAPD and ISSR) that make use of arbitrary primers to amplify random DNA sequences in the genome (Williams et al. 1990; Zietkiewicz et al. 1994). An earlier study by Bhat and Jarret (1995) used a few RAPD markers to study Indian cultivars of banana. Recently, genetic markers have been successfully applied to study diversity in wild bananas of South India, mainly genotypes BB (Uma et al. 2006). Nevertheless, the present report relates to the first study where a large number of both RAPD and ISSR molecular markers have been used to examine genetic diversity in South Indian dessert bananas. The selection of markers is based on extensive analyses for the concentration of magnesium chloride and the annealing temperature. The markers showing less than 3 bands were not selected.

1.4.1. Fingerprinting and cultivar-specific markers

South Indian banana cultivars are identified mainly on phenotypic characteristics of the plants and fruit quality / aroma.

Table 1.5. Genetic key based on the presence or absence of specific marker for different cultivars of banana

Name of the cultivar	Characterized by the presence of the marker		Characterized by the absence of the marker	
	RAPD	ISSR (UBC)	RAPD	ISSR (UBC)
Monthan	OPC-05$_{400}$ OPA-04$_{700}$ OPA-04$_{650}$ OPJ-16$_{750}$ OPJ-20$_{300}$ OPC-08$_{1000}$ OPA-04$_{600}$ OPN-10$_{2000}$ OPN 3$_{2000}$	820$_{2000}$ 826$_{700}$ 845$_{750}$ 848$_{300}$ 863$_{200}$ 890$_{650}$	OPM-20$_{2700}$ OPN-6$_{1200}$ OPN-06$_{1031}$ OPC-05$_{800}$ OPA-03$_{1700}$ OPA-03$_{800}$ OPJ-11$_{1301}$ OPJ-11$_{800}$ OPJ-13$_{1000}$ OPJ-16$_{400}$ OPC-09$_{1200}$ OPC-08$_{1200}$ OPC-08$_{300}$ OPA-14$_{1800}$ OPA-04$_{200}$ OPJ-09$_{400}$	811$_{1200}$ 817$_{800}$ 820$_{800}$ 834$_{800}$ 836$_{2000}$ 845$_{400}$ 890$_{1200}$
Elakki bale	OPN-06$_{850}$ OPA-03$_{500}$ OPC-08$_{3000}$ OPC-08$_{550}$ OPN-10$_{2000}$ OPN 3$_{2000}$	811$_{800}$ 890$_{600}$	OPM-20$_{200}$ OPN-06$_{350}$ OPA-03$_{1000}$ OPD-16$_{1600}$ OPC-08$_{300}$ OPJ-09$_{500}$ OPN-10$_{800}$	811$_{1200}$ 811$_{900}$ 813$_{850}$ 813$_{350}$ 817$_{600}$ 820$_{1000}$ 820$_{900}$ 836$_{1600}$
NR	OPC-09$_{1800}$ OPC-08$_{3000}$ OPC-08$_{400}$ OPA-14$_{600}$ OPC 5$_{800}$	811$_{800}$ 890$_{1600}$ 890$_{600}$	OPM-20$_{600}$ OPN-06$_{1031}$ OPA-03$_{1700}$ OPA-03$_{800}$ OPJ-11$_{800}$ OPD-16$_{1600}$ OPA-04$_{1200}$ OPJ-09$_{500}$ OPN-10$_{800}$	811$_{900}$ 811$_{600}$ 817$_{600}$ 820$_{1000}$ 820$_{800}$ 834$_{800}$ 836$_{1600}$
Ney poovan	OPC-09$_{1800}$ OPC-08$_{3000}$ OPC-08$_{400}$	817$_{400}$ 890$_{1600}$	OPN-06$_{350}$ OPD-16$_{1600}$	813$_{350}$ 836$_{1600}$
CO1	OPN-10$_{2000}$	836$_{1200}$ 863$_{200}$	OPN-06$_{350}$ OPD-16$_{1000}$ OPC-08$_{2000}$	813$_{350}$
Nendran	OPM-20$_{800}$ OPC-04$_{1600}$ OPN 3$_{2000}$	836$_{1200}$ 863$_{200}$ 890$_{1600}$ 817$_{400}$	OPD-16$_{1000}$ OPC-08$_{1800}$	836$_{2000}$
Rasthali	OPA-04$_{650}$ OPA-14$_{600}$	811$_{800}$ 890$_{600}$	OPM-20$_{600}$ OPD-16$_{1600}$ OPD-16$_{1000}$ OPA-14$_{1800}$	811$_{600}$ 836$_{1600}$
Red banana	OPA-04$_{650}$ OPC-08$_{550}$ OPD-10$_{1200}$	817$_{400}$ 890$_{600}$	OPA-03$_{1000}$ OPC-08$_{800}$ OPA-04$_{1000}$	820$_{900}$ 890$_{850}$
Poovan	OPJ-13$_{2000}$ OPA-04$_{600}$	836$_{1200}$	OPC-05$_{800}$ OPJ-11$_{500}$ OPC-04$_{650}$	817$_{800}$
Peyan	OPC-04$_{1600}$	836$_{1200}$ 817$_{400}$	OPJ-11$_{600}$ OPN-10$_{500}$	836$_{2000}$ 890$_{850}$
Karpooravalli	OPC-05$_{400}$ OPC-05$_{400}$ OPA-04$_{700}$ OPJ-13$_{2000}$ OPJ-13$_{1300}$ OPJ-16$_{900}$ OPC-08$_{1000}$ OPC-08$_{550}$ OPA-14$_{600}$ OPA-04$_{600}$	820$_{2000}$ 826$_{700}$ 836$_{1200}$ 840$_{1031}$ 848$_{300}$ 890$_{650}$	OPM-20$_{1200}$ OPC-05$_{800}$ OPC-05$_{300}$ OPA-04$_{1200}$ OPJ-11$_{1500}$ OPJ-11$_{1301}$ OPC-09$_{900}$ OPC-08$_{1250}$ OPC-08$_{300}$ OPA-04$_{1600}$ OPA-04$_{500}$ OPA-04$_{400}$ OPA-04$_{200}$	811$_{1200}$ 813$_{350}$ 817$_{800}$ 817$_{400}$ 820$_{900}$ 820$_{800}$ 836$_{2000}$

Virupakshi	OPN-06$_{850}$ OPA-03$_{2000}$ OPA-03$_{500}$ OPC-04$_{400}$ OPN-10$_{2000}$ OPN 3$_{2000}$	820$_{2000}$	OPA-03$_{1100}$ OPA-03$_{700}$ OPJ-11$_{600}$ OPJ-11$_{500}$ OPD-16$_{800}$ OPN-04$_{1200}$ OPC-04$_{700}$ OPC-04$_{650}$ OPC-08$_{2000}$ OPC-08$_{1800}$ OPC-08$_{1250}$ OPC-08$_{850}$ OPC-08$_{800}$ OPA-14$_{1800}$ OPA-14$_{1200}$ OPN-10$_{800}$	813$_{850}$ 820$_{1000}$ 890$_{850}$
Kunnan	OPA-04$_{700}$ OPJ-13$_{1300}$ OPJ-16$_{900}$ OPA-04$_{600}$	826$_{700}$ 840$_{1031}$ 863$_{200}$ 890$_{650}$	OPM-20$_{1200}$ OPA-03$_{600}$ OPA-04$_{1200}$ OPN-04$_{1200}$ OPC-09$_{900}$ OPC-08$_{1700}$ OPC-08$_{300}$ OPA-04$_{1600}$ OPA-04$_{1000}$	811$_{1200}$ 836$_{2000}$
Kadali	OPA-03$_{500}$ OPJ-16$_{750}$ OPC-09$_{1800}$	845$_{750}$ 890$_{1600}$	OPM-20$_{1200}$ OPM-20$_{600}$ OPN-06$_{1200}$ OPD-16$_{800}$ OPJ-16$_{400}$ OPC-09$_{900}$ OPC-08$_{1700}$ OPA-14$_{1800}$	811$_{1200}$ 813$_{1200}$ 845$_{400}$
Robusta	OPA-03$_{500}$ OPD-10$_{1200}$	817$_{400}$	OPN-06$_{1200}$ OPA-03$_{1700}$ OPA-03$_{1000}$ OPJ-11$_{600}$ OPC-04$_{900}$ OPC-04$_{700}$ OPC-08$_{1700}$ OPN-10$_{800}$	813$_{1200}$ 820$_{900}$
Williams	OPM-20$_{3000}$ OPC-05$_{3000}$	847$_{400}$ 863$_{200}$	OPC-05$_{600}$ OPA-03$_{1700}$ OPC-04$_{700}$ OPA-14$_{1200}$ OPA-04$_{1000}$	817$_{600}$
Motta poovan	OPC-08$_{550}$ OPC-08$_{400}$ OPN-10$_{2000}$ OPN-03$_{2000}$	811$_{800}$	OPM-20$_{900}$ OPA-04$_{1200}$ OPJ-11$_{1500}$ OPJ-13$_{1000}$ OPC-09$_{1200}$	811$_{900}$ 890$_{1200}$
Matti	OPJ-13$_{2000}$ OPC-09$_{1800}$ OPC-08$_{3000}$ OPC-08$_{400}$ OPS-14$_{1800}$ OPA-14$_{600}$	890$_{1600}$	OPM-20$_{900}$ OPM-20$_{600}$ OPA-04$_{1200}$ OPJ-11$_{1500}$ OPJ-11$_{600}$ OPJ-16$_{400}$ OPA-04$_{200}$ OPN-10$_{800}$	811$_{900}$ 811$_{600}$ 845$_{400}$
Laden	OPJ-13$_{2000}$ OPC-04$_{1600}$	817$_{400}$	OPM-20$_{750}$ OPM-20$_{300}$ OPC-05$_{800}$ OPC-05$_{600}$ OPC-05$_{300}$ OPA-14$_{1200}$	817$_{800}$ 817$_{600}$ 817$_{400}$
Anai koomban	OPC-04$_{1600}$	817$_{400}$	OPM-20$_{2700}$ OPM-20$_{300}$ OPC-05$_{900}$ OPJ-11$_{1301}$ OPN-04$_{1200}$ OPC-08$_{2000}$ OPC-08$_{1800}$ OPC-08$_{1700}$ OPC-08$_{1250}$ OPA-14$_{1200}$	890$_{850}$
Sirumalai	OPD-10$_{1200}$	848$_{300}$ 817$_{400}$	OPM-20$_{2700}$ OPC-05$_{900}$ OPA-03$_{1000}$ OPJ-11$_{500}$ OPC-04$_{900}$ OPC-04$_{650}$ OPA-04$_{1200}$ OPN-10$_{800}$	813$_{850}$ 890$_{850}$

Especially in case of NR, people doubted that the exotic fruit qualities such as high fragrance/aroma and sweet fluffy pulp are probably due to the agro-climatic conditions since all other plant phenotypes of NR are similar to those of cv. Elakki. Nevertheless, through the dendrogram data of the present study, it has been established that NR is

altogether a different cultivar with close relationship with cultivars Elakki and Ney poovan. A very high level of polymorphism of the RAPD and ISSR markers existed among the South Indian cultivars of banana (Figures 1.5 and 1.6). A fingerprinting key, based on the bands that are specific to only 3 or lesser number of cultivars, has been developed (Table 1.5). The key thus developed has clearly shown a distinct band OPC 5_{800} for NR and the presence of specific ISSR bands (UBC-848_{300}) for Sirumalai, Monthan and Karpooravalli, and band UBC-820_{2000} for Monthan and Virupakshi. Based on these observations, a specific primer can be picked up for sorting the varietal differences.

1.4.2. Genetic relationships

Although many cultivars included in the present study are popular in Southern states of India, others namely Robusta, Williams, Laden and Rasthali are also grown in various parts of the world and introduced to India probably a couple of centuries ago. The information on genetic relationship would thus be useful for planning breeding programmes to improve the local cultivars. The genetic similarity (GS) coefficients for 21 cultivars obtained with RAPD markers ranged from 0.3177 (between Monthan and Elakki bale, Virupakshi) to 0.7818 (between Robusta and Williams) and for ISSR markers ranged from 0.18 (between Monthan and Virupakshi, Elakki bale) to 0.8462 (between Robusta and Williams) (Table 1.4). Due to this variation, both the markers were found to be equally efficient in establishing the genetic similarity and distance with almost similar grouping. Thus there occurs very high genetic variability among the dessert cultivars in a permutation which was significant at p=0.0001 with a matrix of 10,000 permutations. Similar results have been obtained for RAPD markers in cases such as African plantain core collection (Ude et al. 2003), plantain landraces of 76 accessions of AAB group (Crouch et al. 2000) and South Indian wild *Musa balbisiana* populations (Uma et al. 2006). In another type of study, through inter-retrotransposon amplified polymorphism (IRAP-primers), B-genome-specific primers were used to classify the banana cultivars of South India (Nair et al. 2005). The primers used in this study (Nair et al. 2005) were found useful for identifying the presence of '*bulbisiana*' (B) genome in banana cultivars where the band intensity appeared to be a preliminary indicator of ploidy level.

The dendrogram generated for the RAPD and the ISSR primers (Figures 1.7 and 2.8) showed a clear distinction into major and minor clusters. The dendrogram obtained using GS coefficient (Table 1.4) showed the presence of three main clusters (A, B and C) for RAPD and ISSR analyses. However, the sub-clusters varied for both. The RAPD analyses showing 5, 13 and 2 cultivars in cluster A, B and C respectively whereas, ISSR showed 3, 16 and 2 cultivars in clusters A, B, and C respectively. UPGMA of the data from RAPD analysis resulted in clearly discriminative data for all the genotypes of the present study where the similar genotypes such as those having AAB ranged from 0.6 to 0.7. However, the cultivar Monthan having genotype ABB was distantly related to cultivar Peyan and the other ABB cultivars, Karpoorvalli and Kunnan showed identical genetic distance for RAPD (Figure 1.7). Nevertheless, the UPGMA analysis using ISSR showed clear clustering of these varieties having genotype ABB (cluster 1 of Figure 1.8). If these clusters could be linked to the phenotypic or functional characteristics such as resistance to a particular disease, the marker-based analyses act as powerful tools that are helpful in the improvement of these crops.

1.4.3. Comparison of RAPD and ISSR markers

Owing to the vast advantages offered by DNA markers, there is an increasing trend in adopting such technologies for identifying the genetic diversities, especially in breeding programmes. However, an understanding is important since the type of markers will reflect different aspects of genetic diversity. Similarly, the potential usefulness of molecular techniques in identifying genetic relationships varies from plant to plant because of the uniqueness of each genome. RAPD (Williams et al. 1990) and ISSR (Zietkiewicz et al. 1994) markers have several advantages and a few disadvantages for assessing genetic diversity, and many recent papers have demonstrated the high potential of the two markers for population and species level studies (Wu et al. 2004; Sreedhar et al. 2007; Simmons et al. 2007), including the identification of intra-clonal variants occurring during micropropagation (Martins et al. 2004; Carvalho et al. 2004). Phylogenetic analyses based on RAPD markers have been applied using neighbor joining and/or UPGMA-based Jaccard and raw character difference distances (Pharmawati et al. 2004). RAPD has proven to be efficient in detecting genetic variations, even in closely related organisms, such as two near isogenic lines (NIL) (Martin et al. 1991). Similarly

ISSR-based phylogenetic analyses have variously been conducted in plant varieties (Iruela et al. 2002; Pharmawati et al. 2004). Thus ISSR technique is found to be highly discriminative and reliable (Reddy et al. 2002), and both RAPD and ISSR markers have been found highly economical because a large number of samples can be analysed quickly. Both distance and parsimony analyses are generally conducted in ISSR- (Huang and Sun 2000; Menzies et al. 2003), and RAPD- (Harris 1995; Pharmawati et al. 2004) based studies. Therefore, both RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plant species (Ramage et al. 2004; Martin et al. 2006).

In the present study, the RAPD and ISSR surveys between pairs of 21 cultivars revealed 60.15% and 56.73% of polymorphic bands respectively indicating that both these markers have similar and great discriminating power. They could accurately detect the partition of genetic diversity within the cultivars since each cultivar showed separate data. However, the ISSR markers were found robust since only 12 primers were required for bringing out similarly distinct data as that of 50 RAPD primers.

Most of the earlier studies that addressed the assessment of new methods of cultivar diagnosis have utilized relatively modest collections of plants, although the number and composition of genotypes used varied widely. For instance, Hosaka et al. (1994) used 31 RAPD primers to distinguish 67 of 73 cultivars/breeding lines of potato. In contrast, Sosinski and Douches (1996) screened 16 RAPD primers and used the 10 most informative ones to distinguish between 46 cultivars. Such variations complicate comparisons between studies. An alternative strategy would be to use a function that is strongly correlated to the proportion of genotypes identified but is independent of the number of genotypes studied. One such strategy would be to measure the ability of primers to distinguish between genotypes, namely the resolving power. The primer Resolving power (Rp) provides quantitative data allowing direct comparisons between primers (Prevost and Wilkinson 1999). Resolving power of the primer has been found to correlate strongly with genotype diagnosis and so has potential for a number of applications. It is possible that several primers included in a preliminary study are able to distinguish between all of the genotypes used. Such primers are the most likely to be selected for wider applications although there is currently no basis for comparing between them (Prevost and Wilkinson 1999). Since

cultivar diagnostic capacity of primers is generally based on results from small-scale studies, which may often lead to inconsistencies in primer selection. Therefore, Rp values are often of great help in selection of primers.

In the present study, Rp values of 50 RAPD primers ranged from 0.66 for OPD-04 to 8.19 for OPM-20 and that of 12 ISSR primers ranged from 1.24 for UBC-820 to 3.33 for UBC-836 and 890. When compared to the values reported in other crops (Prevost and Wilkinson 1999; Virk et al. 1995; Teklewold and Becker 2006) the range and the mean Rp observed in our study is high for RAPD and low for ISSR analyses. But Prevost and Wilkinson (1999) pointed out that Rp values could also vary between taxa for a selected set of primers.

Even in case of UPGMA analysis on the basis of ISSR data (Figure 1.8), the varieties Monthan, Karpooravalli and Kunnan (having genotype ABB) grouped into one cluster with an exception of Peyan which is a cooking banana with much different phenotypic characteristics. Similarly Elakki and NR grouped together (Figure 1.8) as they share very similar phenotypic characteristics with only differences in fruit characteristics (data not shown) although they are genotypically AB and AAB respectively. For these cultivars RAPD data (probably due to their large numbers) also showed the close relationship between Elakki and Ney poovan, both having genotype AB and their fruit characteristics are almost similar to NR (AAB) - which is the next member of the cluster A in the dendrogram (Figure 1.7). Contrarily, Kadali and Matti, both having AA genotype grouped together as A2 under cluster 1 in case of RAPD analysis (Figure 1.7) whereas in ISSR data showed their distant relationship (Figure 1.8). However, Poovan (dessert) and Peyan (cooking) that are entirely different from each other in fruit characteristics grouped together in both the analyses indicating their genetic similarity for dominant characteristics. These observations indicate that it is essential to use two different markers for establishing the genetic similarity and distance rather than drawing conclusion with one set of markers. A similar observation has been made for identifying the genetic diversity of *Oryza granulata* (Wu et al. 2004), *Houttuynia* (Wu et al. 2005) and strawberry (Kuras et al. 2004). Based on genetic similarity matrix and the dendrogram data, eight distinct cultivars were identified, which remained similarly distinct in both ISSR and RAPD analyses (Table 1.6).

Table 1.6. List of exclusive cultivars, their genome composition and genetic distances with nearest and farthest cultivars

Sl.No.	Cultivars	Genome composition	Genetic distance(Co efficient)	
			Nearest	Farthest
1.	Monthan	ABB	Kunnan (0.4872)	Elakki bale (0.1800)
2.	Elakki bale	AB	Ney poovan (0.6410)	Laden (0.2917)
3.	Ney poovan	AB	Elakki bale (0.7381)	Monthan (0.4031)
4.	NR	AAB	Elakki bale (0.6503)	Karpooravalli (0.2444)
5.	Kadali	AA	Ney poovan (0.6529)	Monthan (0.4176)
6.	Matti	AA	Ney poovan (0.6798)	Monthan (0.3925)
7.	Karpooravalli	ABB	Nendran (0.5054)	Anai komban (0.2800)
8.	Kunnan	ABB	Karpooravalli (0.6601)	Sirumalai (0.3913)

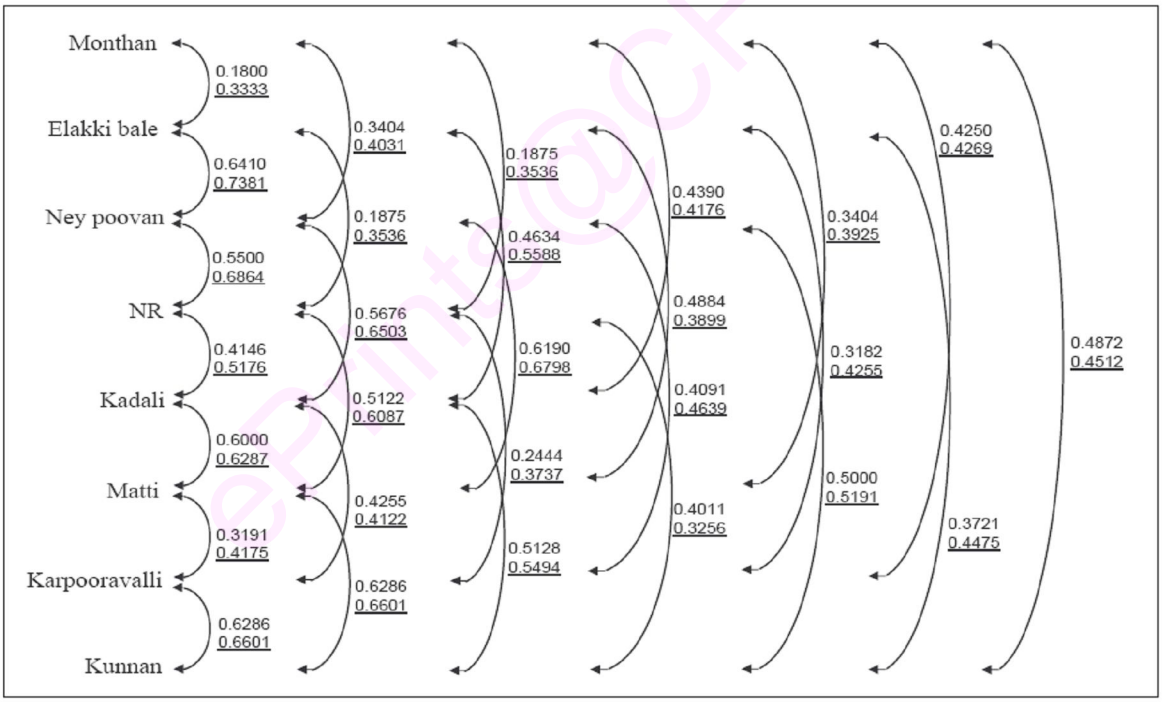

Figure 1.9. Inter- relationships within selected eight cultivars based on ISSR (upper values) and RAPD (values with underline) -derived genetic similarity coefficients

The cultivar Monthan did not fall into any cluster in RAPD dendrogram indicating its distinctiveness. Similarly the cultivars in cluster A and C are the other distinctive cultivars. Nevertheless, the cultivars grouped in cluster B showed distinctly

closer relationship. Among the eight distinctive cultivars identified through the present study, the inter-relationships have been derived based on their similarity co-efficients (Figure 1.9). Through this chart the genetic distance between any two selected cultivars can be quickly referred for both ISSR and RAPD analyses and are highly informative for selecting unique breeding combinations for generating newer genotypes.

1.4.4. Conclusion

In summary, RAPD and ISSR markers are two dominant DNA markers, having high resolution power and hence appear to offer many advantages in establishing genetic distances. They are effective and promising markers for assessing genetic variation in dessert banana cultivars. This study has resulted in the identification and molecular classification of South Indian banana cultivars, of which Robusta and Williams are global and others have either limited geographical distribution or purely endemic to South India. A group of 8 cultivars was identified that are highly distinct from one another. The members of this group may be useful for generating 2X and 4X breeding populations that can be used in breeding secondary triploid hybrids.

CHAPTER - II

SUMMARY

Since edible banana plants are sterile triploids, obtaining variants through various methods would greatly assist in the generation of genetically diverse and agronomically improved clones. The initial step towards this direction is to develop regeneration protocol, which is attempted in the present study for cultivar "Nanjanagudu rasabale". When in leaf explants obtained from shoots cultured in liquid medium were grown on modified medium, cormlet and direct shoot formation was observed. However, similar explants obtained from agar-gelled medium failed to produce any result. Directly shoot buds were initiated on the leaf (sheath) explants after a sequential culture on modified Murashige and Skoog (MS) medium supplemented with different growth regulators. The leaf explants were cultured first on medium with a high level (22.4 µM) of benzyladenine (BA), second on indole-3-butyric acid (IBA) supplemented medium, and third on reduced BA medium under incubation in the dark. The highest adventitious shoot regeneration in 24% of the explants, with the number of shoots ranging from 2-3 per explant, occurred in the explants incubated at the first step in medium with 24.6 µM and 0.198 µM of IBA. Further growth and complete shoot formation occurred under incubation in a 16 h photoperiod. However, when rhizome explants (also obtained from shoots grown in liquid medium) were cultured with various growth regulators in the first step medium containing 2,4,5-T (7.82 µM) produced friable callus that re-differentiated into roots only. Supplementation of various levels of (10-50µM) spermine, spermidine, and putrescine to cultures with secondary embryogenesis showed that about 50% of embryogenic calli rapidly produced secondary embryos only in the presence 40µM Spm but not in other treatments. The crucial role of Spm was further confirmed by the use of 0.1mM each of spermine-inhibitors DFMO and DFMA, which concomitantly inhibited the secondary embryogenesis. The shoots from embryogenic cultures were checked for their performance on solid medium (SM) and partial immersion system (PIS). The rate of shoot multiplication was higher in PIS than in SM throughout six weeks culture period. Uniformity in elongation of all the shoot buds was observed in PIS but not in SM. A large number of plantlets were regenerated from leaf base explants. Simultaneously, a micropropagation protocol was also developed where high levels of Benzylamino purine (BAP up to 53.28 µM) and Kinetin (Kn up to 55.80 µM) were used. The progressive increase of cytokinins levels resulted in concomitant increase in shoot number with a highest of 80 shoot buds per segment in BAP (31.08 µM). The plantlets were analysed 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. A large number of micropropagated plantlets of banana, that were developed from axillary shoot bud explants over 10 years ago were included. Of the 4000 *in vitro* plantlets, 11 were used for screening that included shoot cultures with distinct variation in morphological characteristics (morphotypes), and compared with the mother plant maintained in the field. The absence of any genetic variation indicates that the micro-propagation protocol developed by us for rapid *in vitro* multiplication is appropriate and applicable for clonal propagation NR over a long period.

2.1. INTRODUCTION

2.1.1. Regeneration

Most of the Indian cultivars of banana are susceptible to various bacterial diseases, particularly the Panama wilt disease and a viral disease - the "banana bunchy top" (Navarro et al. 1997). Owing to the parthenocarpic sterile nature of edible bananas, plant-breeding strategies aimed at banana crop improvement are extremely complex and time - consuming. Therefore, virtually all commercial banana production is from clonal derivatives occurring as natural variations. The use of alternative techniques such as tissue culture have been attempted in order to induce variations in banana plants and to select variants having desired qualities, that were in turn multiplied by micropropagation (Novak et al. 1989). Developing a reliable whole - plant regeneration protocol is essential for obtaining a large number of variants as well as to obtain genetic transformants *in vitro*. Reports on the formation of non-organogenic callus (Escalant and Tiesson 1987) and root forming callus (Escalant et al. 1994; Shii et al. 1992) were the initial success stories on developing protocols for regeneration. Subsequently, somatic embryogenesis was achieved using floral explants (Panis and Swennen 1993; Navarro et al. 1997), as were cell suspensions of diploid and triploid cultivars with genomes AA, AAA and ABB (Novak et al. 1989) and AAB (Ganapathi et al. 2001). Regeneration via somatic embryogenesis was reported in diploid and triploid *Musa acuminata* (AA and AAA) using reproductive explants (Jarret et al. 1985; Navarro et al. 1997 and Becker et al. 2000) and rhizome tissue (Bakry and Rossingnol 1985; Novak et al. 1989). Organogenesis has been reported from callus cells (Dhed'a et al. 1991), protoplasts (Panis and Swennen 1993) and leaf-base micro-sections (Okole and Schulz 1996) for certain *Musa* genotypes. Pro-embryogenic callus around the periphery of cultured corm tissue of *Musa acuminata* were observed that had the potential to regenerate into plantlets (May et al. 1995). Shii et al (1992) reported the production of embryogenic cell suspensions of several Cavendish cultivars, using immature male flowers as the explant.

The major objective of this study was to develop a regeneration protocol for the cultivar "Nanjanagudu Rasabale" (NR), the fruits of which have excellent characteristics, such as bright yellow peel, long shelf-life, inviting aroma and delicious creamy-white

pulp. This cultivar is grouped under "Silk" (Wealth of India 1962) and the plants are highly susceptible to both bacterial wilt and bunchy top disease, resulting in the dwindling of their cultivation from 500 hectares in the 1960s to only 5 hectares today. Owing to the best fruit characteristics, there is a high demand for the fruits of the NR cultivar, fetching very high prices for the growers. A suitable protocol for regeneration needs to be developed for this cultivar for improvement of both horticultural and fruit qualities. To the best of our knowledge, there are no reports on the regeneration of adventitious buds from leaf explants in NR, although a few reports on genome AAB exist (Okole and Schulz 1996; Ganapathi et al. 2001). Therefore, the objective of the present study was to identify the explant conditions and culture conditions resulting in the establishment of successful regeneration of shoot buds and cormlets leading to plantlet formation. We also sought to compare such shoot buds and cormlets with those from meristems- derived shoots.

2.1.2. Influence of Polyamines on regeneration

Another class of molecules, namely polyamines (PAs), such as Put, Spd and Spm, the chemically non-protein straight-chain aliphatic amines, are known to play important role in various cellular processes such as DNA replication, cell division, protein synthesis, responses to abiotic stress, rhizogenesis, flower development and *in vitro* flower induction. PAs have also been found implicated in morphogenic process where there are increasing evidences from several studies indicating that, they play a crucial role either directly in somatic embryogenesis and regeneration or indirectly through the release of nitric oxide or inhibition of ethylene biosynthesis (Apelbaum et al. 1981; Bais and Ravishankar 2002; Kumar and Rajam 2004; Silveria et al. 2006).

2.1.3. Micro-propagation using high levels of growth regulators

Propagation of banana through *in vitro* techniques has been reported by several workers using different explant sources as well as regeneration pathways (Cronauer and Krikorian 1983; 1988; Banerjee et al. 1986; Escalant and Teisson 1989; Bhagyalakshmi and Singh 1995; Dhed'a et al. 1991; Navarro et al. 1997). Though a very few exogenous growth regulators have been reported useful for banana micropropagation (Escalant and Teisson 1989; Bhagyalakshmi and Singh 1995; Dhed'a et al. 1991), the sub- and supra-

optimal levels of growth regulators, especially the synthetic ones, were found to induce somaclonal variation (Peres et al. 1999; Smith 1988; Martins et al. 2004; Martin et al. 2006). Even in cases where immediate variations were not noticed, the long-term application of such a high level of cytokinins resulted in somaclonal or epigenetic variations in some of the micropropagated plants (Israeli et al. 1996; Damasco et al. 1996) questioning the very fidelity of their clonal nature. This apart, banana shoots *in vitro* are known to synthesize and accumulate a natural cytokinin, 2iP at the basal portions of shoot clusters resulting in *de novo* bud formation, especially under an exogenous supply of BA (Zaffari et al. 2000; Venkatachalam et al. 2006).

2.1.4. Partial Immersion Systems

For *in vitro* propagation of plants, partial immersion bioreactor systems (PIS) have shown higher advantages compared to semi-solid and liquid medium, in terms of faster multiplication/elongation of shoots. Such improved results were linked to the situation offered by PIS where the latter combine the advantages of both gelled and liquid medium. These advantages are due to intermittent aeration and higher availability of nutrients in liquid. A low-cost Growtek^{TM} bioreactor has been designed and commercialized with unique features like floating and rotating ex-plant holder with perforated ex-plant support and a side tube for medium changing, culture feeding and content monitoring. The usefulness of Growtek^{TM} in terms of enhanced multiplication rates, reduced bioreactor costs, saving on incubation time, the minimization of contamination and plantlet transfer without root injury has been well documented (Dey 2005). Therefore, a set of experiments were designed combining the effects of chemical parameters such as growth regulators and PAs on shoot/embryogenic cultures of banana under normal conditions using solid and liquid medium and compared with the similarly cultured shoots in Growtek^{TM} bioreactors. The effects of these treatments were basically analyzed by checking the morphogenic events of organization of pro-embryogenic masses (PEMs) and further formation of shoot cultures and their performances until they were transferred to soil. In addition, at different stages of experiments, the effects of exogenous PAs were also monitored by analyzing the pools of endogenous PAs.

2.1.5. Genetic analysis of regenerated, micro-propagated and long-term *in vitro* cultured plants

Analyses of isozyme patterns of specific enzymes provide a convenient method for detection of genetic changes, but they are subjected to ontogenic variations. Such methods have restricted applications due to their limited numbers, and only those DNA regions coding for soluble proteins can be sampled. Tissue- and environment-independence in expression of DNA-based markers have made them more reliable over morphological and isozyme markers. Among various DNA-based markers, though Restriction Fragment Length Polymorphism (RFLP) is applicable for screening genetic stability (Valles et al. 1993), the method involves the use of expensive enzymes and hazardous radioactive labeling. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers, on the other hand, require only small samples of DNA without involving radioactivity tests, and are simpler as well as faster than RFLP. In addition, RAPD (Williams et al. 1990) has been proven to be efficient in detecting genetic variations, even in closely related organisms, such as two near isogenic lines (NIL) (Martin et al. 1991). ISSR technique is found to be highly discriminative and reliable (Reddy et al. 2002), and are found highly economical because a large number of samples can be analyzed quickly. Besides, the specific DNA fingerprints obtained are independent of ontogenic expression, and most of the genome can be sampled with an unlimited number of potential markers. Therefore, both RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plant species (Martins et al. 2004; Carvalho et al. 2004; Ramage et al. 2004; Martin et al. 2006).

2.2. MATERIALS AND METHODS

2.2.1. Regeneration

2.2.1.1. Plant material

The rhizomes of sword suckers (Figure 2.1) of *cv.* NR were used to excise the meristem and establish shoot cultures as explained earlier (Bhagyalakshmi and Singh 1995).

2.2.1.2. Culture media and incubation conditions

The aseptic shoot cultures were established according to the method of Bhagyalakshmi and Singh (1995) and maintained on M1 medium containing three–quarter–strength Murashige and Skoog (MS) (1962) basal salts with additional 1g L^{-1} of potassium nitrate and 1 ml l^{-1} of vitamin mixture (Novak et al. 1989) supplemented with ascorbic acid (100 mg l^{-1}), benzyladenine (BA) (4.48 µM), indole butyric acid (IBA, 0.984 µM) and Gelrite® (2.5 g l^{-1}). The pH was adjusted to 5.8 before autoclaving at 105 kPa for 20 min. Cultures were maintained at 25 ± 1°C under a 16 h light (320 µmol m^{-2} S^{-1}) / 8 h dark photoperiod.

2.2.1.3. Liquid medium treatment

Shoot cultures grown on M1 medium were transferred to similar medium (as above) with a higher level of BA (22.4 µM) and without Gelrite® (M1 liquid). Keeping the light and temperature regime unchanged, the shoot cultures in liquid medium were maintained on a gyratory shaker at 90 rpm for 4 wk. Here the sheath explants, i.e., the lower stalk portions of the expanded leaf, became succulent. Leaf sheaths were excised carefully, making sure that any bud or part of the rhizome was not adhering to the explant. The sheathing leaf bases were cut transversely to obtain semicircular pieces of 2-3 mm thickness (Figure 2.1).

2.2.1.4. Effect of light and growth regulator

Initially, four types of explants (Figure 2.2), two each (leaf sheath explant and corm explant) from liquid and solid medium (stage 6 of figure 2.1) were grown in M1 solid medium with different growth regulators as follows: BA (4.48 to 44.8 µM) with one of the auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D, 3.91 to 19.55 µM),

napthalene acetic acid (NAA, 5.37 to 53.7 µM), 2,4-5- trichlorophenoxy acetic acid (2,4-5-T, 3.91 to 19.55 µM), IBA indole-3-butyric acid (IBA, 0.198 to 9.84 µM) and picloram (0.83 - 20.71 µM).

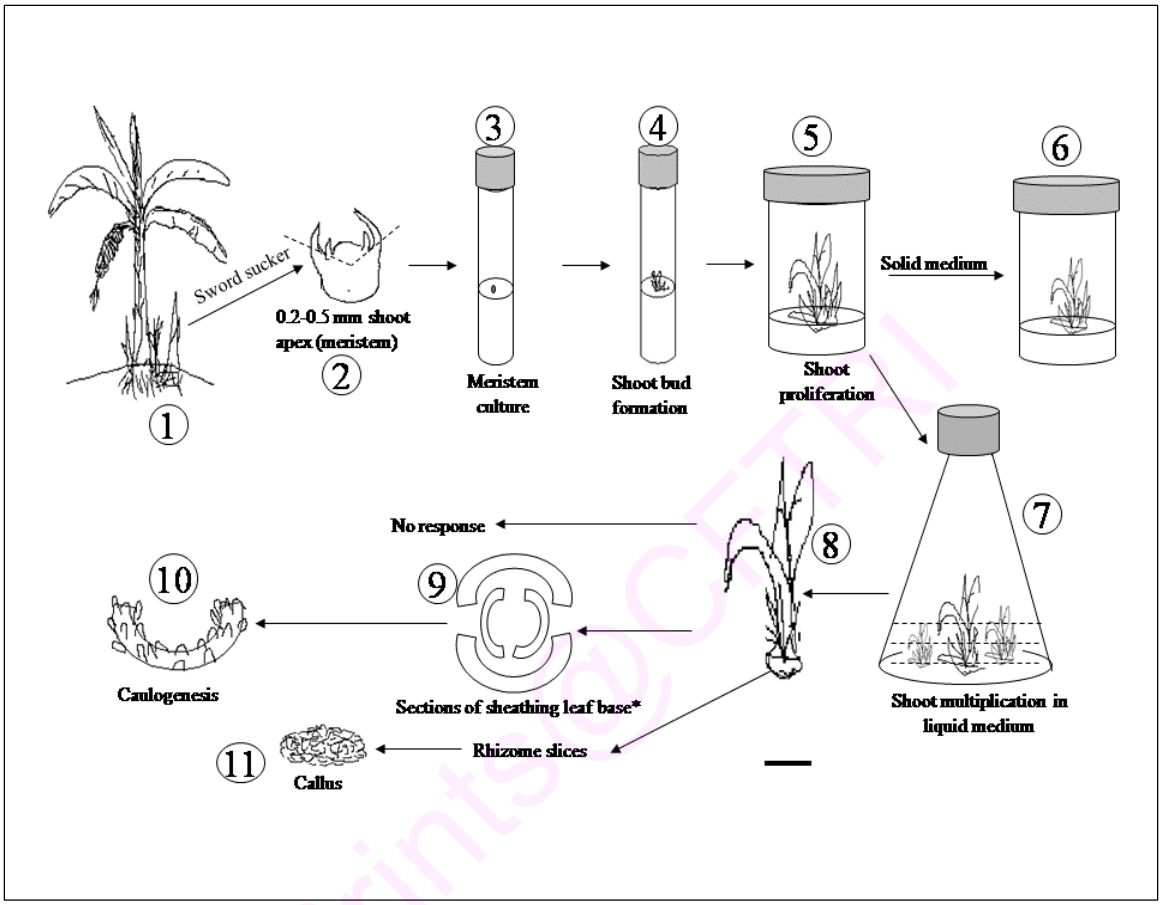

Figure 2.1. Flow diagram of the procedure applied for Banana explant preparation. (1). Mother plant of *cv.* NR with sword sucker from which shoot tip was isolated (bar = 50 cm). (2). Shoot bud showing the meristem (0.2-0.5 mm) that was excised and used for inoculation. (3). Meristem inoculated to M1 medium with BA (4.48 µM) plus IBA (0.198 µM). (4). Formation of shoot bud from meristem explant on M1 medium as in stage 3. (5). Further proliferation of shoot on solid multiplication medium M1 + BA (22.4 µM). (6). Continuous maintenance of proliferating shoots on solid medium as in stage 5. (7). Shoots of stage 5 separated and individually transferred to liquid medium of similar composition as in stage 5 and incubated on gyratory shaker with 320 µmol.m^{-2}.S^{-1}of 16 h photoperiod. (8). Shoot grown in liquid medium showing rhizome with preconditioned shoot and succulent leaf bases that are used for direct shoot induction (bar = 1 cm). (9). Cross sections of leaf base explant having potential for shoot regeneration. (10). Leaf base explant cultured on M1 showing direct regeneration of shoot buds (bar =0.1cm). (11). Rhizome slices resulting in callus formation.

Each type of explant had 10 replicates, and replicated were divided into two groups (i.e. 5 per treatment). One group was incubated in complete darkness and the other in 16 h (320 µmol m^{-2} S^{-1}) light / 8 h dark photoperiod, both maintained at 25 ± 1.0°C. After 3 wk, the explants enlarged and turned dark. Each such explant was cut into pieces and transferred to fresh medium of either the same combination or different media with different auxin-cytokinin combinations and divided into three sets (Figure 2.2). One set was incubated in continuous darkness, the second set in continuous light (320 µmol m^{-2} S^{-1}) and the third set under the above photoperiod, all at 25±1.0°C, for 4 wk. Further culture on different media was done as indicated in Figure 2.2 and Table 2.1.

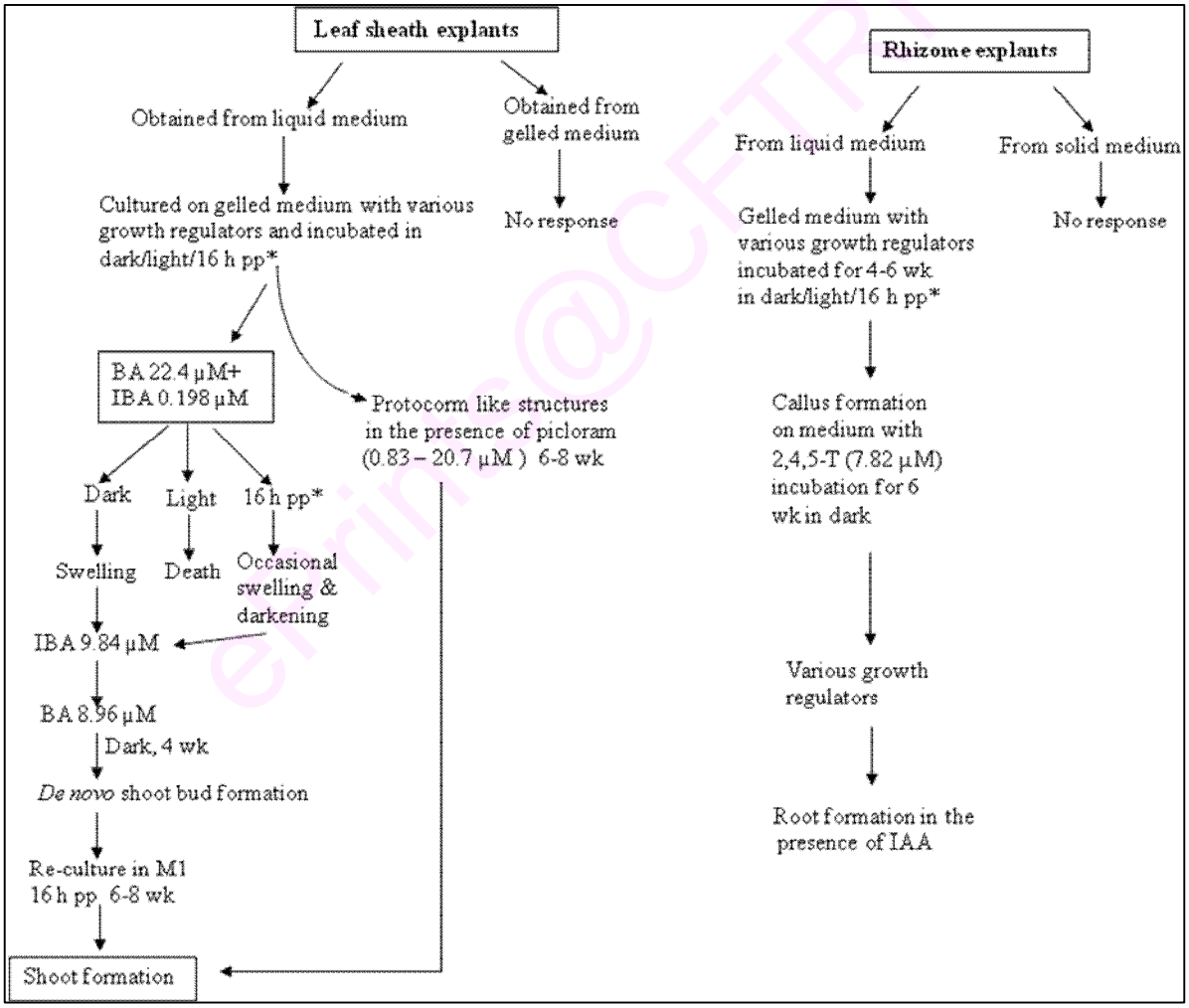

Figure 2.2. The pattern of response of different explants that were pre-treated with different sets of physicochemical conditions. All treatments had M1 as the basal nutrient medium (see text) and incubation temperature of 25 ± 1.0°C. * pp: photoperiod

2.2.1.5. Effect of physical form of the medium

This was studied by culturing the shoots regenerated from leaf explants. Three sets, each with six shoots of approximately 1cm height, were cultured on MS solid, MS liquid on a gyratory shaker (90 rpm) and static MS liquid medium with brief manual agitation (twice-a-day). All the media contained 0 to 22.4 µM of BA and 0.198 µM of IBA as growth regulators. The cultures were incubated under 16 h photoperiod at constant temperature of 25 ± 1.0^{o}C for 4 wk. Data on shoot multiplication and height of individual shoots were recorded, and the percent of *ex vitro* survival was determined 2 months after planting (Bhagyalakshmi and Singh 1995).

2.2.1.6. Polyamine treatment

Concentrations of 10, 20, 30, 40 and 50 µM of each PA *viz*., Spm, Spd and Put (Sigma, St. Louis, Missouri) were tested by adding to control (M2) medium**.** These concentrations were selected based on an earlier study of Laine et al (1988). Culture segments, of uniform size (Ø = 0.5 cm) having embryogenic mass, rhizome with callus and shoot clusters with shoot buds varying in length from 0.2-0.5 cm were selected and cultured in M2 with or without PAs. For each treatment five segments were transferred to each bottle (10 cm in height and 6 cm in diameter) × 5. The observations on growth and morphological changes were recorded periodically, after which the cultures were transferred to fresh respective medium by vertically splitting the culture into three pieces. Three such successive subcultures were made, after which the final morphological observations and endogenous levels of PAs were recorded. Inhibitors of PA biosynthesis (DFMA and DFMO, Sigma, USA) were added (membrane-filtered) to the control medium M2 and to PA-containing medium at concentrations ranging from 0.1 to 0.5mM. Two independent experiments were performed, using at least 5 replicate samples in each and the data obtained were averaged.

2.2.1.7. Extraction and analysis of free PAs

The extraction of PAs and HPLC analyses were conducted according to the method of Flores and Galston (1982) and authentic standards of Put, Spd and Spm (Sigma, USA) were benzoylated following the procedure described by Flores and Galston (1982). Free PAs were extracted by homogenizing the plant materials from two culture

samples (each 100mg of tissue) in 1ml of 5% ice-cold perchloric acid using a pestle and mortar. The homogenate was then centrifuged for 30min at 20,000 ×g. Free PAs in the supernatant were benzoylated as Flores and Golston (1982) and determined using an HPLC (Shimadzu LC6A, Tokyo, Japan). The elution system consisted of MeOH/H_2O (64:36) solvent, running isocratically with a flow rate of 1.0 ml/min. The benzoylated PAs were eluted through a C_{18} column (300 × 4.6mm i.d., with pore size of 5µm), an SLC-6A system controller, and a CR4A data processor was used. Compound detection was done through a UV detector SPD-AV set at a sensitivity of 0.04 AUFC and absorbance at 254 nm. A relative calibration procedure was used to determine the PAs in the samples, using standard of Put, Spd and Spm. Peak areas and retention times were measured by comparing with standard PAs: Put, Spd and Spm. Results were expressed as nanomoles per gram of fresh weight (mean ± SE). Extractions from five different samples per treatment were made independently and each extract was quantified in duplicate.

2.2.1.8. Histological analysis

Portions of leaf sheaths that responded showing the formation of shoot primordia were fixed in FAA (formaldehyde: acetic acid: ethyl alcohol) in the ratio of 5:35:60 for 24 h and then preserved in 70 % alcohol. The fixed tissues were dehydrated in distilled ethanol series (80, 85, 90, 95, 100 %) with three changes per day in each ethanol grade. After dehydration, the ethanol was gradually replaced with xylene, infiltrated in paraffin under 60^oC until the xylene totally evaporated, and were then embedded in paraffin blocks using standard histological procedures. Serial sections of 12 to 15 µm thicknesses were cut using a microtome and mounted onto slides. The de-paraffinized sections were rehydrated and stained with 1 % haematoxylin in propionic acid, dehydrated and slides were prepared by following standard histochemical procedures (Johanson 1940).

2.2.2. Micro-propagation

2.2.2.1. Plant material

The rhizomes of sword suckers of an endangered dessert banana *cv*. NR were used to excise the meristem and establish shoot cultures as described earlier (Bhagyalakshmi and Singh 1995). The mother plant was maintained at the backyard of PCBT department.

2.2.2.2. Culture medium and incubation conditions

The aseptic shoot cultures used in the present study were established as reported earlier (Bhagyalakshmi and Singh 1995), and were maintained on M1 medium based on ¾ strength of MS basal salt mixtures (Murashige and Skoog 1962) with additional 1000.0 mg l^{-1} KNO_3 and 1.0 ml l^{-1} vitamin mixture (Novak et al. 1989) supplemented with 100 mg l^{-1} ascorbic acid, 1.0 mg l^{-1} BA, 0.2 mg l^{-1} IBA, 30 g l^{-1} sucrose and 2.5 g l^{-1} gelrite® (Sigma, USA). The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min. Cultures were maintained at 25 ± 1°C under a 16-h photoperiod (approximately 320 µmol m^{-2} s^{-1} PFD) provided by incandescent lamps (Philips, New Delhi). The *in vitro* multiple shoot cultures were established and maintained on M2 medium based on MS medium supplemented with 1238 mg l^{-1} NH_4NO_3, 2.0 mg l^{-1} BA, 1.0 mg l^{-1} Kinetin, 80 mg l^{-1} adenine sulphate, 25 g l^{-1} sucrose and 2.5 g l^{-1} gelrite®.

2.2.2.3. Growth regulator treatments

In order to improve the rate of shoot multiplication, the growth regulators (cytokinins) in M2 medium were progressively increased to a maximum concentration of 10 mg l^{-1} (in addition to that present in M2), and their effects on both morphology and the genome were studied. The cultures maintained on M2 medium were treated as controls. Various concentrations (0-10 mg l^{-1}) of either BA or kinetin were tested in M2 medium. Culture segments, each having an average of about 4-6 multiple shoot buds of uniform size (ϕ 0.5 cm), were selected, and five such segments were transferred to each bottle (10 cm in height and 6 cm in diameter). The observations on growth and morphological changes were recorded at every 4^{th} week, after which the cultures were transferred to fresh respective medium by vertically splitting the culture into three pieces. Three such successive subcultures were made after which the final morphological observations were recorded. The shoots from each of such treatments were taken for template DNA preparation followed by RAPD and ISSR analyses.

2.2.2.4. Shoot proliferation and rooting

The multiple shoots/ buds obtained from each segment were separated and sub-cultured twice in liquid medium M3 (MS medium supplemented with 1238 mg l^{-1} NH_4NO_3, 0.5 mg l^{-1} NAA and 25 g l^{-1} sucrose) at an interval of four weeks. The liquid

cultures were grown under standard conditions of light and temperature on an orbital shaker at 90 rpm for a period of three weeks. Here the shoot buds grew further proliferating to various degrees. Individual shoots (4-5 cm in length) were separated and cultured on M4 medium containing ½ strength of MS basal salts with 20 g l^{-1} sucrose and 16 g l^{-1} agar (Hi-Media, Mumbai) for four weeks to induce further shoot proliferation and root formation.

Table 2.1. Composition of media used at various stages of micro-propagation of NR

Medium for	Composition with growth regulators	Period of incubation
Establishment and maintenance of stock cultures **(M1)**	¾ strength MS basal salts (MS) with 1 g l^{-1} of additional potassium nitrate + 1 mL l^{-1} of vitamin mixture* + ascorbic acid (100 mgl^{-1}), BAP (4.48 µM), IBA (0.98 µM) and gelrite® (2.5 gl^{-1}) and 3% sucrose.	4 sub cultures at 4 wks interval
Shoot multiplication **(M2)**	MS** + BAP (8.96 µM) + Kn (4.65 µM) + AS (216.80 µM) + sucrose (2.5%) + gelrite® (0.25%)	3 wks
Shoot elongation root initiation **(M3-A)**	MS** + NAA (2.69 µM) + sucrose (2.5%) (Liquid medium)	3 sub cultures at 3 wks interval
Shoot elongation root initiation **(M3-B)**	MS** + NAA (2.69 µM) + sucrose (2.5%) + agar (0.8%) (solid medium)	3 sub cultures at 3 wks interval
Partial hardening of plantlets **(M4)**	½ MS + sucrose (2%) + agar (0.8%)	3 wks

* Vitamin mixture as indicated by Novak et al. (1989)

** Modified MS where the concentration of ammonium nitrate was reduced to 75% of original MS medium (Murashige and Skoog 1962)

2.2.2.5. Shoot Multiplication and Elongation in Partially Immersed System

For PIS, Growtek™ bioreactor (100×150mm (Φ×h)) (Tarsons, India) with unique features like floating, rotating, non-absorbing ex-plant holder with perforated ex-plant support matrix; side-tube with silicon rubber septum for changing media and online monitoring of medium environment was used (Figure 2.3). Each Growtek™ bioreactor

was filled with 200ml of modified MS medium (M3) (MS medium supplemented with 1238 mg l^{-1} NH_4NO_3, and 25 g l^{-1} sucrose (and for SM M3 gelled with 0.8% Agar) with varying concentration of NAA (5,10,15μM) and GA (15,30,45 and 60 μM). Clusters of shoot buds (shoot bud length 0.5-1cm) obtained from embryogenesis experiment were grown for a short period of 2 weeks on M2 medium and used as initial inoculum. Approximately 25g of explant transferred in to both Growtek™ bioreactor and SM. The Growtek™ culture vessels were maintained at 25±2°C under 16h photoperiod, having illumination of 37.5μE $m^{-2}s^{-1}$on a gyratory shaker set at 80 rpm throughout the culturing period. Each treatment had at least 15 replications.

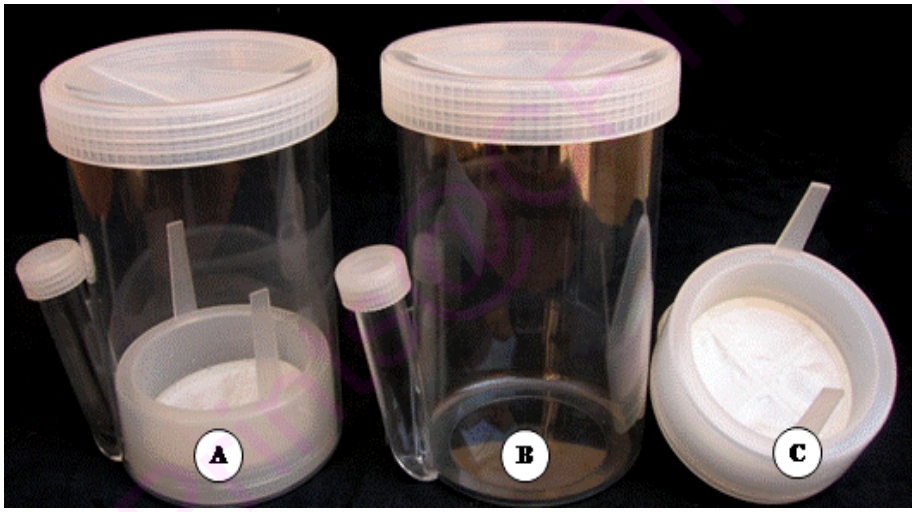

Figure 2.3. Growtek™ bioreactor used for banana shoot multiplication

2.2.2.6. Elongation and rooting

Multiple shoot/buds proliferated from each segment were separated and sub cultured twice with a 4-week interval in liquid medium containing MS basal nutrient with 0.5 mg l^{-1} NAA for further elongation (M3-A in Table 2.1). After 8 weeks, individual shoots with 4-5 cm in length were separated and transferred to solid MS basal medium with 0.5 mg l^{-1} NAA (M3-B) for 4 weeks to induce further shoot elongation and root formation. Further hardening before transferring to soil was done by briefly culturing the plantlets in M4 medium (Table 2.1).

2.2.2.7. *Ex vitro* morphological characterization

The rooted shoots were washed in tap water and planted in polythene bags containing a mixture of sand, vermin-compost and garden soil in equal proportion. The plants were hardened in a moist chamber maintained at 70-80% RH for 3-4 weeks, and subsequently maintained in a greenhouse for one month before planting in the field. Data on percentage of survival during hardening, girth of the pseudo stem, number of leaves and plant height were measured against control plants (grown on M2).

2.2.2.8. Greenhouse transfer and morphological analysis

The rooted shoots were washed in running tap water and were planted in polythene bags containing mixture of sand, vermin-compost and garden soil in equal proportions. The plants were incubated in a mist chamber maintained at 70-80% RH for 3-4 weeks and subsequently maintained in greenhouse for one month before planting in the field.

2.2.3. Genetic analyses

2.2.3.1. Preparation of template DNA from regenerated plants

A large number of plantlets were regenerated from leaf base explants, of which nine plants were randomly selected and another set of eleven plants showing morphological variations such as variegated and abnormal leaves were also included for genetic analyses. The DNA was extracted by using the GenElute^{TM} Plant Genomic DNA Mini prep kit (Sigma, USA). The RNA contamination in all the samples was removed by digesting the extract with 1U RNase-A (Bangalore Genei, Bangalore) for 30 min at 37°C. The quality and quantity of DNA preparations were checked by standard spectrophotometry, and the samples were diluted to a concentration of 25 ng μl^{-1} before use.

2.2.3.2. Preparation of template DNA from micro-propagated plants

For both RAPD and ISSR analyses, leaves from five acclimatized treatment-derived plants were used. In each treatment, the number of plants was reduced to a pool of 5 plants, and 100 mg of young leaves from each plant were used to create the pool. These leaves were frozen in liquid nitrogen and stored at -80°C. The DNA was extracted by using the GenElute^{TM} Plant Genomic DNA Mini prep kit (Sigma, USA). The RNA contamination in all the samples was removed by digesting the extract with 1U RNase-A

(Bangalore Genei, Bangalore) for 30 min at 37°C. The quality and quantity of DNA preparations were checked by standard spectrophotometry, and the samples were diluted to a concentration of 25 ng μl^{-1} before use.

2.2.3.3. Preparation of template DNA from long-term micro-propagated plants

The mother NR plant collected from Nanjanagudu, Karnataka, India was maintained in the inventor's departmental garden and the fresh leaves from this were used as a source of DNA. For testing the long-term effects, the plantlets obtained after repeatedly sub-culturing in M2 medium for 150 times (about 10 years), each after 4 weeks period, were used. Eleven plantlets, including certain morphological variants were chosen from over 4000 micropropagated plantlets for studying DNA fingerprints. The DNA was extracted and examined for quality and quantity as mentioned above.

2.2.3.4. Primer selection and reproducibility

The RAPD primers were selected based on proven results of earlier studies on banana where the variability between and within the genome were distinctly differentiated (Pillay et al. 2001; Ude et al. 2003; Onguso et al. 2004). ISSR primers were designed based on the reported sequences of banana genome. Altogether, 70 RAPD and 25 ISSR primers were selected for screening, and the reproducibility of the PCR amplification was assessed using selected primers with different DNA samples isolated independently from the control cultures and amplified at different times.

2.2.3.5. RAPD analyses

Initially, optimum PCR conditions for both RAPD and ISSR were standardized with various quantities of template DNA (12.5, 25 and 50 ng), dNTPs (100, 200 and 300µM) and $MgCl_2$ (0, 1, 2 and 3 mM). Later, RAPD amplifications were performed routinely using a PCR mixture (25 µl) containing 25 ng of genomic DNA, 1× PCR buffer (Fermentas GmbH, St. Leon-Rot), 200 µM dNTPs (Fermentas GmbH, St. Leon-Rot), 1 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore), 1 µM of each primer (Sigma, St. Louis, Missouri) and various concentrations of $MgCl_2$ (Fermentas GmbH, St. Leon-Rot) depending on the primer. The PCR was performed at an initial denaturation at 93°C for 4 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 36°C and 2

min extension at 72°C with a final extension at 72°C for 10 min using a thermal cycler (Eppendorf, Hamburg).

2.2.3.6. ISSR analyses

In case of ISSR primers, optimal annealing temperature was found to vary according to the base composition of the primers. ISSR amplification was performed using a PCR mixture (25 µl) containing 25 ng of genomic DNA, 1× PCR buffer (Fermentas GmbH, St. Leon-Rot), 200 µM dNTPs (Fermentas GmbH, St. Leon-Rot), 1 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore), 1µM of each primer and various concentrations of $MgCl_2$ depending on the primer. The PCR was performed at an initial denaturation at 94°C for 4 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min at 2°C lower than specified annealing temperature for each primer and 2 min extension at 72°C with a final extension at 72°C for 10 min using a thermal cycler (Eppendorf, Germany) The PCR products were separated on 2% agarose (ICN, California) gel stained with ethidium bromide (0.001%). The number of bands was recorded using a gel documentation system (Herolab GmbH Laborgerate, Wiesloch). The size of the amplification products was estimated using a 100 bp DNA ladder (Fermentas GmbH, St. Leon-Rot). The band intensity histogram of each gel was checked using the Easy Win 32 software (Herolab GmbH Laborgerate, Germany).

2.2.3.7. Statistical and marker analyses

For both RAPD and ISSR analyses, leaves from three acclimatized treatment-derived plants were used. Well-resolved and consistently reproducible fragments ranging from 200 bp to 3.1 kb were scored either as present or absent for each growth regulator treatment using the software Easy Win 32 (Herolab GmbH Laborgerate, Germany). For detecting any genetic change, all the RAPD and ISSR results were compared with each other and also with the control.

