Induction of somaclones in vetiver

[*Chrysopogon zizanioides*(L.)Roberty]

By

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(2010-11-146)

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

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THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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KERALA, INDIA

2013

DECLARATION

I hereby declare that the thesis entitled **"Induction of somaclones in vetiver [*Chrysopogon zizanioides*(L.)Roberty]"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that the thesis entitled **"Induction of somaclones in vetiver "[*Chrysopogon zizanioides*(L.)Roberty]"** is a bonafide record of research work done independently by Miss. Resmi S K (20010-11-146) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Thathwamasi

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Resmi S K

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ABBREVIATIONS

A	Adenine
AFLP	Amplified Fragment Length Polymorphism
AMPRS	Aromatic and Medicinal Plant Research Station
bp	Base pairs
β	Beta
C	Cytosine
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
^{0}C	Degree Celsius
DNA	Deoxyribo Nucleic Acid
DMSO	Dimethyl sulfoxide
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
G	Guanine
GC	Gas Chromatography
Kb	Kilo basepairs
KAU	Kerala Agricultural university
L	Litre
mA	Milli Ampere
Mb	Mega base pairs
Min	Minutes
ml	Millilitre
µg	Microgram
µl	Microlitre
MSAP	Methylation Sensitive Amplification Polymorphism
ng	Nanogram
OD	Optical Density
ODV 23	Odakkali Vetiver 23
pH	Hydrogen ion concentration
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
PVP	Poly vinyl pyrolidine
%	Percentage
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic acid
RNase	Ribonuclease
Rpm	Rotations per minute
Sec	Second (s)

T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UPGMA	unweighted pair group method of arithmetic mean
UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume

Introduction

1. INTRODUCTION

Vetiver [*Chrysopogon zizanioides*(L.) Roberty] is a perennial aromatic grass and native to India belonging to the family Poaceae. The essential oil obtained from the roots of vetiver is the major source of national economy of at least two nations i.e. Haiti Island in the Caribbean and Reunion island in the Indian ocean(Lavania, 2003).

The annual world trade in vetiver oil is estimated to be around 250 tons, with Haiti, Indonesia (Java), China, India, Brazil, Japan being the main producers, and the major consumers being USA, Europe, India, and Japan. Vetiver is a versatile plant, almost all parts of which are used in one or more ways having direct as well as multifarious cultural and industrial applications. It is a gift of India to the modern world and finds its greatest use in modern perfume creations (Lavania, 2003). The unique odour given by vetiver is not duplicated by synthetic counterparts so far. Vetiver oil possesses sedative property and has been traditionally used in aromatherapy for relieving stress, anxiety, nervous tension and insomnia for a long time (Fischer-Rizzi, 1990). It is also called vetivert, khus-khus, khas- vetiverol khas or 'oil of tranquillity'.

Vetiver oil is a fragrance in its own right for which no synthetic substitute is available. It has been manufactured for products like perfumes, deodorants, lotions, soaps etc. It has an important role in aromatherapy and is one of the finest fixatives known. The essential oil distilled from the roots of vetiver is one of the most complex mixtures of sesquiterpene alcohol and hydrocarbon, with an extremely slow rate of volatility. Slow evaporation rate of vetiver oil coupled with its pleasant aroma makes it a perfume by itself.

Vetiver is found to occur in India in wild state throughout the tropical and sub-tropical plains, particularly along the river banks and over the marshy lands. Two distinct morphological complexes of vetiver are found to inhabit spatially separated geographic regions in India: one in the north along the Indo-Gangetic plains and the

adjoining areas mainly in the states of Rajasthan, Madhya Pradesh, Uttar Pradesh and Bihar, and the other in the south along the east and west coasts of the Indian peninsula in the states of Andhra Pradesh, Kanataka, Tamilnadu and Kerala. The two races are distinctly different. The North Indian wild types are profuse flowering and high seed-setting having narrow leaves producing superior quality of laevorotatory root oil (ruh-khus or khus oil) and South Indian cultivated types are low / late flowering, low/non seed-setting with wider leaves producing lower quality of dextrorotatory root oil (Lavania, 2006).

Flowering occur in the South Indian types of vetiver grown in Kerala, but they do not set seed and hence have little scope for genetic recombination and improvement. This has resulted in the degradation of existing germplasm with respect to oil content, oil quality and resistance toward diseases (Hussain, 1982). In most major applications, a large number of plants are required and the quality of the planting material is one of the most important criteria. Induction of somaclonal variation through *in vitro* callus mediated regeneration is an option available for crop improvement. Somaclones have been created in various crops through this method (Keshavachandran *et al*, 1996, Larkin and Scowcroft, 1981 and Saraswathy *et al*, 2011). The present study was taken up for creation of somaclonal variants in vetiver (*Chrysopogon zizanioides*), which could lead to higher oil yield in this crop.

Therefore in this study, attempts have been made to generate variation under *in vitro* culture to further select the variants with desirable qualities with the following objectives:

1.Induction of somaclonal variants through callus mediated regeneration.
2.Morphological evaluation of somaclonal variation
3.Biochemical evaluation of somaclonal variation
4.Molecular evaluation of somaclonal variation

Review of Literature

2. REVIEW OF LITERARTURE

The present investigation was carried out on "Induction of somaclones in *Chrysopogon zizanioides* L.Roberty". The relevant literature on various aspects of the investigation is reviewed in this chapter under various heads.

2.1. Origin and distribution

Vetiver (*Chrysopogon zizanioides* L.Roberty) belonging to the family Poaceae is a grass native to India, and has been in traditional use since ancient times for its perfumery oil obtained from roots and also for contour protection since centuries. It is found occurring in India in wild state throughout tropical and sub-tropical plains, particularly along the riverbanks and over marshy lands. Based on geographical distribution patterns and detailed chromosomal evolutionary parameters, it is suggested that South Indian peninsula is its primary center of origin (Gupta and Pareek, 1995).

The name "vetiver" is derived from the Tamil word "vettiver". Vetiver is known to be in use in India both for its fragrant oil and as traditional medicine since antiquity (Husain, 1994).

2.1.1. Types of vetiver

Two distinct morphological complexes of vetiver such as North Indian races and South Indian races are found to inhabit spatially separated geographic regions in India. The north Indian wild types are profusely flowering and high seed-setting with narrow leaves producing superior quality of laevorotatory root oil and south Indian cultivated types are low / late flowering, low/non seed-setting types with wider leaves producing lower quality of dextrorotatory root oil (Lavania, 2006)

A higher order of genetic diversity with respect to ecological / geographic adaptation, morphometric traits, reproductive behaviour and essential oil concentration and composition is found in the Indian subcontinent, followed by Indonesia (Lal and Sharma, 2000)

2.1.2. Importance of vetiver

2.1.2.1. Economical importance

Vetiver is one of the most versatile crops of the third millennium. The economic importance of the genus Vetiveria depends upon the ability of the species to produce odorous roots, which can be used for the extraction of an essential oil of great economic importance. Since ancient times, the roots are used as a remedy against human and animal disease. The essential oil is produced in secretary cells located inside the mature roots. It consists of a complex mixture of sesquiterpene, hydrocarbons and alcohol (Maffei, 2002).

Vetiver oil possesses sedative property and has been traditionally used in aromatherapy for relieving stress, anxiety, nervous tension and insomnia for a long time (Fischer-Rizzi, 1990).

Khusimol in vetiver oil is a sesquiterpene alcohol which was found to inhibit the binding of vasopressin to rat liver (Rao, 1994). Furthermore, khusimol, epizizanal, a-vetivone, and b-vetivone were found to possess insect repellent properties (Jain, 1982)

2.1.2.2. Ecological importance

Vetiver is a natural barrier against erosion and has been used as a soil conserver since last 20 years. Vetver hedge have been applied for contour protection in

India since centuries. Vetiver is also used for mitigation of carbon emissions. (Gupta and Pareek, 1995)

Vetiver grass has been grown in the tropics over many centuries (NRC, 1993) and has been cultivated longest for the scented oil produced by its roots as well as for preventing soil erosion.

One of the desirable features of most hedgerow vetiver is that it is non fertile (produces no seed or seeds do not produce viable seedlings), hence it must be propagated from clumps of rootstock. Because it does not reproduce by seed, for centuries it has been a very well-behaved grass throughout the tropics and subtropics (Adams and Dafforn, 2000).