2.2.4. Genetic transformation

2.2.4.1. Plant material and culture method

The banana *cv*. NR, was used in the present study and was finger-printed for its genetic difference from other dessert clones of South India (Venkatachalam et al. 2008). The aseptic shoot cultures were multiplied *in vitro* (Venkatachalam et al. 2007b) on M1

medium. Cultures were maintained at 25 ± 1°C under a 16-h photoperiod (approximately 320 µlmol m^{-2} s^{-1} PFD) provided by incandescent lamps (Philips, New Delhi). The *in vitro* multiple shoot cultures were established and maintained on M2 medium. Using leaf explants from shoot cultures grown in M2 liquid medium, shoot buds were regenerated using an earlier protocol (Venkatachalam et al. 2007b). From this, culture segments of uniform size (Ø = 0.5cm) having embryogenic mass and shoot buds varying in length from 0.2-0.5cm were selected and used for sonication-assisted transformation.

2.2.4.2. Establishment of Hygromycin sensitivity of banana cultures

Since transformation involves selection using antibiotics as selectable markers, the sensitivity of shoot bud cultures of NR was ascertained beforehand. For this, sets of about 100 in vitro shoot buds (0.5-0.7cm) were cultured in MS solid medium containing filter sterilized Kanamycin (Duchefa, The Netherlands) at concentrations of 50, 100, 200, 300, 400 mg l^{-1} and hygromycin (Duchefa, The Netherlands) at 2, 4, 6, 8 and 10 mg l^{-1}. The culture sets were continuously monitored for survival of buds inferred by shoot growth. Observations were recorded at the end of third and sixth week, and data expressed as percent survival.

2.2.4.3. *Agrobacterium* culture, plasmid and construction

The *Agrobacterium* strains used to infect banana explants are: (i) a wild type *A. rhizogenes* strain 15834 grown on a solid YEB medium (Vervliet et al. 1975) without antibiotics in the dark at 28°C, (ii) *Agrobacterium rhizogenes* agropine type wild strain A4 (kindly gifted by Dr. Juan B Perez, Spain) containing the binary vector pCAMBIA 1301 containing selectable marker gene hygromycin phosphotransferase (*hpt*II) under the control of the double strength CaMV 35S promoter and CaMV 35S terminator; β-glucuronidase (*uid*A) as reporter gene with a catalase intron under the control of CaMV 35S promoter and NOS terminator (Figure 2.4), and (iii) *A. tumefaciens* strain GV 3101 harboring constructs pPCV002-ABC (kindly donated by Prof. Thomas Schmulling, Freie Universitat Berlin, Angewandte Genetik, Albrecht-Thaer-Weg 6, 14195 Berlin) called *A. tumefaciens rol* ABC with *npt II* gene (Figure 2.4). The second and third strains were maintained on solid YEB medium supplemented with 100 mg l^{-1} carbenicillin and 25 mg l^{-1} kanamycin in the dark at 28°C. Strains of *Agrobacterium rhizogenes* harbouring the binary vector was initially streaked and incubated on solid LB medium supplemented

with 100 mg l^{-1} kanamycin at 26 ± 1°C for 48 h to obtain single colonies. A single colony of this A*grobacterium* was incubated in 10 ml of liquid LB medium containing the same antibiotic at the same concentration at 26 ± 1°C with shaking at 250 rpm for 24 h.

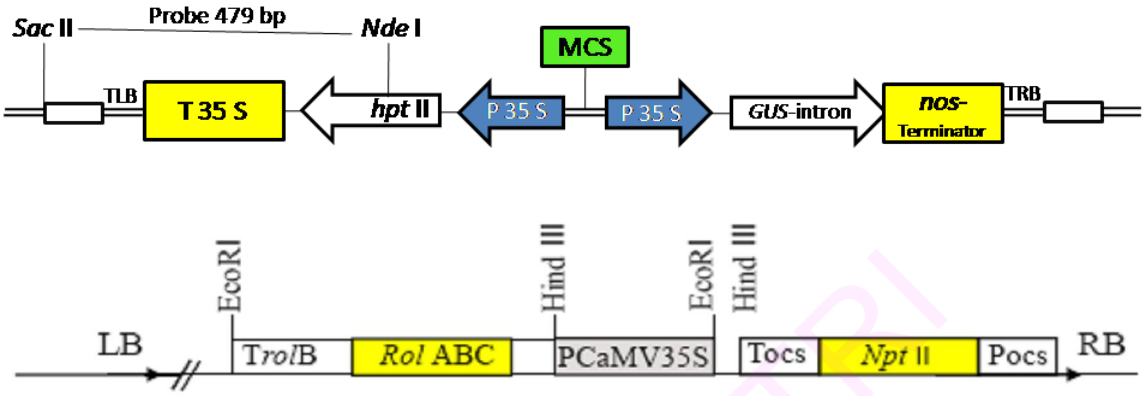

Figure 2.4. Schematic representation of T-DNA region of binary vector pCAMBIA1301 and pPCV002-ABC. LB and RB, left and right borders; p35S and t35S, cauliflower mosaic virus promoter and terminator, respectively; tnos, terminator of nopaline synthase gene

The bacterial culture was then diluted five folds with antibiotic-free LB medium and placed back on the shaker for 3-4 h at 250 rpm and 26±1°C. Finally, the cultured *Agrobacterium* cells were harvested by centrifugation at 4000 rpm for 5 min, re-suspended in infection medium comprising 100 µM acetosyringone (Sigma, USA) and used for co-cultivation.

2.2.4.4. Genetic transformation

Sonication-assisted *Agrobacterium*-mediated transformation (Santarem et al. 1998) was adopted for transformation. Culture segments, of uniform size (0.5cm diameter) having embryogenic mass, rhizome with callus and shoot clusters with shoot buds varying in length from 0.2-0.5 cm were selected and used for co-cultivation. This culture segments and *Agrobacterium* strains were suspended in 10 ml infection medium (identical to that for semi-solid M2 medium except 2.5 g l^{-1} gelrite) in 30 ml Oakridge tubes and ultrasonicated (Bandelin Sonoplus ultrasonicator, Germany) for 30 s at 80% amplitude and then incubated on a gyratory shaker set at 120 rpm for 2 h. The time and frequency of sonication was standardized during a series of preliminary experiments. The

explants were transferred aseptically to petri plate lined with sterile blotters to remove excess bacterial cells adhering to the material surface and co-cultivated on M2 medium of pH 5.6 containing 100 µM acetosyringone, 0.8% agar (for solidification) for 2 days. Subsequently, the explants were washed four times with sterile water where the final wash included 500 mg l^{-1} cefotaxime (Alkem Laboratories, Mumbai, India). The explants were blot dried and cultured on secondary embryogenic selection medium having M2 components, to which 6.0 mg l^{-1} filter-sterilized Hygromycin had been added to assist selection of transformants. Cefotaxime was used along with Augmentin (Glaxo, India), each 100 mg l^{-1} were also present in the medium for the eradication of *A. rhizogenes*.

Sporadic reports indicate that banana meristematic tissues transformed by *Agrobacterium* would lead to regeneration of chimaeric primary plants because the transformation would have occurred in few cells of the bud and the whole bud survives under selection pressure by antibiotic. To eliminate such chimaeric plantlets and to obtain complete transformants, sequentially increasing concentrations of antibiotic hygromycin from 5 to 20mg l^{-1} was used in the fresh shoot multiplication medium and the clusters of buds were isolated by splitting before they are sub-cultured each time. For this, after every 2 weeks, the surviving explants were transferred to fresh medium having sequentially higher concentrations of hygromycin from 10 mg l^{-1}, 15 mg l^{-1} to 20 mg l^{-1}, for a total period of 12 weeks, so that the buds developing from untransformed cells/tissues would be eliminated.

2.2.4.5. PCR analysis

The polymerase chain reaction (PCR) was used to detect the Ri T-DNA integration into the plant genome. The bacteria-free explants grown in M2 medium were isolated, rinsed with sterile distilled water, quickly frozen in liquid nitrogen and powdered. Genomic DNA from putative transformants and normal plant was extracted using GenElute^{TM} Plant Genomic DNA Mini prep kit (Sigma, St. Louis, USA). The RNA contamination in all the samples was removed by digesting the extract with 10 µg of RNase-A for 30 min at 37^{o}C. PCR was performed using rolA gene-specific primer (forward 5' AGA ATG GAA TTA GCC GGA CTA 3' and reverse 5'GTA TTA ATC CCG TAG GTT TGT TT 3') for first transformation and *NPTII* (forward 5' TAT TCG GCTATG ACT GGC A 3' and reverse 5' GCC AAC GCT ATGTCC TGA T 3') (Sigma-

Aldrich, Bangalore, India) for *Agrobacterium* harbouring the binary vector mediated transformation. The PCR mixture (25 µl) contained 50 ng of DNA as the template, 1× PCR buffer, 25 pmoles of each primer, 2.5 mM of dNTPs and 1 U of Taq DNA polymerase. PCR was carried out by amplifying at an initial denaturation temperature of 94^{o}C for 5 min followed by 35 cycles each of 1 min denaturation at 94^{o}C, 1 min annealing at 55^{o}C and 1 min extension at 72^{o}C with a final extension of 72^{o}C for 10 min using a thermal cycler. The PCR products were separated on a 1.5% agarose gel, visualized by staining with ethidium bromide and documented using a gel documentation system.

2.2.4.6. PCR-Southern analysis

A probe size of 479-bp *hpt*II gene, obtained from *pCAMBIA 1301*, was prepared after purification using MinElute^{TM} PCR purification kit (Qiagen GmbH, Hilden) and labeled with psoralen biotin. The homology of *hpt*II gene probe used in this experiment has been checked by earlier workers in authors' lab by sequencing. The isolated genomic DNA (approx. 40 µg quantity) from putative transformants was digested with *NdeI* and *SacII* enzymes, for Southern blot analysis. The digested DNA was electrophoresed on 0.8% (w/v) agarose gel, transferred to Biobond plus nylon membrane (Sigma, USA) and hybridised with the 479-bp *hpt*II gene probe using standard protocols (Sambrook et al. 1989). Hybridization (between digested DNA and labeled probe) and detection were done with Ambion Bio detect kit (Ambion Inc, USA) according to the manufacturer's instructions.

2.2.4.6.1. Restriction digestion of vector and banana genomic DNA

The following were added in a micro centrifuge tube in the order stated:

Nuclease-free water	:	13.8 µl
Restriction enzyme 10X buffer	:	2.0 µl
BSA, acetylated (1mg ml^{-1})	:	0.2 µl
DNA	:	3.0 µl
N*de* I or S*ac* II	:	1.0 µl (10 units)
Final volume	:	20.0 µl

Since the enzymes are not compatible the digestion was carried out separately. Initially the DNA was digested with N*de* I, precipitated, resuspended in water and re-digested with S*ac* II. For digestion, the samples were incubated (after brief centrifugation) at 37°C

for 8 h and the reaction was stopped by heating them at 65°C for 2 min. An aliquot of the digested products were fractionated and observed on agarose gel (0.8%).

2.2.4.6.2. Transfer of DNA to nylon membrane and hybridization

Materials

1. Target DNA
2. Primers for probe preparation (Genosys Sigma, USA)
3. Nylon membrane (Sigma, USA)
4. 20X SSC:

NaCl	:	3M
Sodium citrate pH 7.2	:	0.3M

5. Hybridization buffer

SSC	:	5X
N-lauroylsarcosine	:	0.1% (w/v)
SDS	:	0.02% (w/v)

6. Non isotopic (Psoralen biotin) labeling kit (Ambion, USA)
7. Biodetect kit (Ambion, USA)
8. 5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP-NBT) detection solution (Bangalore Genei, India)

The digested DNA samples (digested with restriction enzymes N*de* I and S*ac* II) were loaded onto the agarose gel 0.8% (w/v) and run at 80 V until the dye front reaches ¾ of the gel. After electrophoresis the gel was stained with ethidium bromide for 15 min and observed on the transilluminator. After examination the gel was soaked for 45 min in several volumes of 1.5 M NaCl and 0.5 N NaOH mixtures with constant gentle agitation. The gel was soaked in several volumes of 0.2 N HCl for 10 min and rinsed briefly with triple distilled water. The DNA was neutralized by soaking in 1 M Tris (pH 7.4) and 1.5 M NaCl at room temperature for 30 min with mild agitation. The neutralization solution was changed three times with 15 min. interval.

2.2.4.6.3. Capillary transfer of DNA from agarose gel to nylon membrane

The transfer tank was filled with 75ml of 10XSSC buffer on each side. A Whatman® No. 3 filter was placed on the platform of the tank. The side of the filter paper was dipped into the buffer. Six Whatman® No. 3 sheets and nylon membrane was cut to the exact size of the gel. The nylon membrane was dipped in deionized water and then incubated in 10X SSC for five min. Two Whatman® No. 3 sheets were dipped in 10X SSC and placed in the middle of the platform. The gel was placed on the top of the filter in inverted fashion. The right side of the gel was nicked to serve as an identification mark. Parafilm strips were placed all around the gel to avoid short circuit of buffer during transfer. The nylon membrane was placed with its right side cut over the gel, so that the cut side matches with that of the gel. Remaining four Whatman® No. 3 filter sheets were dipped in 10X SSC and placed over the membrane. Stacks of paper towels were placed over the filter paper and applied a weight of about 500gms over the entire assembly. The transfer process was allowed for 24hrs with intermittent changes of paper towels and transfer buffer.

2.2.4.6.4. Disassembling the blot

The weight, paper towels and Whatman® No. 3 filter paper were removed. The positions of the wells were marked on the membrane with a pencil. The membrane was peeled off carefully and gently submerged it in 6X SSC. After five min. the membrane was blotted with Whatman® No. 3 filter paper. The gel was restained in ethidium bromide to check the transfer efficiency. After allowing the membrane to dry, it was placed inside a polythene bag and placed it over a UV transilluminator for two min. to allow cross-linking of DNA.

2.2.4.6.5. Pre-hybridization

The membrane was put in a hybridization bag to which 15 ml pre-warmed (68°C) hybridization buffer was added and incubated overnight at 68°C

2.2.4.6.6. Preparation of BrightStar Psoralen-Biotin conjugated probe

A clean untreated 96 well plate was placed on an ice bath. Using Quiagen PCR purification Kit (Quiagen) the PCR product of *hpt*II gene was purified. Purified PCR product was denatured by heating to 100°C for 10 min, and then rapidly cooled the

mixture in a dry ice bath to freeze. Subsequent steps were carried out under low light in a dim room. Centrifuged the vial containing the lyophilized BrightStar Psoralen-Biotin for ~15 seconds at 7,000 x g and reconstituted in 33 µl DMF. The frozen sample was thawed by rolling it between gloved hands, and immediately added 1 µl of the BrightStar Psoralen-Biotin to 10 µl of the nucleic acid solution in a microfuge tube. The sample was mixed and transferred to a well in the 96 well plate (on ice). The nucleic acid solution should have a final concentration of 0.5-50 ng per µl. A 365 nm UV light source was held on the plate and irradiated the sample for 45 minutes. The biotin-labeled nucleic acid was stored at –80°C for long-term storage.

2.2.4.6.7. Hybridization

The probe was diluted 10 fold with 10 mM EDTA, denatured by incubating in boiling water bath for 10 min and snap cooling on ice. The denatured probe was added to the hybridization buffer and mixed it immediately. The membrane was incubated with the probe at 68°C for 6 h with mild agitation.

2.2.4.6.8. Post-hybridization washes

The membrane was washed twice in 50 ml of post hybridization washing buffer I for 5 min at room temperature. Membrane was washed again in 50 ml of post hybridization washing buffer II for 15 min at 68°C under mild agitation.

2.2.4.6.9. Detection of hybridization signals

Detection of hybridization signals were done with Ambion Biodetect Kit (Nonisotopic Detection Kit, Ambion, USA) Membrane was rinsed twice for 10 min at room temperature in Ambion wash buffer. The membrane was subsequently incubated in blocking buffer twice for 20 min. Streptavidin-alkaline phosphatase was prepared by gently and thoroughly mixing 10ml blocking buffer and 1µl Streptavidin-alkaline phosphatase (Ambion USA) (It is important to mix with the blocking buffer before adding to membrane) and incubated the membrane for 45 min, followed by washing three times (15 min each time) in 1X Ambion wash buffer. The membrane was immersed in 10 ml BCIP-NBT detection solution (Bangalore Genei, India). For the development of color, the membrane was kept in the dark without shaking overnight. Reaction was stopped and the results were documented by photography.

2.2.4.7. GUS assay

Histochemical assay of GUS localization in samples was conducted according to the reported protocol (Kumar et al. 2006). Briefly, GUS assay was performed by immersing explants for 12 h at 37°C in a GUS assay buffer containing 100 mM sodium phosphate (pH 7.0), 20 mM EDTA, 0.1% triton X- 100, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 20% methanol, and 1 mM X-Gluc (5-bromo 4-chloro indolyl-d-glucuronide cyclo-hexamonium salt, Sigma, USA) (De Block and Debrouwer 1992). Methanol was added to the reaction mixture to suppress endogenous GUS like activity (Kosugi et al. 1990).

2.2.4.8. Experimental design

For transformation experiments, 400-600 cultured segments were inoculated with *A. rhizogenes* and placed on M2 medium; in each experiment and 25 explants each were cultured as a positive and negative control in respective selection and non-selection media. Thirty sets of such experiments were carried out. The transformation efficiency was recorded by counting the number of putative transgenic cultured segments selected after several sub-cultures on medium with 20 mg l^{-1} hygromycin, each sub-culture period ranging from 10 to 12 weeks. All the observations and calculations were made separately for each set of experiments and were expressed as mean ±SD. The significance ($P \leq 0.05$) of the variables studied was assessed by one-way analysis of variance (ANOVA) using Microsoft Excel XP®. The mean separations were performed by Duncan's Multiple Range Test (DMRT) for segregating means where the level of significance was set at $P \leq 0.05$ (Harter 1960)

2.3. RESULTS

2.3.1. Regeneration

2.3.1.1. Response of leaf sheaths

The explants obtained from shoots cultured on solid medium (stage 6 of Figure 2.1) failed to respond on any of the first set of media combinations and light regimes tested. The leaf lamina explants obtained from liquid cultures showed occasional swelling upon incubation in different set of conditions whereas sheath explants produced several shoot primordia (Figures 2.5A and B) when the explants were first incubated in dark on M1 medium with BA (22. 4 μM) for 3 and IBA (9.84 μM) and then transferred to BA-free medium for 2 wk and subsequently cultured for 4 wk in M1 with reduced BA (8.96 μM) keeping the level of IBA (0.198 μM) constant. The histological study indicated that the shoot primordia originated adventitiously from highly dividing cells that normally occurred as patches in leaf sheath explants, particularly on the upper surfaces of leaves not in contact with medium (Figures 2.5 C and D). On the other hand, the leaf sheath explants produced protocorm like bodies, particularly from the margin when incubated for 6-8 wk in dark on medium with picloram (8.29 μM) (Figures 2.6 A and B). Since several combinations of BA and IBA produced adventitious shoot bud formation from the leaf-base explants, this experiment was repeated twice using M1, and keeping other physicochemical conditions unchanged. Data on the extent of shoot bud formation have been summarized in Table 2.2, which clearly shows that BA played a crucial role in inducing shoot buds directly from the leaf explants. However, the presence of IBA enhanced the percentage response toward adventitious shoot bud formation as well as the number of shoot buds per cm^2 of leaf sheath explants.

2.3.1.2. Response of rhizome explants

The rhizome explants produced callus when incubated in dark on M1 supplemented with 2,4,5-T (7.82 μM) (Figure 2.5E). Transferring the rhizome-derived callus to similar fresh medium resulted in the degeneration of the callus. Therefore, in the subsequent experiments, such rhizome callus was transferred to M1 with different combinations of growth regulators similar to those used for leaf sheath explants. The rhizome callus produced only roots on basal medium and MS with IAA (5.71 to 28.55 μM) and failed to show any response to other treatments

Table 2.2. Response towards shoot bud formation in banana leaf sheath explants***

Treatment No.	Treatments		% of explants forming shoot buds	No. of buds per cm^2 (Mean ± SD)
	BA (µM)	IBA (µM)		
Control	0	0	0	0
1	4.48	0	6	2.5 ± 0.50
2	0	4.92	0	0
3	0	9.84	2	0.8 ± 0.20
4	8.96	9.84	8	3.4 ± 1.10
5	13.44	9.84	13	2.4 ± 0.80
6	22.4	9.84	24	4.7 ± 1.60
7	4.48	14.76	9	0.9 ± 0.50
8	8.96	14.76	8	0.5 ± 0.04
9	22.4	14.76	21	2.7 ± 0.90
10	4.48	14.76	2	0.40 ± 0.04

** Pretreated in liquid M1 with BA (22.4 µM) and IBA (0.198 µM) for 4 wk

*Incubated under 16 h photoperiod (illumination of 320 µmol m^{-2} S^{-1}) at 25 ± 2°C

2.3.1.3. Further growth of shoot buds and protocorms

Shoot buds and protocorms from leaf sheath explants, when transferred to M1 basal medium and incubated in light (320 µmol m^{-2} S^{-1}, 16 h /d), turned green and further growth toward shoot formation took place only in the case of shoot buds. Protocorms subjected to the treatment turned dark and grew larger, with occasional multiplication (Figure 2.6 C). These protocorms never grew into shoots in the presence of light but did so when incubated in dark for 6 wk in liquid MS basal medium. Subsequently, in the presence of light, the shoots from protocorms continued to grow further and formed clusters of shoots (Figure 2.6 D). Root formation from protocorm-derived shoots was observed *in vitro* only in the presence of NAA (2.685 to 5.37 µM) or *ex vitro* after reaching a height of 4 cm.

2.3.1.4. Effect of physical form of the medium

Data shown in Table 2.3 clearly indicate the superiority of liquid forms of media over the gelled form for both shoot multiplication and shoot elongation. However, the percentage of survival *ex vitro* was higher among shoots cultured on gelled medium than

those grown in liquid media. In all forms of media, the increase in BA level progressively increased the shoot number and suppressed the shoot height.

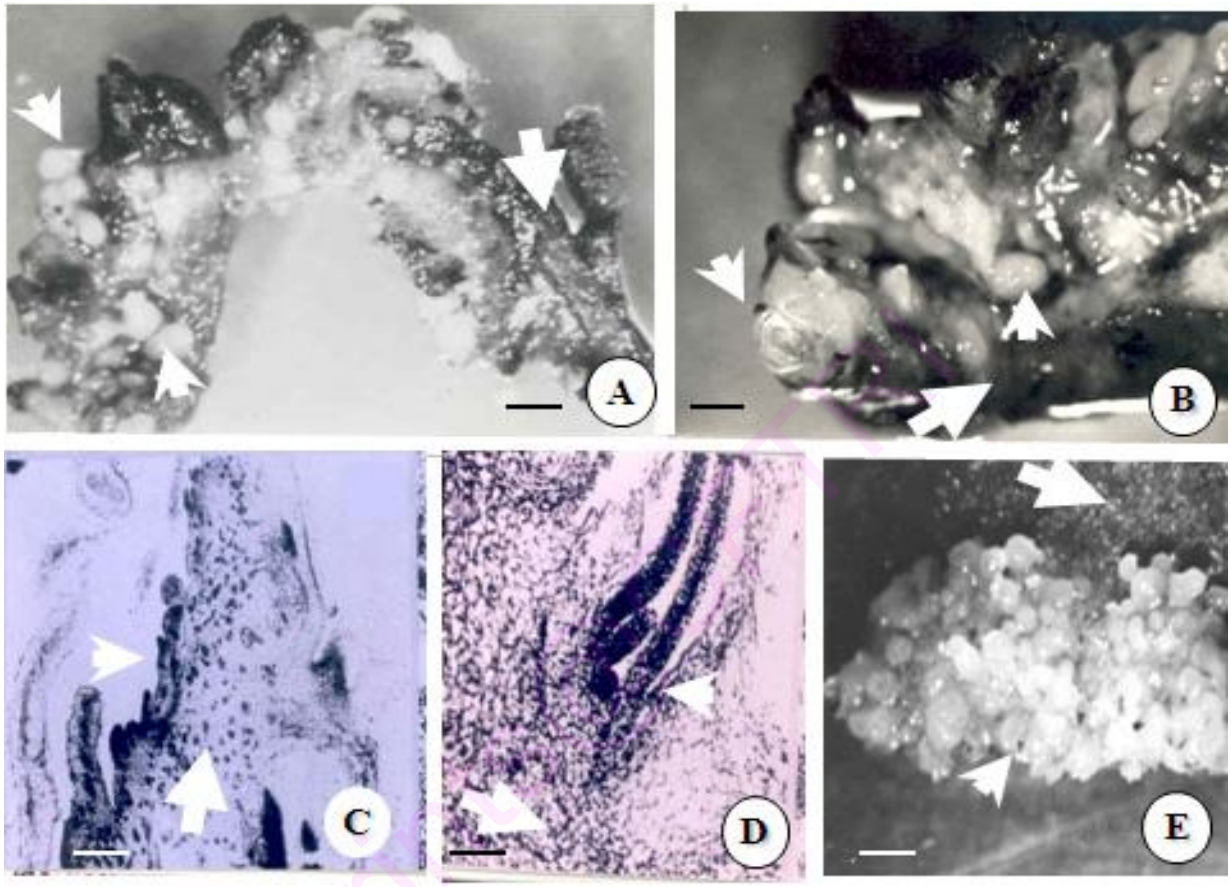

Figure 2.5. Response of banana leaf base and corm explants showing callus/organ formation. (A). Semicircular leaf base explant (obtained as shown in Figure 2.1) showing an array of shoot primordia (short arrow) arising directly from the explant (long arrow) (bar = 0.0245 cm). (B). A portion of the leaf base explant enlarged where organogenesis towards shoot buds (short arrows) is evident from the leaf base explant (long arrow) (bar = 0.0245 cm). (C). Cross section of a leaf base explant showing the adventitious formation of shoot bud from the explant which is callus-like with scattered vascular bundles at the centre and shoot formation mainly from the ventral (inner) surface of the sheathing leaf base explant (bar = 0.0011 cm). (D). A single fully organized shoot bud originating from the leaf base (long arrow) explant (bar = 0.0011cm). (E). Corm explant (long arrow) showing globular callus mass (short arrow) on medium with picloram (8.28 µM). (bar = 0.0245 cm)

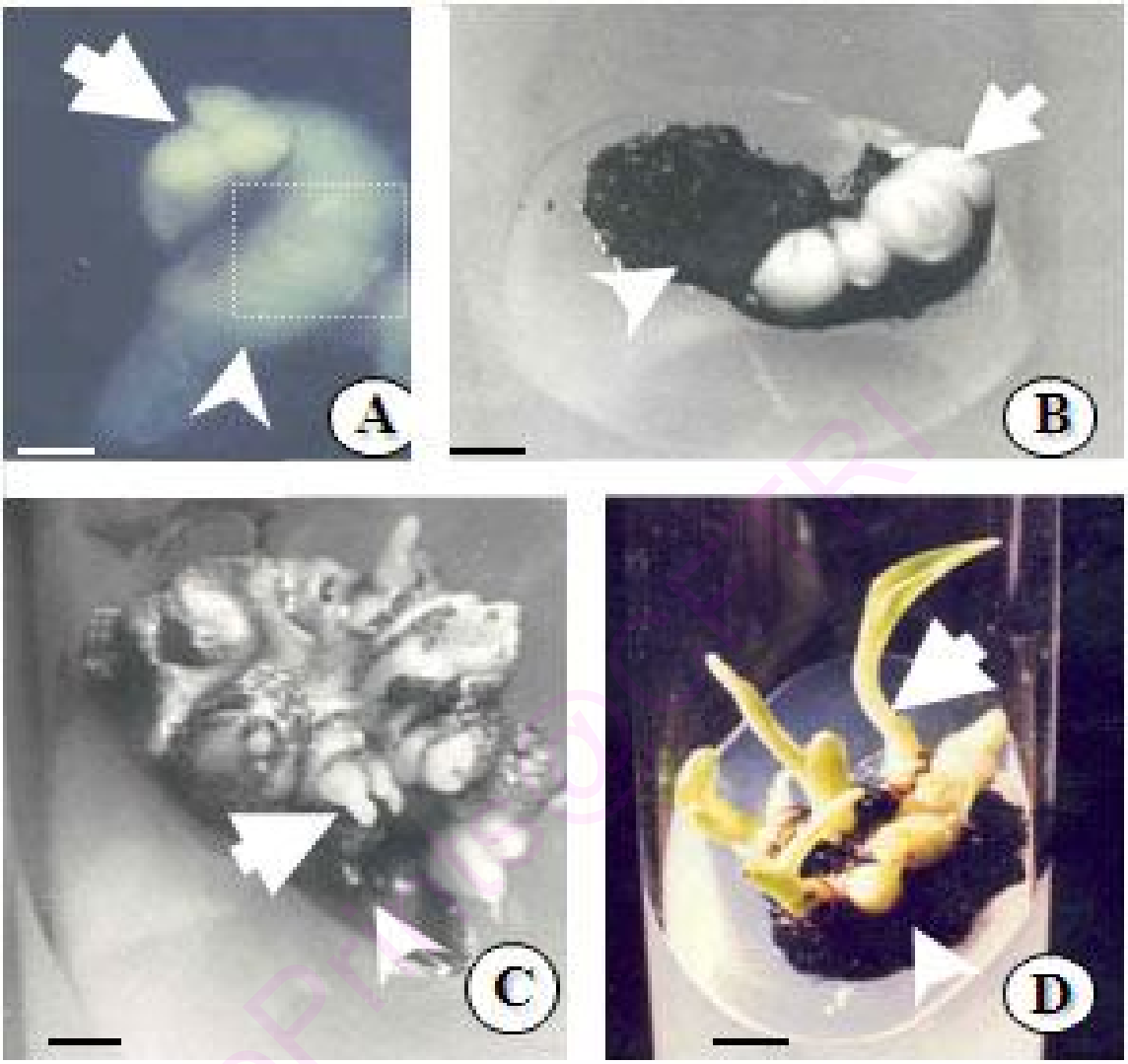

Figure 2.6. Formation of protocorm-like bodies from the leaf base explants. (A). Curled leaf explant showing the adventitious origin of protocorm-like structures (short arrow) and emerging protocorms (long arrow). White patches on the explant in the dotted box are putative organogenic areas (bar = 0.25cm). (B). Degeneration of explant (short arrow) with concomitant further growth of protocorm, like bodies organizing in to hard globular cormlets with shoot primordia (long arrows) (bar = 0.25 cm). (C). Several protocorms (long arrow) from the ventral surface of leaf sheath explant (shoot arrow) (bar = 0.50 cm). (D). Further growth of cormlets into shoots (long arrow) where the leaf base explant (short arrow) undergoes complete degeneration (bar = 0.75 cm).

Table 2.3. Performance of banana shoots regenerated from leaf explants as compared with those from meristem explants

	Meristem-derived				Leaf generated			
BA Conc.	Gelled medium	Agitated liquid	Static liquid	Mean	Gelled medium	Agitated liquid	Static liquid	Mean
	Shoot number							
0	1.2	1.5	1.0	1.2^{a}	1.3	1.5	1.5	1.2^{a}
4.48	2.2	2.5	3.0	2.6^{ab}	2.5	3.0	2.2	2.7^{ab}
8.96	2.5	3.0	2.5	2.7^{ab}	3.4	2.7	4.3	3.5^{b}
13.44	2.5	3.7	5.0	3.7^{b}	2.5	5.0	4.2	3.9^{b}
17.92	3.2	2.5	2.0	2.6^{ab}	2.7	3.4	2.8	3.0
22.4	1.2	2.0	1.5	1.6^{a}	3.1	4.8	5.0	4.3^{c}
Mean	3.6^{x}	2.5^{x}	2.5^{x}	SEm=±0.48	2.6^{x}	3.4^{x}	3.3^{x}	SEm=±0.48
Shoot Height								
0	1.1	1.6	2.8	1.8^{bc}	0.8	3.1	1.2	1.7^{b}
4.48	0.4	2.2	2.7	1.8^{b}	0.8	5.2	1.3	2.3^{c}
8.96	0.3	2.0	2.8	1.7^{b}	0.5	4.9	1.2	2.2^{c}
13.44	0.3	5.4	2.2	2.6^{c}	0.3	3.1	1.1	1.5^{b}
17.92	0.2	3.2	0.5	1.3^{b}	0.1	2.0	1.2	1.1^{ab}
22.4	0.2	0.2	0.4	0.3^{a}	0.1	1.1	0.8	0.7^{a}
Mean	0.4^{x}	2.5^{y}	1.9^{y}	SEm =±0.28	0.4^{x}	3.2^{y}	1.1^{x}	SEm=±0.37
Percent *ex vitro* survival								
0	90	43	97		87	55	85	
4.48	85	25	85		85	40	80	
8.96	65	20	30		67	30	30	
13.44	45	0	10		50	10	0	
17.92	0	0	0		0	0	0	
22.4	0	0	0		5	0	0	

SEm = Standard Error of mean, df = degrees of freedom. Any two means bearing different superscripts a, b, c in columns and x, y, z in rows differ significantly ($P \leq 0.05$)

2.3.1.5. Effect of polyamines on regeneration of cultured banana shoots

When the external PAs were fed to cultures grown in M2, there were significant changes in the response, both in terms of culture morphology as well as levels of endogenous levels of PAs (Figures 2.7 and 2.8). Treatment with low levels (10 µM) of any PA there were no significant difference from that of control. Both Spd and Put at 30 µM showed significant changes in morphology where Spd induced fluffy callus

interspersed with embryonic masses and browning of medium was observed. Whereas, Put at similar levels, resulted in higher non-embryogenic coherent callus with lesser embryogenic masses, although medium browning was not observed (Figure 2.7). Spm, though at 30 µM did not show any significant difference from that of control, a higher level (40 µM) showed very noteworthy effect resulting in enhancement of highly embryogenic culture mass. Further higher levels such as 50 µM of all the PAs showed browning of medium leading to the culture necrosis followed by death. There were much higher differences in the pools of endogenous PAs as a consequence of feeding external PAs (Table 2.4).

Table 2.4. Levels of endogenous total polyamines in banana cultures grown on Basic medium (M2) with different levels of various polyamines

Treatments	Spermidine (nmol gFW^{-1})			Putrescine (nmol gFW^{-1})			Spermine (nmol gFW^{-1})		
	Weeks after inoculation								
	4	8	12	4	8	12	4	8	12
Control Medium (M2)	4.14 ^{d}	7.0 ^{d}	13.4 ^{d}	15.5 ^{cd}	17.4 ^{cd}	112.2 ^{cd}	28.4 ^{c}	86.1 ^{cd}	393.9 ^{c}
M2 +40 µM Spermidine	95.02 ^{bc}	129.0 ^{bc}	92.9 ^{bc}	224.8 ^{bc}	339.1 ^{ab}	62.5 ^{cd}	396.9 ^{bc}	412.3 ^{bc}	202.9 ^{c}
M2 +30 µM Putrescine	48.92 ^{c}	125.8 ^{bc}	45.1 ^{c}	629.0 ^{a}	459.7 ^{a}	991.4 ^{a}	75.8 ^{c}	97.1 ^{cd}	56.3 ^{cd}
M2 +40 µM Spermine	247.39 ^{a}	368.1 ^{a}	237.4 ^{a}	442.5 ^{b}	441.3 ^{a}	564.9 ^{b}	1954.8 ^{a}	2120.5 ^{a}	2542.8 ^{a}

Cultures treated with Spm (40 µM) showed a high endogenous level of Spm accounting for 2500 nmol gFW^{-1} upon 12 weeks of culturing, due to gradual accumulation throughout the culture period (Table 2.4). The next significant effect was that of 30 µM of Put, which invariably increased only endogenous Put pools, which was also highest (1000 nM) on 12 week, although periodic fluctuations were observed during the culture period (Table 2.4). Spd, supplied at 30 µM, though increased Spm pools, (400 nmol g FW^{-1} on 8 week of culturing), this also contributed for the increase in levels of other PAs as well (Table 2.4).

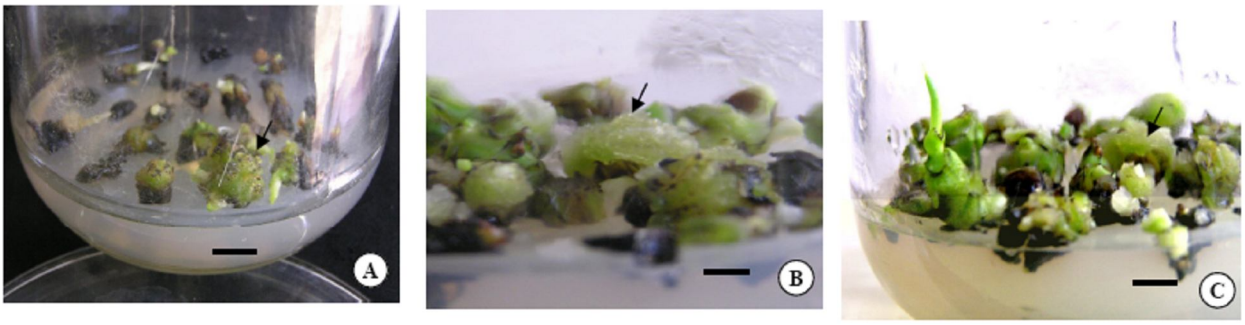

Figure 2.7. Morphological structure of various types of cultures obtained during the process of embryogenesis in M2 medium containing polyamines. A: 30 µM spermidine, B: 30 µM putrescine and C: 40 µM spermine. Bar = 0.8 cm. Arrow indicates meristematic globules produced after 8 weeks

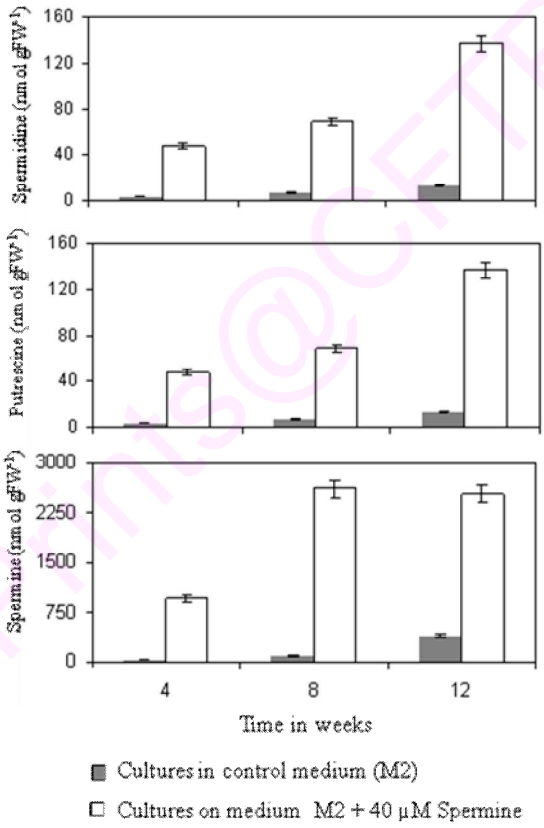

Figure 2.8. Comparison of total endogenous polyamines culture segments grown in control medium (M2) and those grown in M2 + 40 µM Spermine. Data presented as mean of five replicates ± SE

2.3.1.6. Effect of polyamine inhibitors

In earlier studies, DFMO and DFMA have been noted to inhibit the endogenous pool sizes of PAs probably by inhibiting either the biosynthetic pathway or by degrading the accumulated pools of PAs to various degrees in different plant species. Therefore in the

present study it was interesting to check to what extent these inhibitors reduce the pools of PAs in culture segments of banana having numerous regenerated shoot buds. These explants, being organized, showed almost similar levels of PAs as observed in embryogenic cultures mentioned earlier. When both DFMO and DFMA were used at 0.1mM levels, most of the explants turned brown in 8 weeks and by 12th week shoot necrosis was apparent in all the treatments.

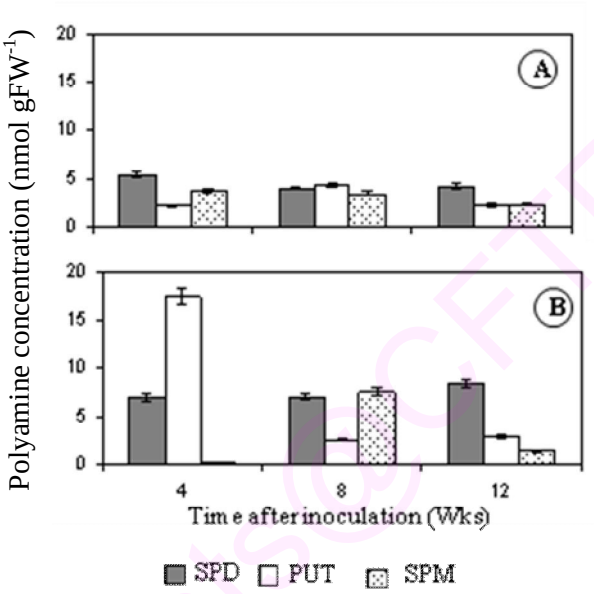

Figure 2.9. Changes in total endogenous polyamines in cultures treated with polyamine inhibitors. A: DFMA at 0.1mM supplemented to basic medium (M2) containing 40 µM Spermine; B: DFMO at 0.1mM supplemented to basic medium (M2) containing 40 µM Spermine

When similar treatments were applied to embryogenic cultures, the already formed embryos also necrosed and failed to form complete shoots. In these treatments, the endogenous pools of PAs were considerably disturbed with a higher suppression of all the PAs by DFMA than by DFMO (Figure 2.9). The rate of suppression of Spd was negligible in both the inhibitor treatments whereas Put was periodically retarded by DMFA. These results confirm that the above effects are, in fact, due to the direct participation of externally fed PAs by way of cellular incorporation and not due to any other effects of indirect nature.

2.3.1.7. Shoot Multiplication and Elongation in Partially Immersed System

NAA and GA have been very useful for rapid elongation of cultured shoots, leading to the generation of high quality shoots fit for acclimatization in case of NR banana (Venkatachalam et al. 2007b). Therefore, the effects of these growth regulators, in combination with two different culture conditions SM and PIS, were studied. Although only increase in growth and shoot height were expected, there was also multiplication as a result of carry-over effects of earlier medium; this parameter was also recorded in addition to shoot height. In SM, all the concentrations of NAA resulted in good shoot multiplication as well as increase in shoot height, (Figure 2.10a). Ten μM of NAA was found producing better results than other levels in terms of higher number/height of shoots, especially when grown for 4 weeks. Whereas in PIS, higher number of shoots as well as shoot height were observed when compared with SM, although 10 μM of NAA was more effective for shoot multiplication (Figure 2.10a). GA was more efficient in supporting shoot multiplication in SM than in PIS, more so when the concentration was 30 μM, although this level did not support as much multiplication as in NAA treatment. Nevertheless, GA very significantly supported shoot height in PIS than in SM. Since shoot height is a decisive factor in better acclimatization of banana shoots (Venkatachalam et al. 2007b), GA appears very useful in obtaining high quality shoots. The Growtek^{TM} bioreactor, functioning on the principle of PIS has been used for micropropagation of other plant species where it was observed to support constant supply of nutrients and aeration to plants combining the advantages of both solid and liquid medium. The results of the present study also showed better utilization of nutrients in PIS than in SM, since the biomass produced will be directly proportional to the nutrient utilization. All in all, almost similar multiplication with better shoot height were observed in PIS than in SM in a period of 4 weeks, suggesting that the observation made through the present study would be of great use for producing better quality shoots of NR banana. Uniform rooting in MS medium with ½-strength NH_4NO_3 supplemented with NAA (10 μM) was also observed. Such shoots appeared more robust than those from SM resulting in 100% survival in green house condition (data not shown).

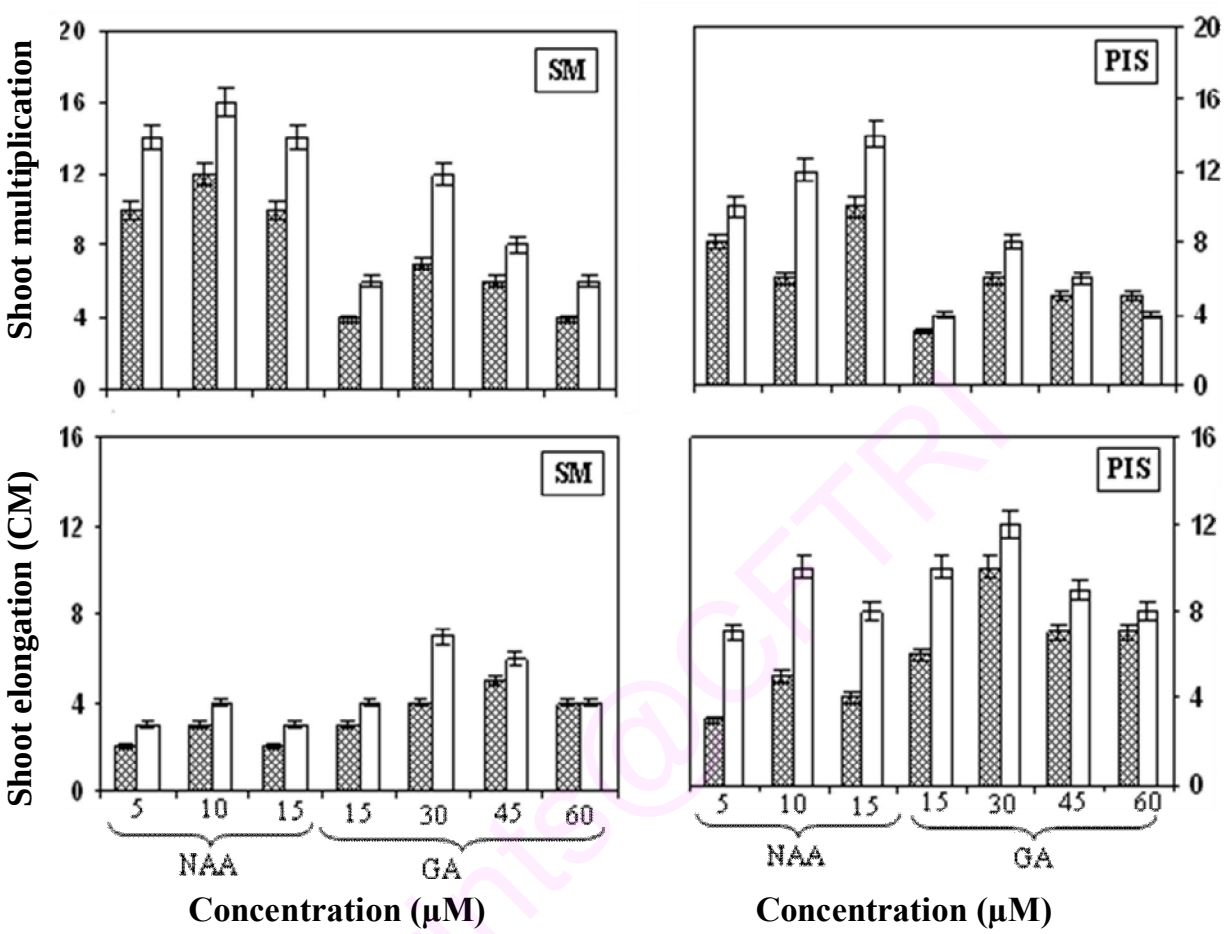

Figure 2.10a. Effects of different levels of NAA and GA (supplemented through M3 medium) on height and number of shoots in two culture systems such as Solid Medium (SM) and Partial Immersion System (PIS) grown for 2 weeks and 4 weeks

It is also worth mentioning that the use of Growtek^{TM} bioreactor works out to be cheaper than any other commonly used apparatus meant for mass cloning of plants (Dey 2005), since liquid form of culture medium is invariably cheaper than the gelled form, with an added advantage of ease in handling.

2.3.2. Micropropagation

2.3.2.1. Morphological changes due to high concentration of cytokinins

For NR, a preliminary screening experiment indicated that a combination of BA (2 mg l^{-1}) and kinetin (1 mg l^{-1}) along with adenine sulphate (80 mg l^{-1}) was suitable for steady multiplication of shoots (Venkatachalam et al. 2006). The results showed that

multiple shoot formation occurred in all the combinations of growth regulators tested, and there were significant differences in the number and quality of shoots/ buds formed under each treatment (Figures 2.10b and 2.11). The number of shoot buds increased with an increasing concentration of BA up to 5 mg l^{-1}, beyond which a suppression of shoot development occurred. BA at low concentrations fostered optimum proliferation of shoots, whereas at higher concentrations (4, 5 and 6 mg l^{-1}) it resulted in clustering of shoots and buds with a maximum number of 80 shoot buds per segment (at 5 mg l^{-1}). However, repeated subculturing on similar treatment up to 5th subculture resulted in a nearly constant number of 52 separable shoots. BA at 5 mg l^{-1} was considered optimal for shoot multiplication as the shoot morphology appeared appropriate for routine application. With higher levels of BA (6-10 mg l^{-1}), a reduction in number as well as length of shoot buds occurred, and exudation of phenolics were observed.

Kinetin at low concentrations induced a lower number of shoot buds and higher shoot lengths than that obtained with similar levels of BA (Figures 2.10b and 2.11). A progressive increment in number of shoot buds occurred with increasing concentrations of kinetin up to 4 mg l^{-1} (62 shoot buds per segment) with a significant reduction in shoot length. An average of 34 shoot buds per segment was formed over repeated sub-culturing (up to 5^{th} subculture) when the medium was supplemented with 3-4 mg l^{-1} kinetin. Therefore, kinetin at 4 mg l^{-1} was considered optimal for shoot multiplication together with control cytokinins as in medium M2. Similar to BA, kinetin at very high concentrations, such as 9-10 mg l^{-1} also caused exudation of phenolics and a reduction in shoot number as well as shoot length.

However, unlike BA, kinetin at various levels did not induce any morphological changes; instead there was more randomness in shoot proliferation pattern. The results further showed that there was an inverse correlation between shoot length and shoot number in each set of growth regulator treatments (data not shown). Since maximum shoot proliferation occurred with particular levels of either BA (5 mg l^{-1}) or kinetin (4 mg l^{-1}), these treatments were found useful for routine application to obtain significantly higher number of shoot buds and thus considered as high-rate multiplication medium (HRMM).

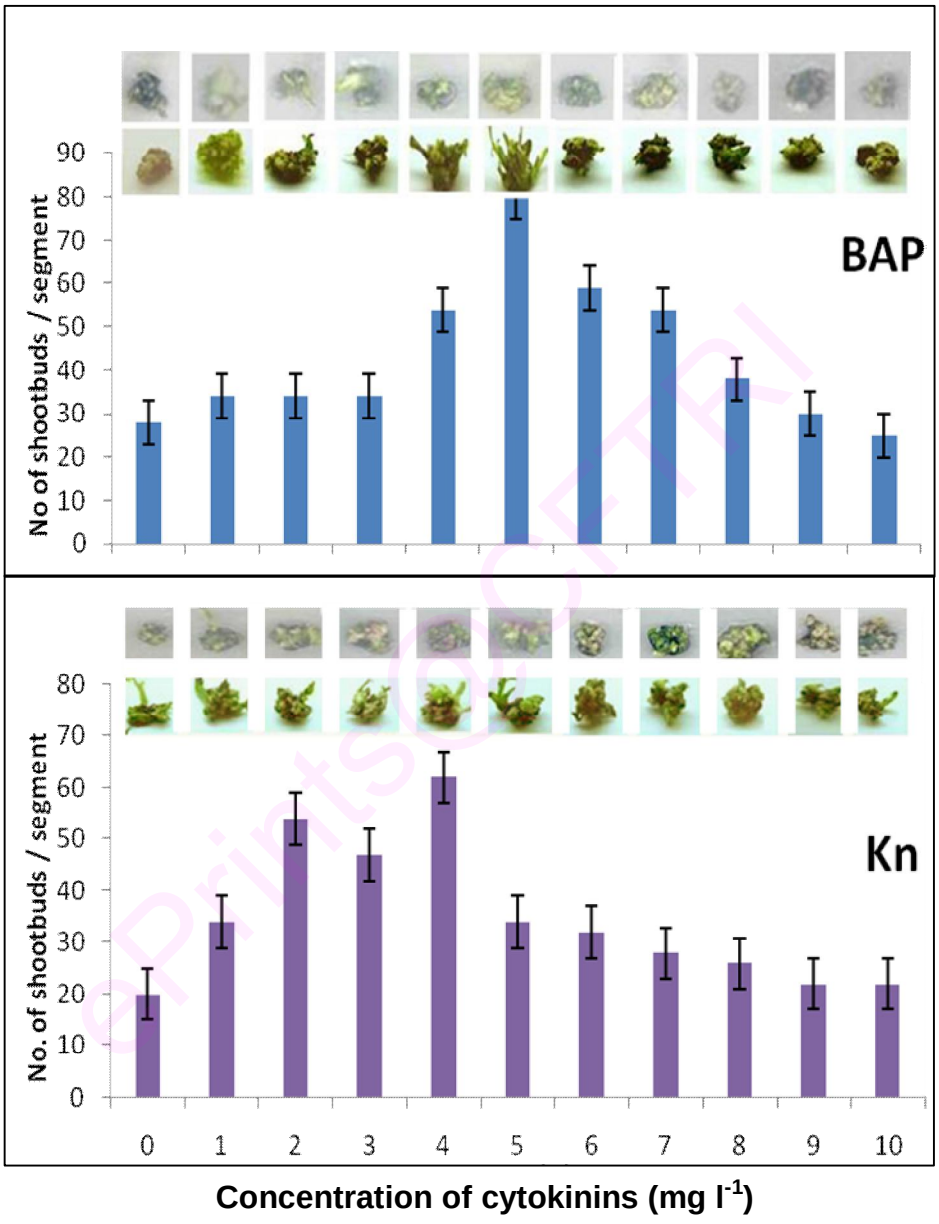

Figure 2.10b. Exogenous cytokinins (BAP, Kn) affecting the rate of shoot proliferation during micropropagation of banana *cv*. NR

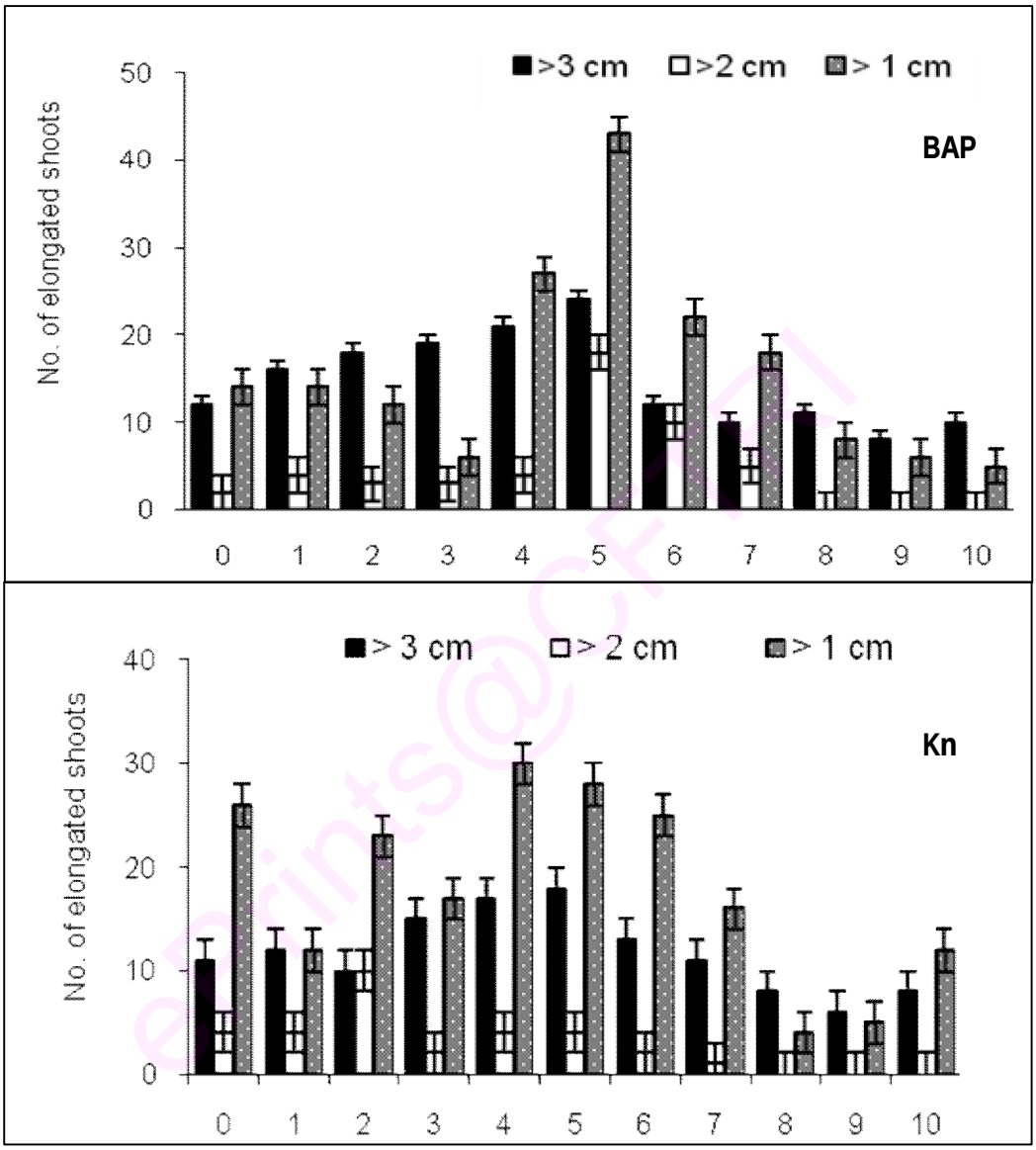

Figure 2.11. Exogenous cytokinins (BAP, Kn) affecting the rate of shoot elongation during micropropagation of banana *cv.* NR

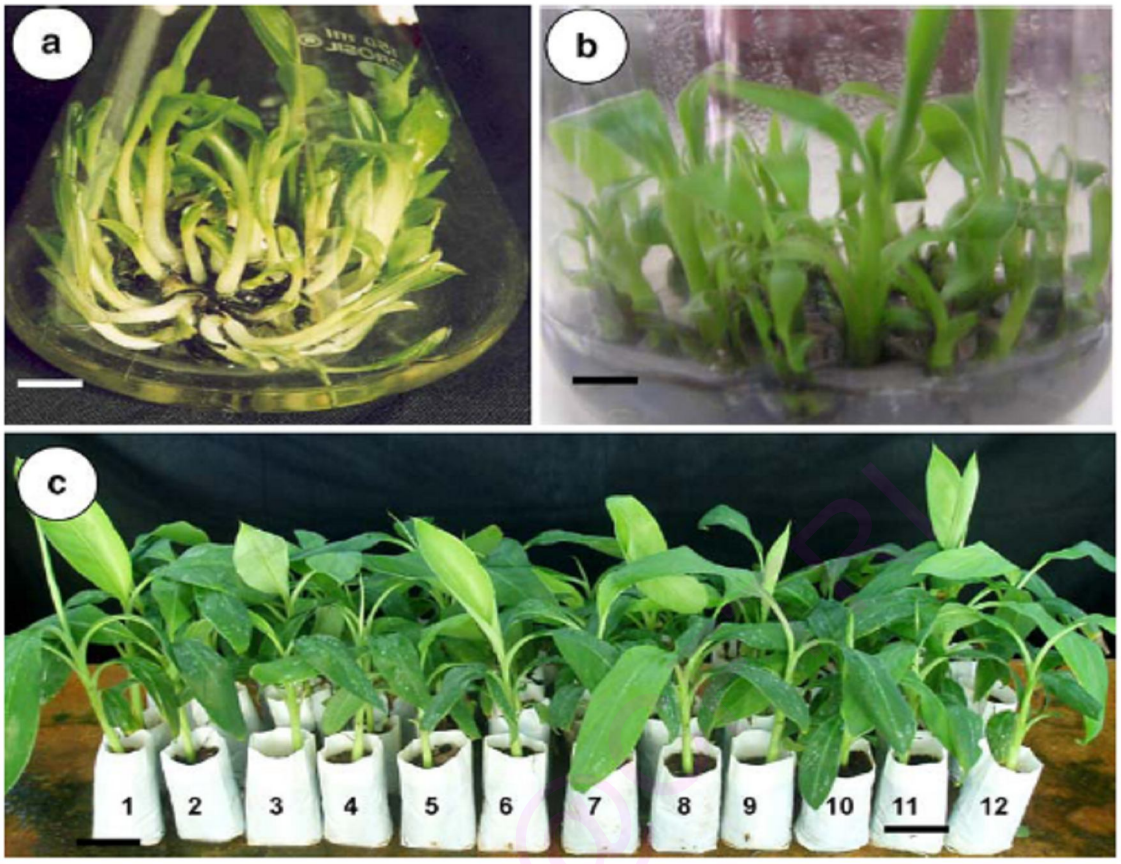

Figure 2.12. Micropropagation in banana *cv.* NR using high levels of cytokinins. (a) multiple shoots induced on HRMM (MS medium supplemented with 1238 mg l^{-1} NH_4NO_3, 5.0 mg l^{-1} BA, 1.0 mg l^{-1} Kinetin, 80 mg l^{-1}adenine sulphate, 25 g l^{-1} sucrose and 2.5 g l^{-1} gelrite®) and proliferated in liquid M3 medium (MS medium supplemented with 1238 mg l^{-1} NH_4NO_3, 0.5 mg l^{-1} NAA and 25 g l^{-1} sucrose) after three weeks (bar = 0.8 cm). (b) shoots acclimatized on M4 medium (½ MS basal salts supplemented with 20 g l^{-1} sucrose and 16 g l^{-1} agar) (bar = 0.8 cm). (c) 2- month-old micropropagated plantlets established under greenhouse conditions (bar=4.6 cm)

In actual, HRMM contained 7 mg l^{-1} BA plus 1 mg l^{-1} kinetin or 5 mg l^{-1} kinetin plus 2 mg l^{-1} BA. In order to evaluate the pattern of further shoot proliferation, the shoot cultures from HRMM were transferred to M3 medium. We observed that shoot buds from various treatments showed rapid proliferation upon transfer to M3 (Figures 2.10b and 2.11). While the proliferation was random in kinetin-treated cultures, there was an uniform concentration-effect in BA-treated cultures with a maximum proliferation recorded at 5 mg l^{-1} BA (Figure 2.12). These responses clearly show that though both BA and kinetin are cytokinins (purine derivatives) causing similar effects in plants, their

effects on shoot proliferation vis-à-vis multiplication are different in NR. Based on these observations, a HRRM was developed for banana micropropagation which resulted in optimum survival of microplants in the soil (Figure 2.12C). Both BA and kinetin significantly affected the number of shoots proliferated per segment when the cultures were grown on medium M3. However, the effects of cytokinins on various morphological features were not evident after two months of acclimatization ex vitro. The number of shoots per segment, shoot length (after three subcultures on liquid medium), number of microplants survived in hardening medium, stem girth of hardened plants (after two months), number of leaves per plant and plant height did not show any variation. Thus the protocol validated through further studies indicated that a high level of multiplication with good quality shoots could be realized leading to cost reduction in commercial micropropagation. By using HRMM a 25% increase in the number of plantlets could be achieved.

2.3.3. Genetic analyses

2.3.3.1. Genetic analyses of Regenerated plants

The 50 RAPD primers resulted in 531 scorable classes, ranging from 200 bp to 3100 bp in size (Table 2.5). The number of bands for each primer varied from 5 (OPJ 14) to 22 (OPM 20) with an average of 10.62 bands per RAPD primer. Of the 25 arbitrary ISSR primers initially screened, 12 produced clear and scorable bands (Table 2.6). Bands for each primer varied from 4 (UBC 863) to 13 (UBC-834) with an average of 7.8 bands per ISSR primer.