2.1.2. Taxonomy

Vetiver belonging to the family Poaceae/Graminae and subfamily Andropogoneae. The genus comprises of ten species, out of which *Vetiveria zizanioides* and *Vetiveria lowsonii* are from Indian subcontinent and *Vetiveria nigritana* is reported from Africa (Maffei, 2002)

The plant is a tufted perennial occurring in large clumps arising from a much branched root stock. The culms are erect, 0.5-3m high. The leaves are basal and cauline with relatively stiff, elongate blades up to 80 cm long and 8 mm wide. The leaves are splitting along the midrib apically and pubescent basally and are sometimes purple. The panicle is loose, irregular, compound, 15-30 cm long and comprise numerous racemous spike like branches (Sreenath *et al* , 1994)

The essential oil obtained by steam distillation from the roots of *Vetiveria zizanioides* (L.) Nash was investigated for their chemical constituents by GC and GC/MS. The major volatile components belong to the sesquiterpene group such as khusimol, a-vetivone, and b-vetivone. The sedative effect of vetiver oil upon

inhalation in rats was studied by observing the number of crossing and rearing motilities (Thubthimthed *et al,* 2001).

2.2. Micropropagation

Improved methods of vegetative propagation are used to preserve crop plants and this is especially true with tropical crops. Selected germlines of *Vetiveria zizanioides* have long been cultivated for their odorous roots, which contain the essential oil of vetiver, used extensively in perfumery and cosmetics. North India type populations show a reluctant tendency to self inbreed so that little interest has been devoted by growers to the screening and selection of new germlines. This fact, together with low seed viability, limits the possibility of applying traditional breeding techniques based on sexual mating and recombination (Sreenath *et al*.,1994).

Tissue culture, also known as micropropagation, is a propagation method used to produce a large number of propagules under sterile conditions. This method uses plant explants that have been sterilized before being placed in containers with a growing medium that has some nutrients added. The explants, the containers and the medium have all been sterilized, and this prevents any cut or torn tissue, or the entire explant itself, from becoming infected with a microorganism of some kind and also from rotting when the plant parts require to become rooted or to multiply (Matt, 2010)

Development of *in vitro* plant propagation and breeding provides a powerful means for the genetic improvement of vetiver grass. Plant propagation using shoot cultures i.e. micropropagation, supplies growers with a highly uniform plant population useful for commercial exploitation and for the maintenance of secure genetic stocks of plant material for breeding, genetic transformation and germplasm conservation. The latter subject is essential for crops which produce short-lived seeds and for those which are normally propagated vegetatively like vetiver grass. To reach these aims, sterile plant material capable of rapid regeneration into mature plants is essential (Sreenath *et al*.,1994).

Keshavachandran *et al* (1996) reported that in vetiver, pre-treatment of immature inflorescence in MS liquid media with 2,4-D(0.5 to 1.0 mgl-1) +BA(0.5 to 1.0 mgl-1) + KIN(0.5 to 1.0 mgl-1) was found to initiate callus in 89 -90 per cent of the cultures when cultured on MS medium with 2, 4-D 2.5 mgl-1 + KIN 1 mgl-1 in seven days. They also reported that BA among the cytokinins induced highest percentage of regeneration and that high percentage of rooting was obtained in solid media with IBA 5 mgl-1.

Sangduen and Prasertsongskun (2009) used young inflorescences of vetiver (*Vetiveria zizanioides* (L.) Nash) which were excised and surface sterilized for callus induction. Aseptic inflorescences were cultured on MS basal medium (Murashige and Skoog 1962) supplemented with 15 μM 2,4-D (2,4- dichlorophenoxyacetic acid) for 3-4 weeks.

Hanping (2001) standardised plant regeneration through *in vitro* somatic embryogenesis in vetiver. He reported MS + 2,4-D (2.0 mgl-1) + KT (0.5 mgl-1) as the suitable medium for establishment and subculture,MS + 6, BA (1.0 mgl-1) as the differentiation medium and 1/2MS + IBA (0.1 mgl-1) as the rooting medium.

2.3. Somaclonal variation

Somaclonal variation has been defined as the genetic and phenotypic variation among clonally propagated plants of a single donor clone (Olhoft and Phillips, 1999)

Tissue culture induced phenotypic and genotypic variations are collectively termed 'somaclonal variation' (Larkin and Scowcroft, 1981). It could result in a range of genetically stable variations, useful in crop improvement (Skirvin et al., 1993; Jain et al., 1998)

Somaclonal variation is manifested as cytological abnormalities, frequent qualitative and quantitative phenotypic mutations, sequence change, and gene

activation and silencing generated by use of a tissue culture cycle (Kaeppler *et al,* 2000)