The screening with 12 ISSR primers generated 94 scorable bands, ranging in size from 200 bp to 3000 bp. A total of 14,175 bands from regenerated plants (numbers of plantlets analyzed × number of bands obtained with ISSR and RAPD primers) were generated, giving rise to monomorphic bands comparing all the plantlets and the mother plant. Examples of the monomorphic bands and their intensity histograms obtained for RAPD and ISSR (Figure 2.13) markers are shown. These analyses clearly indicate that there were no polymorphic bands.

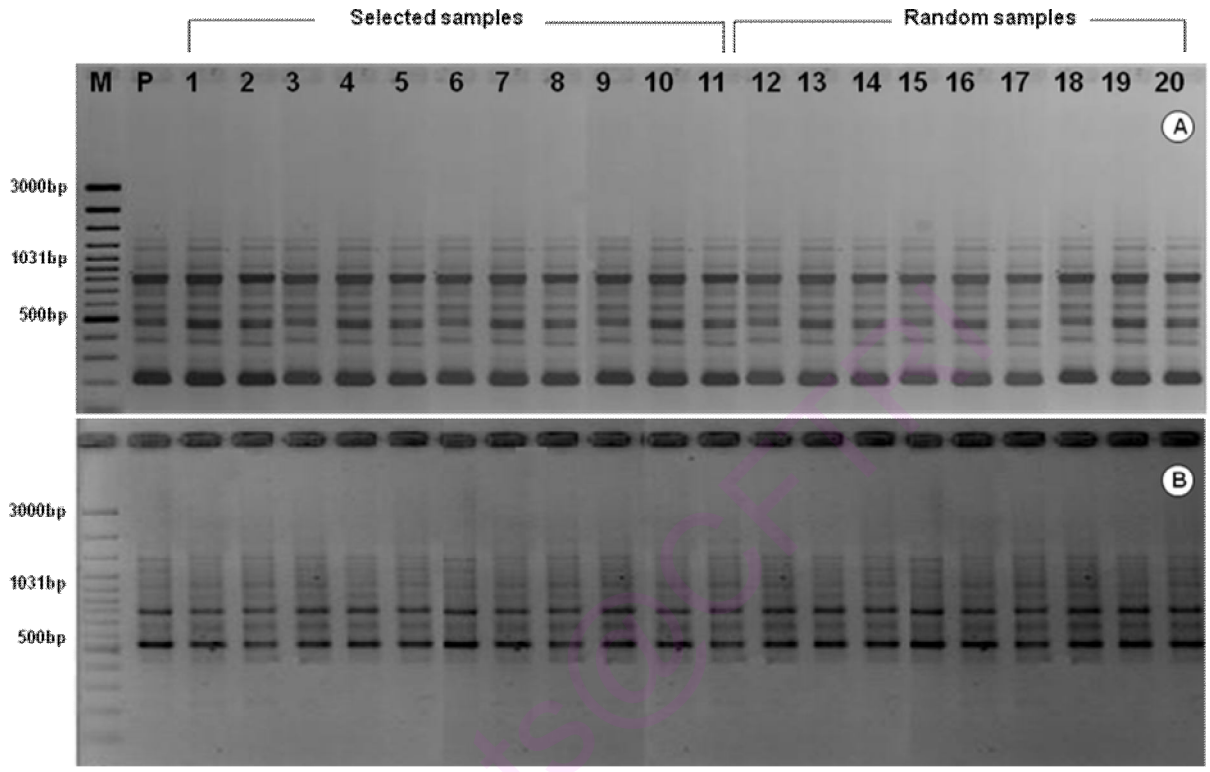

Figure 2.13. RAPD analysis for the primer OPA 14 (A) and ISSR analysis for the primer UBC 836 (B) of regenerated plants. Selected samples - plantlets showing morphological abnormalities (Lanes 1-11), Random samples - randomly selected samples from 4000 plantlets (Lane 12-20), P - sample from mother plant maintained as parental clone. M - GeneRuler ladder

Table 2.5. List of primers, their sequences and size of the amplified fragments generated by RAPD markers for DNA fingerprinting of micropropagated shoots of banana *cv*. NR produced under various levels of high cytokinins *in vitro*

Sl. no.	Primer	Primer sequence (5'–3')[a]	$MgCl_2$ (mM)	Number of scorable bands	Size range (bp)
a) RAPD primers					
1	OPA-03	AGTCAGCCAC	1	8	500-2800
2	OPA-04	AATCGGGCTG	2	12	300-3000
3	OPA-06	GGTCCCTGAC	0	10	300-1031
4	OPA-09	GGGTAACGCC	0	8	400-1800
5	OPA-11	CAATCGCCGT	2	18	300-3100
6	OPA-14	CTCGTGCTGG	0	13	200-3000
7	OPA-20	GTTGCGATCC	1	14	200-2600
8	OPC-01	TTCGAGCCAG	1	12	280-1800
9	OPC-02	GTGAGGCGTC	2	12	300-2800
10	OPC-04	CCGCATCTAC	0	15	220-2000
11	OPC-05	GATGACCGCC	0	14	320-1900
12	OPC-07	GTCCCGACGA	0	16	300-2600
13	OPC-08	TGGACCGGTG	1	14	290-3100
14	OPC-09	CTCACCGTCC	1	10	480-1800
15	OPC-11	AAAGCTGCGG	0	6	200-2000
16	OPC-13	AAGCCTCGTC	0	8	480-1800
17	OPC-20	ACTTCGCCAC	0	7	300-2000
18	OPD-03	GGTCTACACC	0	6	290-1800
19	OPD-04	TCTGGTGAGG	0	10	300-2000
20	OPD-07	TTGGCACGGG	1	13	380-2500
21	OPD-08	GTGTGCCCCA	0	7	290-2400
22	OPD-16	AGGGCGTAAG	1	14	400-2000
23	OPF-12	ACGGTACCAG	0	6	400-1031
24	OPF-15	CCAGTACTCC	1	7	200-2400

25	OPJ-01	CCCGGCATAA	1	8	200-3000
26	OPJ-02	CCCGTTGGGA	2	9	380-2400
27	OPJ-03	TCTCCGCTTG	1	8	500-2400
28	OPJ-04	CCGAACACGG	0	5	500-1031
29	OPJ-06	TCGTTCCGCA	0	10	220-2800
30	OPJ-07	CCTCTCGACA	1	11	300-2000
31	OPJ-08	CATACCGTGG	0	6	400-3000
32	OPJ-09	TGAGCCTCAC	2	8	400-2000
33	OPJ-10	AAGCCCGAGG	1	11	220-1800
34	OPJ-11	ACTCCTGCGA	0	8	400-2200
35	OPJ-14	CACCCGGATG	1	5	500-1031
36	OPJ-15	TGTAGCAGGG	0	9	220-1800
37	OPJ-16	CTGCTTAGGG	1	8	220-2000
38	OPJ-17	ACGCCAGTTC	0	6	500-2200
39	OPJ-20	AAGCGGCCTC	0	7	800-2400
40	OPL-14	TCGTGCGGGT	1	8	1031-2400
41	OPM-12	CACAGACACC	0	12	300-2600
42	OPM-16	GTAACCAGCC	2	18	400-2000
43	OPM-20	AGGTCTTGGG	1	22	320-2000
44	OPM-18	CACCATCCGT	0	7	400-2000
45	OPN-03	GGTACTCCCC	0	18	300-2600
46	OPN-04	GACCGACCCA	0	12	400-2800
47	OPN-06	GAGACGCACA	2	11	340-2000
48	OPN-09	TGCCGGCTTG	1	18	240-3000
49	OPN-10	ACAACTGGGG	1	19	240-3000
50	OPN-12	CACAGACACC	0	7	240-2400
Total				531	

Table 2.6. List of primers, their sequences and size of the amplified fragments generated by ISSR markers for DNA fingerprinting of micropropagated shoots of banana *cv*. NR produced under various levels of high cytokinins *in vitro*

Sl. No.	Primer	Primer sequence (5'–3')[a]	$MgCl_2$ (mM)	Number of scorable bands	Size range (bp)
b) ISSR primers					
1	UBC-811	$(GA)_8C$	0	7	250-2500
2	UBC-813	$(CT)_8$ T	1	5	200-1031
3	UBC-817	$(CA)_8A$	1	8	400-2800
4	UBC-820	$(GT)_8T$	0	11	200 - 2000
5	UBC-826	$(AC)_8C$	0	8	350- 2000
6	UBC-834	$(AG)_8YT$	1	13	700 – 3000
7	UBC-836	$(GA)_8YA$	1	11	200-1000
8	UBC-840	$(GA)_8YT$	1	11	200-1000
9	UBC-845	$(CT)_8RG$	0	6	300-1400
10	UBC-848	$(CA)_8RG$	1	5	200-1400
11	UBC-863	(GAA)6	1	4	500-1800
12	UBC-890	$VHV(GT)_7$	0	5	450-1800
Total				94	

[a] R = A+G, Y = C+T, H = A+T+C and V= A+G+C

2.3.3.2. Micropropagation using high level of cytokinins

Of the 25 arbitrary ISSR primers initially screened, 12 produced clear and scorable bands (Table 2.6). Bands for each primer varied from 4 (UBC 863) to 13 (UBC-834) with an average of 7.8 bands per ISSR primer. The screening with 12 ISSR primers generated 94 scorable bands, ranging in size from 200 to 3000 bp. The 50 RAPD primers resulted in 531 scorable classes, ranging from 200 to 3100 bp in size. Number of scorable bands for each primer varied from 5 (OPJ 14) to 22 (OPM 20) with an average of 10.6 bands per

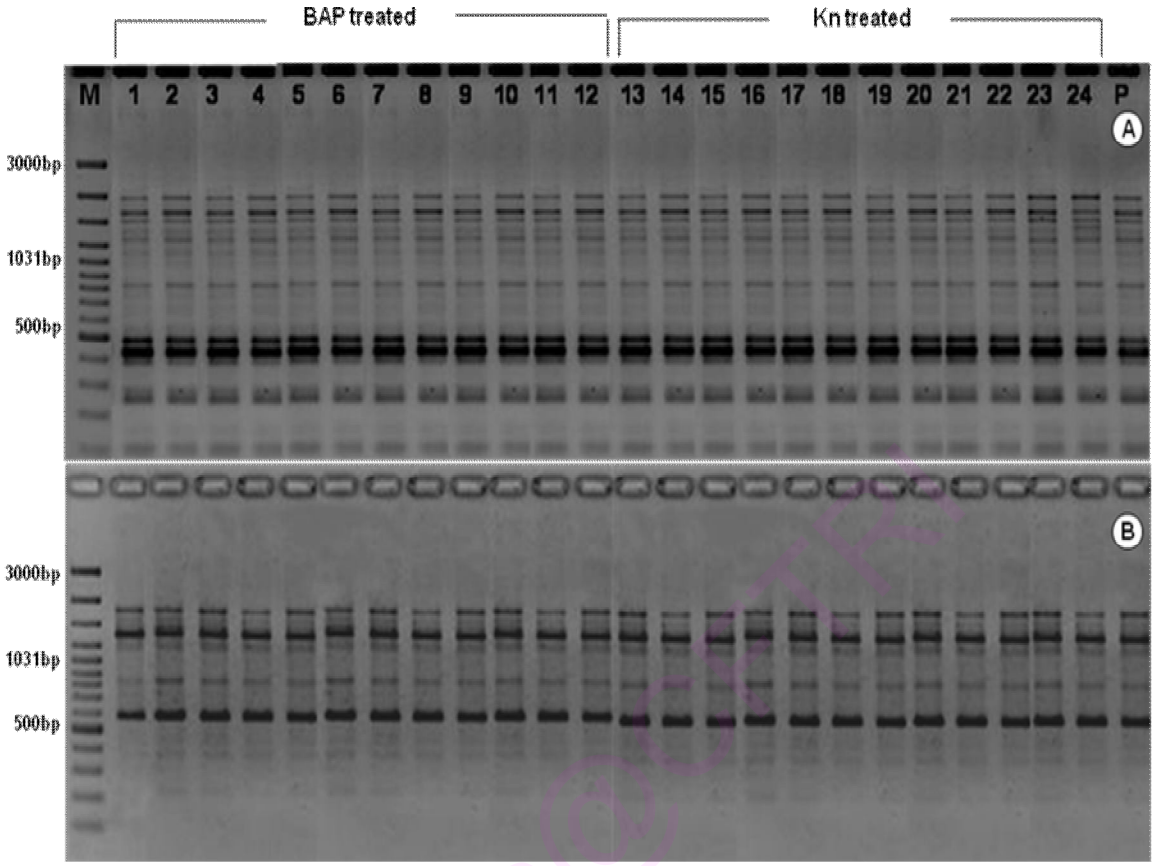

Figure 2.14. RAPD analysis for the primer OPA 04 (A) and ISSR analysis for the primer UBC 811 (B) of micropropagated plants, BAP treated (Lanes 1-12) - representative samples obtained from medium containing various concentration of BAP (0 – 53.76 µM), with 4.65 µM Kn and 216.80 µM Adenine sulphate, Kn treated (Lanes 13-24) - representative samples obtained from medium containing various concentrations of Kn (0 – 55.80 µM), with 8.96 µM BAP and 216.80 µM Adenine sulphate, P - sample from mother plant maintained as parental clone, M - GeneRuler ladder

RAPD primer (Table 2.5). A total number of 17,400 bands (numbers of plantlets analyzed × number of bands obtained with ISSR and RAPD primers) were generated, giving rise to monomorphic bands comparing all the plantlets and the mother plant. Examples of the monomorphic bands obtained for RAPD and ISSR (Figures 2.13, 2.14 and 2.15) markers are shown. These analyses clearly indicate that there were no polymorphic bands.

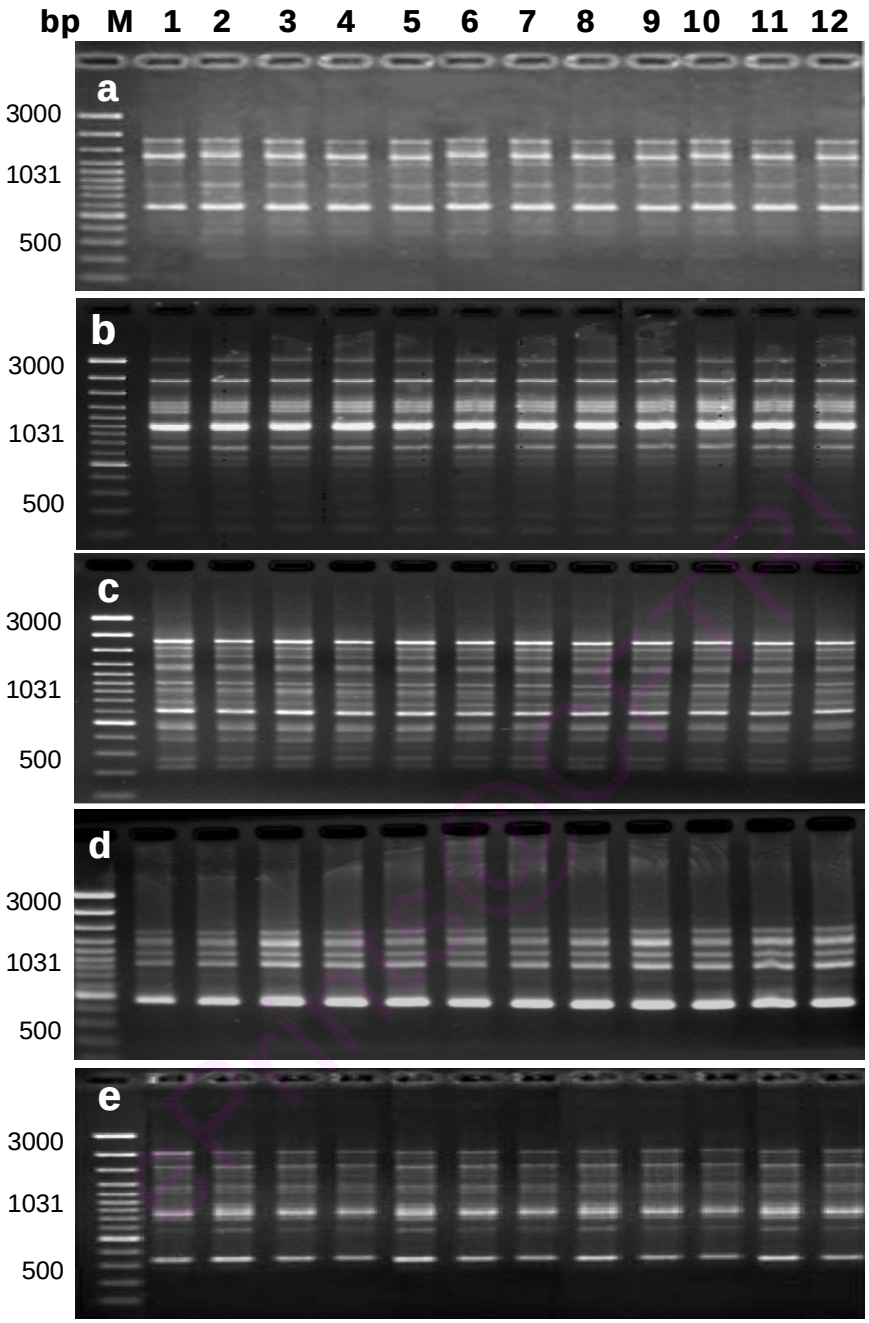

Figure 2.15. DNA fingerprinting of micropropagated shoots of banana *cv*. NR grown on M2 medium supplemented with different concentrations of kinetin. RAPD profiles using the decamer primers OPJ 09 (a), OPC 08 (b) and OPC 01 (c). ISSR profiles using the primer UBC 826 (d) and UBC 890 (e). Lane M represents the 100 bp GeneRuler DNA ladder. Lane 1 represents the control shoots maintained on M2 medium. Lanes 2-11 represent the shoots grown on M2 medium containing 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 mg l^{-1} kinetin, respectively. Lane 12 represents the field-grown mother (source) plant

2.3.3.3. Long-term micro-propagated plants

In order to confirm the genetic fidelity a comparison of RAPD and ISSR patterns of 11 plants chosen from 4000-plus plantlets (Figures 2.16 and 2.17) and a control plant (mother plant) was carried out. Of the 50 arbitrary RAPD primers initially screened, 30 produced clear and scorable amplification products. The 30 RAPD primers resulted in 377 scorable band classes, ranging from 200 bp to 3100 bp in size. The number of bands for each primer varied from 6 (OPJ 08) to 22 (OPM 20) with an average of 12.6 bands per RAPD primer (Table 2.5). The screening with 5 ISSR primers generated 47 scorable band classes, ranging in size from 200 bp to 2200 bp. The number of bands for each primer varied from 7 (UBC 811) to 13 (UBC 834) with an average of 9.4 bands per ISSR primer. A total of 5088 bands (numbers of plantlets analysed × number of band classes with all the ISSR and RAPD primers) were generated, giving rise to monomorphic patterns across all the plantlets analysed. Samples of the monomorphic band classes obtained for RAPD (Figure 2.17) marker is shown. No polymorphic bands were observed.

Figure 2.16. *In vitro* multiplied plants under hardening. The plantlets were obtained after repeatedly sub-culturing in M2 medium for 150 times, each after 4 weeks period

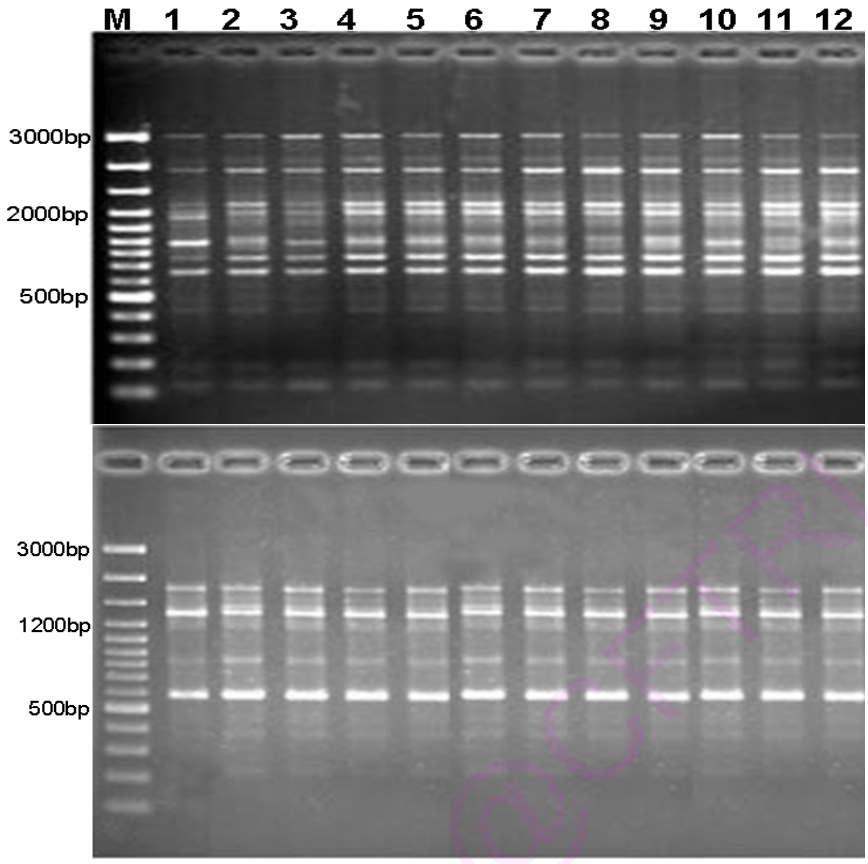

Figure 2.17. RAPD amplification pattern obtained for mother plant (lane 1) and long-term micropropagated shoot cultures (lanes 2-12) generated by primer OPJ 09 and OPN 10. M: GeneRuler^{TM} 100 bp DNA Ladder Plus.

2.3.4. Genetic transformation

2.3.4.1. Sensitivity of explants to antibiotics

Before starting the genetic transformation experiments, the regenerating shoot buds were screened for their sensitivity to various levels of kanamycin and hygromycin in order to arrive at an appropriate concentration for the selection of transformants. Among the various concentrations checked, kanamycin at 200 mg l^{-1} was useful for the eradication of *Agrobacterium* and hygromycin at 6 mg l^{-1} was found ideal for the selection of transformants.

2.3.4.2. Transformation using wild type *A. rhizogenes*

The cultured segments co-cultivated with wild type *A. rhizogenes* remained green and callused slightly (rhizo-callus) in the wounded portions. Hairy roots were initiated

after 23–30 days of culture from *A. rhizogenes* co-cultivated explants. No hairy roots were induced from control-uninfected explants. This experiment demonstrated the susceptibility of banana to *A. rhizogenes* mediated transformation.

2.3.4.3. Transformation using *A. rhizogenes* harbouring a binary vector

Culture segments, of uniform size (Ø = 0.5cm) having embryogenic mass and clusters shoot buds varying in length from 0.2-0.5cm were found ideal for sonication-assisted transformation. While the control explants without sonication did not result in any transformants, the sonication resulted in transformants probably due to uniform micro wounding all over the surface of the shoot buds. In the absence of acetosyringone, the transformation efficiency was extremely low (Table 2.7).

Table 2.7. Efficiency of different treatments in *Agrobacterium rhizogenes* mediated transformation in banana shoot buds

	Treatment with AS (100 µM)	AS + sonication without AS	Sonication + AS
No.of explants co cultivated	1180	1282	1200
No. of explants survived in selection media	17	3	42
Transformation efficiency (%)	1.4	0.24	3.4

2.3.4.4. GUS assay, PCR and Southern analysis

In order to confirm the transformed nature, the putative transformants were checked for the activity of reporter gene through GUS assay. To all the assay mixtures, 20% methanol was added to prevent false positive endogenous "GUS like" activity. While the control tobacco plants showed intense blue colour due to GUS activity, the selected banana plants showed activity of GUS only at vascular bundles, probably xylem parenchyma (Figure 2.18) which was absent in non-co-cultivated shoot buds. Putative transformants obtained in vitro were further cultivated for another 12 weeks on harmone-free M2 medium and were tested for the presence of the *rol*A gene in all such as *rol*A, rol ABC from Ri-T-DNA and the *hpt*II gene from the binary vector T-DNA. PCR, using gene specific primers for *rol*A-specific primer was positive in *rol*A transformed and *rol*ABC transformed plants and hygromycin phosphotransferase resulted in amplification of 479-bp fragment of the *hpt*II gene in all seven hygromycin resistant plants (Figures 2.19 and 2.20). It was interesting to note that of the plantlets obtained from binary vector,

three were negative for *rol*A gene, clearly indicating the independent integration of T-DNA from the pCAMBIA 1301 binary vector and the Ri native plasmid from *A. rhizogenes*. It is evident from our studies that during transformation process, co-transfer of T-DNA from both Ri plasmid and binary vector is not obligatory. Southern analysis further confirmed the transgenic nature and stable integration of T DNA in hygromycin resistant plantlets.

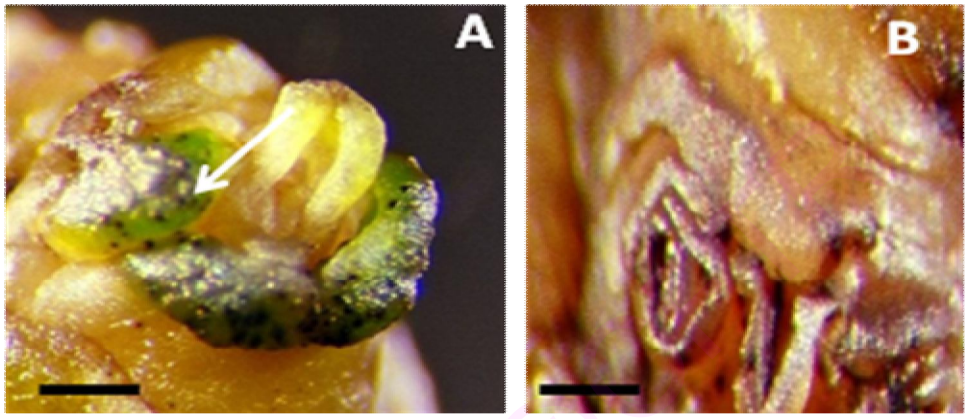

Figure 2.18. A. GUS expression as a result of intron *uidA* gene showing blue colorations as distinct patches at specific growth stage of leaf primordium (shown by white arrow) after twelve weeks of co-cultivation. Bar - 5 mm. B Control untransformed secondary embryos showing no colour formation. Bar - 5 mm

The presence of fragments indicates insertion of the T-DNA into the plant genome (Figure 2.21). The genomic DNA was digested with N*de*I and S*ac*II enzymes. N*de*I cuts once inside the *hpt*II gene and the probe and S*ac*II cuts just outside the T DNA left border. No signals were observed in lanes containing untransformed DNA. The 1.4 kb fragment detected in the Southern blot is the expected size fragment from the pCAMBIA 1301 vector. Since the S*ac*II site is absent in the T-DNA of the vector, all the large size fragments (greater than 1.4 kb) are possibly derived from the plant genome and not from any bacterial contamination.

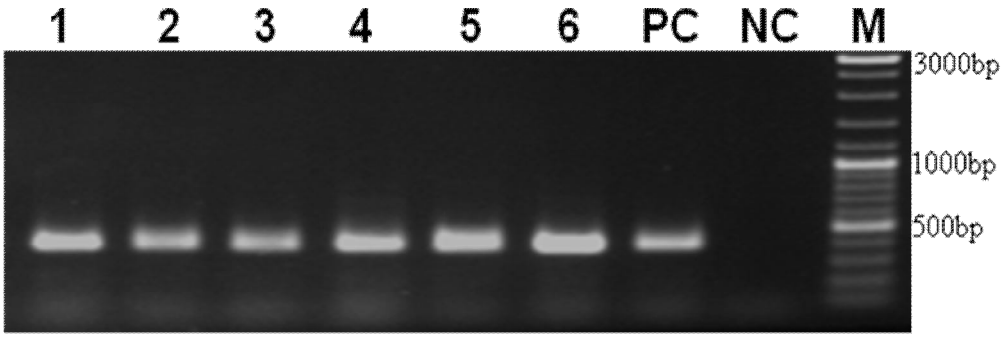

Figure 2.19. PCR for the detection of the *rolA* gene. PCR analysis was carried out using gene-specific primers for the *rolA* gene. Lanes; M-100 bp marker, PC-DNA from *A. rhizogenes* harboring the binary vector, NC-DNA from the untransformed banana. 1–6 DNA from independent transformants selected under kanamycin

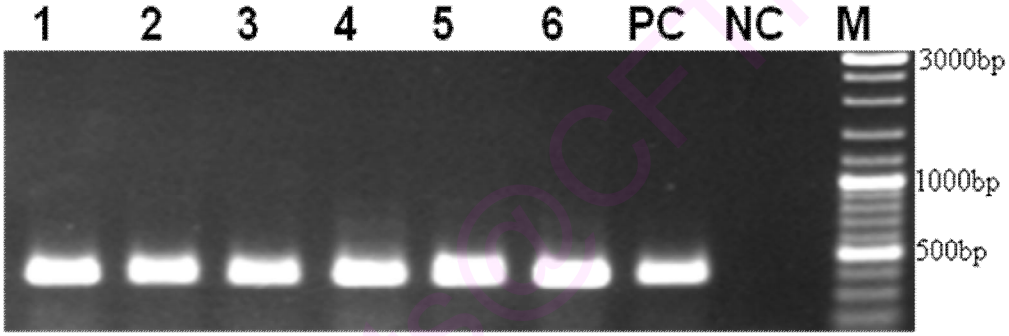

Figure 2.20. PCR for the detection of the *hptII* gene. PCR analysis was carried out using gene-specific primers for the *hptII gene.* Lanes; M-100 bp marker, PC-DNA from *A. rhizogenes* harboring the binary vector, NC-DNA from the untransformed banana. 1–6 DNA from independent transformants selected under hygromycin

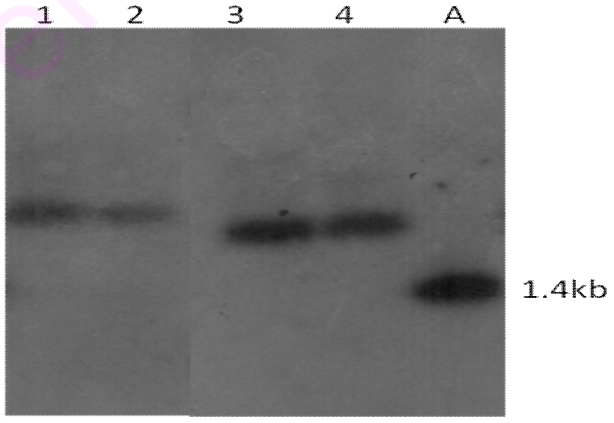

Figure 2.21. Southern blot analysis of putative transformants

2.4. DISCUSSION

2.4.1. Regeneration

Though there have been various reports on the direct regeneration of shoot buds from leaf explants of different plant genera, no such report exists for banana cultivar this NR. In plantains (ABB), regeneration of somatic embryos from the callus, which sloughed off from shoot cultures in liquid medium, was observed (Cronauer and Krikorian 1988). However, further culture of such somatic embryos resulted in the growth of roots only. Similarly, in 'Cavendish' (AAA), spherical masses that developing from leaf base explants which also resulted in root formation (Novak et al. 1989). This study has demonstrated that direct shoot regeneration in triploid banana cultivar (genotype AAB) is achievable (Figure 2.22).

The results indicate that light plays a crucial role during bud induction. A period of darkness is, however, required for organogenesis. This has been observed in many other dicotyledonous plant species but also in some cultivars of banana (Navarro et al. 1997). Similarly, a brief exposure to high cytokinin levels (BA, 22.4 μM), followed by partial withdrawal of the BA and the addition of an auxin (IBA 9.84 μM), probably plays a positive role in supporting regeneration. Overall, our work indicates the need for precise balance among various physico-chemical factors as well as the selection of right explant for the response towards direct shoot regeneration. Contrarily, Okole and Schulz (1996) reported shoot regeneration from shoot base microsections in the presence of a 16 h photoperiod and the addition of one set of growth regulators in genotype AAB. This indicates that within the genotype AAB, clonal variations could be the reasons for variations in response. This further indicates that cultivar NR is a highly recalcitrant.

The failure of response of explants obtained from shoots grown in solid medium and a positive response of those from liquid medium indicates the specific influence of the physical form of the medium on the regeneration potential of banana. This has been further supported by the fact that explants, pre-conditioning the tissue on liquid medium is a requisite, as is the requirement of solid phase (gelled) medium for subsequent morphogenesis. A similar observation was made for suspension cultures of other genotypes of banana where somatic embryos arose from the leaf bases and shoots were formed only in a double-layer medium (Novak et al. 1989). The reason for this is

explicable with the support of a study by Blakesley (1991) where *Musa* shoots (dwarf Cavendish), cultured on solidified medium, absorbed about 52 % of the radio labeled BA, of which 34.6% (i.e., 86% of the absorbed BA) was stored in the pseudostem in an unused form till the end of a 27-d culture period. Probably the high endogenous BA levels in the cells of leaf sheaths are responsible for improving their regeneration competence relative to other parts of the shoot. Since liquid medium supports rapid uptake/excretion of molecules from and to the cells, the interaction of these molecules and establishment of specific chemical gradients appear to play a positive role towards regeneration. It is also important to note from the data presented in table 2.1 that though a high level of BA available in liquid medium could have been accumulated by the leaf-base explants during pretreatment in liquid medium, explants failed to produce shoot buds when growth regulators were completely withdrawn (control, Table 2.2).

It is the requirement for cytokinin rather than for auxin, that supported shoot bud formation. For NR, the quantity of growth regulators was more important than the ratio of cytokinin : auxin, which is also evident from the data in Table 2.2, where treatments 6 and 9 showed the highest number of explants ending toward shoot regeneration. The variations in the mean number of buds per cm^2 of explant may be a result of further splitting of shoot buds, which is also evident in the histological analysis (Figure 2.5). During further multiplication of shoots under conditions with similar growth regulators with variations in the physical form of the medium, there were differences in shoot height and number, though not in a significant manner (Table 2.3). The adventitiously regenerated shoots, when grown in agitated liquid medium, showed higher shoot number (3.4) with more height (3.2 cm) than those derived from meristems. Here, the gelled medium, which is commonly used for large-scale propagation of various plants by commercial tissue culture firms, was less suitable for *cv*. NR.

Banana has been proved to be a highly recalcitrant material for *in vitro* plant regeneration. Banana plants being sterile triploids, improvement via breeding is extremely difficult in bananas. Thus, using the present protocol, the cultivar may be genetically manipulated for improved quality by means of particle bombardment of leaf base explants followed by regeneration. Further, the results indicate possibilities of future work on activating genes that regulate regeneration in bananas. Therefore, the

regeneration protocol developed in the present study may be of great importance in reviving the dwindling NR cultivar for commercial cultivation.

2.4.1.1. Effect of polyamines on regeneration pattern of banana

Table 2.4 summarizes the endogenous contents of PAs in cultures grown on M2 medium where all the PAs increased with time. Such time-dependent accumulation of PAs, and higher accumulation of PAs by organized cultures have also been observed in oat (Rowena et al. 2002) and in red spruce (Minocha et al. 2004) and embryogenic suspension cultures of *Araucaria angustifolia* (Silveria et al. 2006). The higher accumulation of Spm is probably due to the stress condition towards the end of culture period where nutritional stress could play a significant role. Stress-dependent increase in the levels of Spm has been observed in various other studies (Kumar and Rajam 2004; Silveria et al. 2006). The cultures on M2 medium remained mostly except for a slight change in shoot height. The accumulation of Spm in embryogenesis may be essential for the shift from callus to embryogenesis. A fine balance of different PAs may be required for embryogenesis. This is evident from our experimental results that treatment with inhibitors of PAs biosynthesis resulted in drastic reduction towards embryogenesis. Similar kind of response was observed in different plant systems with regard to morphogenic responses (Bais and Ravishankar 2002). PAs are reported to promote shoot multiplication and *in vitro* flowering in *Cichorium intybus* (Bais et al. 2001). Variable results have been observed in various other studies upon external applications of PAs. For example, the application of Put (20 µM) and Spd (40 µM) for callus cultures of *Pinus oocarpa* and *Pinus patula* stimulated the embryogenesis (Niemi et al. 2002) as is the case of the present study. Contrarily, during the growth of embryogenic suspension cultures of *Pinus taeda*, high levels of endogenous Put were associated with reduction in cell division, whereas in oats Put enhanced the formation of somatic embryos (Minocha et al. 2004). In *P. sylvestris*, Spd retarded cell proliferation and growth but enhanced somatic embryo maturation (Niemi et al. 2002). Thus in most of the cultures, these free exogenous PAs supported organization rather than unorganized growth. Accordingly, the PAs were higher in organized cultures, indicating that PAs are invariably organization-dependent.

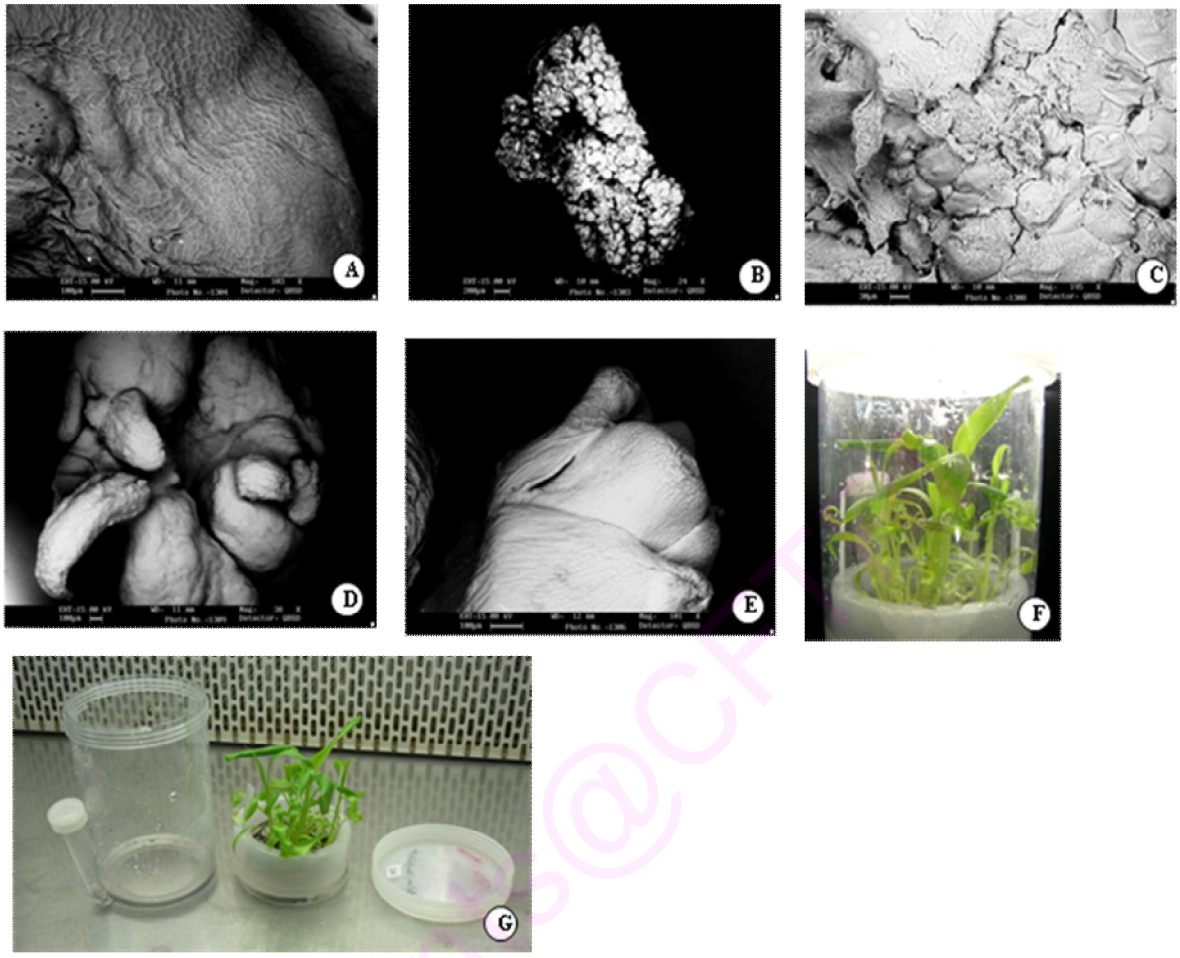

Figure 2.22. Sequential morphological events during plant regeneration. A: Embryonic calli obtained after 4 weeks of culturing on M2 medium supplemented with 40 mM spermine, B: Formation of numerous globular shaped embryogenic mass, C: Enlarged view showing clusters of embryogenic mass indicated by arrow, D; A cluster of pro-embryos and embryos at various stages of development towards shoot formation, F An enlarged view of fully formed embryo showing soot primordium. G. Further growth of embryogenic culture leading to the formation shoots, their further elongation in the Growtek™ bioreactor containing M3 medium with 30 µM of gibberellic acid, G. Different parts of Growtek™ bioreactor

In the present study exogenous supply of Spm resulted in the enhancement of embryogenesis, suggesting the promotive role of PAs for embryogenesis in *banana.* In an earlier study Beatriz et al (1994) studied the effect of exogenous administration of PAs in the concentration range of 0, 50 and 100 µM. The results showed the occurrence of significant differences among embryos indicating that the effects of PAs are not only species dependent but also organ dependent.

2.4.1.2. Shoot Multiplication and Elongation in Partially Immersed System

Since shoot height is a decisive factor in better acclimatization of banana shoots (Venkatachalam et al. 2007a), GA appears very useful in obtaining high quality shoots. The Growtek^{TM} bioreactor, functioning on the principle of PIS has been used for micropropagation of other plant species where it was observed to support constant supply of nutrients and aeration to plants combining the advantages of both solid and liquid medium. The results of the present study also showed better utilization of nutrients in PIS than in SM, since the biomass produced will be directly proportional to the nutrient utilization. All in all, almost similar multiplication with better shoot height were observed in PIS than in SM in a period of 4 weeks, suggesting that the observation made through the present study would be of great use for producing better quality shoots of NR banana. Uniform rooting in MS medium with ½-strength NH_4NO_3 supplemented with NAA (10 µM) was also observed. Such shoots appeared more robust than those from SM resulting in 100% survival in green house condition. It is also worth mentioning that the use of Growtek^{TM} bioreactor works out to be cheaper than any other commonly used apparatus meant for mass cloning of plants (Dey 2005), since liquid form of culture medium is invariably cheaper than the gelled form, with an added advantage of ease in handling.

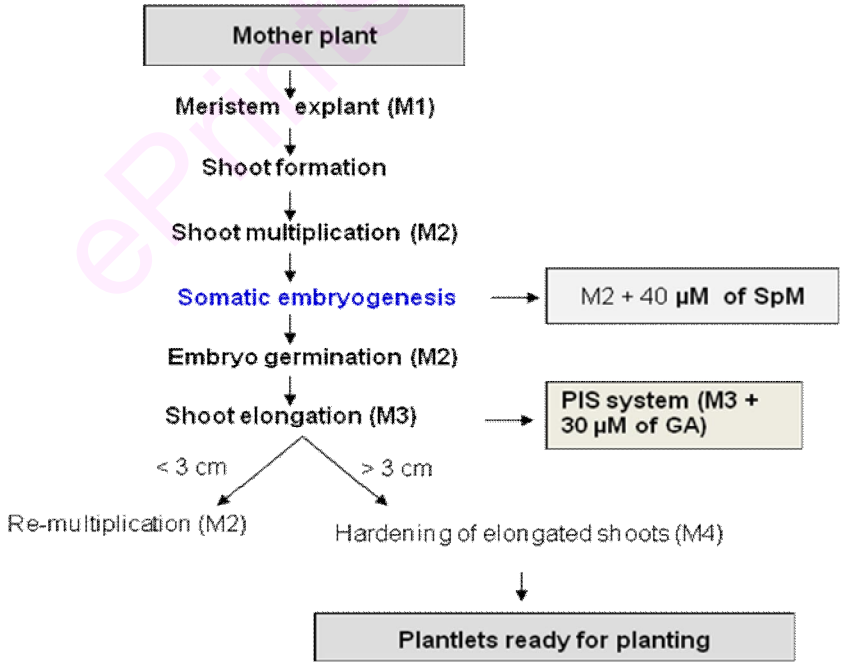

Figure 2. 23. Modified Protocol for rapid multiplication of shoot cultures of NR

2.4.2. Micropropagation

2.4.2.1. Morphological changes due to high concentration of cytokinins

Inclusion of growth regulators into the culture medium becomes obligatory to induce repeated shoot multiplication from shoot buds of banana. Exogenous supply of a single cytokinin was sufficient to induce shoot multiplication to some extent in NR. However, a high rate of shoot multiplication needs high level as well as a precise combination of different cytokinins, because each cytokinin is known to trigger different molecular pathways (Letham and Gollnow 1985; Blackesley and Lenton 1987). The synergistic action of BA and kinetin in inducing multiple shoots has also been reported in other genotypes of *Musa* (Mendes et al. 1999). In the present study, we observed that multiple shoot formation occurred in all the combinations of growth regulators with significant differences in the number of shoots formed under each treatment. Although BA at higher concentrations (≤ 6 mg l^{-1}) fostered an enhanced rate of shoot multiplication, it was detrimental to shoot proliferation at very high concentrations (7-10 mg l^{-1}). This may be due to exudation of phenolics. Similar suppressive effects of BA at very high concentrations have been previously reported in AAB genotype of banana (Vuylsteke et al. 1991; Lee and Phillips 1988). Similar to BA, kinetin at very high concentrations (5-10 mg l^{-1}) also caused exudation of phenolics with a concomitant reduction in shoot number as well as shoot length. Exudation of phenolics due to high levels of cytokinins has not been reported in earlier studies, and hence may form an interesting topic for further physiological studies. We also observed that, unlike BA, kinetin did not show any concentration-linked morphological changes as there was randomness in the pattern of shoot proliferation. Such an effect of kinetin has also been reported in other genotypes of banana, such as AA and AAB (*cv*. silk) (Mukunthakumar and Seeni 2005). Randomness in shoot proliferation in vitro may probably be due to the formation of physiological gradient and/or interaction of exogenously supplied kinetin with other forms of endogenous cytokinins as reported earlier in banana (Zaffari et al. 2000). However, when transferred to liquid shoot proliferation medium, there was uniformity in the morphology of resulting shoots with a difference only in the number of shoots proliferated. Therefore, our study clearly established that tissue culture conditions do not induce somaclonal variation in banana *cv*. NR (AAB) despite exposure to high

levels of cytokinins during micropropagation. In addition, the protocol standardized for rapid multiplication (HRMM) of this elite and endangered cultivar of banana is highly useful for commercial micropropagation as the process not only involves a substantial cost reduction due to high rate of multiplication, but also ensures a high degree of genetic fidelity (Figures 2.23 and 2.24).

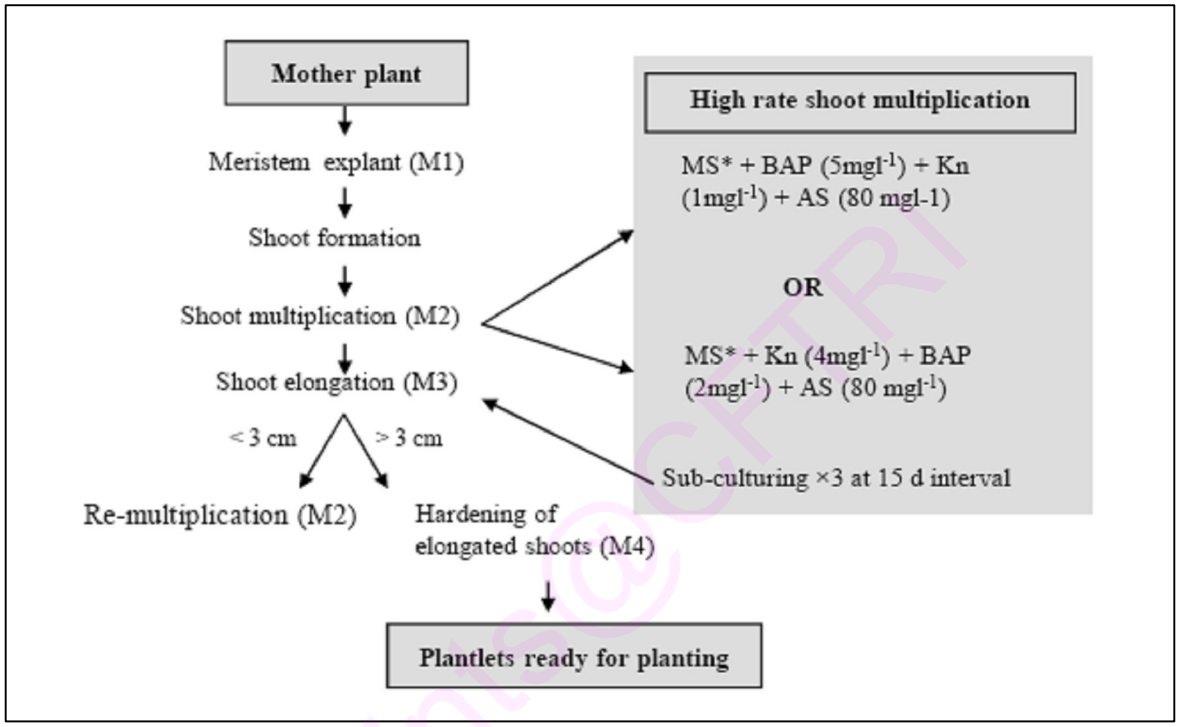

Figure 2.24. Protocol for rapid multiplication of shoot cultures of NR banana

2.4.3. Genetic analyses of plantlets

2.4.3.1. Genetic analysis of regenerated and micro-propagated plants

The present study was done to check the effects of high levels of cytokinins on rapid *in vitro* shoot multiplication of NR where the genetic stability of plantlets was assessed using RAPD and ISSR markers. Inclusion of growth regulators into the culture medium becomes obligatory to induce repeated shoot multiplication from the shoot buds of banana. Exogenous supply of a single cytokinin was sufficient to induce shoot multiplication to some extent in NR. However, a high rate of shoot multiplication needs high level as well as a precise combination of different cytokinins because each cytokinin is known to trigger different molecular pathways (Letham and Gollnow 1985; Blackesley

and Lenton 1987). For NR, an initial screening experiment indicated that a combination of BAP (8.96 µM) and Kn (4.65 µM) along with adenine sulphate (216.80µM) was suitable for steady multiplication of shoots (Table 2.1). The synergistic action of Kn and BAP in inducing multiple shoot formation has also been reported for other genotypes of *Musa* (Mendes et al. 1999). The high levels of growth regulators caused variants like suppression of shoot height creating morphological abnormality and genetic change in other genotypes of banana (Shenoy and Vasil 1992; Martin et al. 2006). The results of the effects of higher levels of BAP indicate that multiple shoot formation occurred in all the combinations tested with significant differences in the number of shoots formed under each treatment. The increase of BAP resulted in concomitant increase in shoot number with a highest of 80 (31.36 µM) shoot buds per segment. At further higher levels of BAP (35.84 µM - 53.76 µM), formation of shoot buds was suppressed which was associated with the exudation of phenolics. Such suppressive effects of BAP, at high concentrations, have been previously reported by Vuylsteke et al (1991). Similar to BAP, Kn at very high concentrations such as 41.85 µM – 55.80 µM, also caused exudation of phenolics and reduction in shoot number as well as shoot height. Such an effect of exudation of phenolics due to high levels of cytokinins has not been noticed in earlier studies and hence may form an interesting topic for further physiological studies.

True-to-type clonal fidelity is one of the most important pre-requisites in the micropropagation of any crop species. A major problem often encountered with the *in vitro* cultures is the presence of somaclonal variation amongst sub-clones of one parental line, arising as a direct consequence of in vitro culture of plant cells, tissues or organs Palombi and Damiano (2002) 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 which amplify different regions of the genome (Martins et al. 2004; Martin et al. 2006; Lattoo et al. 2006; Ray et al. 2006). Hence, in the present study, two PCR based techniques, RAPD and ISSR were adopted for evaluation of clonal fidelity in banana plantlets. The data generated in the form of bands for RAPD (Figures 2.13 and 2.14) markers showed distinct bands. A total of 16875 bands from micropropagated plants and 14,175 bands from regenerated plants

were generated in the present study which is highest compared to all such other studies reported so far (Martins et al. 2004; Bennici et al. 2004; Carvalho et al. 2004; Ray et al. 2006).

In the case of micropropagation of banana, though the protocol is known to yield clonal material, there are reports of somaclonal variation especially in cultivar Williams (AAA) (Israeli et al. 1996; Damasco et al. 1996), Robusta (AAA) and Giant Governor (AAA) (Ray et al. 2006). Such somaclones were relatively stable without generally reverting to normal phenotypes, where the PCR-based tests were found very useful in genotyping such new clones (Ramage et al. 2004). In genus *Musa*, the extent of instability was a result of the interactions between the genotype and the tissue culture conditions (Vuylsteke et al. 1991; Martin et al. 2006). While experimenting with plantains (AB), Cronauer and Krikorian (1983) observed that the frequency of the occurrence of variants in any variety was confined to individual primary explants rather than the genotype. Ray et al (2006) reported complete genetic stability in micropropagated 'Martaman' banana also having genotype AAB. Another common undesirable somaclonal variant observed in 'Cavendish' banana was the mosaic type where the heterogeneity and incidence of variance were epigenetic (Reuveni and Israeli 1990). All these reports inferred that such somaclonal variations were the resultants of tri-way interactions of initial explants, the culture conditions and the genotype. The polymorphism in 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). While genetic variations are exceptions, most of the organized cultures, especially the shoot-tips maintain strict genotypic and phenotypic stability under tissue culture conditions (Bennici et al. 2004), which appears applicable also to NR banana since no variations were observed for the primers tested. The primers selected in the present study were based on the earlier report(s) where such primers were found to be of great use in differentiating the varieties and developing phylogenetic relationships in banana germ-plasm (Pillay et al. 2001; Ude et al. 2003; Onguso et al. 2004). The absence of any variation in the banding pattern for such a set of primers used in the present study

clearly indicate that the extreme growth conditions with high suppression of shoot growth and exudation of phenolics do not induce any genetic variability in NR. Variation in the band intensities for certain fragments, though negligible, is probably due to the fact that in banana DNA competition among RAPD and ISSR loci in the random primed PCR may be a major cause for amplifying results. This phenomenon of variation in the intensity of some fragments has also been observed in other studies (Ryynanen and Aronen 2005). However, the band intensity histograms in the present study did not show any variation. Therefore, it has been clearly established that the tissue culture conditions do not induce somaclonal variation in the banana cultivar NR despite exposure to high levels of cytokinins as well as regeneration from the leaf base explants.

In conclusion, through the present study, for the first time, both micro-propagated and regenerated plants have been genetically analyzed and found that the cultivar NR is genetically stable under *in vitro* conditions. This suggests an urgent need for alternative protocol for developing variants especially to improve the agronomical traits.

2.4.3.2. Genetic stability of long term micro-propagated plants

The exact cause of somaclonal variation in *in vitro* cultures are still unknown, although it is believed that alterations in auxin-cytokinin concentrations and their ratio, duration of *in vitro* culture, *in vitro* stress due to unnatural conditions, altered diurnal rhythm and nutritional conditions (Modgil et al. 2005) together or independently are responsible. Cultured plant tissues are also known to undergo high levels of oxidative stress due to reactive oxygen species formed within the cells and the latter is known to cause DNA damage, including that of microsatellite instability (Jackson et al. 1998). Probably due to some of such reasons, morphological variations have been a common feature as long as the shoot/plantlet cultures are under *in vitro* conditions, with reversal to normal state when such plantlets are transferred to soil. Development of different morphotypes during prolonged *in vitro* culturing was also observed by Goto et al (1998) and Ishii et al (1987) while working on micropropagated morphotypes of *Pinus thunbergii and Pinus radiata* respectively. In these studies, as well as in several other studies, the use of cytokinins, especially the exposure to BAP coupled with altered diurnal rhythm and continuous availability of high levels of nutrients have been noted to induce hyper-hydricity and alterations in morphology. Hyper-hydricity, otherwise known

as vitrification phenomenon, has also been prevalent in xerophytic plants. Such morphological changes, however, were found un-associated with the genetic change (Goto et al. 1998), as also observed in NR banana of the present study. Even at post-transcriptional level, the absence of any change in isozyme patterns in such morphological off-type plants (Ishii et al. 1987) indicates that there could be temporary alterations in the physiological states of the shoots. Therefore, even in the case of NR banana, though certain morphotypes were observed during routine multiplication, the tissue culture protocol developed by us resulted in clonal plantlets with no detectable genetic change.

In conclusion, this study has screened a large number of random primers that are common for higher plants, and some of them are prevalent in monocots. Since there were no changes in the banding pattern observed in tissue culture plants as compared with that of mother plant, we conclude that that our micropropagation protocol for banana var. Nanjanagudu Rasabale can be carried out for a considerable length of time without much risk of genetic instability.

2.4.3.3. Sonication assisted Transformation using *A. rhizogenes* harbouring a binary vector

The use of acetosyringone has been a common feature for accomplishing stable transformants in most of the monocots and a few dicotyledonous plants (Melchers et al. 1989). Thus, both sonication and inclusion of acetosyringone were essential in improving the transformation efficiency to 3.4% in NR. While transforming banana with biolistic method, the actual transformants varied from 11% in AAA (Becker et al. 2000) to 3.4 % in AA. After 24 h of co-cultivation, colonization of *A. rhizogenes* was observed at the wounded regions (Fig not shown). Both control and *A. rhizo*-infected shoot buds were transferred to fresh medium comprising MS medium supplemented with 1238 mg l^{-1} NH_4NO_3, 2.0 mg l^{-1} BA, 1.0 mg l^{-1} Kinetin, 80 mg l^{-1} adenine sulphate, 25 g l^{-1} sucrose, 2.5 g l^{-1} gelrite® and varying concentration of hygromycin. Every 2 months, the surviving embryos were transferred to higher hygromycin concentrations from 5 mg l^{-1} to 10 mg l^{-1}, and up to 20 mg l^{-1}. On this medium the control shoot buds (non-co-cultivated with *A. rhizogenes*) and non-sonicated *A. rhizogenes*-infected cultures failed to grow further due to the presence of hygromycin. Whereas a few buds after sonication / co-

cultivation survived on this medium due to the induction of hygromycin resistance, which were considered as putative transformants.

In the present study, the putative transformant shoots produced secondary shoot buds at the base in 6 weeks of culture on hygromycin selection medium. After 12 weeks of selection, the antibiotic resistant new clones of plants were cultured in medium without BA and Kn to facilitate further growth and elongation. Culture of shoot buds on medium with cytokinins resulted in an increase in the number of shoot buds, and hence more number of putative transformants could be recovered. Based on the requirement, a large number of transformed shoots may thus be obtained.

The use of organized cultures for transformation is appealing because of its relatively short regeneration time. May et al (1995) produced transgenic Cavendish *cv* 'Grand Nain' by co-cultivating wounded meristems with *A. tumefaciens*. However, some workers consider this technique is of limited value because of the potential generation of chimeric plants (Becker et al. 2000) probably occurring as `escapes' on the selection medium. Nevertheless, in the present study, the repeated culture of the putative transformants on increasingly higher concentration of hygromycin resulted in the survival of only complete transformants and no escapes were encountered. Thus the method explained here indicates the feasibility of organized structures such as shoot buds and reap the advantage of faster results.

It is well established that diverse species of bacteria are capable of gene transfer to plants (Broothaerts et al. 2005). However, *Agrobacterium* sp are used widely for genetic modification in plants due to their gene integration capacity to the host genome. Although initially *Agrobacterium*-mediated transformation was thought mainly applicable for dicotyledonous plants, understanding the biochemistry of infection process and finding out the need of acetosyringone for transfection, several monocots have been successfully transformed with *A. tumefaciens.* Whereas, the other species of *Agrobacterium, A. rhizogenes,* has been used for a few dicots such as coffee (Kumar et al. 2006) and broccoli with an antisense ACC oxidase gene (Henzi et al.1999) and *Lithospermum erythrorhizon* with an *uid* A and *hpt*II marker (Yazaki et al. 1998). In case of banana, the present study appears to be the first report on obtaining complete transformants with good frequency using *A. rhizogenes,* especially in genotype AAB.

2.4.3.4. GUS assay, PCR and Southern analysis

The GUS expression is known to vary depending on the site of insertion. For example in Arabidopsis, a large collection of "trap lines" were generated with reporter genes in inserted randomly into the genome. (Bechtold et al. 1993; Topping et al. 1994; Sundaresan et al. 1995; Campisi et al. 1999; Springer 2000). When insertion occurs within or adjacent to a gene or enhancer region, the reporter gene is expressed under the control of the native promoter or enhancer elements. In addition, expression time of GUS also varied in *Arabidopsis* with certain lines expressing GUS activity only at particular stages of root development and vanishing later. Similarly in the present study, GUS expression was both temporary as well as localized.

The *cv*. NR is an endangered plant due to its susceptibility to devastating diseases such as banana bunchy top, *Fusarium* wilt and others. Genetic transformation, in conjunction with pathogen-derived resistance (Fitchen and Beachy 1993), is one potential strategy for developing virus resistance in bananas, which has proven to be difficult to obtain by conventional breeding (Vuylsteke and Swennen 1992). The fruits of NR are highly relished by locals who believe them to be having medicinal properties. The scarcity in meeting the demand may be met by genetic improvement of the cultivar with the present protocol. Since NR is also rich in protein (1.8%), newer proteins of interest may be expressed in fruit pulp with specific promoters. Thus the protocol developed in the present study appears useful for the improvement of NR and probably other cultivars of banana. In case of banana with AAB genotype, the present protocol appears to be the first one for obtaining complete transformants.

CHAPTER - III

Regulation of Ripening Related Genes

SUMMARY

Fruit ripening and softening involve de-polymerization of complex cell wall components where several classes of enzymes and proteins are known to be involved in the process of softening acting either sequentially or synergistically. This chapter deals with studies on the regulation of the fruit softening at biochemical level by following the activities of enzymes as well as by following gene expressions of cell wall loosening (expansins) and hydrolyzing enzymes. The two type cell wall loosening genes, expansin genes, *MaEXPA-1* and *MaEXPA-2*, were found to be fruit-ripening-specific and their expressions significantly and differentially altered by ethylene inducers/suppressors. In control fruit the transcripts of *MaEXPA-1* appeared after 6 days and *MaEXPA-2* appeared 4 days of harvest of mature fruit, although softening initiates immediately after harvest and progresses steadily then onwards. Initial transcript level of *MaEXPA-1* was found higher in control than in treatments that enhance ethylene pathway and was suppressed in ethylene pathway inhibitors, confirming that this gene responds to ethylene pathway. The transcript levels of *MaEXPA-2* were much higher than those of *MaEXPA-1* to various treatments, and high levels coincided with climacteric peak in smoke- and NAA-treatments. Since fruit softening occurred even before the appearance of transcripts of these genes, these genes do not appear to play the initial role in cell wall loosening / fruit softening; nevertheless contributing substantially for post-climacteric softening. Other softening enhancers caused extensive degradation in cell wall assembly, particularly the solublization of pectin. The major activities of pectin methyl esterase (PME), polygalacturonase (PG) and pectate lyase (PEL) in banana *cv*. NR fruit were extensively active over a period of 10 days in ethylene enhancers. Effects of treatments with ethrel, smoking, salicylic acid (SA), abscisic acid (ABA), indole acetic acid (IAA), NAA, GA and MH on activities of cell wall hydrolases showed that, smoking and ethrel stimulated activites PME and PEL but not PG. GA, MH, SA and IAA suppressed ethylene effects resulting in slowing down the ripening. Such suppressions concomitantly suppressed the expression of *PME*, *PG* and *PEL* genes indicating that ripening suppression happens at genetic levels. Thus the chemical treatments play a major role in up- / down-regulating the activities of various cell wall hydrolases.

3.1. INTRODUCTION

Ripening is accompanied by changes in flavour, texture, colour, and aroma. Fruit follow two types of contrasting ripening patterns. Climacteric fruit such as banana, tomato, avocado and apple, show a burst of ethylene biosynthesis and an increase in respiration during peak ripening stage which is often the most appropriate edible stage. Non-climacteric fruits such as strawberry, grape and citrus show no ethylene burst and respiratory peak. Ripening of banana fruit involves a wide variety of coordinated changes which are under strict genetic control and not due to just senescence-related cellular disruption. Such integrity and intracellular compartmentalization of various biochemical activities are maintained throughout the ripening process (Tucker and Grierson 1987; Brady 1987; Palmer 1971). Ethylene formation and the related biochemical networks (Figure G3) are presented under the section "General Introduction".

Banana is a tropical fruit of commercial significance known to undergo substantial textural and color transformations during ripening (Prabha and Bhagyalakshmi 1998; Bhagyalakshmi et al. 2002). Progression in ripening brings about degradation of cell wall components resulting in fruit softening. The cell walls of fruit are generally composed of cellular microfibrils tethered with xylogycans embedded in pectin mesh and glycoproteins (Carpita and Gibeaut 1993). Softening appears to be mainly associated with changes in the pectin fraction of the cell wall (Huber 1983). Thus, a large increase in pectin solubilization has been correlated with softening (Brummell and Harpster 2001). These ultra structural and chemical changes may result from *de novo* synthesis of the cell wall hydrolases such as pectin methyl esterase (PME), polygalacturonase (PG), pectate lyase (PEL) and cellulase.

Pectin Methyl Esterase (PME) (EC 3.2.1.11) demethylates the C6 of the galacturonosyl residue and PG acts preferentially on the demethylated substrate (Koch and Nevins 1989). Thus, PME provides substrate for PG to act upon. Together, they act mainly on the middle lamella (Ahmed and Labavitch 1980; Koch and Nevins 1989; Lohani et al. 2004).

Polygalacturonase (PG) catalyzes the hydrolytic cleavage of α-1,4 galacturonan linkages of pectin. In ripening bananas, polygalacturonase (PG) as either endo-acting

(EC 3.2.1.15) or as exo-acting (EC 3.2.1.67) enzyme has been reported (Pathak and Sanwal 1998 &1999; Pathak et al. 2000; 2003; Asif and Nath 2005). PG has been isolated from tomato (Hobson 1965), avocado (Awad and Young 1979), apple (Bartley 1978), pear (Ahmed and Labavitch 1980), strawberry (Nogata et al. 1993) and other fruits. Multiple forms of PG have been demonstrated in strawberry (Nogata et al. 1993), tomato (Crookes and Grierson 1983), peach (Pressey and Avants 1973) and pears (Pressey and Avants 1976). Pectate lyase (poly [1,4-α-D galacturonide] lyase; EC 4.2.2.2.) catalyzes the cleavage of 1,4-α-D galacturonan linkages of pectate by β-elimination reaction, generating 4,5-unsaturated oligogalacturonates. Pectate lyases (PEL) are widely distributed in bacteria (Pérombelon and Kelman 1980). These enzymes act by depolymerizing cell wall polygalacturonane in the presence of calcium ions, thus destroying the integrity of the plant tissues (Collmer and Keen 1986). High levels of laminarase and xylanase have been recorded in ripening Cavendish banana (Bhagyalakshmi et al. 2002).

The major ripening phytohormone ethylene has been shown to have variable effects on the activities of these hydrolases during ripening (Brummell and Harpster 2001; Lohani et al. 2004). An early rise in respiration and burst of ethylene synthesis occurs during ethylene-induced banana ripening (Jiang et al. 2000; Pathak et al. 2003). Earlier studies on the physiology of banana ripening have indicated that abscisic acid (ABA) and indole 3-acetic acid (IAA) could also modulate ripening (Pathak and Sanwal 1999; Jiang et al. 2000). However the effects of these hormones on the activities of cell wall hydrolases have not been elucidated in banana in detail.

Expansins are the multi-gene family of proteins in plants involved in the induction of needed expansion of cell walls during various physiological and developmental processes and have also been reported to disrupt non-covalent interactions between hemicelluloses / cellulose micro-fibrils. Thus many studies on expansins have focused on their roles in cell division leading to growth. However, specific expansins selectively accomplish irreversible de-polymerization of cell wall polysaccharides in ripening fruit, suggested to provide substrates for the other enzyme, thus playing key role in specific physiological events. In the present study we show that ripening banana fruit *cv*. NR has two types of expansin genes, *MaEXPA-1* and *MaEXPA-2*, found to be fruit-ripening-

specific and their expressions significantly and differentially altered by ethylene inducers/suppressors.

Changes in the activities of cell wall modulating enzymes during banana fruit ripening, over a period of 10 days, after treatments with ethylene inducers/inhibitors are reported herein, emphasizing on enzymes and expressions of major genes involved in cell wall loosening and hydrolysis.

3.2. MATERIALS AND METHODS

3.2.1. Plant materials and treatments

Mature green banana (*Musa acuminata* AAB *cv*. NR) fruit grown for 110 days after anthesis were harvested from the banana plants grown at author's departmental garden. The procedure for treatments was the same as that described by Purgatto et al (2001). Treatments were applied within 24 h of harvest. Each banana bunch was separated into individual hands and individual fingers with uniform size of 14 cm of length and 4 cm of diameter at the central portion and an average mass of 80 g were cleaned with distilled water, surface-sterilized using 1% sodium hypochlorite aqueous solution. The crown surface of each hand was trimmed off using a sterilized knife to make a fresh wound surface for the accessibility of treatment and allowed to air dry for 2-3 h. In each experiment, fingers from the same hand were used as a sample group to avoid variation in ripening behaviors of fingers among different hands (Inaba and Nakamura 1986). The fruits were dipped in aqueous mannitol (120 mM) solution treatment compounds such as SA (1000 mg l^{-1}), ethrel (1%), ABA (25 mg l^{-1}), NAA (500 mg l^{-1}), IBA (500 mg l^{-1}), GA (500 mg l^{-1}) and MH (500 mg l^{-1}) for 30min and air-dried at ambient temperature (29-30°C, 85% RH). Smoking, however, was done directly by exposing the hands for one minute above the fire-doused straw. After treatment, the hands were randomly placed in corrugated cardboard boxes and stored at 29-30°C, 85% RH. Fruit were regularly inspected and samples were collected every 2, 4, 6, 8, 10 days after treatments. Fruit ripening was classified into seven stages (Stover and Simmonds 1987).

3.2.1.1. Texture Measurement

The texture of the fruits at various stages of ripening was measured using an Instron 4301-UTM (Universal Texture Measuring system) by WB Shear at a speed of 100 mm/min and a load of 100 kg. Three fruits were removed from each treatment at 2 day intervals for a total experimental period of 10 days and were used for analysis.

3.2.2. Enzyme assays

3.2.2.1. Enzyme preparation

The method of Pilatzke-Wunderlich and Nessler (2001) for extracting cell-wall-degrading enzymes from the latex of *Papaver somniferum* L. was adapted for extraction

of enzyme from banana pulp. 1 g sample of fresh or frozen (-70°C) pulp tissue was homogenized in a mortar with 1 ml of extraction buffer (cysteine–HCl (20 mM), EDTA (20 mM) and Triton X-100 (0.05%, 0.05 M potassium phosphate pH 7, 2% 2-mercaptoethanol). The sample was then spun in a microcentrifuge at 12000 × *g* for 20 min at 4°C temperature. Samples were used immediately for analysis.

3.2.2.2. PME activity

Pectin methyl esterase activity was measured as described by Hagermann and Austin (1986) with some modification. The reaction mixture was prepared in a 3 ml glass cuvette. It contained 1ml pectin solution (0.01% aqueous solution adjusted to pH 7.5 using 0.1N NaOH), 0.2 ml NaCl (0.15 M), 0.1 ml bromothymol blue solution (0.01%), 0.2 ml water and 0.1 ml homogenate. After adding the enzyme preparation, the cuvette was covered with stopper and inverted gently once and absorbance was measured immediately at 620 nm. The absorbance was again measured after 3 min. The difference in absorbance between 0 and 3 min was the measure of PME activity. Calculation of the activity was carried out against the standard curve drawn as described by Hagermann and Austin (1986). One unit is defined as the amount of the enzyme required for liberating 1 µmol of methyl ester per minute and expressed as unit per mg of the protein.

3.2.2.3. PG activity

Polygalacturonase activity was assayed by the method described by Pathak and Sanwal (1998). The reaction mixture contained 0.2 ml sodium acetate (200 mM, pH 4.5), 0.1 ml NaCl (200 mM), 0.3 ml polygalacturonic acid (PGA, 1% aqueous solution adjusted to pH 4.5) and 0.1 ml of enzyme extract in a total volume of 1.0 ml. The reaction was initiated by the addition of the PGA substrate. The mixture was incubated at 37°C for 1 h followed by addition of DNS. The reaction was terminated by heating the reaction mix in a boiling water bath for 5 min. In control tubes the substrate was added after the heat treatment. The formation of reducing groups was estimated against D-galacturonic acid as the standard after measuring the absorbance at 540 nm. One unit of enzyme is defined as the amount of enzyme required to liberate 1 nmol of galacturonic acid per min under the conditions of the enzyme assay (Miller 1951).

3.2.2.4. PEL activity

The enzyme activity was determined according to the method described by Pitt (1988). The assay system consisting of 2.5 ml polygalacturonic acid solution (1% w/v in 0.05 M Tris–HCl buffer, pH 8.5), 1.0 ml $CaCl_2$ (0.01 M), 1.5 ml enzyme and water was incubated at 37°C for 2 h. The reaction was stopped by the addition of 0.3 ml $ZnSO_4$. $7H_2O$ (9% w/v) followed by 0.3 ml NaOH (0.5 M). The precipitate formed was centrifuged at 3000×g for 10 min. To 2.5 ml supernatant 1.5 ml thiobarbituric acid (0.04 M) was added followed by the addition of 0.75 ml HCl (0.1 M) and 0.25 ml water. The mixture was heated in a boiling water bath for 30min, cooled and absorbance measured at 550nm. The control tubes received enzyme after addition of $ZnSO_4$ and NaOH. One unit of PEL is the amount of enzyme causing a change in absorbance of 0.01 under the conditions of the assay.

3.2.2.5. Extraction and Estimation of Total Protein Content

Total protein was extracted from the banana pulp and estimated according to the method of Lowry et al (1951) using BSA as a standard.

3.2.3. Expression studies

3.2.3.1. RNA isolation

Total RNA was extracted from 5 g pulp tissue of treated banana using CTAB as described by Asif et al (2000) with the following modifications. All the steps were performed on ice and the pellets were obtained by centrifugation at 18000 × g at 4°C.

Reagents:

10M LiCl

3M Na Acetate pH 5.2

2-mercaptoethanol

DEPC treated and autoclaved MilliQ grade water

Absolute ethanol

Chloroform:isoamyl alcohol (24:1)

3.2.3.1.1. Protocol: 1 g of tissue was homogenized in liquid nitrogen and the finely powdered frozen sample was suspended in 5 to10 ml pre-warmed extraction buffer (patent pending for the composition of buffer) and the mixture was transferred to 30 ml

capacity pyrogen –free tubes and incubated for 60 min at 65°C with occasional shaking and cooling to room temperature. To these an equal volume of chloroform and isoamyl alcohol at a ratio of 24:1 was added, mixed thoroughly and then centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was collected, the extraction was repeated with equal volume of chloroform and isoamyl alcohol (at a ratio of 24:1), and centrifuged at 10,000 rpm for 10 min at 4°C. Then to the aqueous phase 8M lithium chloride was appropriately added to make the final concentration of the latter 3M, the mixture incubated overnight at 4°C and the pellet collected by centrifugation at 12000 rpm for 25 to 30 min at 4°C, further by dissolving the pellet in 500µl of DEPC-treated water, the mixture transferred to 1.5mL non-sticky centrifuge tubes to which equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) were added, the contents were mixed by using vortex machine followed by centrifugation at 12000 rpm for 1 min at 4°C, the aqueous phase transferred to non sticky RNAase-free 1.5mL centrifuge tube to which 1/3 volume of 3M sodium acetate (initial concentration of 3M, pH 5.2) and 0.1 volume of 100% ethyl alcohol, mixing the solution and keeping in an ice bath for 25- 45 min, after which the contents were centrifuged at 12000 rpm for 25 min at 4°C where a white jelly like pellet consisting mostly of polysaccharides obtained was discarded, to the supernatant again 1/3 volume of 3M sodium acetate and 3 volumes of absolute ethyl alcohol was added and the precipitate was obtained after storing at -70°C for 3 to 24 hrs, the contents centrifuged at 12000 rpm for 30 min at 4°C, after discarding the supernatant the pellet was washed with 70% ethyl alcohol and pellet dried in vacuum, dissolved in 70 µL of DEPC treated water.

3.2.3.1.2. DNAse treatment

To the 25µl of isolated RNA sample contaminated with DNA, 2.5µl of DNase buffer and 2.0µl of turbo DNase were added in a nuclease free 1.5ml centrifuge tubes and incubated at 37°C for 30 min. Then 2.5µl of DNAse reagent was added and kept at room temperature for 2 min with occasional mixing for the de-activation of DNA if any. Then the mixture was centrifuged at 10,000 × g for 15 min at room temperature or 4°C then the supernatant was transferred to a new tube.

3.2.3.1.3. Characterization of isolated RNA by spectrophotometric method

The yield of isolated RNA was determined spectrophotometrically at 260 nm, where one absorbance unit (A_{260}) equals 40 µg of single stranded RNA per ml. The purity was estimated spectrophotometrically from the relative absorbance at 260 and 280 nm. Pure RNA exhibited and A_{260} / A_{280} ratio of 2.0. Integrity of RNA was checked by electrophoresis in formaldehyde denaturing gels stained with ethidium bromide.

3.2.3.1.4. RNA gel electrophoresis

Reagents

Agarose

37% formaldehyde

10X MOPS stock

a. MOPS : 400 mM; pH 7.0
b. Sodium acetate : 100 mM
c. EDTA : 10 mM pH. 8.0

3.2.3.1.4.1. Protocol: To prepare 200 ml of molten agarose solution, 2.4 g of agarose was suspended in 170 ml of sterile double distilled water and melted in a microwave. When gel was cooled to 55 to 65°C, mixture of 20 ml pre-warmed 10X MOPS buffer and 10 ml of pre-warmed 37% formaldehyde were added and the gel mixture was casted to 0.50 to 0.75 cm thick gel, and allowed to solidify at room temperature. Sample was prepared by mixing RNA with 3 volumes of formaldehyde loading dye (Ambion) and incubated at 65°C for 15 min, and immediately chilled on ice. Solidified gel was placed on the electrophoresis chamber and immersed in 1X MOPS buffer, and allowed for pre-run at 5V/cm for 5 min. Then denatured samples were electrophoresed at a maximum of 5V/cm distance between the electrodes. Electrophoresis was continued until the bromophenol dye front has migrated to within 1-2 cm of the distal edge of the gel. The gel was stained in a solution of 1.5 µg EtBr / ml of sterile water or in a fresh aliquot of 1X MOPS buffer. EtBr staining of formaldehyde gel gives unacceptable high background fluorescence, because of the presence of formaldehyde and the latter was removed by soaking the gel in sterile water for 20 to 30 min. Then gel was documented.

3.2.3.2. Semi quantitative Reverse Transcriptase -PCR analysis

3.2.3.2.1. First strand cDNA synthesis (PCR template generation)

Preparation in a sterile tube

Template RNA	: 0.1-5µg
Oligo $(dT)_{18}$ (MBI Fermentas)	: 0.5µg
Random hexamer (MBI Fermentas)	: 0.2µg
DEPC-treated water (Amresco)	: vol to 11µl

The mixture was incubated at 70°C for 5 minutes and chilled on ice.

Add the following on the order indicated

5X reaction buffer (Ambion)	: 4.0µl
10mM dNTP mix (MBI Fermentas)	: 2.0µl
Ribonuclease inhibitor (Ambion)	: 20.0U
DEPC-treated water	: vol to 18µl

The mixture was incubated at 37°C for 5 minutes. Then 40 units of M-MuLV Reverse Transcriptase was added and incubated at 37°C for 60 minutes. The reaction was stopped by heating the mixture at 70°C for 10 minutes.

3.2.3.2.2. Reverse Transcriptase - PCR analysis

The gene-specific primers for the expansins genes and cell wall hydrolyases were designed using Primer3 software (Table 3.1) and synthesized (MWG biotech, Bangalore, India). First-strand complementary DNAs were synthesized from 1.5 µg of total RNA in 20-µl final volume, using M-MuLV reverse transcriptase and oligo-dT (18 mer) primer (Fermentas GmbH, Germany). PCR amplifications were performed using PCR mixture (15 µl) that contained 1 µl of RT reaction product as template, 1X PCR buffer, 200-µM dNTPs, 1 U of Taq DNA polymerase, and 0.1 µM of each primer depending on the gene. PCR was performed at initial denaturation at 94°C for 4 min, 30 or 22 cycles (1 min at 94°C; 1 min at 55 or 60°C; 1 min at 94°C), and final elongation (10 min at 72°C) using a thermal cycler. The PCR products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%), and documented in a gel documentation system (Herolab GmbH Laborgerate, Germany).

Table 3.1. Gene specific primers and annealing temperatures used for RT-PCR

Primers	Primer sequence (5'-3')	Annealing temperature (°C)	Gene bank ID	Amplified fragment size (bp)
MaEXPA-1F	CACTGCGCTCTTCAACAATG	60	AY083168	502
MaEXPA-1R	CACGTTGTAGCTGGTGATCG			
MaEXP-2F	CTTGTACAGCGACGGGTACG	60	AF539540	500
MaEXPA-2R	AGAGGCTCTGACCAGTGAGG			
PME Forward	ACCGACACGAACTTTTCAGC	61	AY507150	500
PME Reverse	CAGGGTGCCTGTAGACGAAT			
PG Forward	GTCATGGACGCAGAGGAAAT	55	AY603339	423
PG Reverse	GGTCACGGATCTGGTGTTCT			
PEL Forward	CAGTCAAGGCAACCGATACC	61	AF479830	476
PEL Reverse	GGACGGTGTAACCGAGCTAA			
18S F	GGCAGCTATGTGTTTGTCCA	60	AF069226	450
18S R	TGTGGAATTGTGAGCGGATA			

The size of the amplification products was estimated from the 100-bp DNA ladder (Fermentas GmbH). The band intensity of each gel was checked using the Herolab E.A.S.Y Win 32 software (Herolab GmbH, Laborgerate). The transcript levels of each gene in green motile cells were taken for comparison in calculating the transcript abundance of respective genes during regeneration.

3.2.3.3. RNA dot blot

To confirm genomic integration, mRNA blot analysis was performed. The total RNA was precipitated overnight at 4°C by the addition of 0.3 volumes of 10 mM LiCl and then precipitated in ethanol. The quality and quantity of the RNA was checked by electrophoresis as described in the earlier section. 1 µl of total RNA (RNA from the stock 1µg μl^{-1}) was denatured with 2 volumes of denaturing buffer (1X MOPS containing formamide, formaldehyde) by boiling for 5 minutes and immediately chilled on ice and spotted to a Hybond-N nylon membranes (Ambion). The RNA was fixed to the

membrane by using a 302 nm ultraviolet cross linker for 45 min (Ambion). Then the membranes were pre-hybridized for more than 1 h in ULTRAHyb buffer (Ambion) and hybridization was then performed overnight with the same buffer containing the gene-specific DIG-labeled probe at 42°C. The probes were prepared by the cDNAs fragments of *MaEXPA-1* (502 bp), *MaEXPA-2* (500bp) and 18S (450 bp) were amplified (Table 3.1), purified and were labeled by DIG High prime method as described by manufacturer's instruction. After hybridization, the blots were washed with 2.0 × SSC / 0.1% SDS for 15 min at RT and finally with 0.1 × SSC / 0.1% SDS for 15–30 min at 65 °C. The blots were washed and visualized by manufacturer's instruction (Roche). The specific primers used for synthesis of DIG labeled probes are listed in Table 3.1.

3.2.4. Experimental design and data analysis

At each assessment time, three pieces of fruit from control and each of the treatments were assessed for fruit firmness and pool of three fruits used for total RNA extraction.

3.3. RESULTS

3.3.1. Fruit firmness

Firmness is an important internal quality parameter of ripening fruit. In order to clarify the effects of ethylene on fruit softening in banana, fruit of the *cv* 'NR' were treated with different ripening regulators (Figure G3). There were differences in trends for changes in the firmness of banana fruit peel versus pulp during ripening (Figures 3.1 and 3.2). Like other cultivars of banana, the pulp generally was softer than the peel in raw NR. A significant loss in firmness of peel and pulp was observed after 10 d in all the treatments. Such a loss of firmness was rapid in smoke-treatment in both peel and pulp, the loss being rapid from 4th day of treatment in pulp accounting for 50% loss in smoke, followed by 35% loss in ethrel treatment when control had lost only 13% firmness (Figure 3.1). Almost a similar trend for ethrel was observed for peel (Figure 3.1). SA has often been recommended for the control of softening (Srivastava and Dwivedi 2000) and known to act via regulation of major-ripening-specific enzymes. The SA treatment showed more rapid loss of peel firmness than that of pulp where the firmness of pulp throughout the treatment was almost similar to that of control whereas the peel underwent rapid and significant softening accounting for 50% loss by 6th day of treatment when compared the control. All other treatments with growth regulators and MH, except GA, induced steady softening of peel, the highest being in auxin and ABA treatments. The firmness was mostly maintained by MH with significant loss only after 8 days of incubation. The pulp softening was slow in control and MH treatment. Similar to SA, the GA also showed higher peel softening with a rather slow effect on pulp (Figure 3.2). In this set of treatments, the trend was almost similar for peel and pulp (Figures 3.1 and 3.2). MH caused minimum loss in firmness, although such loss in firmness was gradual for both peel and pulp in fruits until 8th day, after which there was a sudden decrease in firmness during subsequent period (Figure 3.2). In general ethrel, smoking, IAA, NAA, IBA and ABA enhanced the ripening process by decreasing the firmness, whereas, GA, SA and MH treatments inhibited fruit softening.

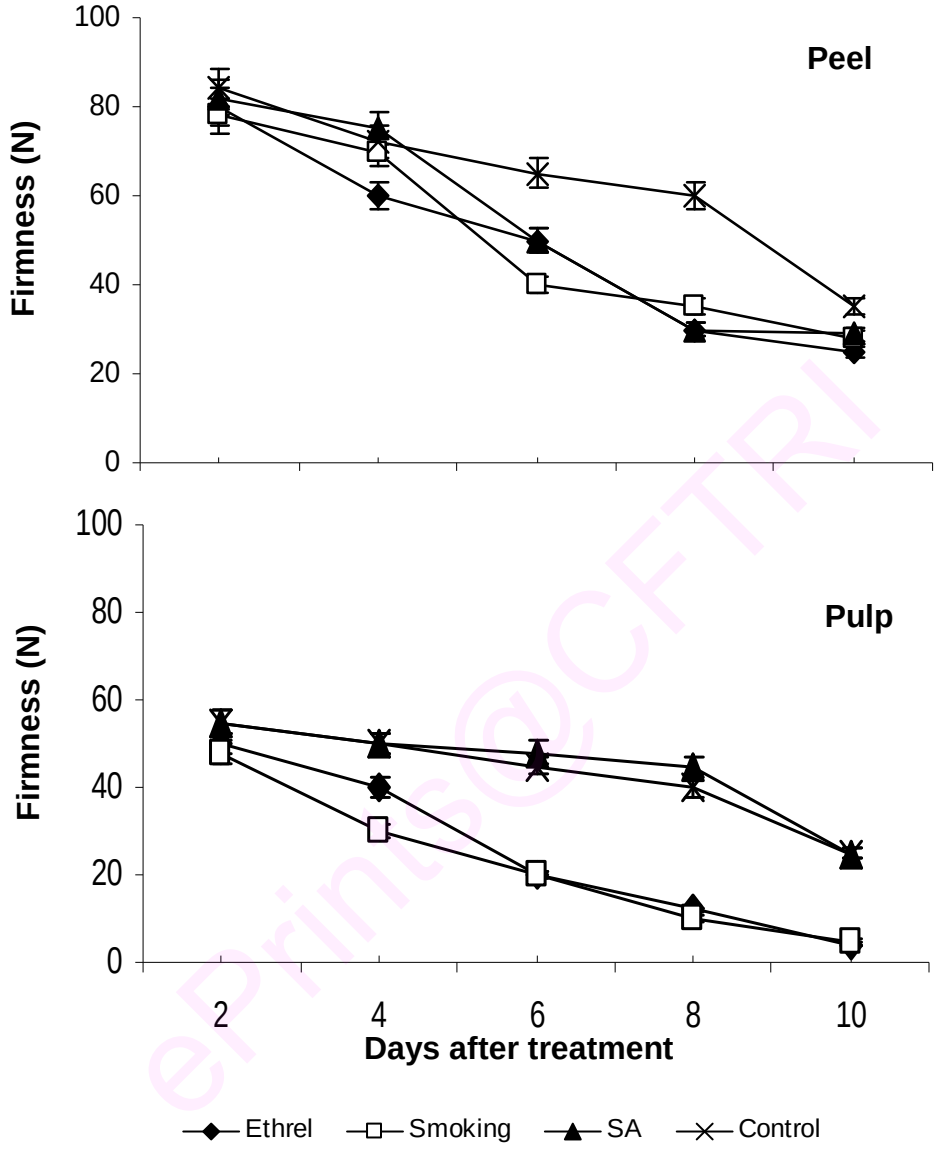

Figure 3.1. Changes in firmness of the peel and pulp of banana fruit pre-treated with ethrel (1%), smoking, 100 mg l^{-1} Salicylic acid (SA) and control fruit. The treatments were performed as described in the materials and methods. Each value is the mean of three six replications. Vertical bars indicate the standard error of the means where they exceed the symbol size

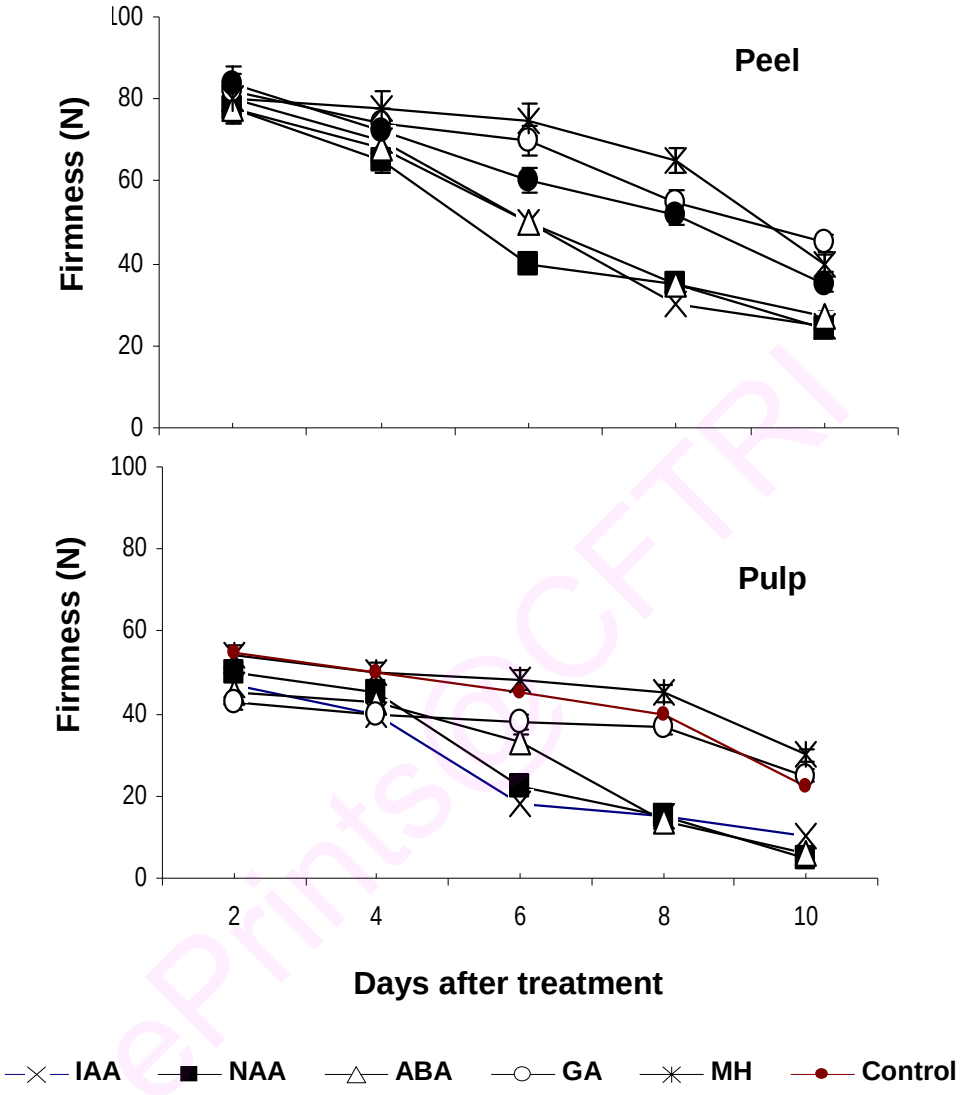

Figure 3.2. Changes in firmness of the peel and pulp of banana fruit pre-treated with different growth regulators (ABA, NAA, IAA, GA and MH) and untreated served as control. The treatments were performed as described in the materials and methods. Each value is the mean of three six replications. Vertical bars indicate the standard error of the means where they exceed the symbol size

3.3.2. Expression of expansin genes

3.3.2.1. Tissue specificity of expansin genes

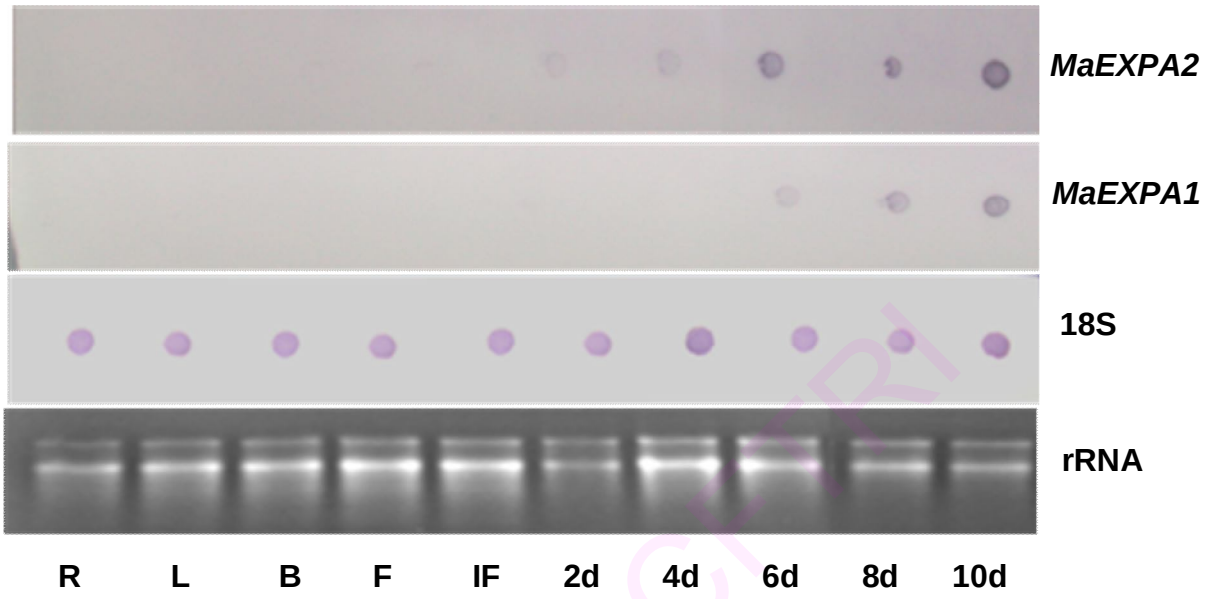

Figure 3.3. RNA dot blot analysis of expansin gene expression in root, leaf, bract, flower, immature fruit, and different stages of ripening fruit. Total 0.1µg RNA isolated from root (R), leaf (L), bract (B), flower (F), immature fruit (IF), various satages of ripening 2d, 4d, 6d, 8d and 10 days after ethrel treatment was spotted on hybond nylon membrane and hybridized with Dig labeled 18S, *MaEXPA-1* and *MaEXPA-2* probes

Expansin genes of various complexities have been reported in plants accomplishing expansion of primary cell wall (Cosgrove et al. 1997; Cosgrove 1999), extension of cell wall (Shcherban et al. 1995) and induction of leaf primordial (Fleming et al. 1997). Fruit-specific expansins have been identified in tomato (Rose et al. 1997), strawberry (Civello et al. 1999) and in Cavendish banana (Asha et al. 2007). In NR banana, transcript accumulation of genes *MaEXPA-1* and *MaEXPA-2* was studied during the course of ethylene induced/inhibited ripening for 10 days. The ripening/softening was characterized by a sharp decrease in fruit firmness. Besides fruit ripening, the mRNA abundance of these expansins was also analyzed in different tissues such as leaf, flower, stem, pseudostem, bract and root and in different developing stages of the unripe fruit by

RNA dot blot as well as RT-PCR. As shown in Figure 3.3. *MaEXPA-1* and *MaEXPA-2* transcription accumulation was not detected in any other tissues except for ripening fruit indicating their fruit as well as ripening specificity.

3.3.2.2. Expression of *MaEXPA-1* and *MaEXPA-2* with reference to fruit firmness

3.3.2.2.1. Northern analysis: The expansin genes have been shown to display direct correlation with the fruit firmness (Rose et al. 1997; Civello et al. 1999). Conversely, the induction of ripening and the consequent softening in banana fruit is induced by ethylene cycle with an outburst of ethylene and CO_2 at climacteric peak. This indicates direct correlation of ethylene induced ripening and softening. Expression of ripening-specific *MaEXPA-1* and *MaEXPA-2* was analyzed by mRNA abundance test in tissues of developing and ripening fruit. There was complete absence of expression of both *MaEXPA-1* and *MaEXP-2* in developing/expanding phase of the fruit as well as immediately after harvest. However, their expressions were noticed in control fruit after 6^{th} day although there was a significant fall in the firmness of peel rather than the pulp.

SA treatment suppressed pulp softening although the relative transcript level of *MaEXPA-2* was also low. Ethylene and smoking enhanced loss of firmness and transcript levels of *MaEXPA-1* higher than that of *MaEXPA-2*. The expression of these two expansins increased steadily without correlating with the increase in softness indicating their probable non-involvement in softening, and hence these genes and their expressions were not observed during the fruit development. However, semi quantitative RT-PCR, using *MaEXPA-1* and *MaEXPA-2* specific primers, revealed strong hybridization signal and their expression in different stages of ripening. These results indicated that the expression of *MaEXPA-1* and *MaEXPA-2* gene is fruit specific and ripening related.

3.3.2.2.2. Semi quantitative RT-PCR analysis of *MaEXPA-1* and *MaEXPA-2*

RT-PCR approach was used to characterize the expression of ripening specific expansin genes *MaEXPA-1* and *MaEXPA-2.* All amplifications were in the linear range between 29 and 32 cycles when cDNA fragments of specific size to be amplified from these two expansin genes using the gene specific primers (Table 3.1) and 29 cycles were used as standard. Control RT-PCR reaction using 18S rRNA primers were also linear between 29 and 32 cycles and produced a single band of similar intensity with all

samples confirming that equal amount of template were present. The conditions for all RT-PCR reactions were identical. The RT-PCR products were separated and all showed identity with the corresponding cDNAs.

3.3.2.2.3. Influence of ethylene inducers / inhibitors on *MaEXPA-1*

Although the presence of *MaEXPA-1* was reported recently by Asha et al (2007), who found this transcript in ripe fruit from Cavendish (genome AAA) var. Harichal, no attempts were made to study its regulation, except for the regulation under the influence of 1-MCP. We analyzed the regulation of transcript accumulation of *MaEXPA-1* under the influence of different ripening enhancers and inhibitors on ripening NR banana (genome AAB) for 10 days. While no transcript accumulation was detected on day 0 in untreated fruit, the transcript accumulation of *MaEXPA-1* started from day 2 in control and treated fruit. Although the expression levels appear to have increased from 6th day onwards in the control fruit (Figure 3.4), the relative transcript levels showed negligible increase (Figure 3.5).

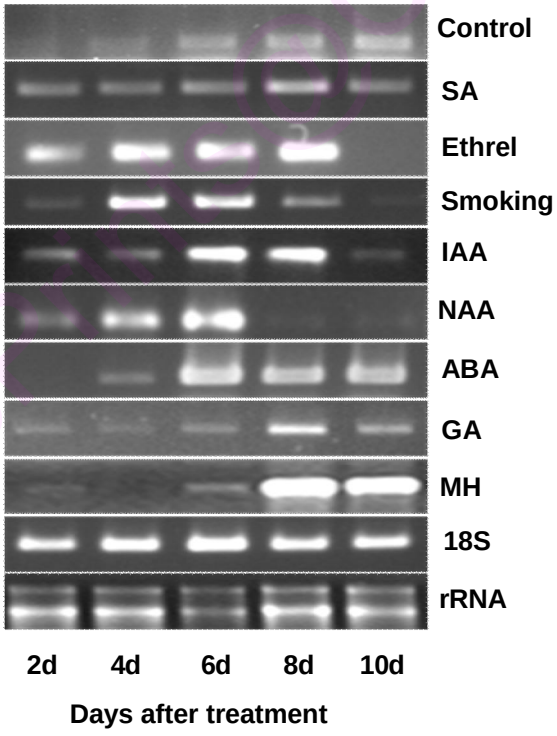

Figure 3.4. Expression of expansin gene *MaEXPA-1* during ripening of banana. The treatments were performed as described in the Materials and methods. The total RNA was extracted in 2, 4, 6, 8 and 10 days after the treatments. RT-PCR was performed as described in the Materials and methods with 5 µg of total RNA. For comparison, total RNA was stained with ethidium bromide (lower panel)

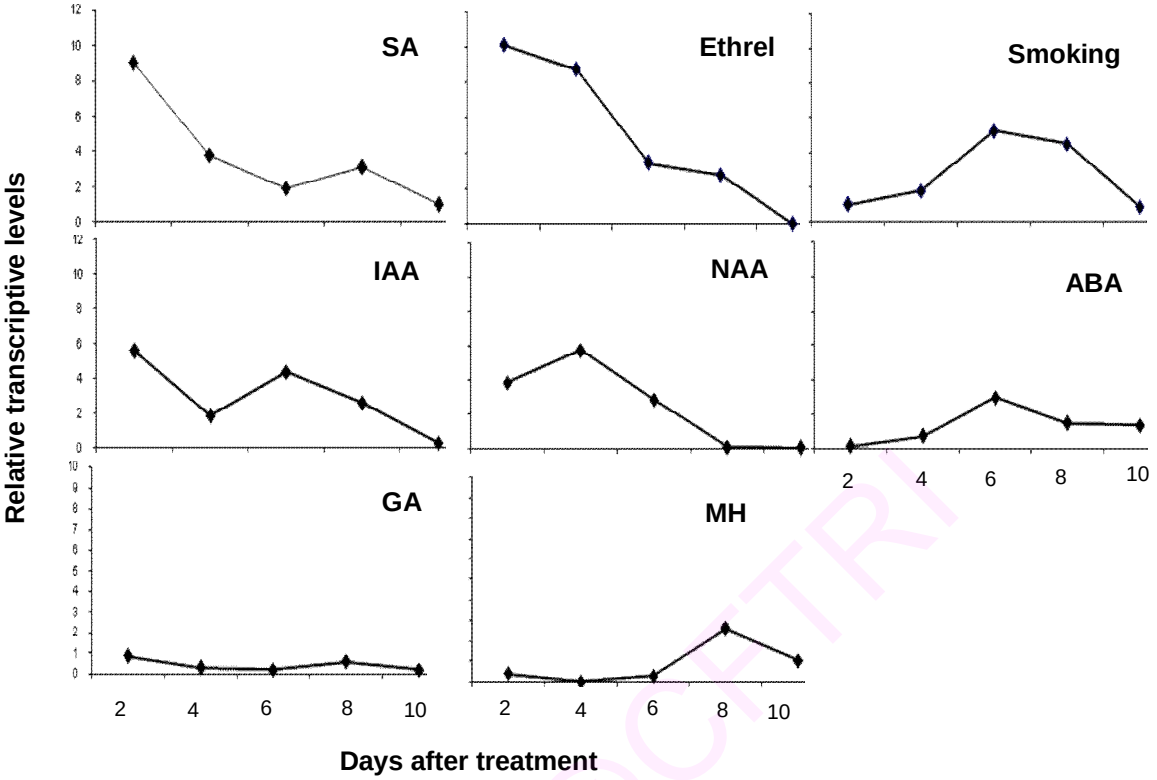

Figure 3.5. Relative (to control) transcript abundance of *MaEXPA-1*. The band intensity of *MaEXPA-1* gene was adjusted with the band intensity of 18S rRNA. Data shown are mean ± SD of three independent experiments expressed as the fold increase in *MaEXPA-1* expression of individual treatments in comparison with control

However 2 days after treatments, there were various levels of expressions of this gene, where high levels of transcripts were recorded in treatments with ethrel (10-folds), SA (9-folds) followed by ABA (over 5-folds) and NAA (nearly 4-folds). SA-treatment maintained the low expression level of *MaEXPA-1* throughout ripening period from 4th to 10th day, with transcription levels of about 3-folds from 4 to 8 days, with a decline after 10th day (Figure 3.5). Exposure to ethrel resulted in the early up-regulation of *MaEXPA-1* and the transcripts levels declined further, although more slowly than in SA treatment. Although smoking is known to impart similar effect as than that of ethrel, the smoke treatment in the present study imparted slower accumulation of this transcript, declining sharply towards the end on 10th day. The auxins, NAA and ABA imparted almost similar effects with NAA showing peak accumulation on 6th day when ABA had just induced

up-regulation of *MaEXPA-1*. MH, the inhibitor of ethylene biosynthesis/transport, although showed strong signals in the gel, the transcript levels were very low and so was the case with NAA and ABA on 8th and 10th day after treatment. Then the transcript level decreased and reached minimum on 10th day. In the exogenous application of SA sharp up-regulation (8.5-folds) of *MaEXPA-1* was observed initially with ceasing transcript levels towards the end. However, in this treatment as well as in GA treatment, the levels of *MaEXPA-1* transcript accumulation were comparatively lower than in all other treatments. The MH treated fruits showed 6-fold, 4-fold increase in *MaEXPA-1* transcript accumulation in 8th day and 10th day respectively.

3.3.2.2.4. Influence of ethylene inducers / inhibitors on *MaEXPA-2*

The expression of *MaEXPA-2* (Figure 3.6 and 3.7) was highest in smoked samples followed by that of ABA and NAA, all with highest expression after 6th day.

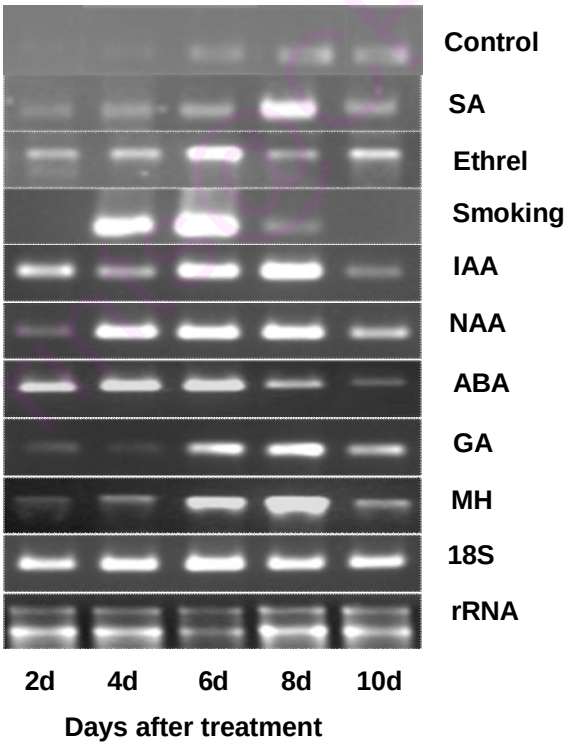

Figure 3.6. Expression of expansin gene *MaEXPA-2* during ripening of banana. The treatments were performed as described under Materials and methods. The total RNA was extracted in 2, 4, 6 8 and 10 days after the treatments. RT-PCR was performed as described in the Materials and methods with 5 µg of total RNA. The PCR products were analysed by 1.2% agarose gel electrophoresis. For comparison, total RNA was stained with ethidium bromide (lower panel)

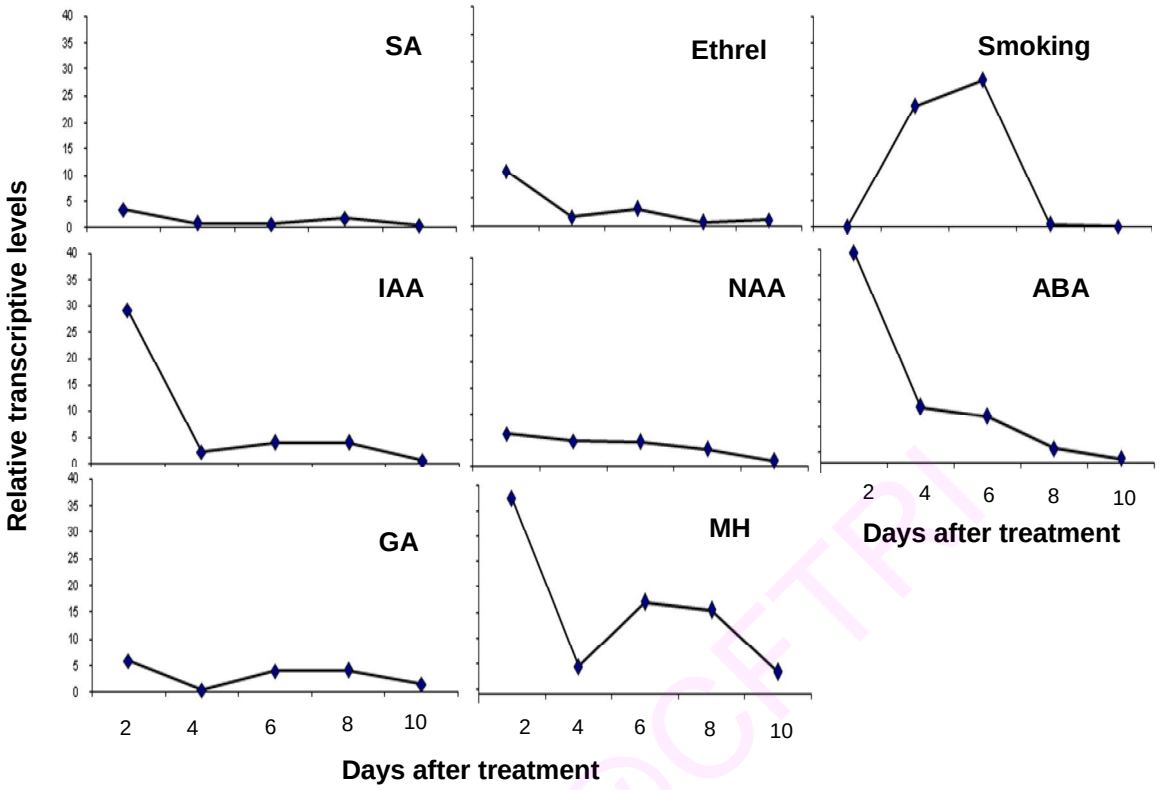

Figure 3.7. Relative (to control) transcript abundance of *MaEXPA-2*. The band intensity of *MaEXPA-1* gene was adjusted with the band intensity of 18S rRNA. Data shown are mean of three independent experiments expressed as the fold increase in *MaEXPA-2* expression of individual treatments in comparison with control

MH and ABA also induced significant levels of expression of *MaEXPA-2* which was high 2 days after treatment. By 10th day the expressions of *MaEXPA-2* was negligible, by which time the fruits were very soft and over-ripe. Moderate expression levels of this protein were found even after 10 days in all the treatments except in smoked fruits where this protein was not detectable towards the end of ripening. When relative transcript levels were checked for *MaEXPA-2,* as in the gel, here too smoke treatment showed nearly 30-fold increase after 4d and over 20-fold increase after 6d of ripening. The transcript levels were almost absent during other ripening periods (Figure 3.7). IBA enhanced the expression of this protein to a highest level of 26-fold higher than that of control in 2 d, which progressively decreased showing negligible activity after 10th day.

The transcript levels were moderate and remained so until complete ripening of 8 d, with traces remaining after 10 d.

3.3.3. Influence of ethylene inducers / inhibitors on cell wall hydrolases

The mature unripe banana fruit treated with exogenous ethylene inducers/inhibitors were monitored for change in appearance over 0-10 days after the treatment. A significant change in peel colour from green towards yellow was observed on day 2 (data not presented) and is due to a decrease in the chlorophyll content of the peel tissue. The inducers treated fruits reached edible softness on day 4. The change in banana fruit firmness during ripening is generally attributed to changes in the activities of various cell wall degrading enzymes. In the present investigation the enzyme activity of PG, PEL and PME under the influence of different ripening enhancers and inhibitors on ripening NR banana (genome AAB) for 10 days was studied. The data expressed as units per 100 mg of fresh weight.

3.3.3.1. Semi quantitative RT-PCR analysis of *PME, PG* and *PEL*

Reverse transcriptase-PCR approach was used to characterize the expression of cell wall hydrolases genes *PG*, *PEL* and *PME*. All amplification were in the linear range between 29 and 32 cycles when cDNA fragments of specific size to be amplified from these genes using the gene specific primers (Table 3.1) and 29 cycles were used as standard. Control RT-PCR reaction using 18S rRNA primers were also linear between 29 and 32 cycles and produced a single band of similar intensity with all samples confirming that equal amount of template were present. The conditions for all RT-PCR reactions were identical. The RT-PCR products were separated and all showed identity with the corresponding cDNAs. For calculating the transcript abundance under stress conditions, the transcript levels of each gene in green mature fruits were considered for comparison.

3.3.3.1.1. Influence of ethylene inducers / inhibitors on *PME* activity

PME activity started increasing sharply after all the treatments when compared to that on day 0 (10 U mg protein^{-1}) (Figure 3.8). Peak PME activity was recorded on day 6 of smoke (200 U mg protein^{-1}), ethrel (180 U mg protein^{-1}), NAA (180 U mg protein^{-1}), IAA (160 U mg protein^{-1}) treated fruits and PME activity started decreasing gradually after day 6 and by day 10 as low as on 0.

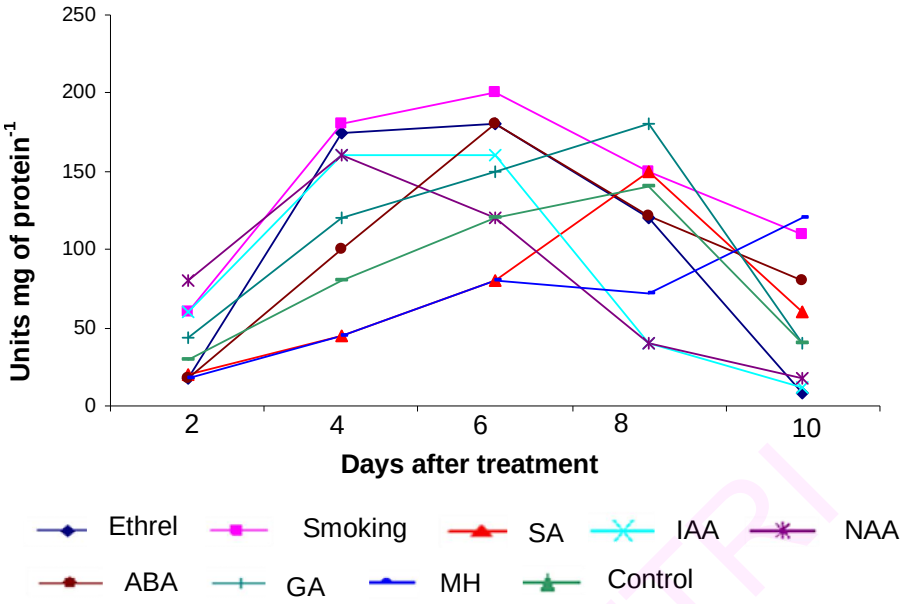

Figure 3.8. Changes in PME activities during ripening of NR banana fruit under the influence of different ethylene inducers and inhibitors. The treatments were performed as described in the Materials and methods. Data shown are mean ± SD of three independent experiments

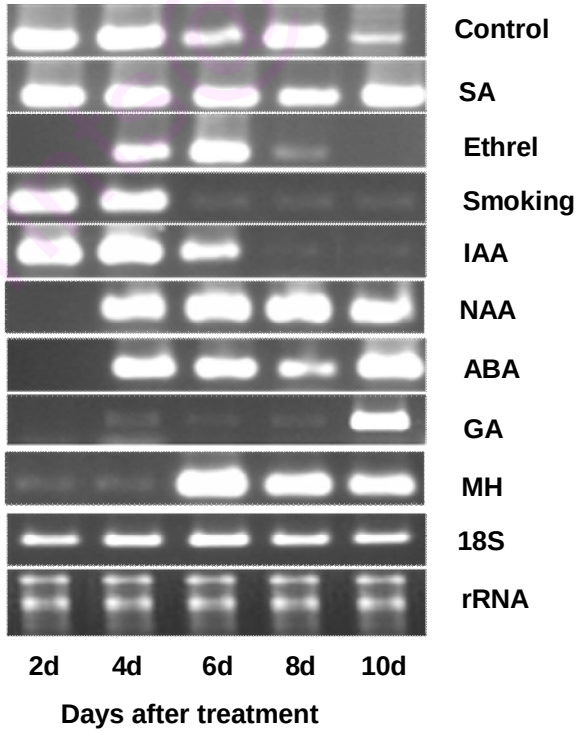

Figure 3.9. Expression of *PME* during ripening of banana. The treatments were performed as described in the Materials and methods. The total RNA was extracted in 2, 4, 6, 8 and 10 days after the treatments. RT-PCR was performed as described in the Materials and methods with 5µg of total RNA

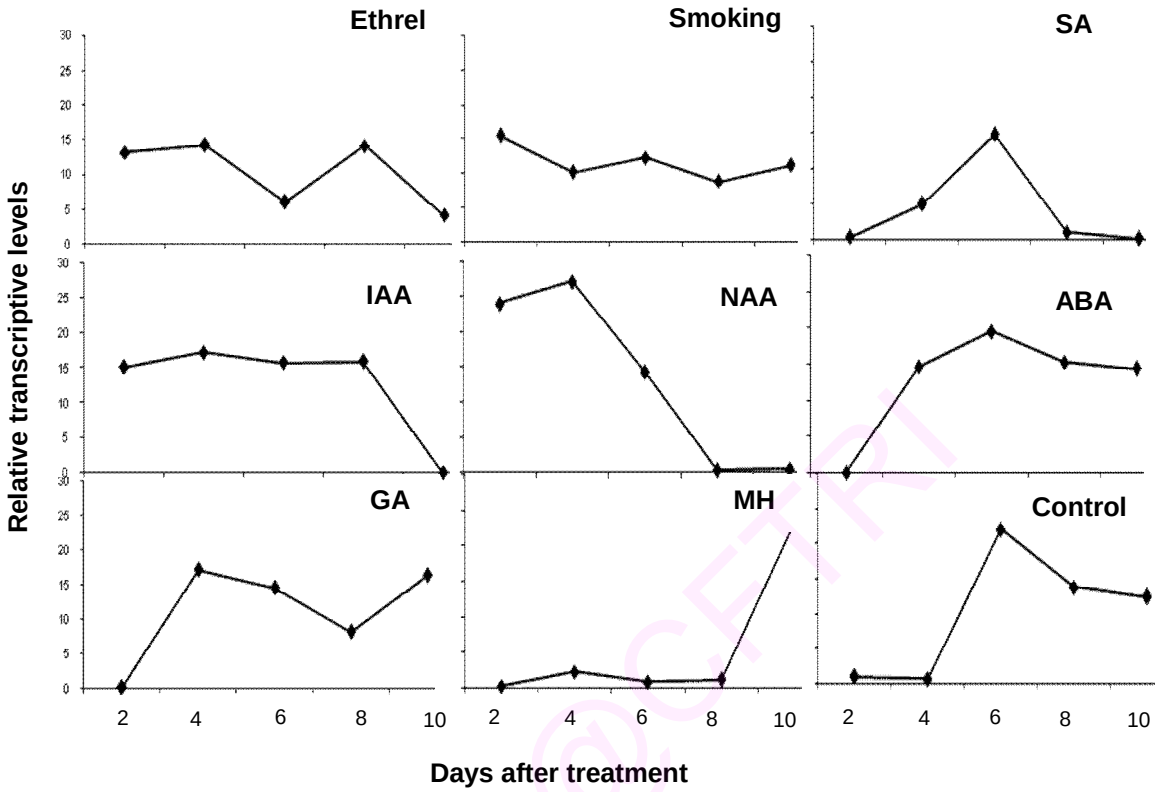

Figure 3.10. Relative transcript abundance of *PME*. The band intensity of *PME* gene was adjusted with the band intensity of 18S rRNA. Data shown are mean of three independent experiments expressed as the fold increase in *PME* expression of individual treatments in comparison with 0 day expression

A similar but markedly reduced pattern was observed when banana fingers were treated with SA, GA and MH treatment. Maximum PME activity by SA (150 U mg protein^{-1}), GA (180 U mg protein^{-1}) and control fruits on day 10. Inhibition of PME activity by SA, MH and GA, shows that these treatments strongly restrict induction of PME by ethylene. However, initial high activity with quick increase was observed in smoke treatment. Although ethrel is known to enhance endogenous ethylene activity, this treatment showed slower and low impact on the increase of PME.

3.3.3.1.2. Expression analysis of *PME*

PME expression levels were maximum on 6th day in control fruits which is 22-fold higher than green fruits. Smoke, SA and ABA showed maximum expression on 6th day of treatment while ethrel, IAA, NAA and GA produced maximum transcript levels

on 4th day and MH on 10th day. Among the treatments, maximum transcript levels were observed by NAA (27.3-fold). However 2 days after treatments, there were various levels of expressions of this enzyme, where high levels of activity were recorded in treatments with ethrel (800 U mg protein^{-1}), ABA (700 U/mg protein^{-1}) and NAA (700 U mg protein^{-1}) followed by Smoke (600U mg protein^{-1}). SA, GA, MH-treatment maintained the low level of enzyme activity throughout ripening period. Exposure to ethrel, IAA, NAA and ABA resulted in the early increase of *PME* activity whereas, SA, GA and MH treated fruits showed delayed ripening (Figure 3.9 and 3.10).

3.3.3.2. Influence of ethylene inducers / inhibitors on PG activity

PG enzyme activity increased gradually from day 0 (40 U mg protein^{-1}) throughout the period of study (Figure 3.11). PG activity increased slowly up to the pre-climacteric and then increased rapidly on day 4, which was the climacteric stage. Maximum activity of PG was observed on day 6 (800 U mg protein^{-1}) of ethrel treated fruits.

3.3.3.2.1. Expression of *PG*

The results revealed that PG transcript levels increased transiently in control and treated fruits, the control fruits showing highest expression after 6 days (5.1-folds). After two days of exposure to various treatments, transcript levels of PG were found reduced to non-detectable levels by smoking, SA and GA, with peak of expression immediately after 6 days in most of the treatments, except in smoking. The expression of PG in smoked fruit was high only during post climacteric stage. However, significant increase was observed for treatments with ethrel (6-folds), IAA (3.2-folds), and GA (1.3-folds) treatments during climacteric stage. Similarly SA (2.0-folds), IAA (3.5-folds), NAA (3.4-folds), ABA (3.9-folds) and GA (1.6-folds) treatments showed maximum expression on 6th day of treatment. While smoke treatment showed maximum transcript level on 10th day, MH on 8th day and ethrel on 4th day which is the maximum transcript level (6-fold) among all the treatments (Figures 3.12 and 3.13).