In Japan, disease resistant lines of rice, tomato, and tobacco were isolated from somaclones (Nakajima, 1991). Ramos-Leal et al. (1996) obtained sugarcane somaclones resistant to eyespot disease. Several reports have also indicated the value of the selected somaclones in plant breeding such as high yield and shattering resistance in Indian mustard (Katiyar and Chopra, 1995)

Mohanty *et* al (2003) studied callus cultures initiated from shoot base explants of *Curcuma aromatica* Salisb. which were maintained on Murashige and Skoog (MS) media supplemented with 2 mg 2,4-dichlorophenoxyacetic acid alone or with 0.5 mg kinetin and were regenerated from 60 and 180 days old callus on MS media supplemented with 3 mgl-1 benzyladenine and 0.5 mgl-1-3 α-naphthalene acetic acid. Out of 113 regenerants analyzed, 85 plants were exclusively diploid and 28 were predominantly diploid revealing presence of polyploid nuclei. Frequency of polyploid cells were more in regenerants obtained from 180 days old callus than from 6 days old callus which could be attributed to the ageing of callus.

Epigenetic aspects of somaclonal variation involve mechanisms of gene silencing or gene activation that were not due to chromosomal aberrations or sequence change. These changes might be unstable or reversible somatically or through meiosis, although certain epigenetic systems outside of tissue culture are quite stable for many generations. Therefore, epigenetic changes induced by tissue culture could be manifested as the activation of quiescent loci or as epimutation of loci sensitive to chromatin-level control of expression (Cubas *et al.*, 1999).

Joshi and Rao (2009) reported the generation of somaclones for two submergence tolerant rice cultivars FR13A and FR43B through gamma irradiation and molecular analysis of the somaclones for the variation in the pyruvate decarboxylase (*pdc*) gene was carried out.

2.4. Analysis of Somaclonal variation

2.4.1. Morphological markers

As per Bhat, *et al*. (2010), morphological markers are those traits that are scored visually, or are those genetic markers whose inheritance can be followed easily. Although they are generally scored quickly, simply and without laboratory equipments, such markers are not put to much use because the genotypes can be ascertained generally at whole plant or plant organ level and frequently the mature plant is used. Such markers frequently cause major alternations in the phenotype which is undesirable in breeding programs. Dominant, recessive interactions frequently prevent distinguishing all genotypes associated with morphological traits. Morphological markers mask the effect of linked minor gene, making it nearly impossible to identify desirable linkages for selecting and are limited in number, influenced by environment and also specific stage of the analysis.

Morphological markers correspond to the qualitative traits that can be visually scored (Chawla, 2002). Many morphological markers like dwarfinism, albinism, plant height, number of tillers, altered leaf morphology, root growth etc have been used as morphological markers in plants. They are used in germplasm collection, identification of elite cultivars and even in finding the phenotypic variability in *in vitro* derived plants.

2.4.2. Biochemical marker

Several metabolites in plant can be used as markers for characterization of germplasm and identification of variations in elite plants

The essential oil distilled from the roots of vetiver, is one of the most complex mixtures of sesquiterpene alcohols and hydrocarbons, and also one of the most viscous

oils with an extremely slow rate of volatility. Chemical composition of vetiver oil is extremely complex, mainly comprising of sesquiterpenes and sesquiterpene derivatives, of which vetiverols, their carbonyl compounds and esters, are the main constituents, and their relative abundance normally establishes the oil quality (Lavania, 2003).

Steam distillation of dried roots of *Vetiveria zizanioides* gave viscous light-brown oil in about 0.3 - 1.0 per cent v/w yield with balsamic earthy and sweet woody odor. The complex mixture of vetiver oil was identified. It can be seen that sesquiterpenes constitute the predominant class of compounds, with khusimol(12.7%), longipinene (4.2 %), valerenol (3.9 %), epizizanal (3.3 %), avetivone (2.0 %) and b-vetivone (1.62 %) being the major ones (Thubthimthed *et al,* 2001).

2.4.2.1. Oil extraction

The dried roots were subjected to steam distillation for 14 hours. The isolated oil was dehydrated by addition of anhydrous sodium sulfate and kept in an amber-colored glass bottle. The oil yield was calculated relative to the dry matter. The steam distillation of dried roots of *Vetiveria zizanioides* gave viscous light-brown oil in about 0.3 - 1.0 per cent v/w yield with balsamic earthy and sweet woody odor (Thubthimthed, 2001).