3.3.3.3. Influence of ethylene inducers / inhibitors on PEL activity

The activity of PEL in control fruit on day 0 was very low (0.4 U mg protein^{-1}) and gradually increased over the ripening period (Figure 3.14). Peak activity was

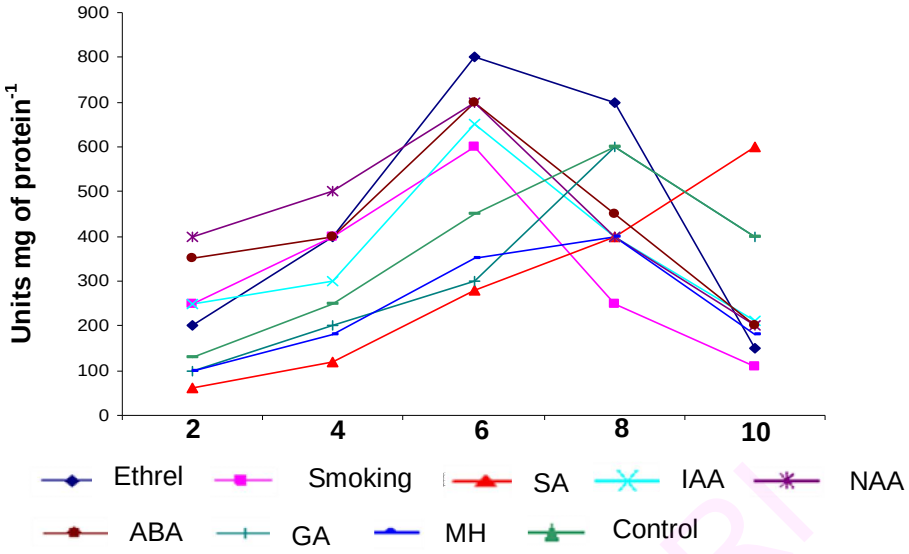

Figure 3.11. Changes in PG activities in ripening of banana treated with different ethylene inducers / inhibitors. Data shown are mean ± SD of three independent experiments

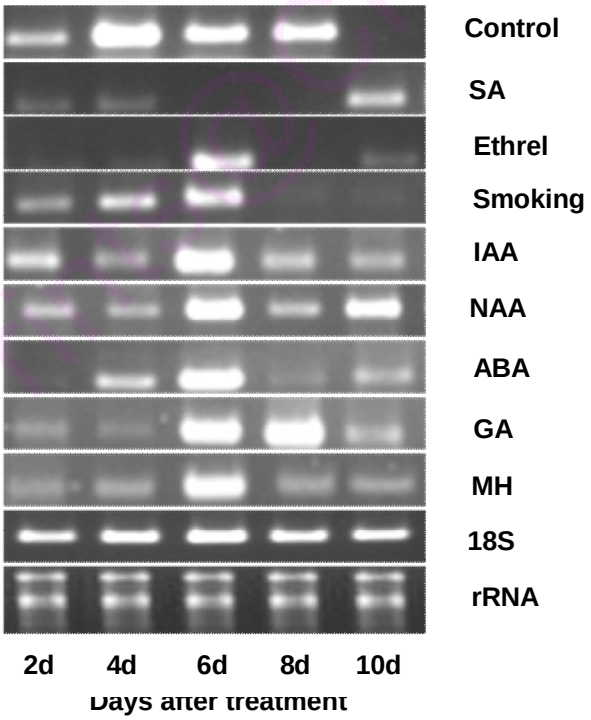

Figure 3.12. Expression of PG gene in ripening banana fruit. The total RNA was extracted after 2, 4, 6, 8 and 10 days of treatments. RT-PCR was performed as described in the Materials and methods with 5µg of total RNA

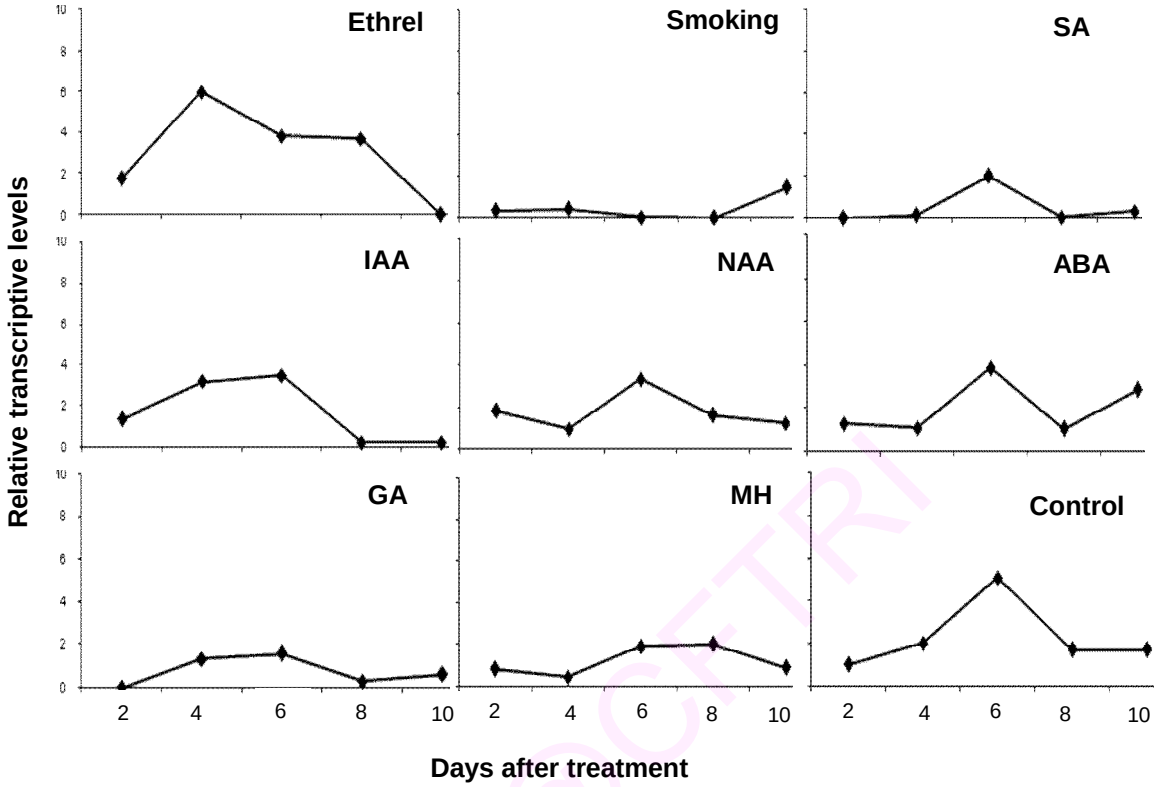

Figure 3.13. Relative transcript abundance of *PG*. The band intensity of *PG* gene was adjusted with the band intensity of 18S rRNA. Data shown are mean of three independent experiments expressed as the fold increase in *PG* expression of individual treatments in comparison with 0 day expression

observed 6 days after treatments with smoke (20 U mg protein^{-1}), ethrel and SA (18/U mg protein^{-1}), ABA and MH (12 U mg protein^{-1}) treated fruits, with a decline in PEL activity between days 8 and 10. A similar trend was observed for NAA and GA-treated fruits although the relative activities were much lesser with maximum activity of 18 U mg protein^{-1}) and 12 U mg protein^{-1}) respectively after 8 days.

3.3.3.3.1. Expression analysis of *PEL*

Compared to PME, up-regulation of PEL by different treatments was to a lesser extent. Control fruit had maximum transcript levels on 6th day, co-inciding with the expressions of PG and PME transcripts. Similarly ethrel, smoke, SA, GA and MH produced maximum levels of transcripts on 6th day, IAA on 2nd day, ABA on 4th day and NAA on 8th day (9.4-fold) which is the maximum compared to all treatments. GA and MH -treatment maintained the low expression level of *PEL* throughout ripening period,

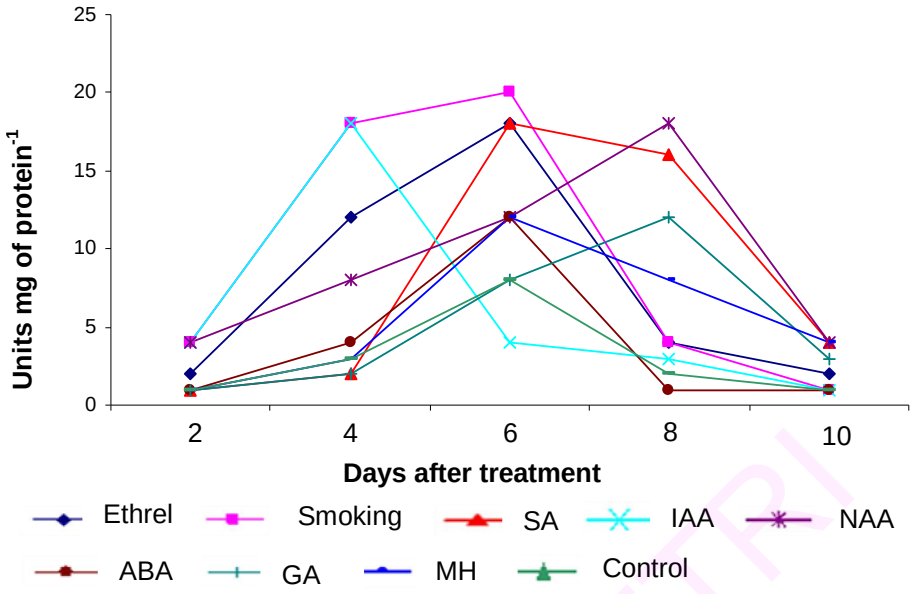

Figure 3.14. Changes in PEL activities during ripening of banana. Different ethylene inducer/ inhibitors. The treatments were performed as described in the Materials and methods. Data shown are mean ± SD of three independent experiments

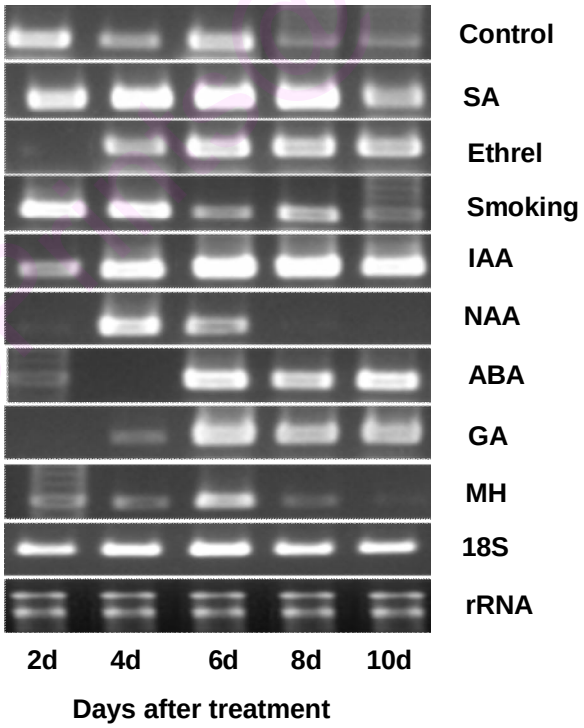

Figure 3.15. Expression of *PEL* during ripening of banana. The treatments were performed as described in the materials and methods. The total RNA was extracted in 2, 4, 6, 8 and 10 days after the treatments. RT-PCR was performed as described in the Materials and methods with 5 µg of total RNA

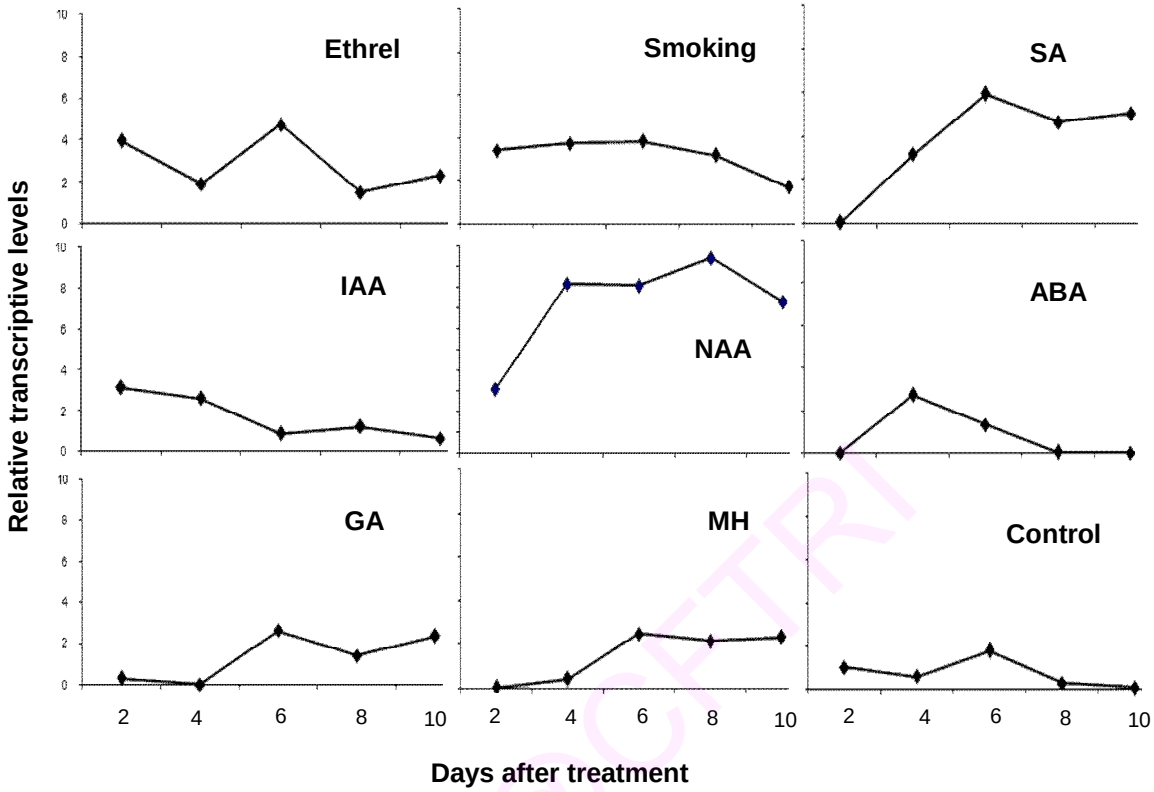

Figure 3.16. Relative transcript abundance of *PEL*. The band intensity of *PEL* gene was adjusted with the band intensity of 18S rRNA. Data shown are mean of three independent experiments expressed as the fold increase in *PEL* expression of individual treatments in comparison with 0 day expression.

where transcript levels were high between 4 and 6 days after treatment second day with substantial expressions of about 2.6-folds (GA) from 4 to 8 days, with a decline after 10th day (Figures 3.15 and 3.16). Exposure to ethrel, GA and smoking resulted in the early up-regulation of *PEL* and the transcript accumulations were highest on 6th day of treatment whereas in smoked fruits the transcript abundance declined steadily. The transcript levels due to ABA treatment was also almost similar when GA and MH were used, showing peak accumulation of PEL transcripts on 6th day when IBA had just induced up-regulation of *PEL by* which time transcript accumulation in ABA treatment had almost vanished (on 10th day). In the exogenous application of ABA, GA, MH and in control fruits the major up-regulation of *PEL* was observed to happen after mostly 6 days after treatment, the quantum of *PEL* transcript accumulation were comparatively much lower than the all other treatments known to induce ethylene enhancement.

3.4. DISCUSSION

In plants the cell wall is often described as a network of cellulose microfibrils embedded in a hemicellulosic polysaccharide matrix. These interact with an additional co-extensive matrix of pectin and other less abundant components, including structural proteins. Structural re-arrangements of these components are needed for cell growth, division and re-organization which is expected through the actions of cellulases. Expansins are the multigene family of proteins in plants involved in the induction of needed expansion of cell walls during various physiological and developmental processes and have also been reported to disrupt non-covalent interactions between hemicelluloses / cellulose microfibrils. The other cell wall components such as pectins and celluloses would probably act after the initiation of cell wall expansin. Studies on expansins are mainly focused on their roles in cell division leading to growth. However, specific expansins selectively accomplish irreversible de-polymerization of cell wall polysaccharides in ripening fruit, suggested to provide substrates for the other enzyme, thus playing key role in specific physiological events. The existence of these genes was reported by Asha et al (2007), who found them in ripe fruit from Cavendish, Genome AAA, *var*. Harichal. The response to ethylene and the degradation of known cell wall components PME, PG and PEL have never been studied in NR. The transcript accumulation of *MaEXPA-1*, *MaEXPA-2*, *PME*, *PG*, and *PEL* under the influence of different treatments has been studied during ripening for 10 days and discussed herein.

3.4.1. Fruit and pulp firmness

Firmness is an important internal quality parameter of fruit. There were differences in trends regarding changes in the firmness of banana fruit peel versus pulp as a response to treatments with different ethylene inducers and inhibitors. Peel firmness decreased steadily up to 8 days of storage with a sharp decline further. In contrast, there was a more marked loss in the firmness of the pulp which also decreased sharply after 8 days (Figure 3.1). Fruit pre-treated with ethylene inducers ethrel, smoke, ABA, IAA and NAA had much lower firmness in both peel and pulp (Figure 3.1 versus Figure 3.2). The difference in the firmness among treatments with ethylene inducers, ethylene inhibitor and control fruit was pronounced greatly with increase in storage period or holding time.

In this investigation, fruit pre-treated with ethylene inhibitors could ripen and soften in a comparatively normal way and slower, supporting the observation of Zauberman and Fuchs (1973), who reported that continuous supply of exogenous ethylene enhanced fruit softening and ripening of avocado even at low temperature. Whereas in NR fruit, the ethylene inhibitors delayed softening of pulp rather than softening of peel.

3.4.2. Regulation of *MaEXPA-1* and *MaEXPA-2*

Fruit development from a mature ovule through final maturity encompasses a wide range of complex and highly regulated physiological processes. Early development in most fruit can be divided into three phases: fruit set, cell division, and cell expansion (Gillaspy et al. 1993). Upon reaching full expansion ripening is initiated, typically involving changes in color, aroma, flavor, and a textural transition that contributes to softening of the tissue. The ripening process in climacteric fruit such as tomato, banana, and apple is highly regulated by the plant hormone ethylene, which is thought to coordinate the numerous metabolic pathways necessary for normal ripening. Expansins are cellular wall proteins that induce extension of cell walls in a pH-dependent manner and are, therefore, considered primary regulators of plant cell enlargement. Expansins have been shown to play important roles in plant development, seed germination and fruit ripening (Cosgrove 1998; 2000; McQueen-Mason and Cosgrove 1994; Whitney et al. 2000).

The first screening was on the specificity of these proteins / genes to fruit. No accumulation of either *MaEXPA-1* or *MaEXPA-2* transcripts was detected in root, leaf, bract, flower and immature fruit (Figure 3.3), suggesting for the first time that the expression of these two genes are fruit ripening specific. When fruit were treated with ethrel, smoke, ABA, IAA and NAA, accumulation of these transcripts increased markedly, (Figures 3.5 and 3.6), which was in agreement with the result of Asha et al (2007), who also noted a similar trend for *MaEXPA-1* and *MaEXPA-2* in banana fruit. Both the genes are expressed abundantly only in mature fruit during ethylene induced ripening and repressed in fruit treated with SA, MH and GA indicating their sensitivity to ethylene. The simultaneous expression of both *MaEXPA-2* and *MaEXPA-1* may be essential for cell wall loosening during banana ripening and these two expansins appear

to act synergistically to enhance softening. In control ripening fruit the transcripts of *MaEXPA-1* appear after 6 days and *MaEXPA-2* appear 4 days after harvest of mature fruit, although softening initiated immediately after harvest and progressed steadily then onwards. Initial transcript level of *MaEXPA-1* was higher in ethylene than in control in treatments enhancing ethylene pathway and suppressed in ethylene pathway inhibitors, confirming the gene's responsiveness to ethylene pathway. The transcript levels of *MaEXPA-2* were much higher than those of *MaEXPA-1* to various treatments, and high levels coinciding with climacteric peak in smoke- and NAA-treatment. Since fruit softening occurred even before the appearance of transcripts of these genes, these genes do not appear to play the initial role in cell wall loosening / fruit softening; nevertheless contributing substantially for post-climacteric softening. A similar influence of hormones on expansins has been reported in other fruit (Cho and Cosgrove 2004). For instance, during ripening of peach fruit, three expansins were detected. All three expansins were fruit specific, but each showed differential pattern of mRNA accumulation during fruit development. Studies on the expression pattern of three expansins in peach showed that *PpEXPA3* playing a role in peach fruit softening where ethylene directly regulates its transcription (Hayama et al. 2003; 2006). Similarly, in strawberry fruit, *FaEXP4* was expressed constitutively throughout fruit development, but with a small reduction in expression at the large green stage (Harrison et al. 2001).

In conclusion, this study reveals the presence of multiple members of the expansin gene family with staggered and differential expression during ripening in NR. banana fruit. It also appears that expression of multiple expansin genes might be required for softening in monocot fruit like banana as has been demonstrated for various dicot fruit like apple and tomato (Lieberman et al. 1977), peach (Pressey and Avants 1973), pear (Ahmed and Labavitch 1980) and strawberry (Archbold and Dennis 1984). Of the four expansins identified, *MaEXPA-2* is specifically expressed in fruit and is abundant during ethylene-induced ripening especially at later stages of rapid softening. Thus, *MaEXPA-2* appears to be an attractive candidate gene for manipulating shelf life of banana. Further studies on the knockouts of individual genes and the regulation by promoters might reveal fine regulation of each gene and their specific role in softening of banana fruit.

3.4.3. Changes of cell wall hydrolases

Phytohormones (ethrel, IAA, NAA, ABA, MH and GA) and other chemicals such as MH and SA play an important role in fruit ripening (Abeles 1973; Biale 1960; Burg and Burg 1965; Srivastava and Dwivedi 2000). There are several indisputable evidences that indicate the role of ethylene in induction/enhancement of ripening of various types of fruits. The change in banana fruit firmness during ripening is generally attributed to changes in the activities of various cell wall degrading enzymes (Jiang et al. 2000; Pelayo et al. 2002; Abeles 1973; Burg and Burg 1965; Payasi et al. 2004) as well as starch degradation (Prabha and Bhagyalakshmi 1998). However, detailed information on these enzymatic changes was not available for NA banana fruit. Before exposure to ethylene treatment with 1-MCP prevented these ripening related changes indicating the involvement of ethylene in accomplishing these changes (Sisler and Serek 1997; Golding et al. 1998; Pathak et al. 2003).

3.4.3.1. Ripening enhancers

Banana is a climacteric fruit and treatment with ethylene stimulates enlargement, accelerates the onset of the rise in respiration, induces endogenous ethylene production and promotes ripening (Tucker and Grierson 1987). The ethylene production preceding ripening occurs during a phase of active cell expansion with a concomitant softening, as revealed by the loss of firmness. Two systems of ethylene regulation have been proposed to operate in climacteric plants. System 1 is functional during normal vegetative growth, is ethylene auto-inhibitory and is responsible for producing basal ethylene levels that are detected in all tissues including those of non-climacteric fruit, while system 2 operates during the ripening of climacteric fruit and senescence of some petals when ethylene production is autocatalytic (Lelievre et al. 1997). As ethylene has been shown to exert both positive and negative feedback regulation on its biosynthesis, studies with various inhibitors of ethylene such as 1-MCP, have provided better understanding of its regulation in such cases as fruit ripening (Blankenship and Dole 2003). Such ethylene production has also been observed as a response to auxin treatment especially ABA, IAA and NAA for a number of plant tissues (Hanson 1966; McGlasson 1970; Pratt and Goeschl 1969). In contrast to this Purgatto et al (2002) reported inhibition of fruit ripening when banana slices were infiltrated with auxins especially IAA. In the present

investigation, treatment of mature green banana (*cv*. NR) with ethylene during the pre-climacteric phase accelerated their ripening supporting the observations by earlier workers (Strydom and Whitehead 1990).

The main ripening-specific enzymes responded variably to different treatments. An interesting observation was the early rise in PME, PG and PEL activity as well as the gene expression levels upon exposure of banana fruits to ethrel, smoke, ABA, IAA, NAA and inhibition of expression by ethylene suppressors such as 1-MCP suggests that the transcription and translation of these genes in *Musa* fruit is regulated by ethylene. Similarly, Dominguez-Puigjaner et al (1997) also reported that *PEL* expression in banana fruit is induced by application of ethylene. In tomato the ethylene-suppression by chemical treatment resulted in the arrest of ripening, and upon re-exposure to ethylene the ripening commenced indicating that the cascade of ripening activity up to even gene expression level (PG activity) are regulated by ethylene. Sitrit and Bennett (1998) also presented evidences for the regulation of PG mRNA in tomato fruits by ethylene. In consistence with these observations, we find advancement of PG activity peak when banana fruit were exposed to ethylene.

PME is known to demethylate the C6 of the galacturonosyl residue and PG acts preferentially on the demethylated substrate (Koch and Nevins 1989). Thus, PME provides substrate for PG to act upon. Together, they act mainly on the middle lamella (Koch and Nevins 1989). Similar changes in PME activity have also been reported for pear (Ahmed and Labavitch 1980). A pattern of continuous increase in PG activity over time with a decrease in PME activity after the climacteric stage in banana is presumably correlated with their substrate preferences. Activity of PME has been reported from tomato and peach fruit (Ray et al. 1988; Zhou et al. 2000) during ripening. The activity of PME in these fruit also preceded the activity of PG, indicating the co-ordinate action of both the enzymes.

In NR, the activity of PEL in variously treated fruit on day 0 was very low (0.4 Umg protein^{-1}) and gradually increased during the ripening phase (Figure 3.14). Peak activity was observed on day 6 (U mg protein^{-1}). Like PG, PEL also catalyses the cleavage of the α(1-4)-galacturonan linkage, but by β-elimination instead of a hydrolytic

cleavage (Pissavin et al. 1998). Parallel changes in PG and PEL activities have been observed during ripening in banana. The PEL gene has been cloned in strawberry and using antisense technology its role in fruit softening has been tangibly demonstrated (Bermudez-Jimenez et al. 2002). Moreover, transgenic tomato fruit carrying antisense to PG retained firmness for much longer periods of time than the control (Sheehy et al. 1988; Smith et al. 1988). The present data for banana suggest that concerted activities of pectin degrading enzymes PME, PG and PEL lead to fruit softening during which PG activity is dependent on PME for making substrate available. Ethylene is responsible for co-ordinating and/or accelerating these effects, since 1-MCP-treated fruit did not exhibit marked changes in firmness or increase in activities of these cell wall hydrolyases. Pelayo et al (2002) suggested that 1-MCP acts by binding irreversibly to ethylene receptors to prevent ethylene-induced ripening in banana. Pathak et al (2003) have suggested that 1-MCP affects expression and activities of ethylene biosynthesis enzymes during banana fruit ripening.

Comprehensively, the result clearly suggests that ethrel, smoke, ABA, IAA and NAA have the ability to modulate the activities of banana fruit softening. These findings support observations made by Jiang et al (2000) where a decrease in fruit firmness occurred in bananas treated with ABA. ABA-treated fruit did not show enhanced activity of hydrolases when treated with 1-MCP before ethylene exposure. Thus, stimulation of banana fruit softening by ABA may be attributed to an ethylene-mediated response. Similarly, Jiang et al (2000) also showed that ABA-induced acceleration of banana fruit ripening did not occur in 1-MCP-treated fruit, probably because of the binding of ethylene receptor sited by 1-MCP. Abscisic acid (ABA) is known to enhance ripening of both climacteric and non-climacteric fruits (Brady 1987; Palejwala et al. 1985; Parikh et al. 1986; Vendrell 1985). The effect of ABA on various ripening parameters may be related to ethylene evolution (Riov et al. 1990). However, the mechanism by which ABA stimulates ethylene production is not clear. Some investigators suggest that ABA has a direct effect on ethylene biosynthesis, predominantly through enhancement of ACC synthesis (Goren et al. 1993; Riov et al. 1990; Tan and Thimann 1989). Others suggest that its stimulative effect is indirect, resulting from acceleration of senescence (Jackson and Osborne 1972; Lieberman et al. 1977). Most studies dealing with stimulation of

ethylene production by ABA have used excised tissues (Goren et al. 1993; Jackson and Osborne 1972; Lieberman et al. 1977; Vendrell 1985). Jiang et al (2000) suggest that ABA facilitates initiation and progress in the sequence of ethylene-mediated ripening events, possibly by enhancing the sensitivity to ethylene. The observation in the present study on NR banana shows that initiation of PME, PG and PEL expression of day 2 and their progression in the sequence of ethylene-mediated ripening events. These results suggest that concerted activities of pectin degrading enzymes PME, PG and PEL lead to fruit softening during which PG activity is dependent on PME for making substrate available whereas the other two enzymes act directly. Since these enzymes are enhanced by ethylene enhancers, the latter appears responsible for coordinating and/or accelerating these effects both at biochemical and gene levels.

3.4.3.2. Delayed ripening

GA: Gibberellins have been shown to delay ripening of bananas (Vendrell and McGlasson 1970; Payasi et al. 2004. We also find delay in ripening of whole banana by GA, which could be due to the opposing effect of GA to ethylene (Scott and Leopold 1967). This study also makes an observation that there occurs a delay in climacteric-respiratory peak in NR fruit treated with GA. Rossetto et al (2003) clearly reported that GA_3 did not delay the ethylene and CO_2 burst, and did not irreversibly block carbohydrate mobilization, it clearly affected triggering of starch breakdown and sucrose accumulation. The impairment of sugar synthesis in bananas is similar to that produced by GA_3 in mango (Khader 1991; Singh et al. 2007), but different from that observed in non-climacteric fruits (Cano-Medrano and Darnell 1997; Zilkah et al. 1997). During banana ripening, Mota et al (2002) observed that GA_3 disturbed starch phosphorylase activity and related protein synthesis. Since GA_3 did not affect ethylene production in banana, it can be concluded that the delay in starch degradation was not caused by inhibition of the climacteric rise but directly by the gibberellins. Thus, starch to sucrose conversion in bananas might have components that are not regulated directly by ethylene signaling. In conclusion, some starch degrading enzymes are inhibited by gibberellins (Purgatto et al. 2001; Mota et al. 2002). In the present investigation it is observed that a delay in climacteric-respiratory peak in NR bananas treated with GA although not to the extent of MH and SA. The advancement of PME, PG and PEL activity peak in fruits treated with

ethrel, smoke, IAA and NAA which hastened banana ripening and their delay fruits treated with GA suggest the role in the synthesis of PME, PG and PEL in ripening.

SA: Salicylic acid, a ubiquitous plant phenolic, as well as other phenolic compounds powerfully inhibited banana fruit PEL thereby delayed ripening (Srivastava and Dwivedi 2000). In *E. chrysanthemi,* PEL is also reported to be inhibited by SA (Tardy et al. 1997; Pissavin et al. 1998). Tannins are decreased in the ripe banana fruit pulp to about one fifth of their value in the green pre-climacteric fruit upon SA treatment (Palmer 1971). To a lesser extent than MH and 1-MCP, the SA also suppressed the activity of pectic enzymes. SA has been a commercial success in the extension of shelf-life of mango (Padmini and Prabha 1997).

IAA: The regulatory mechanisms that control ethylene biosynthesis during fruit ripening have been widely studied and the results have revealed a complex pattern of induction of key enzymes linked to this pathway (Barry et al. 2000). The signaling cascade involved in the climacteric rise of ethylene production, as well as the factors that interfere with this process are not clear. It is possible that IAA did not interfere with ethylene signaling during the climacteric period or even with the expression of the related enzymes.

Considering the data presented herein, where there is a decrease towards the last phase where there is extreme softening, there might be a relationship between critically low levels of free IAA and the onset of starch degradation. Purgatto et al (2002) reported that exogenous IAA delayed the onset of starch degradation, as previously observed, and this delay was not related to the inhibition of ethylene synthesis during the climacteric phase, suggesting that starch degradation could be an ethylene-independent process, at least partially. Another conclusion is that IAA infiltration was not able to mask or mimic the signaling arising as a response to detachment from the mother-plant, since the triggering of ethylene synthesis after harvest was similar to the control slices. IAA levels can be considered as one of the regulatory factors or at least a compound that can efficiently delay the onset of the ripening process.

Similarly in the present investigation, suppression in activities of cell wall hydrolases and their genetic expression levels were observed when banana fruit were treated with IAA. Thus, exogenous IAA may inhibit fruit softening by inhibiting cell wall

hydrolases. Similarly, Purgatto et al (2002) and Pathak and Sanwal (1999) reported a delay in ripening when banana fruit were treated with IAA. Their observations were based on changes in fruit weight, pulp/peel ratio, pulp pH and respiration during banana ripening. Likewise a delay in ripening and softening of banana fruit observed by Pathak and Sanwal (1999) upon treatment with IAA is probably due to inhibition of cell wall degrading enzyme activities.

These results represent another point to validate the experimental model employed for the infiltration of IAA and it seems to be in agreement with the hypothesis that IAA is an inhibition factor of some ripening events in banana, as it is thought to be in strawberry and tomato. In these fruit, decline in free-IAA content represents an *in situ* signal to the development of changes associated with the ripening stage (Archbold and Dennis 1984). Frenkel and Dyck (1973) proposed that a fall of IAA levels could be a determinant factor in the onset of certain events in fruit ripening. In tomatoes, Slovin and Cohen (1993) suggested that low levels of IAA could be a signal for the onset of ripening. In fact, Buta and Spaulding (1994) observed that IAA levels in tomatoes decreased during ripening, reaching the lowest levels before the changes in events correlated to the onset of tomato ripening (Singh et al. 2007). A fall in IAA level in most of the plant tissue heralds' senescence, and the senescence is associated with ethylene production. A contrasting situation was found in melon fruit (Dunlap et al. 1996), where an increase of amide-linked IAA conjugates occurred parallel to an increase of free and ester conjugates. These apparent discrepancies could be considered as examples of the complexity behind the modulation of plant hormone levels and their role in fruit ripening.

NAA: Since this is also an auxin, a similar response as that of IAA may be expected, nevertheless, data of the present study indicate no extensive suppression of ethylene induced softening. This indicates, that NAA being synthetic hormone, is perceived differentially than that of IAA, and hence no similarity response.

CHAPTER - IV

Isolation of Ripening Specific Genes

SUMMARY

To gain a better insight into the molecular regulation of ripening in banana fruit, the mRNA Differential Display technique coupled with silver-staining was used and specific transcripts differentially expressed during ripening were first identified and then isolated. Using 71 primer combinations and four populations of mRNA (Pre-climacteric; Climacteric 1; Climacteric II and post climacteric), a total of 285 bands were obtained, of which 120 were cloned into T/A cloning vector and sequenced. DNA sequence analyses of the clones revealed significant homology to known plant genes and proteins reported in public databases, namely an inner membrane transport protein, sucrose phosphate synthase, heat shock protein, transcriptional regulator, putative senescence protein, a homologue to a protein kinase. These proteins can be associated to biological processes like primary metabolism, secondary metabolism, signal transduction, gene transcription and RNA metabolism, stress responses or defense. Many of the clones have been identified for the first time to be associated with ripening. The northern dot analyses showed the expression of most of the up- and down-regulated genes are specific to fruit and ripening, and never expressed in other tissues. In addition, the homology found with previously described proteins allows disclosing possible biochemical roles for these proteins putatively encoded by the respective gene families and their putative significance in banana ripening is discussed. Since banana lacks information about the molecular regulation of ripening, the results of the present finding provide better insight for the characterization of the changes in gene expression that accompanies the ripening process.

4.1. INTRODUCTION

Fruit ripening is a genetically programmed, developmental process, involving novel gene expression patterns d the coordination of a large number of biochemical and physiological changes. Since fruit ripening is a tightly regulated process (Giovannoni 2001), all the changes are very likely brought about by activation or repression of specific genes. Some of these genes are regulatory and they may control the expression of downstream genes (Adams-Phillips et al. 2004). A few studies have been directed towards isolation and characterization of ripening-specific genes from banana. Consequently, genes encoding enzymes such as malate synthase (Pua et al. 2003), cytochrome P450 (Pua and Lee 2003), acidic chitinase type III (Clendennen et al. 1998), pectate lyase (Dominguez-Puigjaner et al. 1997) and polygalacturonase (Asif and Nath 2005), among others, have been identified. Differential screening of cDNA libraries has also been employed for the identification of banana genes expressed in fruit at early ripening stages (Clendennen and May 1997; Medina-Suarez et al. 1997). The modification of gene expression offers the potential to improve fruit quality by altering biochemical pathways that control the events mentioned above. Even though some genes have been identified, there is still an incomplete knowledge of banana fruit ripening at the molecular level. The pool of banana fruit sequences in public databases is much smaller than the available data for other fruits, such as tomato, grape and peach (Giovannoni 2004).

Various approaches to investigate differential gene expression have been utilized including differential hybridization (Sambrook et al. 1989), subtractive hybridization (Lee et al. 1991), differential display (Liang and Pardee 1992), SAGE (Serial Analysis of Gene Expression), TOGA (Total Gene Expression Analysis), GeneCalling and cDNA miroarray technologies (Schena et al. 1995). These approaches have been employed in order to identify genes that are differentially expressed during ripening and have been very useful in the isolation of a number of genes with differential expression during ripening of kiwifruit (Ledger and Gardner 1994), banana (Clendennen and May 1997; Medina-Suarez et al. 1997; Gupta et al. 2006; Kesari et al. 2007), melon (Hadfield et al. 2000), tomato (Zegzouti et al. 1999), papaya (Devitt et al. 2006) and raspberry (Jones et al. 2000). Transcriptome analyses in apricot (Grimplet et al. 2005) and tomato (Alba et al.

2005) have also been performed in an effort to get an insight into the vast array of genes that may be involved in ripening.

The differential mRNA display, as compared to conventional methods for differential screening, allows simultaneous display of all expression differences, comparison of several samples side-by-side, detection of up-regulated and down-regulated genes and isolation of rare transcripts. In addition, this technique is claimed to be fast, sensitive and reproducible.

The suppression subtractive hybridization (SSH) technique has been successfully employed to isolate differentially expressed genes from fruits in various developmental stages (Choi et al. 2004). Trujillo et al (2007) identified Eighty-four up-regulated unigenes by differential screening of Cavendish banana fruit cDNA subtraction library at a late ripening stage. The expression profiles were also obtained for unigenes annotated as orcinol O-methyltransferase, putative alcohol dehydrogenase, ubiquitin-protein ligase, chorismate mutase and two unigenes with non-significant matches with any reported sequence. Similar expression profiles were observed in banana pulp and peel (Kesari et al. 2007). This result showed the differential expression of a group of genes involved in processes associated with fruit ripening, such as stress, detoxification, cytoskeleton and biosynthesis of volatile compounds. Some of the identified genes had not been characterized in banana fruit. Besides providing an overview of gene expression patterns related to specific metabolic pathways at late ripening stages of banana fruit, this study contributes towards escalating the information available on banana fruit EST. In addition, such information is totally lacking in the local cultivar Nanjanagudu rasabale.

Lately Gupta et al (2006) and Velazquez et al (2007) have used mRNA differential display and identified 22 ripening related genes in banana of which only two have previously been characterized. Transcript analyses of these studies showed that six genes were down-regulated and 16 were up-regulated during the course of ripening with varied patterns of transcript accumulation. Treatment with 1-methylcyclopropene (1-MCP) has been found to inhibit ripening and repress the expression of most of the up-regulated genes, indicating that their expression is directly or indirectly governed by ethylene. Also, the expression of most of the up-/down-regulated genes is specific to fruit and not observed in other tissues. Short time ethylene treatment of banana fruit reveals

the induction of four up-regulated genes within 10-30 min of such treatment and the repression of two down-regulated genes within 30-60 min of ethylene treatment. Sequence analyses have revealed homology to genes that are involved in diverse processes such as gene regulation/signaling, defense, softening and other unknown functions (Gupta et al. 2006).

Though two-dimensional SDS-PAGE of *in vitro* translation products (Medina-Suarez et al. 1997), differential screening of cDNA libraries (Clendennen and May 1997), mRNA differential display (Gupta et al. 2006; Velazquez et al. 2007), cDNA microarray (Xu et al. 2007) and PCR-based suppression subtractive hybridization (SSH) technique (Xu et al. 2007; Kesari et al. 2007) led to the identification of various up- and down-regulated genes during banana fruit ripening, the information has still not been sufficient when compared to other major fruit crops such as tomato, apple, melon and strawberry. Using the PCR-based Differential Display technique, we have identified many genes in ripening NR fruit which have not been identified earlier. A considerable number of the identified genes include those that are involved in defense/stress responses, metabolism and aroma. Most of these genes show ethylene-regulated and ripening-related expression and their further study can help to understand mechanism of banana ripening better. Some of these genes could be good candidates for post harvest biotechnology.

4.2. MATERIALS AND METHODS

4.2.1. Plant materials and treatments

Mature green banana (*Musa acuminata* AAB *cv*. Nanjanagudu Rasabale) fruits, around 110 days after anthesis (of first hand emergence), were harvested at author's departmental garden. The procedure for treatments was the same as that described by Purgatto et al (2001). Briefly, in the present study, treatments were applied within 24 h of harvest. Each banana bunch was separated into individual hands and fingers were separated from hands, individual fingers with uniform size of 14 cm of length and 4 cm of diameter at the central portion where the average fruit weight was about of 80±0.45 g were cleaned with distilled water. The crown surface of each hand was re-cut using a sterilized knife to make a fresh wound surface for treatment and allowed to air dry for 2-3 h. In each experiment, fingers from the same hand were used as a sample group to avoid variation in ripening behaviors of fingers among different hands (Inaba and Nakamura 1986). The fruits were dipped in the 120 mM mannitol containing different concentration of SA (1000mg l^{-1}), ethrel (1%), smoking, ABA (25 mg l^{-1}), NAA (500mg l^{-1}), IBA (500mg l^{-1}), GA (500mg l^{-1}) and MH (500mg l^{-1}) for 30min and air-dried at ambient temperature (29-30°C, 80 to 85% RH). Hands were gently placed in corrugated cardboard boxes and stored at 29-30°C, 85% RH. Fruit were regularly inspected and samples (peel and pulp) were separately collected every alternate day (*i.e.*, 2, 4, 6, 8, 10 days) after treatments. Fruit ripening was classified into seven stages based on the peel appearance according to Stover and Simmonds (1987), and data were thus recorded.

4.2.2. RNA isolation

Total RNA was extracted from 5 g pulp tissue (pooled sample) of treated banana using CTAB as described by Asif et al (2000) with the following modifications. The detailed protocol is described in the chapter 3 under the section 3.2.3.1.

4.2.3. The mRNA Differential Display

The mRNA Differential Display was performed using the GenHunter kit according to the manufacturer's protocol. Differential Display was conducted in four RNA samples, namely RNA isolated from unripe fully expanded fruits, from fruits at

commercial maturity and from overripe fruits, starting from 0.2μg of total RNA from each independent sample. Briefly, poly(A)$^+$–RNA (0.2 μg) was heated at 65°C for 10 min and immediately chilled on ice. First-strand cDNA synthesis was performed in a reaction mixture containing 50 mM Tris–HCl (pH 8.5), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 1mM each dNTP, 40 units of RiboLock™ Ribonuclease inhibitor (MBI (Fermentas GmbH, St. Leon-Rot) and 40 U of H-minus M-MuLV Reverse Transcriptase (MBI Fermentas) and 50 mM of different anchor primer (Table 4.1) for 1 h at 42°C. The reaction was stopped by heating the mixture at 70°C for 10 minutes and chilling on ice. Enzymes were then heat-denatured at 95°C for 5 min.

Table 4.1. List of anchor primers and arbitrary primers used for Differential Display

<table>
<tr><th colspan="3">Anchor primers</th><th>Arbitrary primers</th></tr>
<tr><td>S. No.</td><td>Name of the primer</td><td>Sequence(5'-3')</td><td rowspan="8">GenHunter primers:
H-AP1, H-AP2, H-AP3, H-AP4, H-AP5, H-AP6, H-AP7, H-AP8
Operon Primers:
OPA-03, OPA-04, OPA-06, OPA-09, OPA-11, OPA-14, OPA-20, OPC-01, OPC-02, OPC-04, OPC-05, OPC-07, OPC-08, OPC-09, OPC-11, OPC-13, OPC-20, OPD-03, OPD-04, OPD-07, OPD-08, OPD-16, OPF-12, OPF-15, OPJ-01, OPJ-02, OPJ-03, OPJ-04, OPJ-06, OPJ-07, OPJ-08, OPJ-09, OPJ-10, OPJ-11, OPJ-14, OPJ-15, OPJ-16, OPJ-17, OPJ-20, OPL-14, OPM-12, OPM-16, OPM-20, OPM-18, OPN-03, OPN-04, OPN-06, OPN-09, OPN-10, OPN-12</td></tr>
<tr><td>1</td><td>DD1</td><td>$(T)_{11}AA$</td></tr>
<tr><td>2</td><td>DD2</td><td>$(T)_{11}AC$</td></tr>
<tr><td>3</td><td>DD3</td><td>$(T)_{11}AG$</td></tr>
<tr><td>4</td><td>DD4</td><td>$(T)_{11}CA$</td></tr>
<tr><td>5</td><td>DD5</td><td>$(T)_{11}CC$</td></tr>
<tr><td>6</td><td>DD6</td><td>$AAGC(T)_{11}A$</td></tr>
<tr><td>7</td><td>DD7</td><td>$AAGC(T)_{11}C$</td></tr>
</table>

4.2.3.1. PCR amplification

PCR was performed using a thermal cycler (MWG peqlab, Germany) and the reaction parameters were as follows:

a. Initial Denaturation : 94°C for 4 min

b. Denaturation	: 94°C for 60 sec	
c. Annealing	: 36°C for 45 sec	10 cycles
d. Extension	: 72°C for 2min	
e. Denaturation	: 94°C for 60 sec	
f. Annealing	: 38°C for 45 sec	10 cycles
g. Extension	: 72°C for 2min	
h. Denaturation	: 94°C for 60 sec	
i. Annealing	: 40°C for 45 sec	10 cycles
j. Extension	: 72°C for 2min	
k. Final extension	:72°C for 30 min	

4.2.3.2. Urea formamide denaturing polyacrylamide gel electrophoresis of DNA

Reagents

DNA loading dye

Deionised formamide	:	10ml
Xylene cyanol	:	10mg
Bromo phenol blue	:	10mg
EDTA 0.5M (pH 8.0)	:	200µl

40% acrylamide solution

Acrylamide (Sigma)	:	380g
Bis acrylamide (SRL)	:	20g
Water (Double distilled)	:	vol to 1litre

Binding silane

Binding silane (Sigma)	:	1.5µl
Acetic acid	:	5ml
Ethanol	:	995ml
Repulsion silane	:	Sigmacote/rain X/tween 20:ethanol(1:5)

6% acrylamide gel matrix

40% acrylamide	:	15ml
Urea (Sigma)	:	54g
TBE (10X)	:	10ml

Water (Double distilled)	:	vol to 100ml
APS (SRL)	:	400µl
TEMED (SRL)	:	45µl

TBE: Tris Borate buffer (10X) [500ml]

Tris base (Sigma)	:	54g
Boric acid	:	27.5g
EDTA (pH 8.0)	:	20ml
Distilled water	:	vol to 500ml

4.2.3.3. Preparation of gel and electrophoresis

Large and small glass plates (stored in 10% sodium hydroxide) were washed in double distilled water, wiped well with tissue paper (kimwipes). Three ml binding silane was applied to the small plate and spread uniformly using kimwipe paper towels. To the large plate 3ml of repel silane was applied and smeared. The small plate was placed over the large plate with 4mm side spacers in position. The plates were clamped tightly using bull dog clips/gel casting clamps. 20 ml of 6% polyacrylamide stock solution was taken in a conical flask, to which 60µl of 10% ammonium persulphate (freshly prepared) and 20µl of TEMED were added, mixed thoroughly and poured the mixture uniformly in the space provided in between the two plates without air bubbles using a syringe and needle. The comb was positioned properly and clamps were replaced to hold the plates on the top leaving for the gel for polymerization overnight. The comb was removed carefully and the wells were washed with the help of the syringe and needle by using the gel running buffer. TBE (0.5X) was used as running buffer and the gel was kept for pre run at least for 45 min at 40 W. To each PCR product an equal volume of loading dye was added and denatured at 95°C for 8 min and rapidly cooled on ice. The samples were loaded and run at 40 W until the front dye reached the bottom. The plates were dismantled from the set.

4.2.3.4. Silver staining

Reagents

Fixing (5X)	: 0.5% acetic acid in 10% ethanol
Staining solution	: 0.2% Silver nitrate in 1X fixing solution
Developer (5X)	: 3%NaOH in 0.5% formaldehyde

4.2.3.4.1. Protocol: The gel along with the plate was soaked in fixing solution for 15 min with mild shaking until the dye disappeared, followed by a wash in double distilled water twice for 5 min each. Then the gel was incubated for 15 min in staining solution with shaking followed by a brief wash in double distilled water for 10 sec. The gel was treated with the developer till the bands appeared. The reaction was stopped by soaking in fixing solution for 2 min. The gel was photographed and dried in the drier and preserved for further analysis.

4.2.3.5. Elution of differentially expressed amplicons

Diffusion buffer

Ammonium acetate	:	0.5 M
Magnesium acetate	:	10 mM
EDTA pH 8.0	:	1.0 mM
SDS	:	0.1%

QIAEX II Polyacrylamide Gel Extraction kit (Qiagen)

4.2.3.5.1. Protocol: The gel slice containing the differential DNA band was excised using an aseptic surgical sharp scalpel (no.11) fitted to handle (No. 4). 1-2 volumes of diffusion buffer to 1 volume of gel (*i.e.,* 100-200 µl for each 100 mg of gel) was added and incubated at 50°C for 30 min. The supernatant was transferred into the new 1.5ml tube after centrifugation at 12000 rpm. Further purification was followed according to the manufacturer's instructions using the QIAEX II Polyacrylamide Gel Extraction kit (Qiagen).

4.2.3.6. Re-amplification

For re-amplification, 2 µl of this solution was used in standard PCR reactions using the same primer pair used in the Differential Display of the correspondent reaction. PCR reactions were prepared in a final volume of 20 µl containing 1× Taq buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0), 0.25 mM dNTPs, 0.5 µM each primer and 1 U of Taq DNA polymerase enzyme (Sigma). The cycle program consisted of an initial step of 4 min at 94°C, 35 cycles of 30 s at 94°C, 60 s at 40°C and 45 s at 72°C, followed by a final extension step of 8 min at 72°C.

4.2.4. T/A cloning of isolated differential amplicons

A-tailing: Directly after PCR amplification, the reaction mixture was heated at 95°C for 20 min. Then 1µl of 2mM dATP/ 10 µl of PCR product was added and incubated at 70°C for 15 min. Then the PCR products were purified using HiPura PCR clean-up kit.

4.2.4.1. Ligation of A-tailed PCR product to T-tailed vector

The A-tailed purified PCR product of isolated differential amplicons were cloned by ligating to pKRX-T vector using T/A Clone PCR Product Cloning Kit (SBS, China) and transforming competent cells of *Escherichia coli* strain DH5α̃

The following components were added in a thin-walled 0.2 ml PCR reaction tube:

Plasmid vector pKRX-T DNA	:	1.0 µl
Purified PCR fragment	:	4.0 µl
10X Ligase Buffer	:	1.0 µl
T4 DNA Ligase, 5U/µl	:	0.5 µl
Deionized water	:	vol to 10.0 µl

The reaction components were mixed by pipetting or by a brief spin. The reaction mixture was incubated at 16°C for four hours. The enzyme was inactivated by heating the reaction to 65°C for 10 min.

4.2.4.2. Transformation of *E. coli* using the ligation reaction mix

Reagents

Luria-Bertani broth (LB) (per litre)

Bacto-tryptone	:	10g
Bacto-Yeast extract	:	5g
Sodium chloride	:	10g

The pH was adjusted to 7.0 with 2N NaOH and the total volume was made up to 1 litre with deionized water.

SOB (per litre)

Bacto-tryptone	:	20.0g
Bacto-Yeast extract	:	5.0g
Sodium chloride	:	0.6g

Potassium chloride	:	0.19 g
Magnesium sulphate	:	10.0 mM (added from 1.0 M stock)
Magnesium chloride	:	10.0 mM (added from 1.0 M stock)

The first four components and the magnesium salt were autoclaved separately and then mixed to constitute the SOB medium.

SOC (per 100 ml): To 1.0 ml of SOB, 7 µl of filter-sterilized (Millipore, 0.4 µm) glucose solution (50%w/v) was added.

0.1 M $CaCl_2$ stock solution: 1.47 g of $CaCl_2$ was dissolved in 100 ml of deionized water. The solution was sterilized by filtration and stored as 20 ml aliquots at -20°C.

Ampicillin stock solution: 100 mg ampicillin (Ranbaxy, India) was dissolved in 1.0 ml of deionized water. The solution was sterilized by filtration and stored at 4.0°C and used at a working concentration of 100 µg ml^{-1}

0.1 M IPTG stock solution: 0.12 g of IPTG was dissolved in 5.0 ml of deionized water. The solution was filter-sterilized and stored as aliquots at -20°C.

X-Gal stock solution: 100 mg of X-Gal was dissolved in 2.0 ml of N, N'-dimethylformamide (DMF). The solution was stored in micro centrifuge tube, wrapped in aluminium foil at -20°C.

4.2.4.2.1. Preparation of competent cells using $CaCl_2$

A single colony of *E. coli* (DH5α strain) from a plate, freshly grown for 16-20 h at 37°C was picked and transferred into 50 ml of LB broth in a 250 ml conical flask. The culture was incubated at 37°C with rigorous shaking. The OD_{600} of the culture was determined periodically to monitor cell growth. When the OD_{600} reached 0.4-0.5, the cells were transferred aseptically to 50 ml sterile polypropylene tube. The culture was cooled by storing the tube on ice for 10 minutes. The cells were recovered by centrifugation at 4000 rpm for 8 minutes at 4°C. The media was decanted from the cell pellet. The cell pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice. The cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. The fluid from the cell pellet was decanted and the tubes were kept in an inverted position for 1 minute to allow the last traces of fluid to drain away. The cell pellet was re-suspended in 10.0 ml of ice-cold 0.1 M $CaCl_2$ and cells were stored at 4°C for 4-8 hours.

4.2.4.2.2. Transformation of competent cells

About 200 µl suspensions of competent cells were added to sterile micro-centrifuge tubes. Plasmid DNA (~50 ng) or 4 µl of ligation mixture was added to each tube. The contents of the tubes were mixed by swirling gently and the tubes were stored on ice for 30 minutes. Control samples were included as following: (a) competent cells that received standard supercoiled plasmid DNA and (b) competent cells that received no plasmid DNA. The tubes were transferred to water bath set at 42°C for 90 seconds to subject the cells to heat shock. The tubes were rapidly transferred to ice and the cells were allowed to chill for 45 sec. 800 µl of SOC medium was added to each tube and the cultures were incubated for 45 minutes at 37°C.

4.2.4.3. Selection of transformants/recombinants

About 100 µl of transformation mix was plated onto LB agar plates containing 100 µg ml^{-1} ampicillin, 0.5 mM IPTG and 80 µg ml^{-1} X-Gal. The plates were incubated at 37°C overnight for the colonies to grow.

4.2.4.4. Isolation of plasmid DNA from the transformed colonies

Reagents

Solution I	Glucose	: 50 mM
	Tris-Cl (pH 8.0)	: 25 mM
	EDTA (pH 8.0)	: 10 mM
Solution II	NaOH (freshly prepared)	: 0.2 N
	SDS (freshly before use)	: 1.0%
Solution III	5.0 M Potassium acetate	: 60.0ml
	Glacial acetic acid	: 11.5 ml
	Distilled water	: 28.5 ml

4.2.4.4.1. Protocol: Single colonies of appropriate strain were inoculated in 2 ml of LB broth containing required antibiotic and grown overnight in a shaker incubator at 37°C and 180 rpm. 1.5 ml of the overnight culture was transferred to a 1.5 ml micro centrifuge tube and the cells were harvested by centrifugation at 10,000 rpm for 2 min. After discarding the supernatant, 100 µl of solution I was added and vortexed vigorously until no visible clumps of cells were observed. The samples were kept on ice for 5 min. About

200 µl of freshly prepared alkaline solution (solution II) was added to the tube and mixed gently by inverting the tubes several times until the lysed cell suspension became clear. The samples were kept on ice for 5 min, 150 µl of ice-cold potassium acetate solution (solution III) was added, and tubes were inverted gently. The tubes were centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and equal volume of phenol- chloroform was added and vortexed thoroughly. Centrifugation at 10,000 rpm for 10 min separated the two phases. The upper aqueous phase was transferred to a fresh tube and equal volume of chloroform was added. The tubes were centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a fresh tube and 2 volumes of absolute ethanol were added. The tubes were kept at -20°C for 1 hr to overnight for precipitation. The tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The pellet was washed with 300 µl of 70% ethanol and the air-dried pellet was dissolved in 20 µl of TE buffer. Samples were tested by carrying out agarose gel (0.8%) electrophoresis along with control plasmid.

4.2.5. Sequencing of the clones

DNA sequencing was carried out using M13F and M13R primers by dideoxy chain termination method (Sanger et al. 1977). The reaction was carried out in an automatic DNA sequencer (ABI prism, Applied Biosystems, USA) with fluorescent dideoxy chain terminators at the Bioserve Biotechnologies Pvt Ltd, Hyderabad.

4.2.6. Validation by Northern dot

Four inserts of the clones namely BA4, BA5, BA6 and BA14 from the DD-RT experiments and of the clones used as controls were amplified by PCR either side of the primers T7 and Sp6. The amplification products were spotted in duplicate onto nylon membrane (Hybond N+, Ambion) at a concentration of 300 ng per blot for the cDNAs derived from DD-RT and in the range of 50 ng to 300 ng per blot for the controls. The amplification product corresponding to the region of the pKRX-T plasmid polylinker was used as a negative control. A 18S fragment was used as an internal control of expression. The hybridization, post-hybridization treatments and detection were done as described in the following section.

4.2.7. Quantitative Northern blot analyses of selected amplicons

Reagents

- Transfer apparatus
- 3 MM Whatman® filter paper
- BrightStar plus nylon membranes (Ambion)
- Quick draw paper towels (Sigma)
- Transfer buffer (Ambion)
- ULTRAhyb buffer (Ambion)
- Post-hybridization washing buffer I & II*
- Anti-DIG antibody-alkaline phosphatase conjugate solution*
- Detection buffer: 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5
- Color-substrate solution*

*Supplied with the kit

4.2.7.1. Downward transfer of RNA from formaldehyde gel onto nylon membrane (Modified from Chomczynski 1992)

- The pre-cut paper towels (3cm height) were stacked near the transfer buffer reservoir. The put 3 pieces of filter paper on top of the paper towel. Two more filter papers were soaked in transfer buffer and arranged on the top of the stack
- The membrane was briefly soaked in transfer buffer and placed on top of the stack. Three more wet filter papers were soaked in buffer and placed on the top of the membrane. Then the gel was rinsed and placed over the filter paper
- The membrane was made and assembled as shown in the figure (Figure 4.1)
- Then the stack was covered with plastic paper and a weight (200g) was placed on the top of the stack and transfer was allowed for 15-20 min per mm of the gel

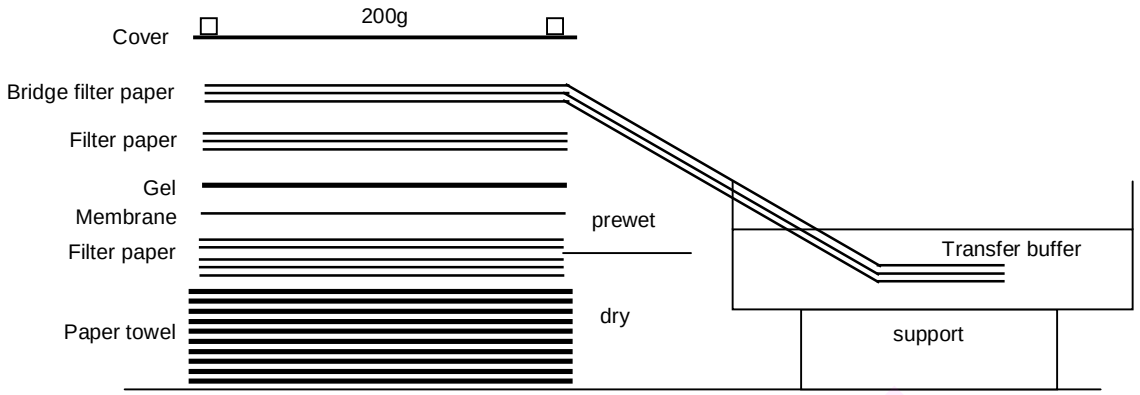

Figure 4.1. Downward transfer setup for the transfer of RNA from formaldehyde gel on to nylon membrane

- The transfer set up was disassembled and the membrane was removed with forceps and briefly rinsed with 1X gel running buffer
- The damp membrane was immediately exposed to UV light for 30 min to crosslink the RNA

4.2.7.2. Pre-hybridization

1. The membrane was placed in a polythene bag and 15 ml pre-warmed (65°C) hybridization buffer was added to it.
2. The bag was sealed and incubated overnight at 42°C with mild agitation

4.2.7.3. Hybridization

1. A 5 µl aliquot of probe for respective gene was heat-denatured by incubating in boiling water for 5 min, followed by snap cooling on ice.
2. A 2 µl aliquot of denatured probe was added to 5 ml of pre-warmed (65°C) hybridization buffer.
3. The hybridization buffer containing the denatured probe was added to the polythene bag containing the membrane
4. The polythene bag was sealed and incubated at 42°C for 6 h with mild agitation in a water bath

4.2.7.4. Post-hybridization washes

1. The hybridization buffer was discarded and the membrane was washed twice in 50 ml of post hybridization washing buffer I for 5 min at room temperature under mild agitation
2. The membrane was washed twice in 50 ml of post hybridization washing buffer II for 15 min at 65°C under mild agitation

4.2.7.5. Detection

1. The membrane was rinsed briefly at room temperature in maleic acid buffer and incubated in 50 ml 1X blocking solution for 30 min
2. The blocking solution was discarded and the membrane was incubated in 10 ml of antibody solution (1:5000 Anti-DIG-AP conjugate in 1X blocking solution) at room temperature for 45 min under mild agitation
3. The membrane was washed twice for 15 min in 50 ml maleic acid buffer and incubated in 20 ml detection buffer for 5 min
4. The detection buffer was discarded and the membrane was incubated overnight in 10 ml freshly prepared color solution
5. The membrane was kept in the dark for color development
6. The reaction was stopped by washing the membrane for 5 min with 50 ml of deionized water The results were documented by photography of the wet membrane following which the membrane was stored at 4°C

4.2.8. Quantitative (relative) reverse transcriptase polymerase chain reaction (qRT-PCR)

Quantitative relative RT-PCR was used to confirm the differential expression of DNA fragments isolated from control and ethylene induced /inhibited ripened fruits. The treatments and RNA isolation were performed as mentioned in Chapter IV. Specific oligonucleotide primers were designed for each DD product for RT-PCR (Table 4.2) and synthesized (Bioserve Biotech, Hyderabad, India). First-strand complementary DNAs were synthesized from 1.5 µg of total RNA in 20-µl final volume, using M-MuLV reverse transcriptase and oligo-dT (18 mer) primer (Fermentas GmbH, Germany). PCR

amplifications were performed using PCR mixture (15 µl) that contained 1 µl of RT reaction product as template, 1× PCR buffer, 200-µM dNTPs, 1 U of Taq DNA polymerase and 0.1 µM of each primer depending on the gene. PCR was performed at initial denaturation at 94°C for 4 min, 30 or 22 cycles (1 min at 94°C; 1 min at 55 or 60°C; 1 min at 94°C), and final elongation (10 min at 72°C) using a thermal cycler. The optimal PCR annealing temperatures and the cycle numbers are shown in Table 4.2. The PCR products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%), and documented. The size of the amplification products was estimated from the 100-bp DNA ladder (Fermentas GmbH). The band intensity of each gel was checked using the Herolab E.A.S.Y Win 32 software. The transcript levels of each gene in control ripening were taken for comparison in calculating the transcript abundance of respective genes during ripening.

Table 4.2. Gene specific primers and annealing temperatures used for RT-PCR

Primers	Primer sequence (5'-3')	Annealing temperature (°C)
BA4 *Forward*	TATTCGGGAACTGGAGGATG	62
BA4 *Reverse*	ATTAGCTCGAGCCGAACCTC	
BA5 *Forward*	GGCTGTCTGTCTCAGGCATT	61
BA5 *Reverse*	CGCATTCTTCAGATGGTTCA	
BA6 *Forward*	CTGGAAGGATCGCCATTAAC	61
BA6 *Reverse*	TTTTCACGGGAATACGGAAG	
BA14 *Forward*	ACATCCGCAATTCTCAAACC	60
BA14 *Reverse*	CCTGACGGCAAAAATAAGGA	
18S *Forward*	GGCAGCTATGTGTTTGTCCA	60
18S *Reverse*	TGTGGAATTGTGAGCGGATA	

4.3. RESULTS

4.3.1. The yield of total RNA

Yields of RNA from different parts of banana plant (root, leaf, flower, bract, immature fruit), and different stages of ripening fruit (Pre-climacteric (PC), Climacteric I (C I), Climacteric II (C II) and Post climacteric (PoC) obtained through all the three protocols varied (Table 4.3). A typical CTAB method yielded 1500 µg of RNA compared to 1500 µg of RNA for Ambion kit (Table 4.3). For all the samples the differences were 1-2 times higher for CTAB method yields when compared to Ambion kit yields. The issue of RNA quality was assessed by calculating the A260/A280 ratio to determine DNA and protein contamination. The closest ratio to 2.0 (indicative of pure RNA) was obtained for all the samples (banana pulp) RNA isolated through Ambion kit. The A260/A230 ratios of all RNA samples were greater than 2.0 indicating that no contamination by polysaccharides or polyphenol occurred (Table 4.3).

Table 4.3. Summary of results from DNA and RNA extractions (100 mg of the frozen powdered sample)

Method	Sample	Yield (µg/g FW)	OD 260/230	OD 260/280	Bench time (hr)	Total time (hr)
Ambion kit	Banana leaf	1500.00	2.12	1.9	6	24
	Banana pulp*	Nil				
Method (CTAB)	Banana root	633.60	2.12	1.89		
	Banana flower	748.80	2.03	1.80		
	Banana bract	1036.80	2.11	1.80		
	Immature fruit	1411.20	2.12	1.90		
	Pre-climacteric fruit (PC)	936.00	2.18	1.89	6	24
	Climacteric I (C I)	1008.00	2.08	1.81		
	Climacteric II (C II)	792.00	2.18	1.92		
	Fully ripened fruit (PoC)	1008.00	2.14	1.88		
	Banana leaf	1500.00	2.00	1.90		

4.3.2. The integrity of the total RNA

The quality of RNA was inspected by agarose gel electrophoresis. All samples showed bands typical of undegraded RNA. For all tested RNA samples, distinct 28S and 18S ribosomal RNA bands were found in ethidium bromide staining 1.2% denaturing agarose gel and the brightness of 28S is probably as equal as that of 18S RNA (Figure 4.2 and Figure 4.3). These showed that the RNA was of high integrity and also relatively free of RNase, and the method was feasible and steady.

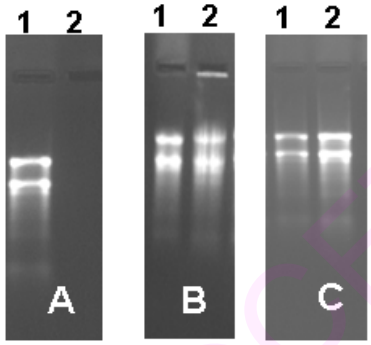

Figure 4.2. Assessment of RNA integrity by agarose gel electrophoresis. Different RNA preparations were isolated with the different protocols described in this chapter, resolved on 1.2% formaldehyde denaturing agarose gel, stained with ethidium bromide (EtBr) and photographed under ultraviolet light A. by using RNAqueous® kit according to instruction manual (Ambion, Austin, TX), B. by phenol chloroform method, C. by NaCl method. Lane 1 - banana leaf and Lane 2 – banana fruit

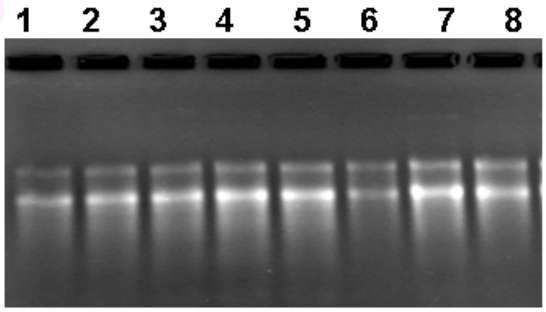

Figure 4.3. Ethidium bromide-stained agarose gel (1.2%) electrophoresis of total RNA (1 µg) isolated from the different parts of banana plant and from fruits at different stages of ripening. Lane 1: Banana root; 2: Banana flower; 3: Banana bract; 4: Immature fruit; 5: Pre-climacteric fruit (PC); 6: Climacteric I (C I); 7: Climacteric II (C II); 8: Fully ripened fruit (PoC)

4.3.3. Analyses of cDNA integrity

cDNA integrity was analysed by the PCR amplification of 18S. The RT- was made using the RT enzyme and oligo dT and random hexamer primers. The forward and reverse primers were designed to amplify the region of 450 bases size. Both forward and reverse primers were having 100% homology with banana18S and the 450 bases amplicon also sharing 100% homology with banana 18S. PCR amplification of 18S in cDNA samples to be used for differential display confirmed cDNA integrity whilst lack of amplification of minus reverse transcriptase (-RT) samples confirmed the absence of genomic DNA contamination (Figure 4.4).

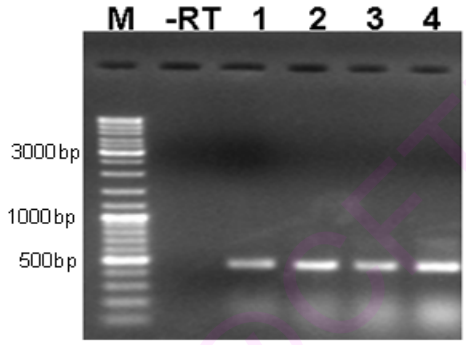

Figure 4.4. Analysis of cDNA integrity by resolving PCR amplified 18S on a 1.2% agarose gel. Lane –RT is a negative control without reverse transcriptase added (no DNA contamination). Lanes 1,2,3,4 are the amplification of 18S from the cDNA of four stages of ripening

4.3.4. Optimization of DDRT- PCR

To determine the concentrations of magnesium chloride, PCR annealing temperature and nucleotides to be used in DDRT-PCR, optimization of these three parameters were carried out. cDNA from the untreated banana fruit was used as template and amplification was carried out using the one anchor (DD1) and two arbitrary primers (OPC 07 and OPJ 18), The optimization series include $MgCl_2$ at 0.5mM increments from 1.0mM to 2.5 mM and dNTPs at 0.1mM increments from 0.1mM to 0.5mM. Except for the $MgCl_2$ concentration of 1.0mM, 1.5 mM to 2.3 mM vary slightly in band patterns but 1.5 mM was found to produce bands of relatively higher intensity at 2.0 mM dNTPs (Figure 5.5). At $MgCl_2$ concentration of 1.5 mM, dNTPs concentrations of 0.1 to 0.3 mM produced a comparable number of bands but 0.2 mM showed the clearest pattern. The

optimal concentrations $MgCl_2$ and dNTPs to be used were determined to be 1.5 mM and 0.2 mM respectively.

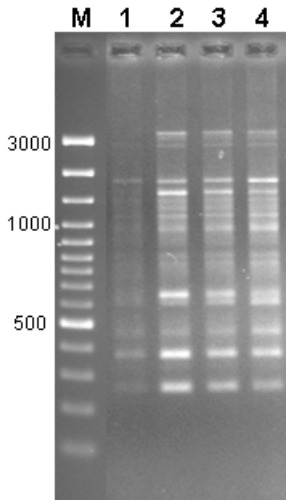

Figure 4.5. Optimization of magnesium chloride concentration for DDRT-PCR

By using the above conditions, the differential display was performed for 7×50 = 350 primer combinations. Three anchor primers (DD1, DD2 and DD3) and 27 random primers produced constant amplifications. These primers combinations were used for differential display reactions.

4.3.5. DDRT-PCR

Differential display was performed as described as above with a degenerate anchored oligo DD1, DD2 and DD3 and twenty eight arbitrary oligos (Table 4.1). Reactions were carried out in duplicate, including nuclease free water controls and the internal experimental minus (-RT) controls. Totally 3×27=71 differential display experiment were successfully done. The use of 71 primer combinations (anchor primers 3 in combination with arbitrary primers 27; Table 4.1) in the four RNA populations resulted in 3927 scorable and distinct fragments in the silver-stained polyacrylamide gels to be analysed. Only the fragments reproducible among the two dilutions used were considered. Fragments in the scorable area of the gels ranged from 50 to 1000 bp, although larger products also existed. However, the great proximity between larger fragments in the gels made them very difficult to score and excise without errors. Figure 4.6 shows an example of a section of a typical Differential Display gel, containing

representative banding patterns among the four template samples used, after amplifications with primer combination DD3 & OPC 07 and DD2 & OPJ 18.

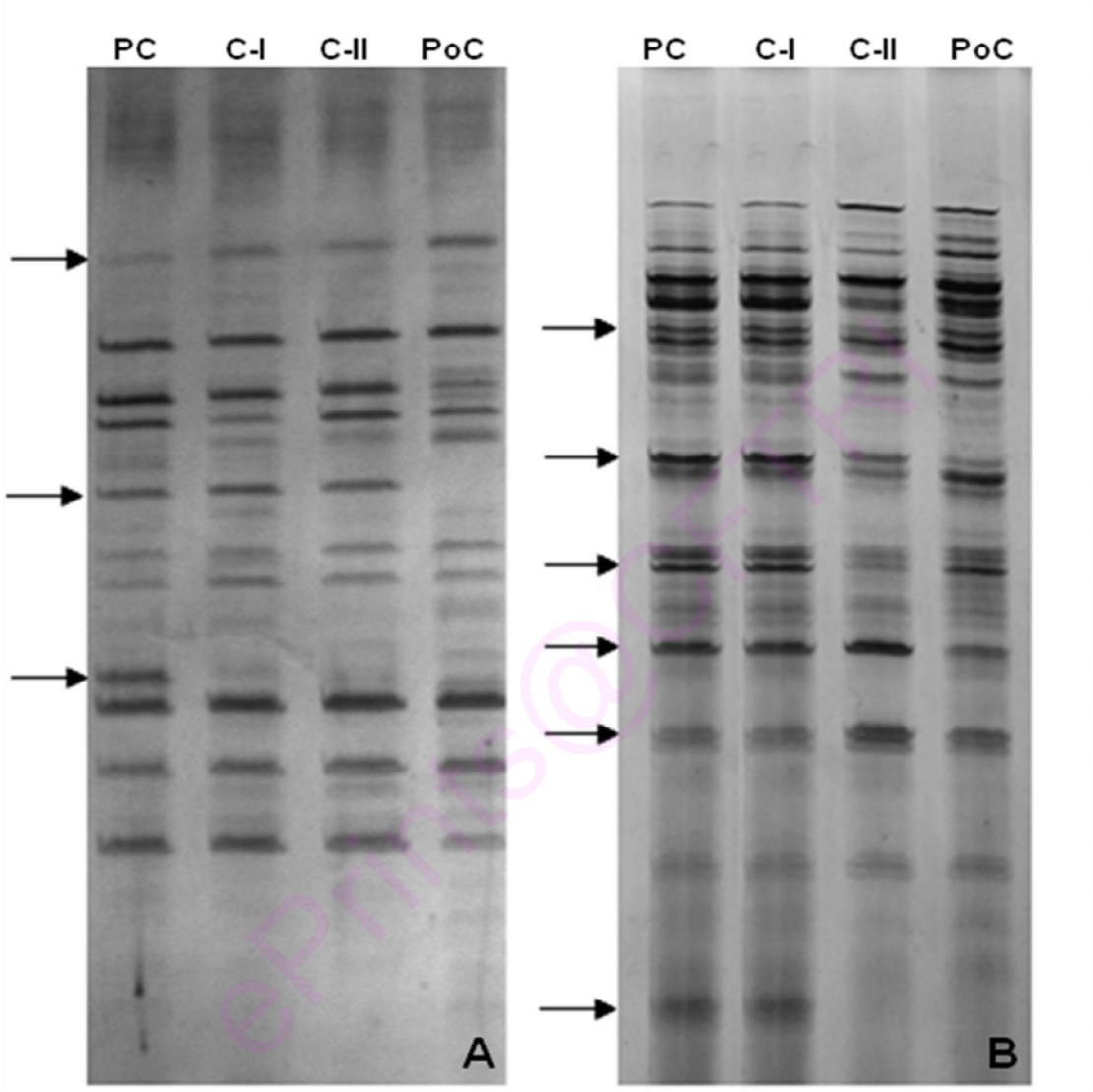

Figure 4.6. Representative DDRT-PCR sequencing gel. cDNA, representing genes differentially expressed at PC, CI, CII and PoC period of ripening, was isolated and identified by RNA differential display. cDNAs were amplified from three separately isolated total RNAs by primer combination of DD3 & OPC 07 (A) and DD2 & OPJ 18 (B). PC, CI, CII and PoC are cDNAs amplified from the fruits sampled at Preclimacteric (PC), Climacteric I (C-I), Climacteric II (C-II) and Post Climacteric (PoC) stages of ripening. Arrows indicate differentially regulated cDNA fragment.

More than 285 bands were identified with a differential display pattern of either present/absent or of higher/lower intensity. Of these, primer combination DD3 & OPC 07 produced highest differential bands of 12 followed by the combination DD2 & OPJ 18 produced 10 differential bands. These amplicons were clustered in different groups on the basis of the following criteria: (i) the intensity of the spots of the individual clones in the northern dot (ii) the correlation between the intensity of the clones in the reverse northern analysis and in the original DD gel; (iii) the correlation between the size of the cloned fragments and the estimated size of the original differential display bands in the DD gels; and (iv) the relative abundance of individual clones after cloning the DD band. The nucleotide sequences of selected clones have been deposited in GenBank. Based on the expression pattern these were grouped as follows:

Group I, consisting of 34 amplicons, showed an increase in their mRNA accumulation (up-regulated); Group II, with 46 amplicons, displayed decrease in gene expression (down regulated) and group III with 14 amplicons, displayed induced expression (Figure 4.6 and Table 4.4)

Table 4.4. List of the isolated bands and their approximate size as determined from manual sequencing gel

S.No	Name	Size (bp)	Expression level	S.No	Name	Size (bp)	Expression level
1.	BA1	211	Up-regulated	46.	BA46	443	Down-regulated
2.	BA2	308	Up-regulated	47.	BA47	334	Down-regulated
3.	BA3	78	Up-regulated	48.	BA48	176	Up-regulated
4.	BA4	307	Up-regulated	49.	BA49	176	Down-regulated
5.	BA5	441	Up-regulated	50.	BA50	217	Up-regulated
6.	BA6	389	Up-regulated	51.	BA51	190	Up-regulated
7.	BA7	390	Up-regulated	52.	BA52	165	Down-regulated
8.	BA8	541	Up-regulated	53.	BA53	250	Down-regulated
9.	BA9	543	Induced	54.	BA54	195	Induced

10.	BA10	240	Down-regulated	55.	BA55	597	Induced
11.	BA11	37	Down-regulated	56.	BA56	231	Induced
12.	BA12	88	Down-regulated	57.	BA57	238	Induced
13.	BA13	88	Induced	58.	BA58	776	Down-regulated
14.	BA14	612	Up-regulated	59.	BA59		Up-regulated
15.	BA15	348	Induced	60.	BA60	93	Down-regulated
16.	BA16	300	Induced	61.	BA61	87	Down-regulated
17.	BA17	355	Down-regulated	62.	BA62	112	Down-regulated
18.	B18	320	Down-regulated	63.	BA63	121	Down-regulated
19.	B19	440	Induced	64.	BA64	178	Induced
20.	BA20	432	Up-regulated	65.	BA65	350	Up-regulated
21.	BA21	454	Up-regulated	66.	BA66	182	Up-regulated
22.	BA22	216	Up-regulated	67.	BA67	130	Down-regulated
23.	BA23	380	Down-regulated	68.	BA68	127	Down-regulated
24.	BA24	229	Down-regulated	69.	BA69	129	Down-regulated
25.	BA25	383	Up-regulated	70.	BA70	500	Down-regulated
26.	BA26	220	Up-regulated	71.	BA71	177	Down-regulated
27.	BA27	192	Up-regulated	72.	BA72	323	Up-regulated
28.	BA28	227	Up-regulated	73.	BA73	115	Induced
29.	BA29	410	Up-regulated	74.	BA74	137	Up-regulated
30.	BA30	334	Induced	75.	BA75	142	Down-regulated
31.	BA31	225	Down-regulated	76.	BA76	173	Down-regulated
32.	BA32	132	Induced	77.	BA77	209	Down-regulated
33.	BA33	774	Down-regulated	78.	BA78	274	Down-regulated

34.	BA34	174	Up-regulated	79.	BA79	109	Down-regulated
35.	BA35	42	Down-regulated	80.	BA80	104	Down-regulated
36.	BA36	40	Down-regulated	81.	BA81	986	Down-regulated
37.	BA37	28	Induced	82.	BA82	159	Down-regulated
38.	BA38	757	Down-regulated	83.	BA83	587	Down-regulated
39.	BA39	421	Up-regulated	84.	BA84	337	Up-regulated
40.	BA40	131	Up-regulated	85.	BA85	105	Up-regulated
41.	BA41	181	Up-regulated	86.	BA86	145	Up-regulated
42.	BA42	406	Down-regulated	87.	BA87	98	Up-regulated
43.	BA43	42	Down-regulated	88.	BA88	821	Down-regulated
44.	BA44	42	Down-regulated	89.	BA89	263	Up-regulated
45.	BA45	800	Down-regulated	90.	BA90	354	Up-regulated

4.3.6. Re-amplification

Re-amplification was carried out to obtain sufficient DNA from differentially display bands for downstream analysis. cDNA from each gel fragment was extracted and re-amplified. Of the 285 cDNA fragments 150 were re-amplified when the same primer combination was used. About half of the candidate bands generated single target PCR product corresponding to the size observed on the original display.

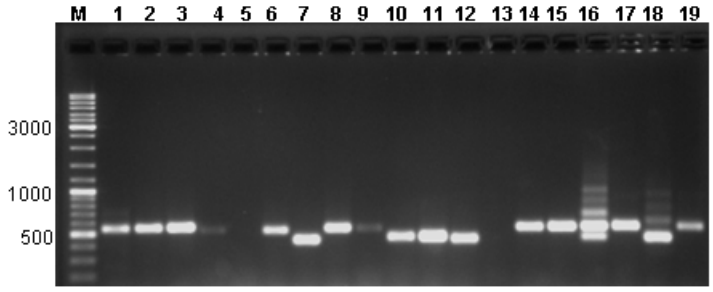

Figure 4.7. Re-amplification of cDNA fragments. Lanes (M) 100 bp molecular marker; Lane no. 16 multiple PCR products were recovered. The estimated size of cDNA bands ranged from 220 bp to 600bp.

However, the remaining candidate bands generated more than one PCR product but in this case; the target PCR product appeared more intense than the non-target product on the gel. (Figure 4.7 Lane no. 16). Cloning was therefore carried out to identify the differentially expressed cDNA. Totally 120 amplicons were selected for cloning.

4.3.7. Differential display fragment cloning

All selected re-amplified amplicons (120) were cloned into pKRX-T cloning systems as they take advantage of the ability of Taq polymerase to add a terminal 3'deoxyadenosine during the PCR. These vectors contain a 3'T overhang at the cloning site, allowing sticky end ligation that is more successful than blunt end ligation. However, the resolution of the re-amplified PCR products on a 1.5% agarose gel was insufficient to detect 5-20 bp differences and multiple products were not observed until cloned fragments were amplified using the universal sequencing T3 and T7 primers.

4.3.8. Cloning confirmation by PCR analyses

Colonies harbouring the recombinant plasmid have a disrupted *lacZ* gene and appeared white while non-recombinant vectors were blue. White colonies were selected from the LB plate and screened by PCR for the presence of insert using the T3 and T7 primers flanking the cloning site. Both the T3 and T7 primers anneal to sites in the pKRX-T vector 60 bp upstream or downstream respectively from the cloning site. Therefore, colonies that contained plasmids with inserts gave a band of 160 plus the size of the insert.

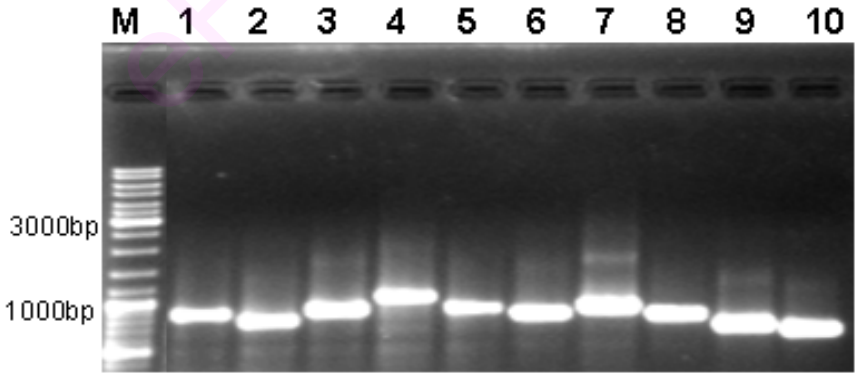

Figure 4.8. PCR screening of recombinant clones. Lane M: molecular weight marker Lanes 1 to 10: PCR amplification of cloned fragments.

Based on the PCR results, clones with the correct insert size were selected (Figure 4.8). All the 120 cloning and transformation reactions yielded recombinant clones. However, the success of obtaining clones with the right insert size varied from 80% to 100%. Of the 120 positive clones, only 90 were selected and plasmid DNA was isolated for sequencing.

4.3.9. Sequence analyses of cDNA fragments

Clones from all the 90 cDNA fragments were sequenced using both T3 forward as well as T7 reverse primer. Of these 75 cDNA fragments produced readable sequences. Sequence length varied from 37bp to 986bp. The size of these cDNA fragments were relatively close to the estimated size from the original display from which these fragments were obtained. All the cDNA fragments were amplified with their respective arbitrary primers on both the 5' and 3' ends as determined by sequencing. Nucelotide sequences of these cloned cDNA fragments are presented in Appendix.

4.3.10. *In silico* analyses of the sequences

BLASTX and BLASTn searches of the 75 nuclotide sequences against NCBI databases identified homologies to 60 known proteins, 15 hypothetical proteins and one each of unknown or unnamed proteins of various species. The sequence identities ranged from % to 100% over amino acid residues. Sequence analysis of these amplified cDNA fragments indicated that they encoded gene products of various functions. The sequence similarities and details of each cDNA clones are shown in Table 4.5. Based on the BLASTX and BLASTn homology search and their putative functions, the amplicons were grouped into nine major groups (Table 4.5).

Group 1. Stress and senescence

Group 2. Transcription and translation

Group 3. Transport

Group 4. Signal transduction

Group 5. Nucleotide repair

Group 6. Metabolism

Group 7. Unidentified functions

Group 8. Unclassified

Group 9. Novel genes

Table 4.5. Isolated transcripts are grouped according to their putative function or to the mechanism to which they have been related

Fragment ID	Size (bp)	Acc. No.	BLAST	Homologous proteins/genes/ domains	Putative function
Group 1. Stress and senescence					
BA57	238	GE47263	213 (2e-52)	Putative senescence-associated protein	Senescence
BA77	209	GE47278	131 (2e-29)	Putative senescence-associated protein (*Pisum sativum*)	Senescence
BA84	337	GE47285	195 (8e-49)	Heat shock protein 70 (*Arabidopsis thaliana*)	Stress response
BA1	211	GE47232	120 (3e-26)	Putative iron-dependent peroxidase (*Escherichia coli*)	Stress response
BA21	454	GE47243	248 (1e-64)	24 kDa seed maturation protein (*Elaeis guineensis*)	seed maturation protein
BA46	443	GE47255	250 (3e-65)	24 kDa seed maturation protein (*Elaeis guineensis*)	seed maturation protein
BA47	334	GE47256	192 (7e-48)	70 kDa heat shock protein (*Phaseolus vulgaris*)	Stress
Group 2. Transcription and translation					
BA56	231	GE47262	33.1 (7.3)	Paired box protein 6a (*Anabarilius grahami*)	Transcriptional regulator
BA63	121	GE47267	48.1 (2e-04)	tRNA(Ile)-lysidine synthase (*Escherichia albertii*)	Translation
BA2	308	GE47233	191 (1e-47)	Transcriptional regulator Cbl (*Escherichia coli*)	Transcription
BA4	307	GE47235	194 (2e-48)	Transcriptional regulator Cbl (*Escherichia coli*)	Transcription
BA50	217	GE47258	82 (2e-15)	Unnamed protein product (*Vitis vinifera*) hypothetical protein (*Oryza sativa*)	Ribosome biogenesis

Group 3. Transport					
BA55	597	GE47261	240 (5e-62)	Outer membrane usher protein fimD homolog	Transport protein
BA27	192	GE47247	33.9 (4.3)	Putative PDR-like ABC transporter (*Oryza sativa* Japonica Group)	Transport protein
BA31	225	GE47249	139 (6e-32)	Monosaccharide transporter/sugar carrier/hexose transporter	Transport protein
BA6	389	GE47237	214 (2e-54)	Putative inner membrane protein (*Shigella flexneri*)	Transport protein
Group 4. Signal transduction					
BA62	112	GE47266	39.2 (3.2)	Arabidopsis thaliana F-box family protein	Signal transduction, cell cycle regulation
BA85	105	GE47286	48 (2e-04)	Protein kinase (*Nicotiana tabacum*)	
BA71	177	GE47272	71 (1e-09)	*Fagus sylvatica* partial mRNA for protein kinase (pk1 gene)	Protein phosphorylation
BA82	159	GE47283	62 (1e-08)	Protein kinase MK6 (*Mesembryanthemum crystallinum*)	ATP binding, protein serine/threonine kinase activity
Group 5. Nucleotide repair					
BA83	587	GE47284	278 (2e-100)	dN 3'phosphatase, 5'polynucleotide kinase Enterobacteria phage	RNA and DNA repair
BA33	774	GE47250	451 (3e-125)	PseT polynucleotide 5'-kinase and 3'-phosphatase (Enterobacteria phage T4)	RNA and DNA repair
Group 6. Metabolism					
BA67	130	GE47269	34.3 (3.2)	Sucrose phosphate synthase (*Musa acuminate*)	Starch and sucrose metabolism
BA68	127	GE47270	73.1 (2e-10)	*Musa acuminata* sucrose phosphate synthase	Starch and sucrose metabolism

BA60	93	GE47260	50.1 (6e-05)	Nitrate reductase, alpha subunit (*E. albertii* TW07627)	Nitrogen metabolism
BA72	323	GE47273	164 (3e-39)	Isopentenyl-adenosine A37 tRNA methylthiolase (*Escherichia coli*)	Catalytic activity, metal ion binding, tRNA methylation
BA73	115	GE47274	48.1 (2e-04)	Uracil phosphoribosyl-transferase (*E. coli* UTI89)	Pyrimidine metabolism
BA76	173	GE47277	34.7 (2.5)	Sucrose phosphate synthase (*Musa acuminate*)	Starch And Sucrose Metabolism
BA78	274	GE47279	101 (2e-22)	Acetylglutamate kinase (*Escherichia coli*)	Arginine synthesis
BA79	109	GE47280	71.6 (2e-11)	Peptidase, U32 family (*Shigella dysenteriae* 1012)	Protein hydrolysis
BA3	78	GE47234	46.6 (6e-04)	Putative 6-phosphogluconolactonase (*Shigella flexneri*)	Pentose Phosphate Pathway
BA17	355	GE47241	85.9 (9e-16)	Succinate dehydrogenase cytochrome b556 small membrane subunit	Component of the tricarboxylic acid cycle
BA24	229	GE47245	44.3 (0.003)	Putative hydrolase (*Solanum commersonii*)	Cell wall degradation
BA25	383	GE47246	33.5 (5.5)	Putative alginate O-acetyltransferase (*Zymomonas mobilis*)	Starch metabolism
BA41	181	GE47253	114 (2e-24)	S-adenosyl-L-homocysteine hydrolase (*Hordeum vulgare* subsp. *Vulgare*)	Maintenance of cellular transmethylation
BA52	165	GE47259	54.7 (2e-06)	Rice starch branching enzyme (*Oryza sativa* Japonica Group)	Starch metabolism
Group 7. Unidentified functions					
BA42	406	GE47254	159 (9e-38)	Unnamed protein product (*Vitis vinifera*)	Unknown function
BA22	216	GE47244	43.1 (0.007)	Hypothetical protein (*Musa acuminata*)	Unknown function

BA10	240	GE47238	150 (2e-35)	Conserved hypothetical protein (*Shigella dysenteriae*)	Unknown function
BA5	441	GE47236	166 (4e-40)	Hypothetical protein (*Escherichia coli*) *gef* membrane toxin	Unknown function
BA90	458	GE47291	80.5 (4e-14)	Hypothetical protein (*Chlamydomonas reinhardtii*)	Unknown function
Group 8. Unclassified					
BA61	87	GE47265	37.4 (7.7)	*Lycopersicon esculentum* aldehyde oxidase (AO5) pseudogene	Aroma related
BA28	227	GE47248	32.7 (9.4)	Transposase IS630 (*Listonella anguillarum*)	DNA modification/evolution
BA81	986	GE47282	39.3 (0.48)	Novel protein similar to vertebrate atrophin 1 (ATN1)	Unknown
BA74	137	GE47275	73.6 (5e-12)	Predicted outer membrane biogenesis protein (*E. coli*)	Membrane biogenesis
Group 9. Novel genes					
GE47239; GE47240; GE47242; GE47251; GE47250; GE47257; GE47260; GE47231; GE47268; GE47271; GE47276; GE47281; GE47287; GE47288 and GE47289					

4.3.11. Verification of the DD cDNAs expression by RNA blot analysis

Confirmation of the observed differential display expression levels was necessary to eliminate possible false positive differential display results. At present there are several techniques that allow fast corroboration of gene expression, we used Northern dot blot hybridizing as a first approach to verify differential expression followed by Northern blot of the selected genes to verify the changes in gene expression. The cloned differential display products were used to probe with total RNA isolated from different ripening stages of banana fruit. High prime DNA-dig labeled probes effectively

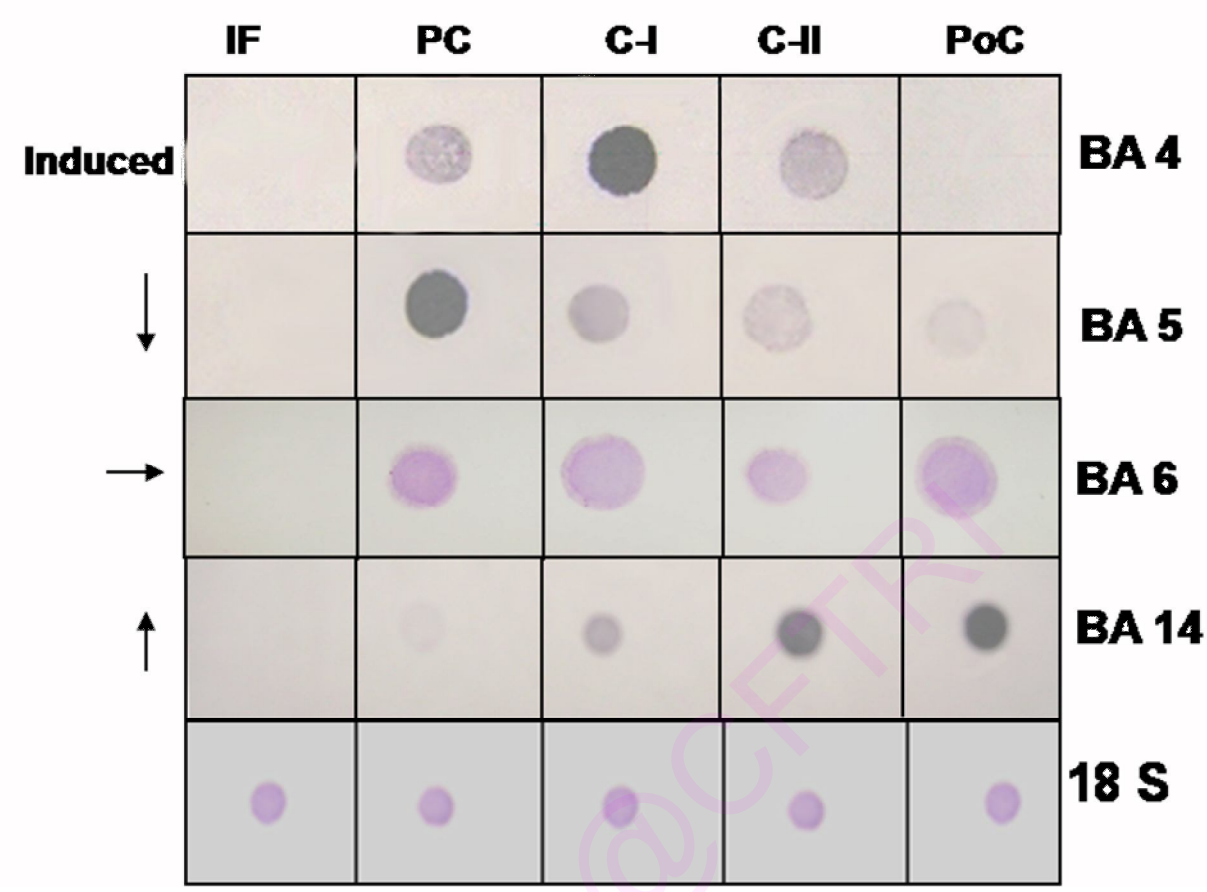

Figure 4.9. Confirmation of differential accumulation of BA4 (Lane 2), BA6 (Lane 3), and BA12 (Lane 4) of pulp of ripening banana fruit. Total RNA (1 µg per dot) was used for RNA gel blot analysis and hybridized with Dig-labeled probes

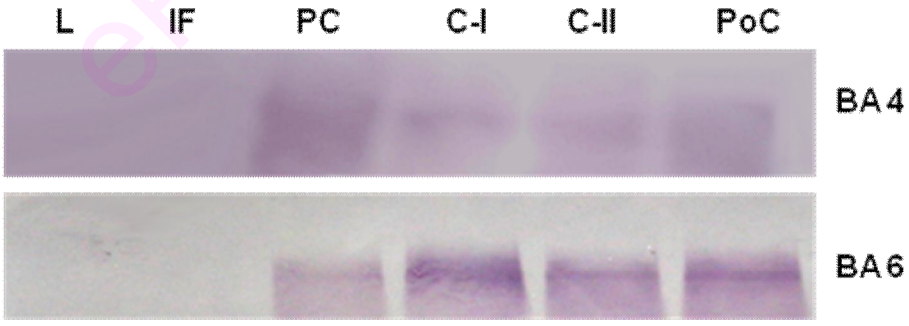

Figure 4.10. RNA northern-blot analysis showing BA4 and BA5 mRNA abundance in different tissues. Total RNAs were extracted from leaf and immature fruit and four different stages of fruit ripening of banana. RNA from each sample was separated by electrophoresis and hybridized with BA4 and BA5 specific dig-labelled cDNA probes

incorporated the digitoxin and provided sufficient sensitivity for detecting their target mRNAs. Firstly, four selected clones were assessed for regulation by RNA blots using total RNA isolated from immature fruit and four stages of ripening banana fruit under normal condition. Results of Dot blot hybridization confirm ethylene induced cDNA differential expression in pulp of banana fruits (Figure 4.9). In the case of banana pulp the clone BA4 (Putative inner membrane protein), BA5 (Putative inner membrane protein of *Shigella flexneri*), BA6 (Transcriptional regulator Cbl of *E. coli*) and BA14 novel gene give a positive strong-labeled signal, the clone 18 S (*Musa acuminata* 18S ribosomal RNA gene) was used as a control (Figures 4.9 and 4.10 and Table 4.2)

4.3.12. Tissue specific expression of genes by Northern blot

In order to identify genes that are fruit specific and ripening related, tissue specific expressions were studied by northern dot analyses in different stages of developing fruit, ripening fruit and in different tissues such as leaves and immature fruit (30 days after flower emergence). RNA samples from 0 day and 2, 4 and 6 days of ripening banana fruit were used as control. Blots were hybridized to various genes (BA4 and BA5) and exposed to colour substrate solution for a longer time as compared to the time course studies so as to detect faint signals. As shown in Figure 4.10, for the up-regulated gene (BA14), no expression was detected in any other tissue even on long exposure. The down-regulated gene BA5, although not expressed in different vegetative tissue and expressed only during ripening, showed fruit-specific expression.

4.3.13. Transcript patterns after ethylene inducers/inhibitors treatment

In order to study if these genes are ethylene-induced, ripening-related and fruit specific, the expression pattern of some of these genes namely BA4, BA5, BA6 and BA14 were monitored during the entire course of ripening. Expression was also monitored in fruit where ripening were regulated by different ethylene inducers and inhibitors. Four unigenes most of the genes studied, treatment with GA and IAA had almost completely blocked the expression of genes in the fruit indicating that their transcription was ripening related and influenced by ethylene either directly or indirectly (Figure 4.11). In order to distinguish between direct and indirect ethylene regulation we treated mature green fruit with ethrel for a short duration (30 min) and RNA from these

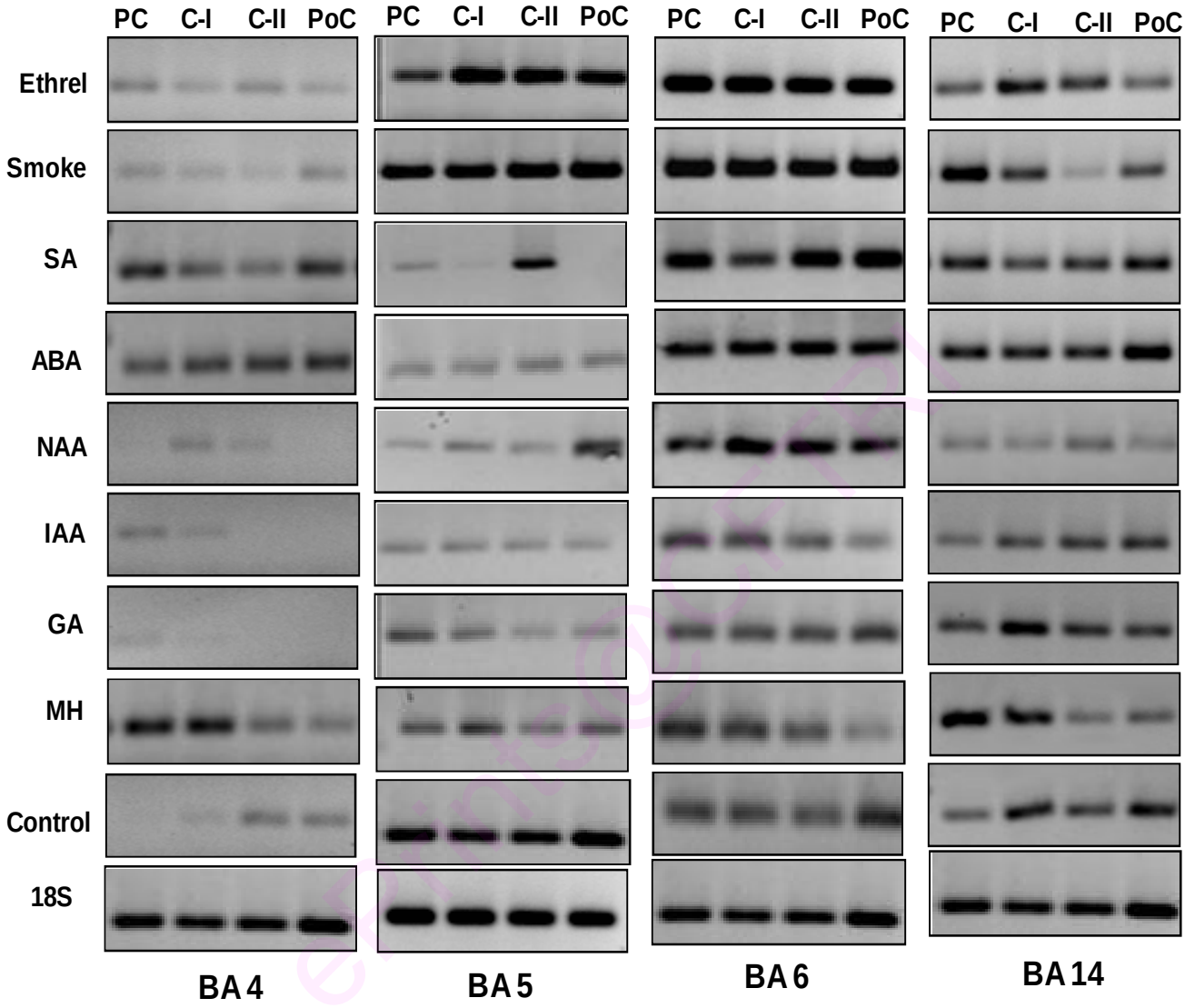

Figure 4.11. Transcript accumulation pattern of the unigenes isolated from DDRT-PCR during ripening of banana. Expression of four unigenes namely, BA4, BA5, BA6 and BA 14 was studied through RT-PCR. The treatments of different ethylene inducers/inhibitors were performed. The total RNA was extracted in 2, 4, 6 and 8 days after the treatments. RT-PCR was performed as described in the Materials and methods with 5µg of total RNA. The PCR products were analysed by agarose gel electrophoresis. The treatments were control, Ethrel, SA, smoke, ABA, NAA, IAA, GA and MH treatments

samples was tested for gene expression. Since ripening had not progressed much in these samples, this treatment allowed us to study any expression that is governed primarily by ethylene and not by secondary ripening related changes. The expression of one highly induced gene (BA4; whose expression could be detected from the 2nd day itself and increased as time progressed), one down-regulated gene (BA5), one constituently expressed gene (BA6) and one up-regulated gene was studied. As shown in figure 4.11, the expression of three of the genes tested was highly responsive to ethylene. Transcription of BA4 and BA5 was triggered differentially and regulated by different ethylene inducers/inhibitors treatment. No change was observed in the expression pattern of BA6 while transcripts of BA14 also were not much regulated by these nine ethylene inducers/inhibitors treatment.

4.4. DISCUSSION

The aim of this study was to obtain a preliminary and global overview on fleshy fruit development and climacteric ripening gene expression patterns, which allow a comprehension of processes implicated at each ripening stage. Approaches like subtractive hybridization and differential library screening have been employed in climacteric fruits like kiwifruit (Ledger and Gardner 1994), banana (Clendennen and May 1997; Medina-Suarez et al. 1997; Gupta et al. 2006; Caamal-Velázquez et al. 2007) and melons (Hadfield et al. 2000) and in non-climacteric fruits like strawberry (Nam et al. 1999) and grapes (Davies and Robinson 2000). Whereas mRNA differential display has been used to study ripening in tomato (Zegzouti et al. 1999) and raspberry (Jones et al. 2000). In an effort to get an insight into the vast array of genes that may be involved in ripening, transcriptome analyses in apricot (Grimplet et al. 2005) and tomato (Alba et al. 2005) have also been performed

In banana previous studies have provided important information on the expression of ripening related genes by using cDNA subtraction (Clendennen and May 1997; Trujillo et al. 2007) and two dimensional protein resolution (Medina-Suarez et al. 1997) of *in vitro* translated products during ripening of banana. Gupta et al (2006) and Caamal-Velazquez et al (2007) successfully employed the differential display and identified the ethylene inhibitor, 1-MCP regulated genes, and low temperature induced genes respectively. Recently Mbeguie-A-Mbeguie et al (2007) used suppression subtractive hybridization approach and identified genes differentially expressed during early banana fruit development undergoing changes in ethylene responsiveness. Kesari et al (2007) identified 37 EST-unigenes from banana which are expressed differentially during ripening by using suppression subtractive hybridization (SSH).

The present study has used mRNA differential display to analyze NR banana fruits treated with ethrel an ethylene inducer, the first set of unigenes differentially expressed at different stages of banana fruit ripening has been obtained from a pulp cDNA, with a comparative view of PC, C-I, C-II and PoC (i.e., Pre-climacteric, Climacteric I, Climacteric II and Post climacteric) ripening stages. The advantages of Differential Display over alternative methods are: i). Minimal amount of starting material

is required (Differential Display can be performed with as little as 200 ng of total RNA per sample.); ii). Multiple samples can be analyzed simultaneously. (Alternative methods are limited to comparing two samples, whereas Differential Display is limited only by the number of lanes available on the gel apparatus.); iii). Sensitivity (As Differential Display is a PCR- based procedure, transcripts present at very low copy numbers are included in the analysis.) and speed.

To understand post harvest ripening changes in banana fruit at molecular level, present study employed DDRT-PCR technique and isolated ethylene responsive cDNA. These cDNAs were classified into several categories based on results of BLASTX searches in the NCBI database. cDNAs isolated by DDRTPCR were involved in a variety of physiological and molecular events including: metabolism; protein destination and storage; protein synthesis; signal transduction; molecular transport and intracellular transport; transcription and post-transcription; cell structure, growth, and division; and disease and defense. These results imply that many complicated molecular processes occur in the early stage of postharvest banana ripening. It is anticipated that the information obtained from this study will guide further exploration to establish the connection between differentially expressed genes, the corresponding translated proteins, and their biological functions in the early stages of banana ripening. Based on their biological functions they have been grouped into nine groups (Table 4.5).

4.4.1. Stress and senescence related genes and role of ethylene on Climacteric ripening

Climacteric fruit ripening is characterized by a respiratory peak with concomitant ethylene production. The ethylene burst is autocatalytic and accelerates the ripening process by affecting the transcription and translation of many ripening-related genes. Many components of biochemical pathways involved in cell wall, sugar and fatty acids metabolism, pigmentation, volatile production and signal transduction have been identified as being affected by ethylene production (Alexander and Grierson 2002).

In response to many stresses, including heat, oxidizing conditions all cells produce a common set of heat shock proteins (Hsps). In the Hsp70 group *vis1 -1* isolated from tomato fruits was found involved in regulation of fruit ripening and hence significantly affect fruit quality and thus play important roles in fruit ripening process by

way of protecting fruit from environmental and ripening associated stresses (Ramakrishna et al. 2003).

Thus the earlier studies as well as our own results suggest that degradation processes which ensue on the onset of ripening produce a general stress and defense related response and several genes belonging to this process are triggered, and hence expressed. In tomato (Fei et al. 2004) and watermelon (Levi et al. 2006) only 7% and 4% of ESTs were correlated to defense/stress response, respectively during fruit development and natural ripening. The higher percentage (18%) of such ESTs reported in our study would be due to pre-exposure of fruit to ethylene, a known stress hormone, in order to initiate ripening; in which case the treatment evoked most of the early responsive genes leading towards natural ethylene burst, which might not have been traceable in cases where samples were taken from naturally ripening fruit, which is a slower and longer process. Present study has shown that ethrel treatment of ripening banana fruit evokes a general stress response and genes belonging to this class are expressed during ripening as well.

Genes coding (homologous) for enzymes of the senescence associated proteins, heat shock proteins and seed maturation proteins (BA57, BA77, BA84, BA1, BA21, BA46 and BA47) are induced in ethylene treated fruits at different stages of ripening. Although metabolites synthesized by these enzymes, namely HSPs, seed maturation proteins, lignins and sesquiterpenes, have been mainly implicated in defense reactions (Table 4.5), they are also found associated with ethylene induced ripening.

4.4.2. Transcription and translation regulated genes

A significant number of ESTs (BA56, BA63, BA2, BA4 and BA50) were homologous to genes involved in transcription, translation or regulation of gene expression. In this study, it was possible to isolate ESTs homologous to paired box protein, tRNA lysidine synthase, transcriptional regulator protein and un-named protein product of *Vitis vinifera* which have been shown to be involved in a variety of functions including protein–protein interactions, in tRNA identity, codon recognition, transcription and ribosome biogenesis to regulate gene expression (Table 4.5). (Pena et al. 2006; Kesari et al. 2007)

4.4.3. Transport and signal transduction

Apart from stress/defense response and transcriptional regulator related, other genes were also isolated which are known to be involved in monosaccharide transport, inner membrane protein.

4.4.4. Primary metabolism

Primary metabolism includes a broad range of vital processes for the cell maintenance as energy production and generation of metabolites that can be redirected for diverse pathways. Several genes putatively involved in energy production or transfer were induced during ripening. The present study has been able to isolate 13 such ESTs. One of the EST homologous to *S*-adenosyl-l-homocysteine hydrolase, which is needed for methyl group recycling and has been shown to regulate gene expression by DNA methylation-dependent gene silencing. Apart from regulation of gene expression, this methyl recycling leads to generation of SAM, which is a methyl donor to many transmethylation reactions including those involved in synthesis of pectin, phosphotidylcholine as well as ethylene and polyamine biosynthesis (Rocha et al. 2005; Kesari et al. 2007; Fonseca et al. 2004). The key features of the regulation of sugar metabolism in fruit are rapid continuous degradation and re-synthesis of sucrose, which is mainly catalysed by Sucrose synthase or sucrose phosphate synthase (Quoc and Foyer 2001). The present study has also been able to isolate such gene, which was up-regulated during ripening.

4.4.5. Differential expression of selected unigenes

Several unigenes expressed during ripening are listed in table 4.5. In order to know if these genes are ethylene-regulated, ripening-related and fruit-specific, the expression pattern of some of the genes was monitored during the entire course of ripening and in fruit where ripening was regulated by different treatments. Expression of genes was also studied in different tissues (Figures 4.9 and 4.10). Transcripts levels of most of the genes studied were undetectable in immature fruits and was induced on day 2 after induction of ripening, except for BA 6. Transcripts of BA 6 were detected throughout the ripening period and not influenced by the various treatments. Ripening as well as transcript accumulation of the genes BA 4 and BA 5 were inhibited in fruit pre-

exposed to NAA, IAA and GA. Transcripts of these genes were present only in the fruit tissue and no detectable transcripts were observed in leaf and immature fruit. These data represent only one developmental stage of different tissues and expression of these genes at different developmental stages or to environmental stimuli cannot be ruled out. Taken together, the present expression studies suggest that these genes are ethylene-regulated and directly involved in ripening of banana fruit. Although these genes showed fruit-specific expression, that may be due to ethylene response because other tissues were not exposed to ethylene.

4.4.6. Conclusion

This study reports changes in the expression of genes, of ripening banana fruit, functioning at different levels, such as primary metabolism, signaling, stress responses, pigmentation and aroma biosynthesis and cell wall modifications, supporting the hypothesis that banana ripening is a cell senescence process closely linked to stress-response mechanisms regulated by ethylene. Although, the information generated in this study will be used as a basis and a starting point for subsequent research enabling one to establish the connection between differentially expressed unigenes, their corresponding translated proteins and their biological functions, allowing the correlation of biochemical events involved in the different stages banana ripening. Knowledge on these genes is expected to contribute to increase pools of public EST collections of banana, one of the weakest public EST collection among the most consumed fruits (Zegzouti et al. 1999), for (i) a large-scale expression analysis and identification of candidate genes and/or (ii) providing molecular markers usable to improve banana fruit quality through conventional breeding or biotechnological approaches.

Conclusion and Future Prospects

Brief background

Bananas and plantains (*Musa* sp.) are a major stable food for millions of the people in the tropical world. World banana production has been estimated to be 80.6 Mt annually and India is the major producer accounting for 23% of the World produce. India, especially the southern part, is a unique place of origin for several cultivars of banana where Nanjanagudu rasabale (NR) (genotype 'AAB') is an important endangered cultivar of Mysore which was not characterized by earlier studies. Therefore, the present study focuses on genetic characterization, cultivar rejuvenation through tissue culture technique and fruit characterizations.

To study the genetic relationship of NR with other banana cultivars, morpho-taxonomic characters were first developed and optimized for bananas and 119 descriptors were defined to characterize *Musa* germplasm. Modern molecular techniques of DNA analysis through markers where RAPD and ISSR analyses for distinguishing *Musa* germplasm are reported appropriate (Howell et al. 1994; Onguso et al. 2004; Crouch et al. 2000; Uma et al. 2006). However, the information on genetic diversity and phylogenetic relationships within the south Indian banana cultivars is scarce and particularly that of NR was non-existent, before this study. The information developed on genetic relationships is essential and decisive for developing an efficient breeding programme.

Bananas are propagated through vegetative means and any genetic improvement remains stable with no risk of lateral gene flow since there is no sexual reproduction in the edible bananas. In fact there are very few reports on regeneration protocols which are restricted to somatic embryogenesis in diploid and triploid *Musa acuminata* ('AA' and 'AAA') (Novak et al. 1989; May et al. 1995). Organogenesis has been reported from callus cells, protoplasts and leaf-base micro-sections for certain *Musa* genotypes. A very few exogenous growth regulators have been reported useful for banana micropropagation (Bhagyalakshmi and Singh 1995), the sub- and supra-optimal levels of growth regulators, especially the synthetic ones, were found to induce somaclonal variation (Martin et al. 2006). Even in cases where immediate variations were not noticed, the long-term application of high levels of cytokinins resulted in somaclonal or epigenetic variations in some of the micro-propagated plants questioning the very fidelity of their clonal nature.

Another class of molecules, namely polyamines (PAs), are known to play important role in various cellular processes (Bais and Ravishankar 2002). PAs have also been found implicated in morphogenic process and play a crucial role either directly in somatic embryogenesis and regeneration or indirectly through the release of nitric oxide or inhibition of ethylene biosynthesis (Kumar and Rajam 2004). Considering these intriguing information, it was necessary to study the effects of different growth regulators both during regeneration and micro-propagation.

Fruit ripening constitutes one of the most important developmental processes in higher plants and involves progressive changes that are governed both developmentally and hormonally. Most of the changes observed during ripening are governed by expression and regulation of hundreds of genes. Molecular aspects of ripening are studied in great detail in tomatoes and strawberries (Reviewed by Giovannoni 2001). Many of these studies have concentrated either on gene involved in ethylene biosynthesis and ethylene receptors or on downstream target genes involved in ripening. However, there are many other genes which are involved in the process are yet to be identified, which is important not only to understand how a complex ripening process is governed but also for their tremendous potential for biotechnological applications. Approaches like subtractive hybridization and differential library screening have been employed in climacteric fruit like kiwifruit and melons, while mRNA differential display has been used to study ripening in tomato. Recently, a transcriptome analysis in tomato (Alba et al. 2005) and in banana (Gupta et al. 2006) has also been performed in an effort to get an insight into the vast array of genes that may be involved in ripening. These studies have been successful in identifying several novel genes related to fruit ripening whose action in the ripening process was not obvious earlier.

Objectives of the study

- Developing biochemical and genetic markers to differentiate NR
- Developing regeneration and genetic transformation protocol for banana
- Identifying the genes expressed to the maximum during ripening

Highlights of the findings

Entire work carried out and the results obtained are summarized under the following headings.

Molecular fingerprinting of NR

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. A strong linear relationship was observed between the Resolving power (Rp) of the primer and its ability to distinguish genotypes. Based on these data, a genetic similarity matrix was established and a dendrogram for each set of primers was developed by UPGMA. The genetic similarity coefficients in RAPD analysis ranged from 0.3177 to 0.7818 and in ISSR analysis from 0.1800 to 0.8462. A fingerprinting key was generated where the presence/absence of specific RAPD/ISSR bands were recorded for each cultivar. The presence of a specific RAPD ($OPC\text{-}5_{800}$) band was observed for an endemic cultivar-Nanjanagudu Rasabale (NR). The study resulted in the identification and molecular classification of South Indian banana cultivars of which Robusta and Williams are global and others have either limited geographical distribution or purely endemic to South India. A group of eight cultivars was identified that are highly distinct from one another. The members of this group may be useful for generating 2X and 4X breeding populations for further use in breeding secondary triploid hybrids.

Regeneration

The response for both cormlet and direct shoot formation was observed only in leaf explants obtained from shoots cultured in liquid medium but not in similar explants obtained from shoots grown on gelled medium. In the sequence, the leaf explants were cultured first on medium with a high level (22.4mM) of benzyladenine (BA), second on indole-3- butyric acid (IBA) supplemented medium, and third on reduced BA medium

under incubation in the dark. The highest adventitious shoot regeneration in 24% of the explants, with the number of shoots ranging from 2 to 3 per explant, occurred in the explants incubated at the first step in medium with 22.4 and 0.198 mM IBA. Further growth and complete shoot formation occurred under incubation in a 16-h photoperiod. While keeping the culture conditions constant and replacing BA with picloram (0.83–20.71mM) in the initial step, adventitious origin of cormlets occurred in 12% of the explants. However, when rhizome explants (also obtained from shoots grown in liquid medium) were cultured with various growth regulators in the first step, medium containing 2,4,5-trichlorophenoxyacetic acid (7.82 mM) produced friable callus that re-differentiated into roots only. Physical forms of the medium, i.e., agar-gelled and liquid, imparted specific effects on the extent of multiplication of leaf-regenerated shoots with no differences in morphology and growth patterns when compared with those of meristem-derived plants. Contribution of exogenous polyamines and polyamine-inhibitors on plantlet regeneration patterns of banana was also studied. The rhizome explants (without shoot bud) of *in vitro* shoots produced a mixture of embryogenic and non-embryogenic calli on modified MS medium. The analyses of endogenous pools of polyamines showed higher levels of PAs in embryogenic than in non-embryogenic calli. Supplementation of various levels of (10-50µM) spermine, spermidine, and putrescine to cultures with secondary embryogenesis showed that about 50% of embryogenic calli rapidly produced secondary embryos only in the presence 40µM Spm but not in other treatments. The crucial role of Spm was further confirmed by the use of 0.1mM each of DFMO and DFMA along with Spm where the presence of inhibitors concomitantly inhibited the secondary embryogenesis.

The regenerated plantlets were analysed for their genetic stability using RAPD and 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 regenerated plantlets analyzed.

Micropropagation

Cytokinins, such as BA and kinetin were added to the routine shoot multiplication medium at concentrations up to 10 mg l^{-1}. After 12 weeks of culture involving three

subcultures, the maximum number of shoot buds were 1 produced in cultures receiving either 5 mg l^{-1} BA (80 shoot buds) or 4 mg l^{-1} kinetin (62 shoot buds). Certain morphological abnormalities observed during proliferation of shoot buds in vitro were not observed during acclimatization ex vitro. To check the genetic stability, RAPD and ISSR profiles of micropropagated plantlets obtained from different cytokinin-treatments were compared with control microplants maintained on MS medium as well as the field-grown mother plant. A total of 17,400 bands were generated showing homogeneous RAPD and ISSR patterns. Band intensity histogram of each gel confirmed their monomorphic nature with no genetic variation in all the plantlets analyzed.

The shoots obtained from the embryogenic cultures were checked for their performance on solid medium (SM) and partial immersion system (PIS). The rate of shoot multiplication was higher in PIS than in SM throughout six weeks culture period. Uniformity in elongation of all the shoot buds was observed in PIS but not in SM. Evaluation for the acclimatization, survival under greenhouse conditions revealed the better performance of PIS-derived plants than those from SM.

Genetic transformation

A protocol for stable genetic transformation of banana has been developed by co-cultivating shoot bud explants with *Agrobacterium rhizogenes* harbouring a binary vector. *A. rhizogenes* strain A4 harbouring plasmid *pCAMBIA 1301* with an intron *uid*A reporter and hygromycin phosphotransferase (*hpt*II) marker gene under the control of *CaMV35S* promoter was used for sonication-assisted transformation of shoot bud explants. Transformation frequency up to 3.4% was obtained depending on the medium used. The use of hygromycin in the multiple shoot induction medium allowed the selection of transgenics having Ri T-DNA along with the T-DNA from the *pCAMBIA 1301* binary vector. The putative transformants were positive for the expression of the *uid*A gene. PCR and Southern blot analysis confirmed the independent, transgenic nature of transformants and indicated single and multiple locus integrations.

Regulation of ripening related genes

The induction of ethylene synthesis is a foremost essential step in the onset of ripening in climacteric fruit- banana. Quantification of genes that are up- and down-regulated during banana fruit (*cv*. NR) ripening under the influence of ethylene inducers

and inhibitors was completed. For this, mature green banana fruits were harvested from plants and treated with ethylene inducers/inhibitors such as ethrel, smoking, salicylic acid (SA), indole acetic acid (IAA), naphthalene acetic acid (NAA), gibberellic acid (GA), maleic hydrazide (MH), abscisic acid (ABA) and untreated served as control. The pulp and peel of fruit samples representing different ripening (pre-climacteric, climacteric, post-climacteric and ripened) stages were quick frozen in liquid N_2 and ground to a fine powder. Total RNAs were extracted and complete cDNAs were synthesized. The gene specific primers for ripening related enzymes *viz.,* pectin methyl esterase (PME), polygalacturonase (PG), pectate lyase (PEL), *MaEXPA-1* and *MaEXPA-2* were designed, Reverse Transcriptase-PCR was performed for these genes. Each band was normalized against the intensity obtained with the same cDNA using the internal 18S primers. Exposure of fruits to ethylene inducers as well as smoking hastened fruit ripening and increased the transcript levels of *PG, PEL* and *MaEXPA-1* whereas in fruits treated with ethylene inhibitors showed increased and constant level of expression. It is concluded that ethylene induced ripening of banana having more than one mechanisms operating during ripening which are tightly controlled at various levels.

Isolation of ripening specific genes

To gain a better insight on the molecular regulation of banana fruit ripening, the mRNA Differential Display technique (DDRT-PCR) coupled with silver-staining was used to identify and isolate transcripts differentially expressed during ripening. Using 71 primer combinations and four populations of mRNA (Pre-climacteric; Climacteric 1; Climacteric II and Post climacteric), a total of 285 bands were obtained, of which 120 were cloned into T/A cloning vector and sequenced. DNA sequence analyses of the clones revealed significant homology to known plant genes and proteins reported in public databases, namely an membrane transport protein, sucrose phosphate synthase, heat shock protein, transcriptional regulator, putative senescence protein, a homologue to a protein kinase. These proteins are known to be associated to biological processes like membrane transport, secondary metabolism, signal transduction, gene transcription and RNA metabolism and stress responses or defense. In addition to these reported proteins, several unknown gene fragments were also identified and are reported for the first time in NR ripening. The RNA dot analyses showed that the expression of most of the up- and

down-regulated genes being very specific to fruit and not observed in other tissues. The homology found with previously described proteins allows disclosing possible biochemical roles for the proteins putatively encoded by these gene families and their putative significance in banana ripening is discussed. The present study provide a contribution to characterize the changes in gene expression that accompanies the ripening process in banana; a species which lacks information about the molecular regulation of ripening.

Conclusion

- Twenty one different edible south Indian clones of banana were collected and their genetic relationships were established using genetic markers, where it was found that Nanjanagudu rasabale (NR) - an endangered landmark variety of Mysore is a unique one and is genetically different from others.
- A protocol for regeneration of plantlets from the leaf sheath explant was worked out and regenerated plantlets were found genetically similar to the mother plant.
- A micropropagation protocol was fine -tuned and has been checked at pilot level by producing 4000 plantlets in 6 months and transferred to field. Since it is an endangered variety, a substantial number of plants have been given to several farmers and Research Institutions**.** The cultivation of NR variety which had dwindled to 5 ha in the year 2003 has now increased to 15 ha
- Genetic markers RAPD (50) and ISSR (12) markers were selected to screen genetic variants among micropropagated and regenerated shoot cultures of NR. However, no variants were obtained in both the sets indicating that the protocol developed through this study is efficient for the production of clonal material as well as stable.
- NR banana is a highly recalcitrant material, not responding easily to produce callus and plantlet regeneration. A protocol was developed for the induction of shoots and cormlets directly from the leaf surface of NR where the involvement of polyamines in regeneration pattern was established.
- Transformation of banana was achieved by *A. rhizogenes* harboring Ri plasmid and binary vector pCAMBIA 1301. Differential T-DNA integration patterns were observed. Integration of T-DNA from the binary vector was observed in all the

transgenic plants. However, the integration of Ri T-DNA was not observed in all the tested transgenic plants

- The present results suggested that activities of the cell wall hydrolases PME, PG, and PEL were largely dependent on ethylene production and perception by banana fruit tissue. Ehrel, smoke, ABA, might enhance the effect of ethylene. IAA GA, SA, and MH on the other hand, inhibits activities of all three cell wall hydrolases enzymes.
- Present study has provided comprehensive information that ethylene-induced ripening in banana fruit is accompanied by the expression of stress, defense and detoxification related genes as well as genes that are involved in ethylene biosynthesis, cell wall loosening and hydrolases.
- The novelty of the present study lies in the generation of enormous data on the regulation ripening genes by external chemicals.

Future lines of work

The extension of studies carried out in this thesis could be in the following lines.