2.4.2.2. Gas Chromatography

Keshavachandran *et al* (1996) reported gas chromatic analysis of various tissues of vetiver and compared them with the profiles of standard vetiver oil. The profiles of oil from the different tissues revealed that the oil from calli, *in vitro* roots, *in vivo* roots and the standard oil were more or less similar. They conducted the analysis in a Schimadzu GC-15A gas chromatography with a column of carbowax 20 M 20 per cent, CW 60/80 AW-DMCS mesh, of column length-3M.

Heller and Milen (1983) investigated vetiver oil using capillary Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometry (GC/MS). GC analysis was performed using a Fisons gas chromatograph model 8000 series equipped with a FID detector and a DB-5 capillary column (30 m X 0.25 mm; film thickness 0.25 mm). The operating conditions were as follows; carrier gas: helium with a flow rate of 2 ml / min; column temperature: 50 - 220 ^{o}C at 4 ^{o}C / min : injector and detector temperatures: 230^{0}C.

In vitro studies with rhizome explants of *Vetiveria zizanioides* obtained through organogenetic pathway was focused on the difference in essential oil content with that of control plants. There was variation exhibited by *in vitro* regenerated plants in their morphological growth and were referred as "morphotypes". Two types of morphotypes were identified , long and short based on differences in their height. Essential oil was extracted and analyzed by Gas chromatography. The two morphotypes showed varied essential oil content of 2.1 per cent (long) and 1.9 per cent (short) with the control showing 1.8 per cent. The plants showed marked differences in percentage composition of essential oil with respect to important compounds like khusimol, valencene, vetiverol, vetivone, vetivenene, vetiselinol and nootketone as well (Saraswathy *et al*, 2011).

Adams (2001) performed GC/MS analysis in vetiver on a VG Quattro mass spectrometer operating at 70 eV ionization energy, equipped with a DB-wax column (60 m x 0.3 mm x 0.25 mm). The oven temperature was programmed from 35 ^{o}C for 5 min to 220 ^{o}C for 45 min at 3^{o}C/min increase, with helium as carrier gas. The identification of the oil components was accomplished by comparing their GC retention indices as well as their mass spectra with corresponding data of authentic compounds or published spectra

Adams *et al* (2004) analysed normal and tissue cultured vetiver plants. Analyses of the oil compositions revealed that the oil from the tissue cultured plant is not very similar to normal vetiver oil. They also noticed that large number of alkanes

and alkanols were present- the C19–C29 alkanes, which were completely absent in the vetiver oils from normal plants. They reported that, the tissue cultured vetiver does not produce components like b funebrene, prezizaene, a-amorphene, or b-vetispirene, which are found in the non-cleansed (normal) vetiver.

Weyerstahl *et al* (2000) have exhaustively examined vetiver oil from Haiti. They stated that the composition was so complex (most GC peaks contained 2–4 components) that general, routine analyses of vetiver oils were probably not possible.

2.4.3. Molecular markers

DNA based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, sex determination, characterisation, varietal identification, clonal fidelity testing, assessment of genetic diversity, validation of genetic relationship, phylogenetic and evolutionary studies, marker assisted selection, mapping of mutations, characterisation of transformants, population genetics, molecular taxonomy and evolution, forensic analysis, estimation of genomic size etc. Somaclonal variations can also be characterized by a variety of molecular methods (Henry, 1998).

Molecular markers have diverse applications in fruit crop improvement, particularly in the areas of genetic diversity and varietal identification studies, gene tagging, disease diagnostics, pedigree analysis, hybrid detection, sex differentiation and marker assisted selection (Bhat *et al*., 2010).

Several morphological and biochemical markers have been used traditionally for the study of genetic diversity in plants. But these are influenced much by the environmental conditions and stages of growth of the plant. Recently molecular markers have emerged as a powerful tool for unambiguous identification of germplasm and characterization of plant species, varieties and ecotypes. Molecular

markers consist of specific molecules, which show easily detectable differences among different species (Singh, 1998).

According to Joshi *et al*., (1999), genetic polymorphism could be defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. DNA based markers mostly in use include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Variable Number of Tandem Repeats or Short Sequence Repeats (SSR) or micro satellite Inter Simple Sequence Repeat (ISSR) etc. The discovery of PCR was a landmark in the effort and proved to be a unique process that brought about a new class of DNA profiling markers.

Each marker system is characterised by a unique combination of advantages and disadvantages and the choice of marker system dictated to a significant extent by application.