- A group of 8 cultivars was identified that are highly distinct from one another. The members of this group may be useful for generating 2X and 4X breeding populations that can be used in breeding secondary triploid hybrids.
- The study gives an insight to the *in vitro* morphogenetic behavior of banana and biochemical basis for the embryogenic and non-embryogenic nature of the callus, involvement of polyamines in plant morphogenesis.
- The study demonstrated the regeneration of normal transgenic plants from *A. rhizogenes* mediated transformation system. The transgenic plants, which contain Ri T-DNA, may be adopted for breeding purpose in order to eliminate the Ri T-DNA, which possibly segregates in subsequent generations.
- The studies have identified several candidate genes whose promoters could be used for isolation of specific *cis* elements involved in fruit specific expression as well as those responsible for ethylene induction and repression.
- The study has provided a deeper biochemical / genetic regulation of ripening-specific genes, based on which, chemical formulations may be developed to achieve controlled ripening as well as shelf life extension of banana.

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Publications

Papers Published

1. **Venkatachalam L**, Thimmaraju R**,** Bhagyalakshmi N and Sreedhar RV (2006). Direct shoot regeneration in banana (AAB 'silk') from leaf base explants and field performance of the regenerated shoots. **In Vitro Cellular and Developmental Biology-Plant.** 42: 262-269

2. **Venkatachalam L**, Sreedhar RV and Bhagyalakshmi N (2007). Effect of high levels of cytokinins on genetic stability during micropropagation of banana *cv*. Nanjanagudu rasabale (AAB) as determined by RAPD and ISSR markers. **Plant Growth Regulation**. 51: 193-205

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5. **Venkatachalam L,** Sreedhar RV and Bhagyalakshmi N (2008). The use of genetic markers for detecting DNA polymorphism, genotype identification and phylogenetic relationships among banana cultivars. **Molecular Phylogenetics and Evolution.** 47: 974-985

6. **Venkatachalam L** and Bhagyalakshmi N (2008). Spermine-induced morphogenesis and effect of temporary immersion system on the shoot cultures of banana. **Applied Biochemistry and Biotechnology.** DOI 10.1007/s12010-008-8226-z

Papers for publication

1. **Venkatachalam L** and Bhagyalakshmi N. *Agrobacterium* mediated transformation of banana (AAB silk)-Nanjanagudu rasabale. **Communicated**

2. **Venkatachalam L** and Bhagyalakshmi N. Differential expression of ripening-related genes in banana var. Nanjanagudu Rasabale (AAB)

3. **Venkatachalam L** and Bhagyalakshmi N. Identification of differentially expressed genes under the influence of ethylene inducers/inhibitors by DD RT-PCR

4. **Venkatachalam L** and Bhagyalakshmi N. Differential expression of expansin genes during ripening of banana and their regulation by ethylene inducers and inhibitors

Papers presented in symposia / conferences:

1. **Venkatachalam L,** Thimmaraju R, Sreedhar RV, Ravishankar GA, Bhagyalakshmi N (2004). Engineering Banana for therapeutic proteins. Poster presented at Colloquium on plant proteins and National nutrition security, held on 30th March 2004 at Central Food Technological Research Institute, Mysore, India

2. **Venkatachalam L,** Thimmaraju R, Vinod kumar, Sreedhar RV and Bhagyalakshmi N (2004). *Agrobacterium* mediated transformation of banana cv. Silk (AAB) - a triploid monocotyledonous fruit crop. Poster presented at 16th Indian Convention of Food Scientists and Technologists (ICFOST), held on 9th & 10th December 2004 at Central Food Technological Research Institute, Mysore, India

3. **Venkatachalam L,** Sreedhar RV, Kaunain Roohie, Thimmaraju R and Bhagyalakshmi N (2005). Effect of high concentration of growth regulators on genetic stability in banana as determined RAPD and ISSR markers. Poster presented at National symposium on Plant Biotechnology: New Frontiers, held between 18th to 20th November at Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

4. **Venkatachalam L,** Bahgyalakshmi N and Sreedhar RV (2006). Analyses of genetic diversity of Indian dessert banana cultivars and fidelity of long - term micropropagated plants at Global Horticulture: Diversity and Harmony, International Horticultural Congress-2006 Seoul, South Korea

5. **Venkatachalam L,** Bahgyalakshmi N and Sreedhar RV (2007) Influence of polyamines on regeneration patterns in banana var. Nanjanagudu Rasabale (AAB). 8th Agricultural Science Congress, TNAU, Coimbatore, Tamil Nadu

6. **Venkatachalam L** and Bhagyalakshmi N (2007) Spermine-induced morphogenesis and effect of partial immersion system (PIS) on the shoot cultures of banana: International conference on "Biotechnology New Horizon" Thiruvanathapuram, Kerala

Appendices

List of the differential amplicons, accession numbers and their sequences

Frag. ID	Acc.No.	Sequences (5′-3′)
BA1	GE470232	TGTGAGGCGTCGCGAAGTGGCGGTTATCAAGACGGCGTGGATGCGGGCGGCAGCTATGTG TTTGTCCAGCGTTGGGAACACAACCTGAAGCAGCTCAACCGGATGAGCGTTCACGATCAG GAGATGGTGATCGGGCGCACCAAAGAGGCCAACGAAGAGATCGACGGCGACGAACGTCCG GAAACCTCTCACCTCACCCACCTGTCACTCA
BA2	GE470233	TGTGAGGCGTCCCCTGGATTAGCTCGAGCCGAACCTCCGGGAAAAGTTCGCGAAAAGCTT TAATGACCTCTGGCAAGCTATAACGTGCCTGAGTATGCGTCGTTGCAATAGTGAGAACGC CAGACGTATCGTTGGTAAACAGGTCTGCAAGCCGACGAACATTACTGGCTTCATTCAGAA TACGTTCTGCAATGACCAGTAATGCTTTGCCCGGTTCAGTCATGCCCAGCAGTCGCTTAC CTCGTCGAACAAATATTTCGATGCCAAGTTCATCCTCCGGTTCCCGAATATGACGGCTGA CGCCTCAC
BA3	GE470234	TACAGGTGGGTGAGGTATTCATACATGCCTTTGGGCGTGCTACCGGCGGTAATTGCCAGG TTAACACGACGCCTCACA
BA4	GE470235	GTGAGGCGTCAGCCGTCATATTCGGGAACTGGAGGATGAACTTGGCATCGAAATATTTGT TCGACGAGGTAAGCGACTGCTGGGCATGACTGAACCGGGCAAAGCATTACTGGTCATTGC AGAACGTATTCTGAATGAAGCCAGTAATGTTCGTCGGCTTGCAGACCTGTTTACCAACGA TACGTCTGGCGTTCTCACTATTGCAACGACGCATACTCAGGCACGTTATAGCTTGCCAGA GGTCATTAAAGCTTTTCGCGAACTTTTCCCGGAGGTTCGGCTCGAGCTAATCCAGGGGAC GCCTCAC
BA5	GE470236	GTGAGGCGTCTTCAGGATTAGAATCGATAAAACAAAATGGGTTAAAGAGAGCGATATAAT AGCGGCGGGTGCTTGAGGCTGTCTGTCTCAGGCATTAGCTGAACGGCAGATAGAGAAAAG CCCCGAGTGATATTTTACCATCAACCCGAGGCCTCCTATATGCTGAACACATGTAGAGTG CCTCTTACTGACCGTAAGGTCAAGGAGAAGAGAGCAATGAAGCAGCATAAGGCGATGATT GTCGCCCTGATCGTCATCTGTATCACCGCCGTAGTGGCGGCGCTGGTAACGAGAAAAGAC CTCTGTGAGGTTCACATCCGAACTGGCCAGACGGAGGTTGCTGTTTTCACGGCTTACGAA TCCGAGTAAGAGCAACGGCGGGGAGTGATCCCATAAGCGCTAGCTTAAGGGTTGAACCAT CTGAAGAATGCGACGCCTCAC
BA6	GE470237	GTGAGGCGTCCATGCTGAAGAGTGGGGTTACTTTAAAATTATCCATCTGGAAGGATCGCC ATTAACCCGCATCGACGGGATGATGATCCTCGGTATGTTTGGCGGCTGCTTTGCCGCAGC GCTGTGGGCCAACAATGTCAAACTGCGAATGCCGCGCAGCCGTATCCGCATTATGCAGGC CATCATTGGCGGCATTATCGCCGGTTTTGGCGCGCGTCTGGCAATGGGCTGTAACCTGGC GGCGTTCTTTACCGGTATTCCTCAGTTCTCGCTGCATGCCTGGTTCTTTGCCATCGCCAC TGCCATTGGTTCATGGTTTGGCGCGCGCTTTACCCTGCTGCCCATCTTCCGTATTCCCGT GAAAATGCAGAAAGTTTCTGACGCCTCAC
BA10	GE470238	GTGCCTGTGTGTACTGGAAGGACATCGCGCGGAAAAAATCATTCTCGATCAAGGGGGCGG CCTGTTGGTTAATGGGACAACCTCAGCGGTCGTGGTAGATGAAGGTGGTGAATTGTTGGT GTATCCAGGTGGGGAAGCCAGCAATTGTGAGATTAATCAGGGCGGCGTTTTTATGCTGGC CGGGAAAGCCAGTGATACGTTGCTTGCTGGTGGCACCATGAATAATCTCGGTGGCGAAGT
BA12	GE470239	GCTTGCCCTGGACGAGTTACGATGACCTGACAGAAGTATCAGACGTCTTCTGCGGTTTCT ATGATAGAGCAATTCTGTGGCGAAGT
BA13	GE470240	ACTTCGCCACAGAATTGCTCTATCATAGAAACCGCAGAAGACGTCTGATACTTCTGTCAG GTCATCGTAACTCGTCCAGGGCAAGCT
BA17	GE470241	TCGCATGCTCCCGGCCGCCATGGTTACTTCGCCACATGATGATGGATTTTGGCTATCTGG AAGAAACATTCGAAGCGGGTAAACGCTCCGCCAAAATCTCCTTTGTTTTTACTGTCGTGC TTTCACTTCTCGCAGGAGTCCTCGTATGGTAAGCAACGCCTCCGCATTAGGACGCAATGG CGTACATGATTTCATCCTCGTTCGCGCTACCGCTATCGTCCTGACGCTCTACATCATTTA TATGGTCGGTTTTTTCGCTACCAGTGGCGAAGTATCCATGGCCGCGGGATATCACTAGTG

		CGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGC
BA20	GE470242	TAGCTTGCCCTGGACGAGTTAGAAAAAAGATCATGTTTGTTTATATTTCTGCAAAAGTGT TCATTATTCTGTTAATCATCATTGTTATCTAAGAGTTATGATATTAGTTATGTAGCATCA CTCAACTTTGAAATGGCACTAGAGTAAATTTAAATTTTTCTAATAATTTCATAATCGGGA GTCAATCCTCTCAAGGTTGAGTCAACTTTTTAATTAAGGCAAATCTAAGTAAATAAAATC ATTGTGAAATCTAGTTTGCTGGCAATAATAAGTATCTTGTTAGAGCAGTTCAAGCAAACA AAGGGCAATCATAAAATTTTCCTGTAGGTTGGAATTCAAGAAAATGAGGGCACAGAGAAA TAATGAAATAAACTAATAAAATAGGAATTTTCTATGGGGACCAAATAAATTGATTACACA CAGGCACCGACC
BA21	GE470243	GAATTAGGTCTTGGGCGGGGGGAAACCTGCTGATGTTTTCCTATGGAGGAATAAGAAGGT CTCGGCTGCCACGCTCGGTGGAGCGACGGCCATCTGGGTCTTGTTTGAGTTGATGGAGTA CCACTTGCTGACCTTTGTTTGCCATAGTCTCATCCTGTCCCTGGCCATCATGTTCCTCTG GTCAAACGCATCAACTTTCATCAACAAGTCCCCTCCACGGATTCCAGAGGTAAGCATTCC GGAAGATTTGACTGTGAACATTGCACTTTCGCTTCGATATGAGATCAACTGGGCTTTTGC TGCTCTGAGGGACATCGCATTAGGGCGTGACCTTAAGAAATTCCTTTCTGTCATTGCTAG CCTGTGGGTTTTCTCAATCATCGGCAATTGCTGCAATTTCTTGACCTTATTTCATATAGC GTTTGTCACACTACACACAGGCACCGACCCGAGT
BA22	GE470244	TAGCTTGCCCTGGACGAGTTGCAGTGACGACAGCTGAGCTGTAGCAGTGAACGAGACTAG ACGAGGTTGTCGACGACAGCTAATAGAGCAAACAAAGGCGACATATGTAGGGTTTCATTT CACCCAAGGTTCGCAATACCGTACCGTACCGGTGTTTTGACGTTGGCTCGGTACGGTATG GTACCAGCATACCAAGCAGTACACACAGGCACCGACC
BA24	GE470245	TAGCTTGCCCTGGACGAGTTATAAACGGCTAGAAGATTGCTTGCCACACCATTGTTCTTG TTGGTCAATGTCAATGGAGTTCCTATCATGAGCCATCCGGCTTGCTTCTCTTGCATGTTG GACCACTCTTCAGGCTGAAGCTTGGATTTTGAAGCATTTATCAGCTCCAAAAGAGAAAGA ATAACCTGCTAGGACAGCATTAGCTCTAGGTTTTGTTTGGCAATCAAGCTT
BA25	GE470246	GCTTGCCCTGGTACGAGTTGTTGTAAAGTTCATAAGGAATCAAGATGATATGAAAGACTC TATAACCCAGGAAAAGAATATAGCTATCCACCATATAGAAGCAAAATGAGTAAAAACACT GCGTTGCTTCGCTTCCTAAATATATATAGGACTGATTGAAGGAGATGCTATATTCATAAT CATTTATGCATTCGGGTCGGGATGCGGTGCGCTAACGCTGACTTTCGGGCTATTTGAATT GATTACGCTTTGCTTGGCTTTCTGCTTTCGAGGTGCTAATGTTAGTTCTAGTTTTCGGCA TTAGTGAGTTCTTGTTAGAGTATCGGCAGCCTCAACCTCTACCTCTACATTAACTAAGGG ACATAGGCATAGGCACCGTTATC
BA27	GE470247	GCTTGCCCTGGACGAGTTCTCCAACACTTCTCCGGTTAGATTGGCGTGTTTCTCCTTTAA GGTCTCTTCAGTATAGAGGTGGCTGAGATTTCCCATAAGCTGCAAAATACGATATGGTCG ATCAAGATCTCAGTTGTCGATTCAGGAACTTCAAGTCAAACAAACTTGCTTGTGGACTAT ACTCACGATCGA
BA28	GE470248	TCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATCGATTAGCTTGATTGCCCCCTACT GAAGTAGCAAAGCTCTGCCCGGTGACTCTTAATGAGTTTTCCACTTCCAGAAGTCGGGCT CCCGAACAAAGGACTTGGTTATAACTTGGAAGGAATTATAGGAAGCAGCCCTGCCTAGCA ACGTTAGTAGTCCAATGACTCCCAAAAACATCTATTGTAATTAAAAGAGTGGAGCGGCCC CAAAAGCATCTATTGTAATAACTCGTCCAGGGCAAGC
BA31	GE470249	GGTCTTGGGGTCCACTTGGCTGGTTGATTCCTAGTGAAACTTTCCCCCTGGCGACGAGGA CCGCCGGCTATGCCTTTGCTGTCAGCTCCAACATGCTTTTCACCTTTGTCATTGCCCAAG CTTTCCTATCTATGATGTGTCATCTACGTGCCGGGATCTTCTTCTTCTTTGGTGCATGGA TTGTGGTGATGGGTCTGTTCGTCATCTTCTTATTGCCCAAGACCT
BA33	GE470250	CTCGCGAATGCATCTAGATTACATCCGGATATTCAATCATTAGTTGAGAAAAAATAAATG AAAAAGATTATTTTGACTATTGGCTGTCCTGGTTCTGGTAAGAGTACTTGGGCTCGTGAA TTTATTGCTAAGAATCCCGGGTTTTATAATATCAATCGTGATGACTATCGCCAATCTATT ATGGCGCATGAAGAACGCGATGAGTACAAGTATACCAAAAAGAAAGGAGGTATCGTAACT GGTATGCAGTTTGATACAGCTAAAAGTATTCTGTACGGTGGCGATTCTGTTAAGGGAGTA ATCATTTCAGATACTAACCTGAATCCTGAACGTCGCCTAGCATGGGAAACTTTTGCCAAA GAATACGGCTGGAAAGTTGAACATAAAGTGTTTGATGTTCCTTGGACTGAATTGGTTAAA

		CGTAACTCAAAACGCGGAACTAAAGCAGTACCAATTGATGTTTTACGTTCAATGTATAAA AGCATGCGAGAGTATCTCGGTCTTCCAGTATATAATGGGACTCCTGGTAAACCAAAAGCA GTTATTTTTGATGTTGATGGTACACTAGCTAAAATGAATGGTCGTGGTCCTTATGACCTT GAAAAATGCGATACCGATGTTATCAATCCTATGGTTGTTGAACTGTCTAAGATGTATGCT CTTATGGGTTATCAAATCGTAGTCGTTTCAGGTCGTGAAAGTGGGAACTAAAGAAGACCC AACGAAATATTATCGTATGACCGTAAATGGGTTTGAGGACATTGCTGGCGTTCC
BA34	GE470251	GGTCGGTGCCTGTGTGTAGGAGGGGGTAGCTAGTGGATAGATGTCGCGGAATTCGTCGTT GTCGCTGGGGCCTTCATGATCATCATCATCATCCGCTGGTGCTGCTGTAATAGTAGTGGG AGTAACAGCGTTGATGCGATGCCTTTGATCATTCAACTCGTCCAGGGCAAGCT
BA38	GE470252	CTCGCGAATGCATCTAGATTACATCCGGATATTCAATCATTAGTTGAGAAAAAATAAATG AAAAAGATTATTTTGACTATTGGCTGTCCTGGTTCTGGTAAGAGTACTTGGGCTCGTGAA TTTATTGCTAAGAATCCCGGGTTTTATAATATCAATCGTGATGACTATCGCCAATCTATT ATGGCGCATGAAGAACGCGATGAGTACAAGTATACCAAAAAGAAAGGAGGTATCGTAACT GGTATGCAGTTTGATACAGCTAAAAGTATTCTGTACGGTGGCGATTCTGTTAAGGGAGTA ATCATTTCAGATACTAACCTGAATCCTGAACGTCGCCTAGCATGGGAAACTTTTGCCAAA GAATACGGCTGGAAAGTTGAACATAAAGTGTTTGATGTTCCTTGGACTGAATTGGTTAAA CGTAACTCAAAACGCGGAACTAAAGCAGTACCAATTGATGTTTTACGTTCAATGTATAAA AGCATGCGAGAGTATCTCGGTCTTCCAGTATATAATGGGGACTCCTGGTAAACCAAAAGC AGTTATTTTTGATGTTGATGGTACACTAGCTAAAATGAATGGTCGTGGTCCTTATGACCT TGAAAAATGCGATACCGATGTTATCAATCCTATGGTTGTTGAACTGTCTAAGATGTATGC TCTTATGGGTTTATCAAATCGTAGTCGTTTCAGGTCGTGAAAGTGGAACTAAAGAAGACC CAACGAAATATTATCGTATGACCCGTAAATGGGTTGAG
BA41	GE470253	GTCCCGTGGTCTGTGGCTATGGCGATGTGGGCAAGGGCTGTGCTGCTGCAATGAAGCAAG CTGGTGCCCGTGTGATTGTTACTGAAATTGACCCCATCTGTGCTCTTCAGGCCCTCATGG AGGGCATTCCTGTTCTCACACTGGAGGATGTTGTTTCTGAGGTCTACATCTTCGTGACCA CCACGGGAC
BA42	GE470254	GGTCTTGGGCTTGGCAAAGCAGTGGAGGTACTGGACACACTTGGTAGTAGCATGACGAAT TTGCACCTCAGTGGTACTTTTGTATCAGGAGGGTCAACAAAAGGAAATAAAATATCGATT TTATCTTTTGAAGTTGCCAACACAATAGTTAAGGGGTTCAATCTTATGCAATCACTTTCA AAGGAGAACATAAAGCATTTAAAGGAAGTGGTTCTTCCATCAGAAGGTGTACAACACCTA ATATCCAAAGACATAGATGAGTTGCTAAGGATCGCTGCTGCTGACAAAAGGTTCATTCCT TGTAGCTTCGTTGTTCATTTATTCGGCCACTTGATAAATAAAATTATATAACTTCAATAG TTTCTGAGATTTTCTCCTTACACACACGTACACACAGGCACCGACC
BA46	GE470255	GGTCGGTGCCTGTGTGTAGTGTGACAAACGCTATATAAAATAAGGTCAAGAAATTGCAGC AATTGCCGATGATTGAGAAAACCCACAGGCTAGCAATGACAGAAAGGAATTTCTTAAGGT CACGCCCTAATGCGATGTCCCTCAGAGCAGCAAAAGCCCAGTTGATCTCATATCGAAGCG AAAGTGCAATGTTCACAGTCAAATCTTCCGGAATGCTTACCTCTGGAATCCGTGGAGGGG ACTTGTTGATGAAAGTTGATGCGTTTGACCAGAGGAACATGATGGCCAGGGACAGGATGA GACTATGGCAAACAAAGGTCAGCAAGTGGTACTCCATCAACTCAAACAAGACCCAGATGG CCGTCGCTCCACCGAGCGTGGCAGCCGAGACCTTCTTATTCCTCCATAGGAAAACATCAG CAGGTTTCCCACCGCCCAAGACC
BA47	GE470256	GCTTGATTGCCGTCTTTGATTAGGGTGGTGGTACTTTTGATGTCTCCGTCCTGGAAATCT CCAATGGGGTGTTTGAGGTCAAGGCTACCAACGGGGACACATTCCTTGGTGGTGAAGACT TTGACAATGCATTGCTGGACTATCTGGTCAGTGAATTTAAAAGAACCGATAACATCGATT TGTCCAAAGACAGATTGGCATTACAAAGGCTAAGAGAAGCAGCTGAGAAAGCAAAAGTGG AGTTGTCATCAACCACGCAGACGGAGATAAACCTTCCTTTTATTACTGCTGATGCTTCTG GAGCAAAGCACTTCAATACACACAGGCACCGACC
BA48	GE470257	GGCACGGGTGCATACCTTGTTGAAGTTGGGTGTGGCAAGACCACCAAGGACCACGACCGC CTTCTTTTGACTGTCATTAAGGCTGCTGATGGCTCATACCCTACTGTTGCAGAGGTGCAA GCAGTCCACGGCGCTGCCAAGGCAGCTGACCAGCAGTTGGCCAAGGCCCGTGCC
BA50	GE470258	AGTCAGCCACCTAGCAAGATTGACTTGCAACTTGAAAGTGGTGAGTATTTCATGAGCGAC AAAAAGAAATCAGCCAAGAAATGGCATGAGAAACAGGAAAAGCAGGCAGAGAAGACAGCT

		GAGAATAAGAGAAAAAGAGAAGCTGCATTTATTCCACCAAAGGATCCTACCCGTGATAAT ACCAAGTCTGTTGATGACAAAGATGATGTGGCTGACT
BA52	GE470259	GTCTTGGACTCAGATGCTGGACTCTTTGGTGGATTTGGCAGGATCCATCACACTGCAGAG CACTTCACTGCCGTAAGTCTTGCTCAGATGAAATTGCGTACCGTATATTGTGTGCTCTTT ATTAACCTCTGTTGTGCTCATTCCTTGCAGGATTGTTCACATGAC
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BA55	GE470261	TGACCGCTATCTACTCACTATAGGCCCCACNGGTGNTCGGGCCCCCCCTCAGGTCACCAG GAGCGTGAAGTAACGCCAGAGAAGGGGATTGCCGCCTGTTTGACGACTGAAAGCCTGGAT GCAATGGGTGTTAATACTGATGCGTTTCCGGCTTTTAAACAACTGGACAAACAAGCGTGT GTGCCATTGGCGGAGATTATTCCGGATGCCAGCGTAACGTTTAATGTGAATAAACTCCGT CTGGAAATTTCAGTACCGCAAATCGCCATCAAAAGTAATGCCCGTGGTTATGTCCCCCCT GAACGTTGGGATGAAGGGATCAACGCGCTATTACTGGGATATTCGTTTAGCGGGGCTAAC AGTATTCATAGCAGCGCAGACAGTGATTCTGGCGACAGCTATTTTCTGAATTTAAACAGT GGCGTTACCCAACGGCTGGTGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGG CCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTG GCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCA
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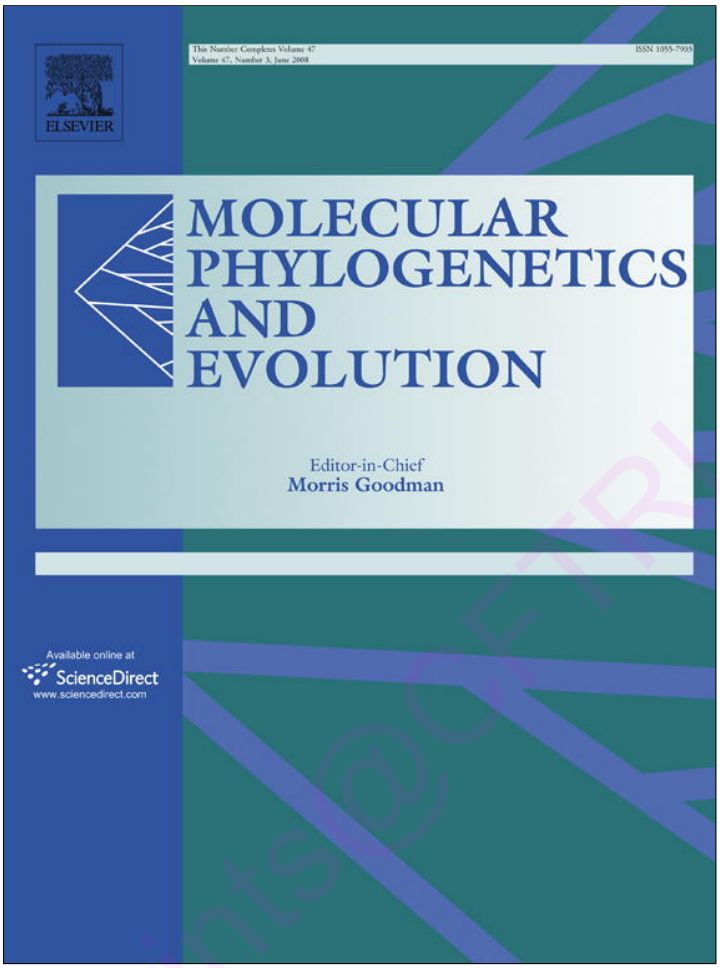
BA57	GE470263	AGTACTCATATAGGCCCNGGGAGGTAGGGCCCCCCCGCTGAGGCTGACAGACGNTGACAC CACTCGTGATGTGAGCGAGTTAATTAGTCTCATGTCTTATGGAATGATGGTAATATGTTT TCCAACAGGCATTAGTATTCTTTATTGCTCTAATATTTATAGGGACTGTATCAGACAGTG GTGTCC
BA60	GE470264	ATGGCGTAAGATGAAGAACATTGACTGGTTAGATGGCGAAGGCAATGACCAGGTACAGGA GAGCGTAAAATGAAAATTCGTTCACAAGCCGGC
BA61	GE470265	GGTCGCAGAGGGAAGGGAATCTACTAGACTAAAGCCATCATTTGTTACCACTGCTAATTT GACATCTCAAACTCGTCCAGGGCAAGCT
BA62	GE470266	CCCCCCCCTGAGGCTGACCAGACGATCTTGCCCTGGACGAGTTTGAGATGTCAAATTAGC AGTGGTAACAAATGATGGCTTTAGTCTAGTAGATTCCCTTCCCTCTGCGACC
BA63	GE470267	CTGAGCTGACCAGACGGATGGNTGATAGAAGGGTTGGCTGAAGGTGAGAATGGCGTAAGT TTTGTCACGTAGCCACGGCGGCACGCCCAGCTCTTGCCAGATTTTCTTTAGCTTACGCCCT
BA64	GE470268	AGGGACACCACTACATTAAGGGGCAGCAAGAACAAAGAGCAATGGAATTCCTAGTTTCAT CCGGTATCGGTCATCCTATATATAGAGAAATACTGAATTTGTAGTTTCATAGTAGGGACC AAGCAACTAGGCATGTAACTTATAATGTGCAGCGTCAGTGAACTCGTCCAGGGCAGCT
BA67	GE470269	AGGGCGTAAGCTGTTATGGCCGTTATATGCCTCGTATGGTTGTAAGTATTAATTATTCCA TTTATTTATTTAAGAAATACATTGTATATCTTCTTTTGATTGATGCTTTGTTCTTGTAGT CTTACGCCCT
BA68	GE470270	GGCGTAAGCTGTTATGGCCGTTATATGCCTCGTATGGTTGTAAGTATTAATTATTCCATT TATTTATTTAAGAAATACATTGTATATCTTCTTTTGATGATGCTTTGTTCTTGTAGTCTT ACGCCCT
BA69	GE470271	AGGGCGTAAGCTGTTATGGCCGTTATATGCCTCGTATGGTTGTAAGTATTAATTATTCCA TTTATTTATTTAAGAAATACATTGTATATCTTCTTTTGATGATGCTTTGTTCTTGTAGTC TTACGCCCT
BA71	GE470272	AGGTGCGACCGCTATCTACTCACTATGAGGCCCNGGGNTCCGGGCCCCCCCTGCAGGTGC ACCCAGGACGTAAACTTGCCCTGGACGAGTTATTTTGAACATGGTGAGGGGAAGGTAAAT

		GCTGTGGATACTCTACATGAATGGATGGTTGATCTTTCCCAGTTGTACCTCGGGCTT
BA72	GE470273	GGCGTAAGCATTGCAATAAAGATGGGGATAAAGAGAGAAAAAACAAGGCCCACCGGAACG GCAGGCCTGAGAATTACGGCTGATAATAACCCACGCCAAGGTCGTTTTCTTTGCGGGTAC GGGCAATCACTGATTCCGGTGTTTCTGCCACGCGCAGACCCATTTCATCTTCAGTACGCA CCACTTTACCGCGCAGAGAGTTCGGGTAGACGTCGGTAATTTCTACATCGACGAATTTAC CGATCATATCCGGCGTGCCTTCGAAGTTGACCACGCGGTTATTTTCCGTACGCCCGGAAA GCTCCATGATGCTCTTACGCCCT
BA73	GE470274	GCCGGCTTGAAGAAAGGAGGCATAATCCGTCGATTTTTTTTGTGGCTGCCCCTCAAAGGA GAAAGAGTATGAAGATCGTGGAAGTCAAACACCCACTCGTCAAACACAAGCCGGC
BA74	GE470275	CTGAGGCTACCCCGACGGTGGCCGGCTGTGTCGTTTCGGCTGGATAATATTCTTTTCGAT CAAGGGCGGATCGCCATTGATGACAAAGTAAGCAAAGCGGATCTGGAGATTTTTGTTGAT CCCTTAGGCAAGCCGGC
BA75	GE470276	GCCCGAGGCGTATGAACATGACGGCGGGATTCAAATTTTGCAGGGTTGGCCAGCAGTTAG TTCCGCCACCCGGCGTTAAGGGGAGAGATAAGATGGTGCATTACGAAGTAGTTCAGTATT TGATGGATTGTTCCTCGGGCTT
BA76	GE470277	GCCCNNGGGGGCTTGGGCCCCCCCGCGTAGGCTGACCAGACGGATAGGCGCTAGCTGTTA TGGCCGTTATATGCCTCGTATGGTTGTAAGTATTAATTATTCCATTTATTTATTTAAGAA ATACATTGTATATCTTCTTTTGATGATGCTTTGTTCTTGTAGTCTTACGCCCT
BA77	GE470278	GGACACCACTCGTGATGTGAGCGAGTTAATTAGTCTCATGTCTTATGGAATGATGGTAAT ATGTTTTCCAACAGGCATAGTATTCTTTATTGCTCTAATATTTATAGGGACTGTATCAGA CATTATTGGCGTAAGGATTGATAGTAAGTATATTATGGCGATAATAATATGGCTTTATTT TCTGTCAGGAGGGTATATTAGTGGTGTCC
BA78	GE470279	GATGACCGCCGCGAAAGCAGAACAACTGATTGAGCAGGCATTATTACTGACGGCATGATA GTGAAAGTGAACGCGGCGCTGGATGCGGCCCGCACGCTGGGCCGTCCGGTAGATATCGCC TCCTGGCGTCATGCGGAGCAGCTTCCGGCACTGTTTAACGGTATGCCGATGGGTACGCGG ATTTTAGCTTAAGTTTTGTTGGCCGGAGGCGCAGCTTTCCGGCATTGAATTTCAAAATAA GGAAACAGAGTTATGGCACTTTGGGGCGGTCATC
BA79	GE470280	GATGACCGCCCGAAAGATGAGTGTGAAACCTGCTGCATTAAGTATCCGAACGGGCGCAAC GTGCTGTCGCAGGAAAACCAACAAGTGTTTGTACACACAGGCACCGACC
BA80	GE470281	GCTTGCCCTGGACGAGTTGTACGAGGCAGACAGGATGGAGAGCGGGAGGAGAAAGACTTG GATCTGCGAAGCTCAATTCCCACCGTTACACACAGGCACCGACC
BA81	GE470282	CCCCCGTAGCTACCCAACGATGATTGCCGGTGACTGAGTTATTTTGAACATGGTGAGGGG AAGGTAAATGCTGTGCGATACATCTACAATAGAATGGATGGTTGATCGTTATCCCANATT GTACCTCGTGGACATTAAGTGCTGGTGAATTCCTGCGAGCCNGGGGGATCCACGTAATTC TAAGAGCTGGGCGACCACCCGCGGGTGGCANNGCTCCNAACCTCTTTGTTCCCCTTTAGT GAGGGTTAACTTGCGCGCCTTGGGCCGTAATCATTGGTCACTATCTTGTTTATCCTTGTG TTGAAAATTTGTTTATTCCGTCTCACAAAATTTCCACACAACATACCGAGTCCGGAAGCA TTAATGTGTAAANGCCTGGGTGCCTAAATGAGTGAAGCTTAACTTCATCATTNAATTTTG CGTTTCGCGCCTTCCACTGGCCCCGCTTTTCAAGTCGGGGGAAAACCCTGGTCGGTGGCC AGCTGCCATTTAATTGAATTCGGGCCCAAACCGCGGCCGGNGGGAATGAAGGCGGGTCTT TGGCTGTATTTGGGGCGCACTCTTTCCGGACTTTCCTCGGCGTCCAACTGACCTTCGCTG CGCCTCTCGGTCGGTTCCGGCCTGCGGGGCGCACCGGGTTATTCAGCCTCCACCTTCAAG GGCGGTGATTACGGGTTATCCCACAAGAAAATTCAGGGGGGAATTACACCCCCAGGAAAA GAAACATTGTTGAAACCAAACAGGGCCAAACAANNGGCACAAAGAAACCCGGGAAAAAAA AGGACCGGGTTTGGTGGGGGGTTTTTCCAAAAGGGCTCACACCCCCCCCTAAAAAGGAAC CATTTACAAAAAATCTAAAGCCCAATTAAAAGGNGGCCCAAACACCAAAGAAGGAAAAAA TAAAAATACCACACGGGCTCCACCCGTGAACCCCCCGGGCACCCTCTCCATGTTCCCAAC CCTTCCCCTATACGANACCCNTTCCG
BA82	GE470283	TAGGCGTAAGGATACATTGACAGAGGATGAGGCCAGATTTTATGTTGCAGAAACAGTATT AGCAATTGAATCTATTCATAAACATAATTACACACAGGCACCGACCAAAGGCTTGGTGAA TTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCC

BA83	GE470284	ATATTCAATCATTAGTTGAGAAAAAATAAATGAAAAAGATTATTTTGACTATTGGCTGTC CTGGTTCTGGTAAGAGTACTTGGGCTCGTGAATTTATTGCTAAGAATCCCGGGTTTTATA ATATCAATCGTGATGACTATCGCCAATCTATTATGGCGCATGAAGAACGCGATGAGTACA AGGTATACCAAAAAGAAAGGAGGTATCGTAACTGGTATGCAGTTTGATACAGCTAAAAGT ATTCTGTACGGTGGCGATTCTGTTAAGGGAGTAATCATTTCAGATACTAACCTGAATCCT GAACGTCGCCTAGCATGGGAAACTTTTGCCAAAGAATACGGCTGGAAAGTTGAACATAAA GTGTTTGATGTTCCTTGGACTGAATTGGTTAAACGTAACTCAAAACGCGGAACTAAAGCA GTACCAATTGATGTTTTACGTTCAATGTATAAAAGCATGCGAGAGTATCTCGGTCTTCCA GTATATAATGGGACTCCTGGTAAACCAAAAGCAGTTATTTTTGATGTTGATGGTACACTA GCTAAAATGAATGGTCGTGGTCCTTATGACCTTGAAAAATGCGATAC
BA84	GE470285	AAGCTTGATTGCCGTCTTTGATTTGGGTGGTGGTACTTTTGATGTCTCCGTCCTGGAAAT CTCCAATGGGGTGTTTGAGGTCAAGGCTACCAACGGGGACACATTCCTTGGTGGTGAAGA CTTTGACAATGCATTGCTGGACTATCTGGTCAGTGAATTTAAAAGAACCGATAACATCGA TTTGTCCAAAGACAGATTGGCATTACAAAGGCTAAGAGAAGCAGCTGAGAAAGCAAAAGT GGAGTTGTCATCAACCACGCAGACGGAGATAAACCTTCCTTTTATTACTGCTGATGCTTC CGGAGCAAAGCACTTCAATACACACAGGCACCGACCA
BA85	GE470286	GGCGTAAGGATACATTGACAGAGGGATGAGGCCAGATTTTATGTTGCAGAAACAGTATTA GCAATTGAATCTATTCATAAACATAATTACACACAGGCACCGACC
BA86	GE470287	ATCGTGGACCCGGGGNAGGGCCCCCCCGCGTAGGCTGACCAGACGATGGGCGATAGCAGT GTATGATCACGAAGTGTCCTCTGTTTGGAATGATGTTCCTCCAGGTCAATGTTTGTAATT CGGGTACCGATTGTAGTGTATCGGT
BA87	GE470288	GGGCGTAAGCAGTGTATGATCACGAAGTGTCCTCTGTTTGGAATGATGTTCCTCCAGGTC AATGTTTGTAATTCGGGTACCGATTGTAGTGTATCGGT
BA88	GE470289	TCGAGGTCACCAGACGAGGGCGGTGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGA GCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCG CTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC ACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTA ACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCA GCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTC CGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGC TCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACAT GTGAGCAAAAGGCCAGCGAACAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTT TCCATAGGCTCCGCCCCCCTGACGAGCATCACGAAAATCGACGCTCAAGTCAGAGGTGGC AAACCCGACAGGACTATAAAANATACCAGGGGCGGTTTTCCCCCCTTGGAAAGCATTCCC CTCGTGCGCTCTTCACTGTTTCCGACCCTGACGGTTTAACCNGGATACTGTTCAGCATTT CTTCCTTCNGGACAAGAGATAGGGTTTTTTAATAACTAACGGGATAGGTTTTTAATGGAT ATATGTTATATAAATGAAGAGACAAAACCCACAATCGAAAA
BA89	GE470290	GTCAGCCACTGGTAACGATGACCATGTATTTGATATGGACGATGTGCTACCCTAATTAAT TCAGGCCCAGTACGCAAGCTGACATCTGGATGTTTTATCATCATGGCCTTCATAAAAGCT TGAAGCAGGATGCAGCTTTTAACAAACATGACGATCTCATTAACAAACCGGTGTTAGTAT AAAGCAGTGCCTTTTTGTTAGATTTGAGTGATCACTGAACTCTTATAACTCGCTGAAGGA TTTGCCTGTTTGGGTGGCTGACT
BA90	GE470291	CGGCTACCACATCCAAGGAAGGCAGGCAGCGCGCGCAAATTACCCAATCCTGACACGGGG AGGTAGTGACAATAAATAACAATACCGGGCTCCACGAGTCTGGTAATTGGAATGAGTACA ATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAA TTCCAGCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGACCTT GGTTTGGGTCGGTCGGTCCGCCTTTTGGTGTGCACCGCCCGCCCTGATCCTTTTGTCGAC GATGCGGTCTGGCCTTAGCTGGCCGGGTCGTGCCCTCGGCGTCGTTACTTTGAAGAAATT AGAGTGCTCAAAGCAAGCCCACGCTCTGGATACATTAGCATGGGATAACATCACAGGATT TCGATCCTATTGTGTTGGCCTTCGGGATCGGAGTAATG



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The use of genetic markers for detecting DNA polymorphism, genotype identification and phylogenetic relationships among banana cultivars

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ABSTRACT

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. A strong linear relationship was observed between the Resolving power (Rp) of the primer and its ability to distinguish genotypes. Based on these data, a genetic similarity matrix was established and a dendrogram for each set of primers was developed by UPGMA. The genetic similarity coefficients in RAPD analysis ranged from 0.3177 to 0.7818 and in ISSR analysis from 0.1800 to 0.8462. A fingerprinting key was generated where the presence/absence of specific RAPD/ISSR bands were recorded for each cultivar. The presence of a specific RAPD (OPC-5_{800}) band was observed for an endemic cultivar—Nanjanagudu Rasabale (NR). The study resulted in the identification and molecular classification of South Indian banana cultivars of which Robusta and Williams are global and others have either limited geographical distribution or purely endemic to South India. A group of eight cultivars was identified that are highly distinct from one another. The members of this group may be useful for generating 2X and 4X breeding populations for further use in breeding secondary triploid hybrids.

1. Introduction

Bananas are the most important among the tropical fruits as they constitute a major portion of income for poor in developing countries with the world annual production of around 40 million tonnes (FAO Yearbook, 2003). Banana belongs to the genus *Musa* L. (Family Musaceae, order Gingiberales) having a wide range of diploids, triploids ($2n = 3x = 33$) and tetraploid cultivars/landraces that evolved from inter-specific hybrids of the two wild diploid species, *Musa acuminata* Colla (contributes for 'A' genome) and *Musa balbisiana* Colla (contributes for 'B' genome) (Simmonds and Shepherd, 1955). Most of the edible cultivars of banana are thus sterile triploids or tetraploids, propagated mainly by vegetative means. Certain seedless diploid varieties are also cultivated in India and relished as delicious dessert bananas by local people. Due to their sterile nature, commercial varieties of banana are least adaptive to environmental challenges and are continuously under the threat of extinction. Therefore, any genetic analysis and propagation/conservation studies on this crop are of utmost importance.

India is recognized as one of the major centres of origin and diversity of *Musa* at a global level, along with South East Asian countries. The diversity of bananas spreads across southern states of India (Simmonds, 1962; Singh and Uma, 2000). The earliest attempt made to evaluate the clonal situation in South India resulted in a monograph published by Jacob in 1952 (cited by Uma et al., 2006). Since then, attempts have been made in the states of Tamil Nadu and Kerala to evaluate accessions, resolve synonymy, identify mutants and classify cultivars based on cytological and morphotaxonomic characters. While the presence of genetic diversity is most important in any crop improvement programme, prospection, collection and conservation of clones need attention to save the erosion of gene pool and ensure its utilization in breeding programme. Such an effort has resulted in the collection of a total of 525 accessions from all over India, including the major field gene-banks, and the clones have been pooled at the National Research Centre for Banana (NRCB), Tamil Nadu. While some clones have been evaluated partially for their reaction to Sigatoka, *fusarium* wilt and nematodes, the others have been evaluated for better yield and fruit qualities. Because most of the cultivated banana varieties are the result of natural mutation and selection, it is

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necessary to locate promising clones through intensive evaluation. *In vitro* propagation of such selected clones holds a great promise for future genetic improvement programmes on banana.

Several methods have been used to investigate the genetic variability present in *Musa* germplasm. Morpho-taxonomic characters were first developed and optimized for bananas and 119 descriptors were defined to characterize *Musa* germplasm (Horry and Arnaud, 1997). Edible bananas are generally the hybrids between *M. acuminata* (represented as 'A' genome) and *M. balbisiana* (represented as 'B' genome). Thus the various commercial clones used in the present study (Table 1) are the hybrids between the two, some being sterile diploids (AB), or triploids (AAB, ABB) or autopolyploids (AAA). The genome-type is decided on the basis of morphological descriptors as well as preliminary cytological studies. Therefore, more of "*balbisiana*" and lesser "*acuminata*" characteristics are named as ABB and so on. In addition, genetic changes may be expected because many vegetative characteristics are influenced by the environmental variations and a high degree of plasticity of the genome. The development and application of technologies based upon molecular markers provide the powerful tools that are adequate enough to reveal polymorphism at the DNA sequence level, and are often robust to detect genetic variability within the populations and between individuals (Kresovich et al., 1995; Simmons et al., 2007). Therefore, these markers have also been routinely applied for genetic analysis for formulating taxonomic, phylogenic and heterotic groupings, which may be of considerable value for defining core collections and developing efficient breeding schemes. DNA analysis through markers has been found extremely useful for predicting the breeding value of parental genotypes (Crouch et al., 1997, 1998a; Creste et al., 2004). One of the most important marker-based characteristics identified is that for parthenocarpy (Crouch et al., 1998b), which has tremendously benefited the breeders.

Genetic diversity in plants can be estimated in several ways. The RAPD[1] (Random Amplified Polymorphic DNA, Williams et al., 1990) technique has been used extensively for this because of the ease and rapidity with which data can be generated in a less laborious manner than other methods such as RFLP (Restriction Fragment Length Polymorphism) and AFLP (Amplified Fragment Length Polymorphism). RAPD technique has been used to assess genetic relationships in many plants and banana itself for distinguishing *Musa* germplasm (Howell et al., 1994), African bananas (Ude et al., 2003; Pillay et al., 2001; Onguso et al., 2004), landraces of AAB group of *Musa* (Crouch et al., 2000) and in BB *Musa* (Uma et al., 2006). Several reasons have been given to justify the use of these dominant markers for phylogenetic analysis rather than sampling sequences or morphological characters. The dominant markers, RAPD and ISSR, sample multiple loci from across the different genomes, in contrast to single gene analysis (Albertson et al., 1999; El-Rabey et al., 2002). Therefore, these PCR-based analytical tools are robust to address problems of introgression, lineage and unrecognized paralogy (Simmons and Webb, 2006). Therefore, the successful use of dominant markers to resolve phylogenetic relationships has lead to widespread application of ISSRs (Joshi et al., 2000; Wolfe and Randle, 2001) and RAPDs (Bowditch et al., 1993; Spooner et al., 1996).

A dominant marker is also expected to result in high band informativeness helpful in distinguishing among the genotypes as well as between primers. This functional property, commonly known as Resolving power (Rp) of a primer, has been found to correlate strongly with its ability to distinguish between genotypes. This function is well suited for comparing primers, primer-enzyme combinations or probe-enzyme combinations generated by RAPD, ISSR, AFLP and RFLP analyses. Essentially, Rp provides quantitative data allowing comparisons between primers (or probe-enzymes etc.), including those that are able to distinguish all genotypes examined in a study. Rp can also be used to predict the performance of group of primers (Prevost and Wilkinson, 1999).

The information on genetic relationships is essential and decisive for developing an efficient breeding programme. Advances in *Musa* breeding have established that crossing of divergent genotypes and subsequent selection of improved hybrids are the essential steps for the production of new banana cultivars (Ortiz and Vuylsteke, 1996). However, the information on genetic diversity and phylogenetic relationships within the South Indian banana cultivars is scarce.

The present study was aimed at using RAPD and ISSR markers to assess the levels of genetic diversity in popular cultivars of South India and to construct a dendrogram demonstrating the genetic relationships amongst them. Since the present evaluation involves commercially important diploid, triploid and tetraploid cultivars, the information generated is expected to be of great help in finding genetic relationships and planning breeding programmes.

Table 1
List of cultivars, type of utilization, ploidy and genome composition of banana plants used in the present genetic diversity study

Serial No.	Cultivars	Type	Ploidy	Genome composition
1	Monthan	Cooking	3X	ABB
2	Elakki bale	Dessert	2X	AB
3	NR	Dessert	3X	AAB
4	Ney poovan	Dessert	2X	AB
5	CO1	Both	4X	AABB
6	Nendran	Both	3X	AAB
7	Rasthali	Dessert	3X	AAB
8	Red banana	Dessert	3X	AAA
9	Poovan	Dessert	3X	AAB
10	Peyan	Cooking	3X	ABB
11	Karpooravalli	Dessert	3X	ABB
12	Virupakshi	Dessert	3X	AAB
13	Kunnan	Cooking	3X	ABB
14	Kadali	Dessert	2X	AA
15	Robusta	Dessert	3X	AAA
16	Williams	Dessert	3X	AAA
17	Motta poovan	Both	3X	AAB
18	Matti	Dessert	2X	AA
19	Laden	Both	3X	AAB
20	Anai koomban	Both	2X	AA
21	Sirumalai	Dessert	3X	AAB

2. Materials and methods

2.1. Plant material

The banana suckers of the most popular cultivars of southern part of the India were collected from germplasm maintained at Tamil Nadu Agricultural University, Tamil Nadu, India. The suckers were planted and maintained at Plant Cell Biotechnology Department of Central Food Technological Research Institute, Mysore, India for conservation and research purposes. Twenty-one cultivars were used for the present study (Table 1). The young unfurled leaves were collected from five different plants of each cultivar and used for DNA extraction.

2.2. Genomic DNA extraction

Each cultivar was reduced to a pool of five plants, and 100 mg of young leaves from each plant were used to create the pool. These leaves were frozen in liquid nitrogen and stored at −80 °C. The

[1] *Abbreviations used:* RAPD, random amplified polymorphic DNA; ISSR, inter simple sequence repeats; RFLP, restriction fragment length polymorphism; AFLP, amplified fragment length polymorphism; UPGMA, unweighed pair-group method with arithmetical averages.

DNA was extracted using the GenElute™ Plant Genomic DNA Mini prep kit (Sigma, St. Louis, USA). The RNA contamination in all the samples was removed by digesting the extract with 10 μg of RNase-A (Bangalore Genei, Bangalore, India) for 30 min at 37 °C. Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to a concentration of 25 ng μl^{-1} before use.

2.3. Selection of primers and band profile reproducibility

Initially, 75 RAPD and 25 ISSR primers were screened against genomic DNA of two banana cultivars for their ability to amplify DNA fragments. Three replicate DNA extractions (pool of five plants) from leaves of two different banana cultivars, Nanjanagudu Rasabale (NR) and Robusta, were used to assess the consistency of the band profiles. Of all the primers, 50 RAPD and 12 ISSR primers produced robust amplification patterns and no band was detected in any negative control amplification. The reproducibility of the RAPD and ISSR amplifications were assessed using selected primers (OPA-14, OPC-05, OPC-09, OPM-20, UBC-811, UBC-826 and UBC-890) with different DNA samples isolated independently from same cultivars (NR and Robusta) and amplified at different times.

2.4. RAPD and ISSR analyses

The RAPD primers were decamer oligonucleotides (Operon Technologies, Alameda, CA, USA) and for ISSR analyses, 12 primers based on di-nucleotide or tri-nucleotide repeats were utilized (Table 2) following the previous criterion (Venkatachalam et al., 2007a,b,c). These oligonucleotides were obtained from UBC primer set 100/9 (University of British Columbia).

2.5. PCR amplification

The protocol for RAPD analysis was adapted from Williams et al. (1990) and for ISSRs, the protocol of Zietkiewicz et al. (1994) was adapted. Initially, optimum PCR conditions for both RAPD and ISSR were standardized with various quantities of template DNA (12.5, 25 and 50 ng), dNTPs (100, 200 and 300 μM) and $MgCl_2$ (0, 1, 2 and 3 mM). Later, RAPD amplifications were performed routinely using PCR mixture (25 μl) which contained 25 ng of genomic DNA as template, 1× PCR buffer (Fermentas GmbH, Germany), 200 μM dNTPs (Fermentas GmbH, Germany), 1 unit (U) of *Taq* DNA polymerase (Fermentas GmbH, Germany) and 1 μM of each primer (Table 2). PCR was performed using a thermal cycler (Eppendorf, Germany) at initial denaturation at 93 °C for 4 min followed by 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 36 °C and 2 min extension at 72 °C with a final extension at 72 °C for 10 min.

In case of ISSR primers, optimal annealing temperature was found to vary according to the base composition of the primers. PCR mixture (25 μl) contained 25 ng of genomic DNA as template, 1× PCR buffer (Fermentas GmbH, Germany), 200 μM dNTPs (Fermentas GmbH, Germany), 1 U of *Taq* DNA polymerase (Fermentas GmbH, Germany). PCR was performed at initial denaturation temperature of 94 °C for 4 min followed by 40 cycles of 1 min denaturation at 94 °C, 1 min at 2 °C lower than specified annealing temperature for each primer and 2 min extension at 72 °C with a final extension at 72 °C for 10 min using a thermal cycler (Eppendorf, Germany).

The PCR products obtained were separated on 2% agarose (ICN, USA) gel, stained with ethidium bromide (0.001%) and the number of bands was recorded using a gel documentation system (HeroLab, Laborgerate, Germany). The size of the amplification products was estimated from 100 bp DNA ladder (Fermentas GmbH, Germany). The band intensity histogram of each gel was checked using Easy-Win 32 software (HeroLab, Laborgerate, Germany).

2.6. Resolving power (Rp)

Resolving power (Rp) for each primer was calculated following the method of Prevost and Wilkinson's (1999) for selecting primers that can distinguish a maximal number of accessions. Resolving power (Rp) of a primer is = $\sum Ib$ where *Ib* (band informativeness) takes the value of: $Ib = 1 - [2 \times |0.5 - p|]$, *p* being the proportion of the 21 genotypes (banana cultivars analyzed) containing the bands.

2.7. Data analysis

For both RAPD and ISSR profiles, the well-resolved and consistently reproducible fragments ranging from 200 to 3100 bp were scored as present (1) or absent (0) for each analysis. Bands with the same migration distance were considered homologous. A pair-wise similarity matrix was computed and analysed with NTSYS (Rohlf, 1998) version 2.02 using the simple matching coefficient (Sokal and Michener, 1958). The similarity matrix was used to construct a dendrogram by the un-weighed pair-group method with arithmetical averages (UPGMA).

3. Results

3.1. Selection of primers and reproducibility

To begin with, only two cultivars of banana, i.e., Robusta and NR (the first one is fairly studied and the second one is never studied), were subjected for genetic analysis to screen the primers for their ability to amplify DNA fragments. The bands were scored by eye in two independently prepared sets and were un-affected by DNA extraction method and PCR replication. Based on the results of their ability to produce good number of distinct bands, 50 RAPD and the 12 ISSR primers were selected. DNA samples from each of the 21 cultivars were amplified using the decamers listed in Table 2, where all the primers produced distinctly robust bands varying in numbers. For any of the primers used, band scores did not differ between repeat assessments or between gels. The majority of band positions varied between cultivars. Each of the primers produced large numbers of polymorphic bands. The data on the total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (%PB), Resolving power (Rp), amplified fragment size (AFS) obtained for each primer are shown in Table 2. The total amplified products of 50 RAPD primers was 537 (average of 10.74 bands per primer) ranging from 200 to 3100 bp, of which 323 were polymorphic (60.15%). The number of bands for each RAPD primer varied from 5 (OPJ 04 and OPJ 14) to 22 (OPM-20). The resolving power (Rp) of the 50 RAPD primers ranged from 0.66 for primer OPD-04 to 8.19 for primer OPM-20. Samples of RAPD analysis are shown in Fig. 1.

For ISSR analyses, after screening 25 primers, 12 were selected for the PCR amplifications of DNA samples from 21 cultivars resulting in highly reproducible amplification products. TNB, NPB, %PB, Rp and AFS obtained for each primer are shown in Table 2. The total amplified products of 12 ISSR primers was 104 (average of 8.67 bands per primer) ranging from 200 to 3000 bp, 59 of them polymorphic (56.73%). Number of bands for each primer varied from 4 (UBC-863) to 15 (UBC-890). The Rp of the 12 ISSR primers ranged from 1.24 for primer UBC-820 to 3.33 for primers UBC-836 and UBC-890. A sample of ISSR results is shown in Fig. 2.

3.2. Molecular analysis and fingerprinting of banana cultivars

The genetic similarity (GS) coefficients for 21 cultivars obtained with RAPD markers ranged from 0.3177 (between the cultivars

Table 2
List of RAPD and ISSR primers selected from Operon and UBC, respectively

Serial No.	Primer	Primer sequence (5′–3′)	TNB	NPB	%PB	Rp	AFS
1	OPA-03	AGTCAGCCAC	8	7	87.50	2.48	500–2800
2	OPA-04	AATCGGGCTG	12	11	91.66	6.19	300–3000
3	OPA-06	GGTCCCTGAC	10	5	50.00	3.33	300–1031
4	OPA-09	GGGTAACGCC	8	4	50.00	2.48	400–1800
5	OPA-11	CAATCGCCGT	18	12	66.66	4.57	300–3100
6	OPA-14	CTCGTGCTGG	13	6	46.15	2.10	200–3000
7	OPA-20	GTTGCGATCC	14	4	28.57	0.95	200–2600
8	OPC-01	TTCGAGCCAG	12	5	41.66	3.72	280–1800
9	OPC-02	GTGAGGCGTC	12	5	41.66	2.10	300–2800
10	OPC-04	CCGCATCTAC	15	9	60.00	3.33	220–2000
11	OPC-05	GATGACCGCC	14	13	92.86	6.10	320–1900
12	OPC-07	GTCCCGACGA	16	10	62.50	4.48	300–2600
13	OPC-08	TGGACCGGTG	16	15	93.75	5.33	290–3100
14	OPC-09	CTCACCGTCC	14	12	85.71	5.91	480–1800
15	OPC-11	AAAGCTGCGG	6	2	33.33	1.14	200–2000
16	OPC-13	AAGCCTCGTC	8	3	37.50	1.71	480–1800
17	OPC-20	ACTTCGCCAC	7	3	42.86	1.14	300–2000
18	OPD-03	GGTCTACACC	6	3	50.00	1.16	290–1800
19	OPD-04	TCTGGTGAGG	10	3	30.00	0.66	300–2000
20	OPD-07	TTGGCACGGG	13	5	38.46	3.14	380–2500
21	OPD-08	GTGTGCCCCA	7	5	71.43	2.19	290–2400
22	OPD-16	AGGGCGTAAG	14	13	92.86	7.53	400–2000
23	OPF-12	ACGGTACCAG	6	3	50.00	1.62	400–1031
24	OPF-15	CCAGTACTCC	7	4	57.14	2.00	200–2400
25	OPJ-01	CCCGGCATAA	8	6	75.00	2.48	200–3000
26	OPJ-02	CCCGTTGGGA	9	8	88.88	3.81	380–2400
27	OPJ-03	TCTCCGCTTG	8	6	75.00	3.05	500–2400
28	OPJ-04	CCGAACACGG	5	3	60.00	1.43	500–1031
29	OPJ-06	TCGTTCCGCA	10	4	40.00	2.95	220–2800
30	OPJ-07	CCTCTCGACA	11	5	45.45	3.14	300–2000
31	OPJ-08	CATACCGTGG	6	3	50.00	0.76	400–3000
32	OPJ-09	TGAGCCTCAC	8	5	62.50	2.48	400–2000
33	OPJ-10	AAGCCCGAGG	11	5	45.45	2.95	220–1800
34	OPJ-11	ACTCCTGCGA	8	3	37.50	1.14	400–2200
35	OPJ-14	CACCCGGATG	5	1	20.00	0.86	500–1031
36	OPJ-15	TGTAGCAGGG	9	5	55.55	3.62	220–1800
37	OPJ-16	CTGCTTAGGG	8	2	25.00	1.05	220–2000
38	OPJ-17	ACGCCAGTTC	6	3	50.00	1.24	500–2200
39	OPJ-20	AAGCGGCCTC	7	4	57.14	2.38	800–2400
40	OPL-14	TCGTGCGGGT	8	7	87.50	3.81	1031–2400
41	OPM-12	CACAGACACC	12	9	75.00	2.48	300–2600
42	OPM-16	GTAACCAGCC	18	13	72.22	4.29	400–2000
43	OPM-20	AGGTCTTGGG	22	16	72.22	8.19	320–2000
44	OPM-18	CACCATCCGT	7	5	71.42	2.10	400–2000
45	OPN-03	GGTACTCCCC	18	12	66.66	4.57	300–2600
46	OPN-04	GACCGACCCA	12	6	50.00	3.71	400–2800
47	OPN-06	GAGACGCACA	11	9	81.81	4.95	340–2000
48	OPN-09	TGCCGGCTTG	18	13	72.22	5.33	240–3000
49	OPN-10	ACAACTGGGG	19	5	26.32	1.91	240–3000
50	OPN-12	CACAGACACC	7	3	42.86	1.72	240–2400
Total			537	323	60.15	151.76	
51	UBC-811	$(GA)_8C$	7	6	85	3.24	250–2500
52	UBC-813	$(CT)_8T$	5	4	80	1.90	200–1031
53	UBC-817	$(CA)_8A$	8	6	75	3.05	400–2800
54	UBC-820	$(GT)_8T$	11	4	36.36	1.24	200–2000
55	UBC-826	$(AC)_8C$	8	5	62.50	2.48	350–2000
56	UBC-834	$(AG)_8YT$	13	5	38.46	2.67	700–3000
57	UBC-836	$(GA)_8YA$	11	6	54.54	3.33	200–1000
58	UBC-840	$(GA)_8YT$	11	3	27.27	1.33	200–1000
59	UBC-845	$(CT)_8RG$	6	6	100	3.24	300–1400
60	UBC-848	$(CA)_8RG$	5	4	80	2.48	200–1400
61	UBC-863	(GAA)6	4	3	75	2.48	500–1800
62	UBC-890	$VHV(GT)_7$	15	7	46.66	3.33	450–1800
Total			104	59	56.73	30.76	

Total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (%PB), resolving power (Rp) and amplified fragment size (AFS) obtained per RAPD primer and ISSR primer.
Key to symbols: R = A + G, Y = C + T, H = A + T + C, V = G + A + C.

Monthan and Virupakshi) to 0.7818 (between Robusta and Williams). The UPGMA analysis made it possible to discriminate all of the genotypes of this study. The dendrogram, based on RAPD data, showed a clear distinction into major and minor clusters (Fig. 3A). The dendrogram obtained using GS coefficient (Table 3) showed the presence of 3 main clusters (A, B and C) having 5, 13 and 2 cultivars in each, respectively. The cultivar Monthan did not group with any other variety and hence can be considered as

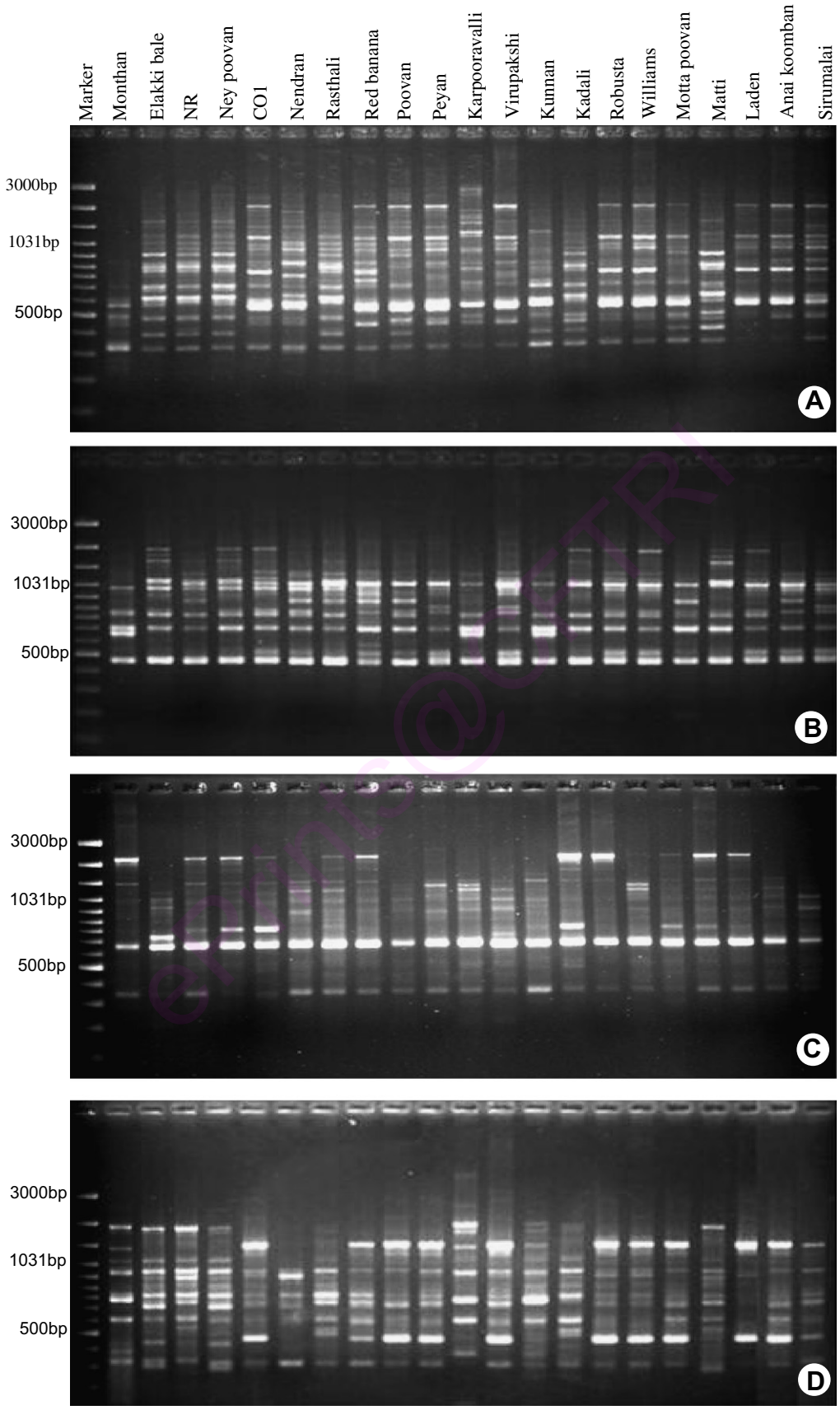

Fig. 1. RAPD profile of DNA from 21 cultivars of South Indian banana cultivars using primers OPA-14 (A), OPN-06 (B), OPM-20 (C) and OPC-05 (D). Lane Marker represents 100 bp GeneRuler DNA ladder.

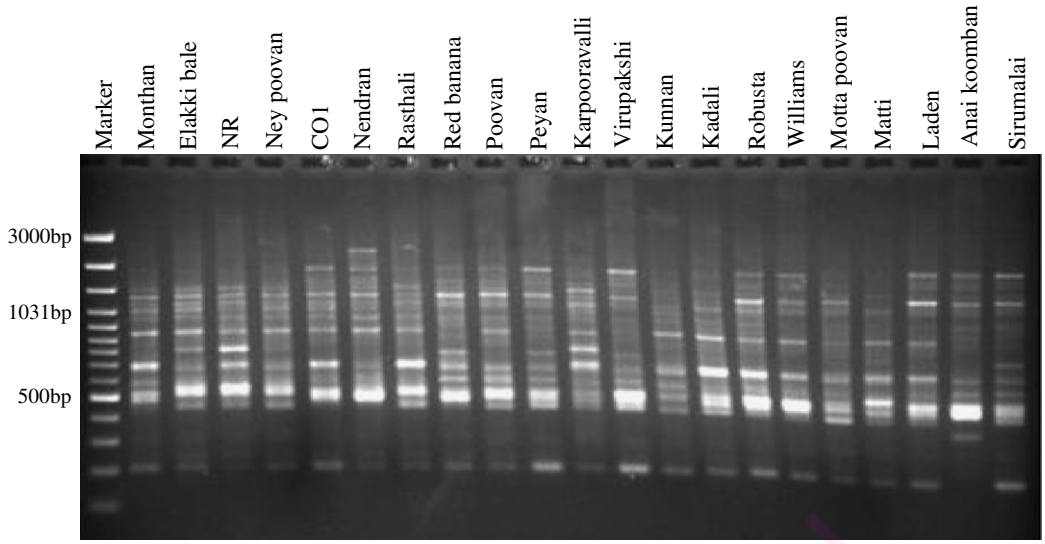

Fig. 2. ISSR profile of DNA from 21 cultivars of South Indian banana cultivars using primer UBC-810. Lane Marker represents 100 bp GeneRuler DNA ladder.

"isolate". The cluster A had 2 sub-clusters (A1 and A2), where the subcluster A1 showed the presence of 3 cultivars, Elakki, Ney poovan and NR; and the subcluster A2 represented 2 cultivars—Kadali and Matti. The cluster B showed the presence of two subclusters (cluster B1 and B2) where the subcluster B1 included 11 cultivars and the subcluster B2 showed 2 cultivars—Nendran and Rasthali. The cluster C showed the presence of 2 cultivars—Karpooravalli and Kunnan (Fig. 3A).

The GS coefficients for 21 cultivars obtained with ISSR markers ranged from 0.1800 (cultivar Monthan and Elakki bale) to 0.8462 (Robusta and Williams). The UPGMA analysis clearly showed phenetic discrimination for all the genotypes (21 cultivars) of this study. The dendrogram derived on the basis of ISSR analyses using GS coefficient (Table 3) showed a clear distinction into 3 main clusters (A, B and C) with 3, 16 and 2 cultivars in each, respectively (Fig. 3B). The cluster A showed 2 subclusters (A1 and A2), where subcluster A1 showed one cultivar—Monthan and subcluster A2 included the two cultivars—Karpooravalli and Kunnan. The cluster B showed 2 subclusters (B1 and B2), where subcluster B1 included 3 cultivars (Ney poovan, Rasthali and Kadali) and the subcluster B2 showed the presence of 13 cultivars. The cluster C showed two cultivars—Elakki bale and NR (Fig. 3B).

4. Discussion

Morphological markers for identifying the cultivars of banana are limited in number and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of the traits (Smith and Smith, 1989). In contrast, DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationships studies (Reddy et al., 2002). RAPD-based phylogenetic analyses have been conducted using neighbor-joining and/or UPGMA with Jaccard and raw-character-difference distances (Pharmawati et al., 2004; Simmons and Webb, 2006). Similarly, ISSR-based phylogenetic analyses have variously been conducted in plant varieties (Iruela et al., 2002; Pharmawati et al., 2004). In our earlier studies, the genetic markers of di-, tri- and tetra-nucleotide repeat primers were used to ascertain the genetic fidelity in long-term micropropagated (Venkatachalam et al., 2007c) and regenerated banana plants (Venkatachalam et al., 2007b). The present study was conducted to determine the extent of genetic diversity in the most popular cultivars of banana, including certain rare cultivars (such as NR) of South India that were not analysed earlier. We chose to analyse the banana germplasm, based on marker systems (RAPD and ISSR) that make use of arbitrary primers to amplify random DNA sequences in the genome (Williams et al., 1990; Zietkiewicz et al., 1994). An earlier study by Bhat and Jarret (1995) used a few RAPD markers to study Indian cultivars of banana. Recently, genetic markers have been successfully applied to study diversity in wild bananas of South India, mainly genotypes BB (Uma et al., 2006). Nevertheless, the present report relates to the first study where a large number of both RAPD and ISSR molecular markers have been used to examine genetic diversity in South Indian bananas. The selection of markers is based on extensive analyses for the concentration of magnesium chloride and the annealing temperature. The markers showing less than 3 bands were not selected.

4.1. Fingerprinting and cultivar-specific markers

South Indian banana cultivars are identified mainly on phenotypic characteristics of the plants and fruit quality/aroma. Especially in case of NR, people doubted that the exotic fruit qualities such as high fragrance/aroma and sweet fluffy pulp are probably due to the agro-climatic conditions since all other plant phenotypes of NR are similar to those of Elakki. However through the present study it has been established that NR is altogether a different cultivar with close relationship with cultivars Elakki and Ney poovan (based on the dendrogram data shown later). The high level of polymorphism of the RAPD and ISSR markers existed among the South Indian cultivars of banana (Figs. 1 and 2). A fingerprinting key, based on the bands that are specific to only 3 or lesser number of cultivars, has been developed (Table 4). The key thus developed has clearly shown a distinct band OPC-5_{800} for NR and the presence of specific ISSR bands (UBC-848_{300}) for Sirumalai, Monthan and Karpooravalli and band UBC-820_{2000} for Monthan and Virupakshi. Based on these observations, a specific primer can be picked up for sorting the varietal differences.

4.2. Genetic relationships

Although many cultivars included in the present study are popular in southern states of India, others namely Robusta, Williams, Laden and Rasthali are also grown in various parts of the world and introduced to India probably a couple of centuries ago. The

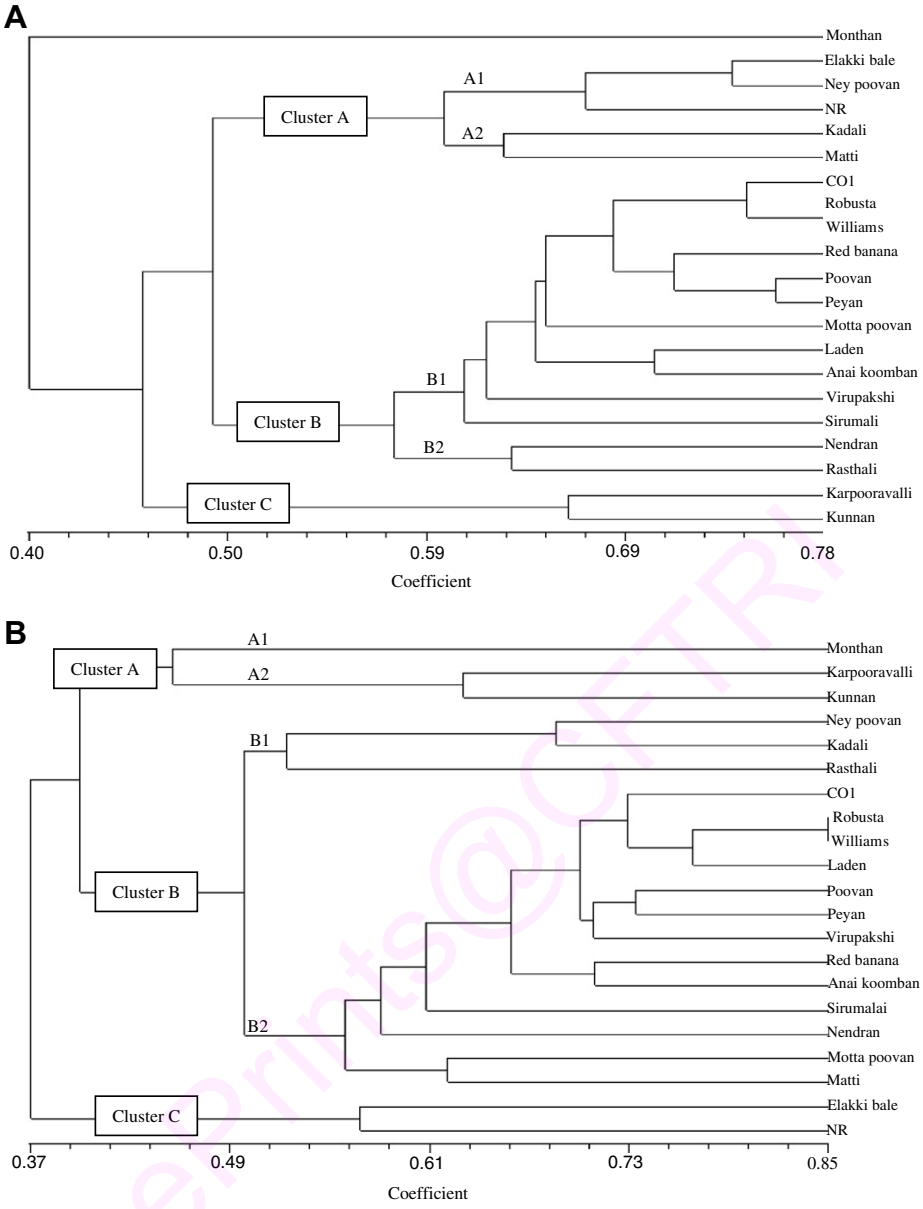

Fig. 3. (A) Dendrogram displaying the genetic distances among banana cultivars obtained from cluster analysis of RAPD data. (B) Dendrogram displaying the genetic distances among banana cultivars obtained from cluster analysis of ISSR data.

information on genetic relationship would thus be useful for planning breeding programmes to improve the local cultivars. The genetic similarity (GS) coefficients for 21 cultivars obtained with RAPD markers ranged from 0.3177 (between Monthan and Elakki bale, Virupakshi) to 0.7818 (between Robusta and Williams) and for ISSR markers ranged from 0.18 (between Monthan and Virupakshi, Elakki bale) to 0.8462 (between Robusta and Williams) (Table 3). Due to this variation, both the markers were found to be equally efficient in establishing the genetic similarity and distance with almost similar grouping. Thus there occurs very high genetic variability among the dessert cultivars in a permutation which was significant at $p = 0.0001$ with a matrix of 10,000 permutations. Similar results have been obtained for RAPD markers in cases such as African plantain core collection (Ude et al., 2003), plantain landraces of 76 accessions of AAB group (Crouch et al., 2000) and South Indian wild *M. balbisiana* populations (Uma et al., 2006). In another type of study, through inter-retrotransposon amplified polymorphism (IRAP-primers), B-genome-specific primers were used to classify the banana cultivars of South India (Nair et al., 2005). The primers used in this study (Nair et al., 2005) were found useful for identifying the presence of '*balbisiana*' (B) genome in banana cultivars where the band intensity appeared to be a preliminary indicator of ploidy level.

The dendrogram generated for the RAPD and the ISSR primers (Fig. 3) showed a clear distinction into major and minor clusters. The dendrogram obtained using GS coefficient (Table 3) showed the presence of 3 main clusters (A, B and C) for RAPD and ISSR analyses. However, the sub-clusters varied for both. The RAPD analyses showing 5, 13 and 2 cultivars in clusters A, B and C, respectively, whereas, ISSR showed 3, 16 and 2 cultivars in clusters A, B and C, respectively.

UPGMA of the data from RAPD analysis resulted in clearly discriminative data for all the genotypes of the present study where the similar genotypes such as those having AAB ranged from 0.6 to 0.7. However, the cultivar Monthan having genotype ABB was distantly related to cultivar Peyan and the other ABB cultivars, Karpoorvalli and Kunnan showed identical genetic distance for RAPD (Fig. 3A). Nevertheless, the UPGMA analysis using ISSR showed

Table 3
Genetic similarity co-efficient among the commercially important South Indian banana cultivars based on ISSR (top wedge) and RAPD (bottom wedge) analyses

Cultivars	Monthan	Elakki bale	NR	Ney poovan	CO1	Nendran	Rasthali	Red banana	Poovan	Peyan	Karpooravalli	Virupakshi	Kunnan	Kadali	Robusta	Williams	Motta poovan	Matti	Laden	Anai koomban	Sirumalai
Monthan	1	0.1800	0.1875	0.3404	0.4167	0.3913	0.3556	0.3125	0.4130	0.3913	0.4250	0.3750	0.4872	0.4390	0.4651	0.4889	0.3696	0.3404	0.4186	0.3830	0.3333
Elakki bale	0.3333	1	0.5676	0.6410	0.4082	0.3542	0.4762	0.3617	0.3750	0.3542	0.3182	0.3958	0.3721	0.4634	0.3333	0.3600	0.3617	0.4884	0.2917	0.3200	0.3830
NR	0.3536	0.6503	1	0.5500	0.3400	0.3696	0.4286	0.3478	0.3913	0.3696	0.2444	0.3265	0.3256	0.4146	0.2917	0.3469	0.4419	0.5122	0.2766	0.3617	0.3404
Ney poovan	0.4031	0.7381	0.6864	1	0.5870	0.5682	0.5714	0.5111	0.5556	0.5333	0.4091	0.4792	0.5000	0.6842	0.5111	0.5319	0.4167	0.6190	0.4348	0.4894	0.4681
CO1	0.4409	0.5052	0.4394	0.6211	1	0.6444	0.4490	0.5532	0.7045	0.7209	0.4565	0.6889	0.5111	0.4681	0.7381	0.7907	0.5532	0.5208	0.6512	0.6304	0.6444
Nendran	0.4194	0.4922	0.4712	0.5916	0.6612	1	0.5581	0.5682	0.6512	0.5909	0.3696	0.5652	0.4884	0.5116	0.5682	0.5870	0.4681	0.5333	0.5227	0.5778	0.5217
Rasthali	0.4294	0.5829	0.5882	0.6461	0.5260	0.6328	1	0.6098	0.5455	0.4565	0.2766	0.4375	0.4186	0.4762	0.5000	0.5217	0.5349	0.5714	0.4545	0.5814	0.4565
Red banana	0.3842	0.4794	0.4660	0.5538	0.6117	0.6519	0.6842	1	0.7073	0.6429	0.2653	0.6512	0.3696	0.4545	0.7000	0.6364	0.5814	0.5111	0.5714	0.7073	0.5682
Poovan	0.4402	0.5052	0.4921	0.5885	0.6944	0.6538	0.6201	0.7143	1	0.7317	0.3913	0.6977	0.4773	0.5000	0.7500	0.7209	0.6279	0.5556	0.7000	0.6744	0.5778
Peyan	0.4086	0.4896	0.4531	0.5729	0.7159	0.6464	0.5598	0.7069	0.7588	1	0.4318	0.7143	0.5238	0.5116	0.6829	0.6977	0.6429	0.5333	0.5952	0.6512	0.5556
Karpooravalli	0.4269	0.4255	0.3737	0.4639	0.4869	0.5054	0.4084	0.4162	0.4789	0.5027	1	0.3542	0.6286	0.3810	0.3778	0.3750	0.3191	0.3191	0.3333	0.2800	0.2857
Virupakshi	0.3177	0.4352	0.3568	0.4798	0.6461	0.5538	0.4479	0.6089	0.6292	0.6590	0.3795	1	0.4348	0.4255	0.7317	0.7442	0.5435	0.4792	0.6429	0.6591	0.6744
Kunnan	0.4512	0.4475	0.4011	0.5191	0.5027	0.5393	0.4693	0.4599	0.5193	0.5367	0.6601	0.4140	1	0.5128	0.5000	0.5227	0.4318	0.4000	0.4186	0.4444	0.3913
Kadali	0.4176	0.5588	0.5176	0.6529	0.4635	0.5137	0.5376	0.4603	0.4787	0.4628	0.4199	0.4074	0.5494	1	0.4884	0.4783	0.4222	0.6000	0.4091	0.4043	0.3830
Robusta	0.4358	0.4410	0.4124	0.5469	0.7168	0.5838	0.5243	0.6591	0.6897	0.6534	0.4368	0.6395	0.5085	0.4917	1	0.8462	0.5814	0.5111	0.7368	0.7073	0.5682
Williams	0.4402	0.4670	0.4242	0.5641	0.7733	0.6270	0.5676	0.6760	0.7062	0.7283	0.4789	0.6763	0.5278	0.4632	0.7818	1	0.6364	0.5652	0.7949	0.7209	0.5870
Motta poovan	0.4302	0.4737	0.4757	0.5179	0.6264	0.5450	0.5611	0.6348	0.6743	0.6763	0.4316	0.5272	0.4778	0.4385	0.6102	0.6743	1	0.6190	0.6098	0.7073	0.5333
Matti	0.3925	0.5899	0.6047	0.6798	0.5255	0.5285	0.5955	0.5155	0.5417	0.5260	0.4175	0.4416	0.4309	0.6287	0.4845	0.5337	0.5769	1	0.5349	0.5556	0.5000
Laden	0.4181	0.4249	0.4031	0.5156	0.6724	0.5769	0.5249	0.5889	0.6744	0.6286	0.4202	0.5862	0.4341	0.4270	0.6471	0.7349	0.6316	0.5243	1	0.7000	0.5952
Anai koomban	0.3474	0.4141	0.4145	0.4874	0.6354	0.5956	0.5189	0.6257	0.6278	0.6384	0.3807	0.6149	0.4536	0.4010	0.6011	0.6743	0.6416	0.4718	0.7012	1	0.6905
Sirumalai	0.3552	0.4624	0.4247	0.4845	0.6180	0.5519	0.5000	0.6264	0.6193	0.6207	0.3968	0.5780	0.4333	0.4032	0.5829	0.6102	0.5682	0.4762	0.6036	0.6727	1

clear clustering of these varieties having genotype ABB (cluster 1 of Fig. 3B). If these clusters could be linked to the phenotypic or functional characteristics such as resistance to a particular disease, the marker-based analyses act as powerful tools that are helpful in the improvement of these crops.

4.3. Comparison of RAPD and ISSR markers

Owing to the vast advantages offered by DNA markers, there is an increasing trend in adopting such technologies for identifying the genetic diversities, especially in breeding programmes. However, an understanding is important since the type of markers will reflect different aspects of genetic diversity. Similarly, the potential usefulness of molecular techniques in identifying genetic relationships varies from plant to plant because of the uniqueness of each genome. RAPD (Williams et al., 1990) and ISSR (Zietkiewicz et al., 1994) markers have several advantages and a few disadvantages for assessing genetic diversity, and many recent papers have demonstrated the high potential of the two markers for population and species level studies (Wu et al., 2004; Sreedhar et al., 2007; Simmons et al., 2007), including the identification of intra-clonal variants occurring during micropropagation (Martins et al., 2004; Carvalho et al., 2004). Phylogenetic analyses based on RAPD markers have been applied using neighbor-joining and/or UPGMA-based Jaccard and raw-character-difference distances (Pharmawati et al., 2004). RAPD has proven to be efficient in detecting genetic variations, even in closely related organisms, such as two near isogenic lines (NIL) (Martin et al., 1991). Similarly ISSR-based phylogenetic analyses have variously been conducted in plant varieties (Iruela et al., 2002; Pharmawati et al., 2004). Thus ISSR technique is found to be highly discriminative and reliable (Reddy et al., 2002), and both RAPD and ISSR markers have been found highly economical because a large number of samples can be analysed quickly. Both distance and parsimony analyses are generally conducted in ISSR- (Huang and Sun, 2000; Menzies et al., 2003), and RAPD-based (Harris, 1995; Pharmawati et al., 2004) studies. Therefore, both RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plant species (Ramage et al., 2004; Martin et al., 2006).

In the present study, the RAPD and ISSR surveys between pairs of 21 cultivars revealed 60.15% and 56.73% of polymorphic bands, respectively, indicating that both RAPD and ISSR markers have similar and great discriminating power. They could accurately detect the partition of genetic diversity within the cultivars since each cultivar showed separate data. However, the ISSR markers were found robust since only 12 primers were required for bringing out similarly distinct data as that of 50 RAPD primers.

Most of the earlier studies that addressed the assessment of new methods of cultivar diagnosis have utilized relatively modest collections of plants, although the number and composition of genotypes used varied widely. For instance, Hosaka et al. (1994) used 31 RAPD primers to distinguish 67 of 73 cultivars/breeding lines of potato. In contrast, Sosinski and Douches (1996) screened 16 RAPD primers and used the 10 most informative ones to distinguish between 46 cultivars. Such variations complicate comparisons between studies. An alternative strategy would be to use a function that is strongly correlated to the proportion of genotypes identified but is independent of the number of genotypes studied. One such strategy would be to measure the ability of primers to distinguish between genotypes, namely the resolving power. The primer Resolving power (Rp) provides quantitative data allowing direct comparisons between primers (Prevost and Wilkinson, 1999). Resolving power of the primer has been found to correlate strongly with genotype diagnosis and so has potential for a number of applications. It is possible that several primers included in a pre-

Table 4
Genetic key based on the presence or absence of specific marker for different cultivars of banana

Name of the cultivar	Characterized by the presence of the marker		Characterized by the absence of the marker	
	RAPD	ISSR (UBC)	RAPD	ISSR (UBC)
Monthan	OPC-05$_{400}$ OPA-04$_{700}$ OPA-04$_{650}$ OPJ-16$_{750}$ OPJ-20$_{300}$ OPC-08$_{1000}$ OPA-04$_{600}$ OPN-10$_{2000}$ OPN 3$_{2000}$	820$_{2000}$ 826$_{700}$ 845$_{750}$ 848$_{300}$ 863$_{200}$ 890$_{650}$	OPM-20$_{2700}$ OPN-6$_{1200}$ OPN-06$_{1031}$ OPC-05$_{800}$ OPA-03$_{1700}$ OPA-03$_{800}$ OPJ-11$_{1301}$ OPJ-11$_{800}$ OPJ-13$_{1000}$ OPJ-16$_{400}$ OPC-09$_{1200}$ OPC-08$_{1200}$ OPC-08$_{300}$ OPA-14$_{1800}$ OPA-04$_{200}$ OPJ-09$_{400}$	811$_{1200}$ 817$_{800}$ 820$_{800}$ 834$_{800}$ 836$_{2000}$ 845$_{400}$ 890$_{1200}$
Elakki bale	OPN-06$_{850}$ OPA-03$_{500}$ OPC-08$_{3000}$ OPC-08$_{550}$ OPN-10$_{2000}$ OPN 3$_{2000}$	811$_{800}$ 890$_{600}$	OPM-20$_{200}$ OPN-06$_{350}$ OPA-03$_{1000}$ OPD-16$_{1600}$ OPC-08$_{300}$ OPJ-09$_{500}$ OPN-10$_{800}$	811$_{1200}$ 811$_{900}$ 813$_{850}$ 813$_{350}$ 817$_{600}$ 820$_{1000}$ 820$_{900}$ 836$_{1600}$
NR	OPC-09$_{1800}$ OPC-08$_{3000}$ OPC-08$_{400}$ OPA-14$_{600}$ OPC-5$_{800}$	811$_{800}$ 890$_{1600}$ 890$_{600}$	OPM-20$_{600}$ OPN-06$_{1031}$ OPA-03$_{1700}$ OPA-03$_{800}$ OPJ-11$_{800}$ OPD-16$_{1600}$ OPA-04$_{1200}$ OPJ-09$_{500}$ OPN-10$_{800}$	811$_{900}$ 811$_{600}$ 817$_{600}$ 820$_{1000}$ 820$_{800}$ 834$_{800}$ 836$_{1600}$
Ney poovan	OPC-09$_{1800}$ OPC-08$_{3000}$ OPC-08$_{400}$	817$_{400}$ 890$_{1600}$	OPN-06$_{350}$ OPD-16$_{1600}$	813$_{350}$ 836$_{1600}$
CO1	OPN-10$_{2000}$	836$_{1200}$ 863$_{200}$	OPN-06$_{350}$ OPD-16$_{1000}$ OPC-08$_{2000}$	813$_{350}$
Nendran	OPM-20$_{800}$ OPC-04$_{1600}$ OPN-3$_{2000}$	836$_{1200}$ 863$_{200}$ 890$_{1600}$ 817$_{400}$	OPD-16$_{1000}$ OPC-08$_{1800}$	836$_{2000}$
Rasthali	OPA-04$_{650}$ OPA-14$_{600}$	811$_{800}$ 890$_{600}$	OPM-20$_{600}$ OPD-16$_{1600}$ OPD-16$_{1000}$ OPA-14$_{1800}$	811$_{600}$ 836$_{1600}$
Red banana	OPA-04$_{650}$ OPC-08$_{550}$ OPD-10$_{1200}$	817$_{400}$ 890$_{600}$	OPA-03$_{1000}$ OPC-08$_{800}$ OPA-04$_{1000}$	820$_{900}$ 890$_{850}$
Poovan	OPJ-13$_{2000}$ OPA-04$_{600}$	836$_{1200}$	OPC-05$_{800}$ OPJ-11$_{500}$ OPC-04$_{650}$	817$_{800}$
Peyan	OPC-04$_{1600}$	836$_{1200}$ 817$_{400}$	OPJ-11$_{600}$ OPN-10$_{500}$	836$_{2000}$ 890$_{850}$
Karpooravalli	OPC-05$_{400}$ OPC-05$_{400}$ OPA-04$_{700}$ OPJ-13$_{2000}$ OPJ-13$_{1300}$ OPJ-16$_{900}$ OPC-08$_{1000}$ OPC-08$_{550}$ OPA-14$_{600}$ OPA-04$_{600}$ OPC 5$_{3000}$	820$_{2000}$ 826$_{700}$ 836$_{1200}$ 840$_{1031}$ 848$_{300}$ 890$_{650}$	OPM-20$_{1200}$ OPC-05$_{800}$ OPC-05$_{300}$ OPA-04$_{1200}$ OPJ-11$_{1500}$ OPJ-11$_{1301}$ OPC-09$_{900}$ OPC-08$_{1250}$ OPC-08$_{300}$ OPA-04$_{1600}$ OPA-04$_{500}$ OPA-04$_{400}$ OPA-04$_{200}$	811$_{1200}$ 813$_{350}$ 817$_{800}$ 817$_{400}$ 820$_{900}$ 820$_{800}$ 836$_{2000}$
Virupakshi	OPN-06$_{850}$ OPA-03$_{2000}$ OPA-03$_{500}$ OPC-04$_{400}$ OPN-10$_{2000}$ OPN 3$_{2000}$	820$_{2000}$	OPA-03$_{1100}$ OPA-03$_{700}$ OPJ-11$_{600}$ OPJ-11$_{500}$ OPD-16$_{800}$ OPN-04$_{1200}$ OPC-04$_{700}$ OPC-04$_{650}$ OPC-08$_{2000}$ OPC-08$_{1800}$ OPC-08$_{1250}$ OPC-08$_{850}$ OPC-08$_{800}$ OPA-14$_{1800}$ OPA-14$_{1200}$ OPN-10$_{800}$	813$_{850}$ 820$_{1000}$ 890$_{850}$
Kunnan	OPA-04$_{700}$ OPJ-13$_{1300}$ OPJ-16$_{900}$ OPA-04$_{600}$	826$_{700}$ 840$_{1031}$ 863$_{200}$ 890$_{650}$	OPM-20$_{1200}$ OPA-03$_{600}$ OPA-04$_{1200}$ OPN-04$_{1200}$ OPC-09$_{900}$ OPC-08$_{1700}$ OPC-08$_{300}$ OPA-04$_{1600}$ OPA-04$_{1000}$	811$_{1200}$ 836$_{2000}$
Kadali	OPA-03$_{500}$ OPJ-16$_{750}$ OPC-09$_{1800}$	845$_{750}$ 890$_{1600}$	OPM-20$_{1200}$ OPM-20$_{600}$ OPN-06$_{1200}$ OPD-16$_{800}$ OPJ-16$_{400}$ OPC-09$_{900}$ OPC-08$_{1700}$ OPA-14$_{1800}$	811$_{1200}$ 813$_{1200}$ 845$_{400}$

Table 4 (*continued*)

Name of the cultivar	Characterized by the presence of the marker		Characterized by the absence of the marker	
	RAPD	ISSR (UBC)	RAPD	ISSR (UBC)
Robusta	OPA-03_{500} OPD-10_{1200}	817_{400}	OPN-06_{1200} OPA-03_{1700} OPA-03_{1000} OPJ-11_{600} OPC-04_{900} OPC-04_{700} OPC-08_{1700} OPN-10_{800}	813_{1200} 820_{900}
Williams	OPM-20_{3000} OPC-05_{3000}	847_{400} 863_{200}	OPC-05_{600} OPA-03_{1700} OPC-04_{700} OPA-14_{1200} OPA-04_{1000}	817_{600}
Motta poovan	OPC-08_{550} OPC-08_{400} OPN-10_{2000} OPN-03_{2000}	811_{800}	OPM-20_{900} OPA-04_{1200} OPJ-11_{1500} OPJ-13_{1000} OPC-09_{1200}	811_{900} 890_{1200}
Matti	OPJ-13_{2000} OPC-09_{1800} OPC-08_{3000} OPC-08_{400} OPS-14_{1800} OPA-14_{600}	890_{1600}	OPM-20_{900} OPM-20_{600} OPA-04_{1200} OPJ-11_{1500} OPJ-11_{600} OPJ-16_{400} OPA-04_{200} OPN-10_{800}	811_{900} 811_{600} 845_{400}
Laden	OPJ-13_{2000} OPC-04_{1600}	817_{400}	OPM-20_{750} OPM-20_{300} OPC-05_{800} OPC-05_{600} OPC-05_{300} OPA-14_{1200}	817_{800} 817_{600} 817_{400}
Anai koomban	OPC-04_{1600}	817_{400}	OPM-20_{2700} OPM-20_{300} OPC-05_{900} OPJ-11_{1301} OPN-04_{1200} OPC-08_{2000} OPC-08_{1800} OPC-08_{1700} OPC-08_{1250} OPA-14_{1200}	890_{850}
Sirumalai	OPD-10_{1200}	848_{300} 817_{400}	OPM-20_{2700} OPC-05_{900} OPA-03_{1000} OPJ-11_{500} OPC-04_{900} OPC-04_{650} OPA-04_{1200} OPN-10_{800}	813_{850} 890_{850}

liminary study are able to distinguish between all of the genotypes used. Such primers are the most likely to be selected for wider applications although there is currently no basis for comparing between them (Prevost and Wilkinson, 1999). Since cultivar diagnostic capacity of primers is generally based on results from small-scale studies, which may often lead to inconsistencies in primer selection. Therefore, Rp values are often of great help in selection of primers.

In the present study, Rp values of 50 RAPD primers ranged from 0.66 for OPD-04 to 8.19 for OPM-20 and that of 12 ISSR primers ranged from 1.24 for UBC-820 to 3.33 for UBC-836 and 890. When compared to the values reported in other crops (Prevost and Wilkinson, 1999; Virk et al., 1995; Teklewold and Becker, 2006) the range and the mean Rp observed in our study is high for RAPD and low for ISSR analyses. But Prevost and Wilkinson (1999) pointed out that Rp values could also vary between taxa for a selected set of primers.

Even in case of UPGMA analysis on the basis of ISSR data (Fig. 3B), the varieties Monthan, Karpooravalii and Kunnan (having genotype ABB) grouped into one cluster with an exception of Peyan which is a cooking banana with much different phenotypic characteristics. Similarly Elakki and NR grouped together (Fig. 3B) as they share very similar phenotypic characteristics with only differences in fruit characteristics (data not shown) although they are genotypically AB and AAB, respectively. For these cultivars RAPD data (probably due to their large numbers) also showed the close relationship between Elakki and Ney poovan, both having genotype AB and their fruit characteristics are almost similar to NR (AAB)—which is the next member of the cluster A in the dendrogram (Fig. 3A). Contrarily, Kadali and Matti, both being of AA genotype grouped together as A2 under cluster 1 in case of RAPD analysis (Fig. 3A) whereas in ISSR data showed their distant relationship (Fig. 3B). However, Poovan (dessert) and Peyan (cooking) that are entirely different from each other in fruit characteristics were grouped together in both the analyses indicating their genetic similarity for dominant characteristics. These observations indicate that it is essential to use two different markers for establishing the genetic similarity and distance rather than drawing conclusion

Table 5
List of exclusive cultivars, their genome composition and genetic distances with nearest and farthest cultivars

Serial No.	Cultivars	Genome composition	Genetic distance (Coefficient)	
			Nearest	Farthest
1	Monthan	ABB	Kunnan (0.4872)	Elakki bale (0.1800)
2	Elakki bale	AB	Ney poovan (0.6410)	Laden (0.2917)
3	Ney poovan	AB	Elakki bale (0.7381)	Monthan (0.4031)
4	NR	AAB	Elakki bale (0.6503)	Karpooravalli (0.2444)
5	Kadali	AA	Ney poovan (0.6529)	Monthan (0.4176)
6	Matti	AA	Ney poovan (0.6798)	Monthan (0.3925)
7	Karpooravalli	ABB	Nendran (0.5054)	Anai komban (0.2800)
8	Kunnan	ABB	Karpooravalli (0.6601)	Sirumalai (0.3913)

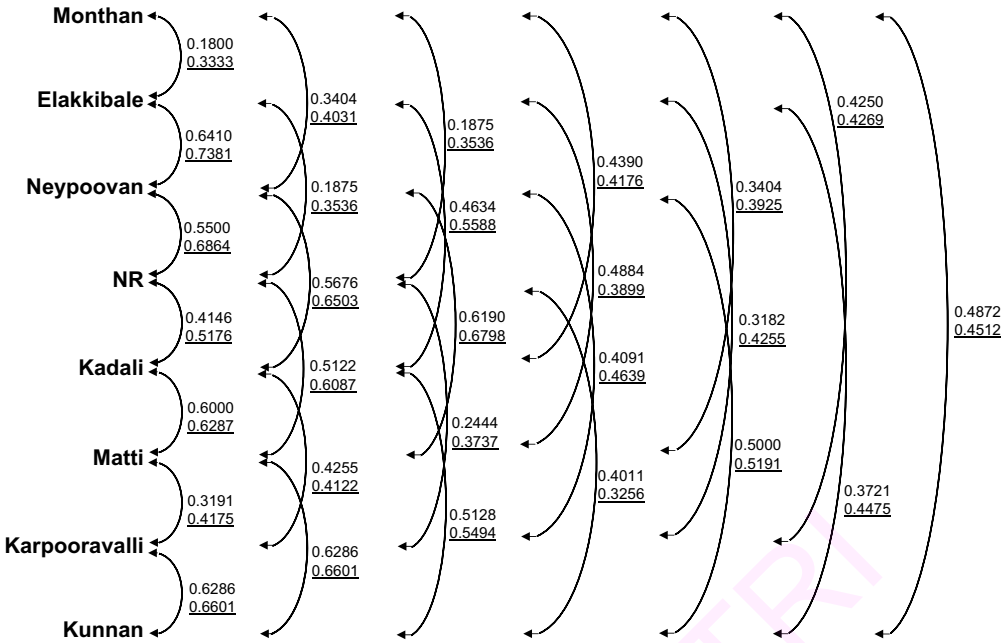

Fig. 4. Inter-relationships within selected eight cultivars based on ISSR- (upper values) and RAPD-derived (lower values with underline) genetic similarity coefficients.

with one set of markers. A similar observation has been made for identifying the genetic diversity of *Oryza granulata* (Wu et al., 2004), *Houttuynia* (Wu et al., 2005) and strawberry (Kuras et al., 2004).

Based on genetic similarity matrix and the dendrogram data, eight distinct cultivars were identified, which remained similarly distinct in both ISSR and RAPD analyses. These eight cultivars are listed in Table 5, showing near and farthest relationship, irrespective of RAPD/ISSR. The cultivar Monthan did not fall into any cluster in RAPD dendrogram indicating its distinctiveness. Similarly the cultivars in clusters A and C are the other distinctive cultivars. Nevertheless, the cultivars grouped in cluster B showed distinctly closer relationship. Among the eight distinctive cultivars identified through the present study, the inter-relationships have been derived based on their similarity coefficients (Fig. 4). Through this chart the genetic distance between any two selected cultivars can be quickly referred for both ISSR and RAPD analyses and are highly informative for selecting unique breeding combinations for generating newer genotypes.

In summary, RAPD and ISSR markers are two dominant DNA markers, having high resolution power and hence appear to offer many advantages in establishing genetic distances. They are effective and promising markers for assessing genetic variation in banana cultivars. This study has resulted in the identification and molecular classification of South Indian banana cultivars, of which Robusta and Williams are global and others have either limited geographical distribution or purely endemic to South India. A group of 8 cultivars was identified that are highly distinct from one another. The members of this group may be useful for generating 2X and 4X breeding populations that can be used in breeding secondary triploid hybrids.

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Spermine-Induced Morphogenesis and Effect of Partial Immersion System on the Shoot Cultures of Banana

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Abstract Contribution of exogenous polyamines (PAs) and polyamine-inhibitors on plantlet regeneration patterns of banana (*cv.* Nanjanagudu Rasabale-AAB) was studied and the performance of regenerated shoots in temporary immersion system was evaluated. The rhizome explants (without shoot bud) of in vitro shoots produced a mixture of embryogenic and nonembryogenic calli on modified MS medium. The analyses of endogenous pools of polyamines showed higher levels of PAs in embryogenic than in nonembryogenic calli. Supplementation of various levels of (10–50 μM) spermine (Spm), spermidine (Spd), and putrescine (Put) to cultures with secondary embryogenesis showed that about 50% of embryogenic calli rapidly produced secondary embryos only in the presence 40 μM Spm but not in other treatments. The crucial role of Spm was further confirmed by the use of 0.1 mM each of α-DL-Difluromethylornithine and α-DL-Difluromethylarginine along with Spm where the presence of inhibitors concomitantly inhibited the secondary embryogenesis. The shoots obtained from the embryogenic cultures were checked for their performance on solid medium (SM) and partial immersion system (PIS). The rate of shoot multiplication was higher in PIS than in SM throughout 6 weeks culture period. Uniformity in elongation of all the shoot buds was observed in PIS but not in SM. Evaluation for the acclimatization, survival under greenhouse conditions revealed the better performance of PIS-derived plants than those from SM.

Keywords Embryogenesis · *Musa* · Partial immersion system · Polyamine

Abbreviations

2iP	N^6-(2-isopentenyl)adenine
BA	Benzyladenine
DFMA	α-DL-Difluromethylarginine
DFMO	α-DL-Difluromethylornithine
GA	Gibberellic acid
HPLC	High performance liquid chromatography
PAs	Polyamines

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PIS	Partial Immersion System
Put	Putrescine
SM	Solid Medium
Spd	Spermidine
Spm	Spermine

Introduction

Edible banana cultivars are sterile triploids or tetraploids, and they are normally propagated by vegetative means. The progress made over the last decades in in vitro-based technologies has contributed immensely for the production of high quality planting material of desired clones. Propagation of banana through in vitro techniques has been reported by several workers using different explant sources as well as regeneration pathways. Diverse procedures have been originally described for somatic embryo induction in banana, differing from each other mainly due to the genotypic differences and use of various types of explants such as zygotic embryos [1], foliar bases and corm slices [2], in vitro-cultured meristems [3–6], and immature male [7, 8] and female [9] flowers.

A variety of exogenous growth regulators have been reported useful for banana micropropagation [3, 6, 10, 11]. This apart, banana shoots in vitro are known to synthesize and accumulate a natural cytokinin, 2iP at the basal portions of shoot clusters resulting in de novo bud formation, especially under an exogenous supply of benzyladenine (BA) [12, 13].

Another class of molecules, namely polyamines (PAs), such as Putrescine (Put), Spermidine (Spd), and Spermine (Spm), the chemically nonprotein straight-chain aliphatic amines, are known to play important role in various cellular processes such as DNA replication, cell division, protein synthesis, responses to abiotic stress, rhizogenesis, flower development, and in vitro flower induction [14, 15]. PAs have also been found implicated in morphogenic process where there are increasing evidences from several studies indicating that they play a crucial role either directly in somatic embryogenesis and regeneration or indirectly through the release of nitric oxide or inhibition of ethylene biosynthesis [14–17].

For in vitro propagation of plants, partial immersion bioreactor systems (PIS) have shown higher advantages compared to semisolid and liquid medium, in terms of faster multiplication or elongation of shoots. Such improved results were linked to the situation offered by PIS where the latter combines the advantages of both gelled and liquid medium. These advantages are due to intermittent aeration and higher availability of nutrients in liquid. A low-cost Growtek™ bioreactor has been designed and commercialized with unique features like floating and rotating ex-plant holder with perforated ex-plant support and a side tube for medium changing, culture feeding, and content monitoring. The usefulness of Growtek™ in terms of enhanced multiplication rates, reduced bioreactor costs, saving in incubation time, the minimization of contamination, and plantlet transfer without root injury has been well documented [18]. Therefore, a set of experiments were designed combining the effects of chemical parameters such as growth regulators and PAs on shoot or embryogenic cultures of banana under normal conditions using solid and liquid medium and compared with the similarly cultured shoots in Growtek™ bioreactors. The effects of these treatments were basically analyzed by checking the morphogenic events of organization of pro-embryogenic masses and further formation of shoot cultures and their performances until they were transferred to soil. In addition, at different stages of experiments, the effects of exogenous PAs were also monitored by analyzing the pools of endogenous PAs. To our knowledge, this is the first report in banana on the use of PAs for

the propagation where involvements of spermine in enhancing the rate of shoot multiplication and plantlet regeneration from somatic embryos have been documented.

Materials and Methods

Plant Material

The rhizomes of sword suckers of an endangered dessert banana *cv.* Nanjanagudu Rasabale (AAB) were used to excise the meristem and establish shoot cultures as described earlier [11]. The mother plant was maintained at the backyard of authors' department.

Culture Medium and Incubation Conditions

The aseptic shoot cultures used in the present study were established as reported earlier [11] and were maintained on M1 medium based on strength of MS basal salt mixtures [19] with additional 1,000.0 mg l^{-1} KNO_3 and 1.0 ml l^{-1} vitamin mixture [2] supplemented with 100 mg l^{-1} ascorbic acid, 1.0 mg l^{-1} BA, 0.2 mg l^{-1} IBA, 30 g l^{-1} sucrose, and 2.5 g l^{-1} gelrite (Sigma, St. Louis, USA). The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Cultures were maintained at 25±1 °C under a 16-h photoperiod (approximately 320 μmol m^{-2} s^{-1} PFD) provided by incandescent lamps (Philips, New Delhi, India). The in vitro multiple shoot cultures were established and maintained on M2 medium, designed based on MS medium supplemented with 1,238 mg l^{-1} NH_4NO_3, 2.0 mg l^{-1}BA, 1.0 mg l^{-1} Kinetin, 80 mg l^{-1} adenine sulphate, 25 g l^{-1} sucrose, and 2.5 g l^{-1} gelrite. The shoots and the rhizome explants obtained from these cultures were used subsequently for embryogenesis, and callusy rhizome formations were induced as reported earlier [13]. For plantlet formation and shoot elongation, M2 medium was used.

Polyamine Treatment

Concentrations of 10,20,30,40, and 50 μM of each PA viz., Spm, Spd, and Put (Sigma, St. Louis, USA) were tested by adding to control (M2) medium**.** These concentrations were selected based on an earlier study of Laine et al [20]. Culture segments, of uniform size (∅= 0.5 cm) having embryogenic mass, rhizome with callus and shoot clusters with shoot buds varying in length from 0.2–0.5 cm were selected and cultured in M2 with or without PAs. For each treatment, five segments were transferred to each bottle (10 cm in height and 6 cm in diameter) ×5. The observations on growth and morphological changes were recorded periodically; after which, the cultures were transferred to fresh respective medium by vertically splitting the culture into three pieces. Three such successive subcultures were made; after which, the final morphological observations and endogenous levels of PAs were recorded. Inhibitors of PA biosynthesis (DFMA and DFMO, Sigma, St. Louis, USA) were added (membrane-filtered) to the control medium M2 and to PA-containing medium at concentrations ranging from 0.1 to 0.5 mM. Two independent experiments were performed, using at least five replicate samples in each; the data obtained were averaged.

Extraction and Analysis of Free PAs

The extraction of PAs and high performance liquid chromatography (HPLC) analyses were conducted according to the method of Flores and Galston [21] and authentic standards of

Put, Spd, and Spm (Sigma, St. Louis, USA) were benzoylated following the procedure described by Flores and Galston [21]. Free PAs were extracted by homogenizing the plant materials from two culture samples (each 100 mg of tissue) in 1 ml of 5% ice-cold perchloric acid using a pestle and mortar. The homogenate was then centrifuged for 30 min at 20,000×*g*. Free PAs in the supernatant were benzoylated as Flores and Galston [21] and determined using an HPLC (Shimadzu LC6A, Tokyo, Japan). The elution system consisted of MeOH/H_2O (64:36) solvent, running isocratically with a flow rate of 1.0 ml/min. The benzoylated PAs were eluted through a C_{18} column (300×4.6 mm i.d., with pore size of 5 μm), an SLC-6A system controller, and a CR4A data processor was used. Compound detection was done through a ultraviolet detector SPD-AV set at a sensitivity of 0.04 AUFC and absorbance at 254 nm. A relative calibration procedure was used to determine the PAs in the samples, using standard of Put, Spd, and Spm. Peak areas and retention times were measured by comparing with standard PAs: Put, Spd, and Spm. Results were expressed as nanomoles per gram of fresh weight (mean ± SE). Extractions from five different samples per treatment were made independently and each extract was quantified in duplicate.

Shoot Multiplication and Elongation in Partially Immersed System

For PIS, Growtek™ bioreactor (100×150 mm (Φ×h); Tarsons, India) with unique features like floating, rotating, nonabsorbing ex-plant holder with perforated ex-plant support matrix; side-tube with silicon rubber septum for changing media and online monitoring of medium environment was used. Each Growtek™ bioreactor was filled with 200 ml of modified MS medium (M3), MS medium supplemented with 1,238 mg l^{-1} NH_4NO_3, and 25 g l^{-1} sucrose (and for SM M3 gelled with 0.8% Agar) with varying concentration of naphthalene acetic acid (NAA; 5, 10, and 15 μM) and gibberellic acid (GA; 15, 30, 45, and 60 μM). Clusters of shoot buds (shoot bud length 0.5–1 cm) obtained from embryogenesis experiment were grown for a short period of 2 weeks on M2 medium and used as initial inoculum. Approximately 25 g of explant transferred in to both Growtek™ bioreactor and SM. The Growtek™ culture vessels were maintained at 25±2 °C under 16 h photoperiod, having illumination of 320 μmol $m^{-2}s^{-1}$ PFD on a gyratory shaker set at 80 rpm throughout the culturing period. Each treatment had at least 15 replications.

Rooting, Hardening, and Green House Cultivation

Rooting of the elongated Growtek™ bioreactor and SM grown banana shoots was achieved on MS medium with 1/2-strength NH_4NO_3 supplemented with NAA (1 mg l^{-1}) and 15 g l^{-1} sucrose (M4). Well-rooted plantlets were, then, planted in soil containing equiproportion of red soil, sand, and vermi-compost, which was found to be most suitable through our earlier trials.

Scanning Electron Microscopy

The samples treated with different PAs, consisting of primary or secondary explants, derived structures, and embryos at different developmental stages, were processed for scanning electron microscopy, according to Larry et al. [22]. The samples were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) for 6 h, dried in alcohol series up to 100%, sputter coated with gold, and examined in a LEO Scanning Electron Microscope 435 VP (Leo Electron Microscopy Ltd., Cambridge, UK).

Statistical Analysis

All data presented are the mean values of five replicates, and the data have been presented as means with standard errors of the means. All the observations and calculations were made separately. The data were analyzed by one-way analysis of variance, and post-hoc mean separations were performed by Duncan's Multiple Range Test at $P \leq .05$ [23].

Results and Discussion

Table 1 summarizes the endogenous contents of PAs in cultures grown on M2 medium where all the PAs increased with time. Such time-dependent accumulation of PAs and higher accumulation of PAs by organized cultures have also been observed in oat [24], in red spruce [25] and embryogenic suspension cultures of *Araucaria angustifolia* [15]. The higher accumulation of Spm is probably due to the stress condition towards the end of culture period where nutritional stress could play a significant role. Stress-dependent increase in the levels of Spm has been observed in various other studies [17, 15]. The cultures on M2 medium remained mostly unchanged except for a slight change in shoot height. When the external PAs were fed to these cultures, there were significant changes in the response, both in terms of culture morphology as well as levels of endogenous levels of PAs (Fig. 1). There was no significant difference of treatment with low levels (10 μM) of any PA from that of control. Both Spd and Put at 30 μM showed significant changes in morphology where Spd induced fluffy callus interspersed with embryonic masses, and browning of medium was observed whereas Put, at similar levels, resulted in higher nonembryogenic coherent callus with lesser embryogenic masses, although medium browning was not observed. Spm, though at 30 μM, did not show any significant difference from that of control; a higher level (40 μM) showed very noteworthy effect resulting in enhancement of highly embryogenic culture mass. Further higher levels such as 50 μM of all the PAs showed browning of medium leading to the culture necrosis followed by death.

A detailed investigation of morphological events occurring towards embryogenesis in Spm (40 μM)-treated cultures was recorded using scanning electron microscope. There were sequential events occurring towards embryogenesis, starting from globular masses passing though further stages of pro-embryos and embryos at various stages of development towards shoot formation. These observations suggest the usefulness of PAs for enhancing embryogenic events in in vitro cultures of banana. Contrarily, in other studies, Put was more useful in supporting organized cultures of oat (*Avena* sp.) and its cultures obtained after reciprocal crosses [24].

There were much higher differences in the pools of endogenous PAs as a consequence of feeding external PAs (Table 1). Cultures treated with Spm (40 μM) showed a high endogenous level of Spm accounting for 2,500 nmol gFW^{-1} upon 12 weeks of culturing due to gradual accumulation throughout the culture period (Table 1). The next significant effect was that of 30 μM of Put, which invariably increased only endogenous Put pools, which was also highest (1,000 nM) on 12 weeks, although periodic fluctuations were observed during the culture period (Table 1). Spd, supplied at 30 μM, though increased Spm pools (400 nmol g FW^{-1} on 8 weeks of culturing), contributed for the increase in levels of other PAs as well (Table 1). Variable results have been observed in various other studies upon external applications of PAs. For example, the application of Put (20 μM) and Spd (40 μM) for callus cultures of *Pinus oocarpa* and *Pinus patula* stimulated the embryogenesis [26] as is the case of the present study. Contrarily, during the growth of

Table 1 Levels of endogenous total polyamines in banana cultures grown on Basic medium (M2) with different levels various polyamines.

Treatments	Spermidine (nmol gFW^{-1})			Putrescine (nmol gFW^{-1})			Spermine (nmol gFW^{-1})		
	Weeks after inoculation								
	4	8	12	4	8	12	4	8	12
Control Medium (M2)	4.14 d	7.04 d	13.44 d	15.46 cd	17.44 cd	112.17 cd	28.39 c	86.10 cd	393.88 c
M2 + 40 μM Spermidine	95.02 bc	129.00 bc	92.85 bc	224.75 bc	339.08 ab	62.51 cd	396.98 bc	412.30 bc	202.88 c
M2 + 30 μM Putrescine	48.92 c	125.82 bc	45.06 c	629.00 a	459.67 a	991.37 a	75.80 c	97.05 cd	56.31 cd
M2 + 40 μM Spermine	247.39 a	368.14 a	237.40 a	442.51 b	441.31 a	564.89 b	1954.77 a	2120.52 a	2542.82 a

Data presented as mean of five replicates. Means with common letters are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test

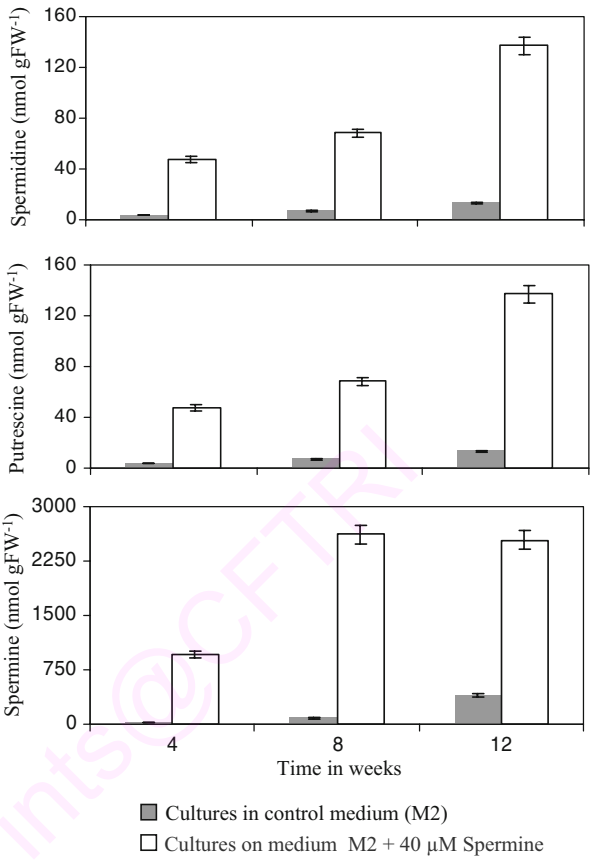

Fig. 1 Comparison of total endogenous polyamines culture segments grown in control medium (M2; *filled bar*) and those grown in M2 + 40 μM Spermine (*unfilled bar*). Data presented as mean of five replicates ± SE

embryogenic suspension cultures of *Pinus taeda*, high levels of endogenous Put were associated with reduction in cell division whereas in oats, Put enhanced the formation of somatic embryos [24]. In *Pinus sylvestris*, Spd retarded cell proliferation and growth but enhanced somatic embryo maturation [26]. Thus, in most of the cultures, these free exogenous PAs supported organization rather than unorganized growth. Accordingly, the PAs were higher in organized cultures, indicating that PAs are invariably organization-dependent.

In earlier studies, DFMO and DFMA have been noted to inhibit the endogenous pool sizes of PAs probably by inhibiting either the biosynthetic pathway or by degrading the accumulated pools of PAs to various degrees in different plant species. Therefore, in the present study, it was interesting to check to what extent these inhibitors reduce the pools of PAs in culture segments of banana having numerous regenerated shoot buds. These explants, being organized, showed almost similar levels of PAs as observed in embryogenic cultures mentioned earlier. When both DFMO and DFMA were used at 0.1 mM levels, most of the explants turned brown in 8 weeks and by the 12th week, shoot necrosis was apparent in all the treatments. When similar treatments were applied to embryogenic cultures, the already-formed embryos also necrosed and failed to form complete shoots. In these treatments, the endogenous pools of PAs were considerably disturbed with a higher suppression of all the PAs by DFMA (Fig. 2A) than by DFMO (Fig. 2B). The rate of

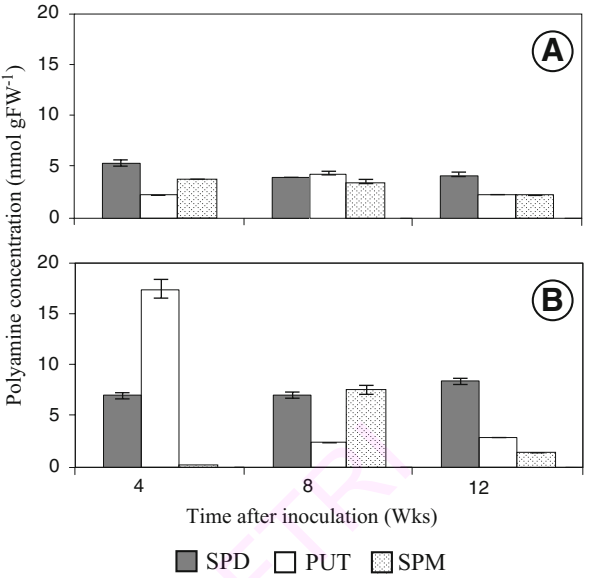

Fig. 2 Changes in total endogenous polyamines in cultures treated with polyamine inhibitors. **A** DFMA at 0.1 mM supplemented to basic medium (M2) containing 40 μM Spermine. **B** DFMO at 0.1 mM supplemented to basic medium (M2) containing 40 μM Spermine

suppression of Spd was negligible in both the inhibitor treatments whereas Put was periodically retarded by DMFA. These results confirm that the above effects are, in fact, due to the direct participation of externally fed PAs by way of cellular incorporation and not due to any other effects of indirect nature.

The accumulation of Spm in embryogenesis may be essential for the shift from callus to embryogenesis. A fine balance of different PAs may be required for embryogenesis. This is

Table 2 Effects of different levels of NAA and GA (supplemented through M3 medium) on height and number of shoots in two culture systems such as Solid Medium and Partial Immersion System grown for 2 weeks and 4 weeks.

Growth regulator (μM)	Shoot multiplication				Shoot elongation (cm)			
	SM		TIS		SM		TIS	
	Weeks after inoculation							
	2	4	2	4	2	4	2	4
NAA								
5	10 b	14 ab	8 b	10 c	2 d	3 d	3 e	7 e
10	12 a	16 a	6 c	12 b	3 e	4 c	5 cd	10 b
15	10 b	14 ab	10 a	14 a	2 d	3 d	4 de	8 cd
GA								
15	4 d	6 c	3 d	4 f	3 c	4 c	6 bc	10 b
30	7 c	12 b	6 c	8 d	4 b	7 a	10 a	12 a
45	6 c	8 c	5 c	6 e	5 a	6 b	7 b	9 bc
60	4 d	6 c	5 c	4 f	4 b	4 c	7 b	8 cd

Data presented as mean of five replicates. Means with common letters are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test

SM Solid medium, *PIS* partial immersion system

evident from our experimental results that treatment with inhibitors of PAs biosynthesis resulted in drastic reduction towards embryogenesis. Similar kind of response was observed in different plant systems with regard to morphogenic responses [14]. PAs are reported to promote shoot multiplication and in vitro flowering in *Cichorium intybus* [27]. In the present study, exogenous supply of Spm resulted in the enhancement of embryogenesis, suggesting the promotive role of PAs for embryogenesis in banana. In an earlier study, Beatriz et al. [28] studied the effect of exogenous administration of PAs in the concentration range of 0, 50, and 100 μM. The results showed the occurrence of significant differences among embryos indicating that the effects of PAs are not only species dependent but also organ dependent.

NAA and GA have been very useful for rapid elongation of cultured shoots leading to the generation of high quality shoots fit for acclimatization in NR variety of banana [29]. Therefore, the effects of these growth regulators, in combination with two different culture conditions SM and PIS, were studied. Although only increase in growth and shoot height were expected, there was also multiplication as a result of carry-over effects of earlier medium; this parameter was also recorded in addition to shoot height. In SM, all the concentrations of NAA resulted in good shoot multiplication as well as increase in shoot height (Table 2). Ten micromolar was found producing better results than other levels in terms of higher number or height of shoots, especially when grown for 4 weeks. Whereas in PIS, higher number of shoots as well as shoot height were observed when compared with SM, although 10 μM of NAA was more effective for shoot multiplication (Table 2). GA was more efficient in supporting shoot multiplication in SM than in PIS, more so when the concentration was 30 μM, although this level did not support as much multiplication as in NAA treatment. Nevertheless, GA very significantly supported shoot height in PIS than in SM. Because shoot height is a decisive factor in better acclimatization of banana shoots [29], GA appears very useful in obtaining high quality shoots. The Growtek™ bioreactor, functioning on the principle of PIS, has been used for micropropagation of other plant species where it was observed to support constant supply of nutrients and aeration to plants combining the advantages of both solid and liquid medium. The results of the present study also showed better utilization of nutrients in PIS than in SM because the biomass produced will be directly proportional to the nutrient utilization. All in all, almost similar multiplication with better shoot height were observed in PIS than in SM in a period of 4 weeks, suggesting that the observation made through the present study would be of great use for producing better quality shoots of NR banana. Uniform rooting in MS medium with 1/2-strength NH_4NO_3 supplemented with NAA (10 μM) was also observed. Such shoots appeared more robust than those from SM resulting in 100% survival in green house condition (data not shown). It is also worth mentioning that the use of Growtek™ bioreactor works out to be cheaper than any other commonly used apparatus meant for mass cloning of plants [18] because liquid form of culture medium is invariably cheaper than the gelled form with an added advantage of ease in handling.

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