RFLP and RAPD analysis have been applied widely in the analysis of somaclonal variation (Piccioni et al., 1997; Henry, 1998). Veilleux et al. (1998) used both RAPD and SSR techniques to characterize the genetic composition of anther-derived potato plants. Wolff et al. (1995) used RAPD, SSR, and RFLP markers to evaluate somaclonal variation in vegetatively propagated chrysanthemum cultivars.

2.4.3.1. Restriction Fragment Length Polymorphism (RFLP)

RFLPs being co-dominant markers can detect coupling phase of DNA molecules, as DNA fragments from all homologous chromosomes are detected (Botstein *et al*., 1980). However, their utility has been hampered due to the large amount of DNA required for restriction digestion and Southern blotting. They have their origin in the DNA rearrangements that occurred due to evolutionary process, point mutation within the restriction enzyme recognition site sequences, insertion or

deletions within the fragments and unequal crossing over. Restriction enzymes are useful when it used with AFLP technique.

2.4.3.2. Diversity Arrays Technology (DArT)

Diversity Arrays Technology (DArT) is a DNA hybridisation-based molecular marker technique that can detect simultaneously variation at numerous genomic loci without sequence information. This efficiency makes it a potential tool for a quick and powerful assessment of the structure of germplasm collections. Risterucci *et al*., (2009) demonstrated the usefulness of DArT markers for genetic diversity analyses of 168 *Musa* genotypes from two of the most important field collections of *Musa* in the world viz., Cirad (Neufchateau, Guadeloupe) and IITA (Ibadan, Nigeria).

2.4.3.3. Sequence Tagged Sites (STS)

RFLP probes specifically linked to a desired trait can be converted into PCR based STS markers based on the nucleotide sequence of the probe. Using this technique, tedious hybridisation procedures involved in RFLP analysis can be overcome. This approach is extremely useful for studying the relationship between various species (Parrish *et al*., 1999) when these markers are linked to some specific traits.

2.4.3.4. Amplified Fragment Length Polymorphism (AFLP)

AFLP is a technique based on the detection of genomic restriction fragments by PCR amplification. The fingerprints are produced, without any prior knowledge of sequence, using a limited set of generic primers. AFLP has medium reproducibility but is labour intensive and has high operational and development costs (Karp *et al*., 1997).This technique thus shows an ingenious combination of RFLP and PCR techniques and is extremely useful in detection of polymorphism between closely related genotypes.

2.4.3.5. Variable Number Tandem Repeats (VNTR)

This is one of the powerful techniques for studying diversity utilising hyper variable regions of the gene comprised of tandemly repeated simple sequences. These repeats vary in number, and are therefore generally called VNTRs. It identifies repeated DNA regions of differing lengths resulting from variable numbers of serial repeats of a core DNA sequence (Crouch *et al*., 1999). These core sequences are referred to as mini-satellites or micro-satellites. Parasnis *et al*., (1999) developed microsatellite (GATA)*n* which reveals sex-specific differences in papaya.

2.4.3.6. Microsatellites

Microsatellites or simple sequence repeats (SSRs) are among several molecular markers, which are the most informative to characterise and assess the genetic variability of the genus *Musa* (Grapin *et al*., 1998), since these are highly polymorphic, multi-allelic, co-dominant, reproducible, easy to interpret, and amplified via polymerase chain reaction (PCR). The main disadvantage of these markers is the cost required to design oligonucleotide primers specific to the flanking sequences of the microsatellite DNA regions. Microsatellites are specific and highly polymorphic (Karp *et al*., 1997, Jones *et al*., 1997), but they require knowledge of the genomic sequence to design specific primers and thus are limited primarily to the economically important species.

2.4.3.7. Simple Sequence Repeats (SSRs)

Simple sequences repeats (SSRs), also called microsatellites, are tandem repeat motifs of di-, tri-, or tetra nucleotides and are abundant in all eukaryotic genomes (Hamada *et al*., 1982). Gillespie *et al*., (2005) determined genetic diversity between lines of *Vigna unguiculata* subspecies by AFLP and SSR markers.

2.4.3.8. Single Nucleotide Polymorphism (SNP)

SNPs occur frequently throughout the plant genome as they offer enormous potential in the discovery and detection of important genes in crops. Just as the name indicates, SNPs are identified by a single nucleotide base change in the genetic code at a specific location on the chromosome. Once discovered, these single-base differences can be used as molecular markers. Single nucleotide variations in genome sequence of individuals of a population are known as SNPs. They constitute the most abundant molecular markers in the genome and are widely distributed throughout genomes although their occurrence and distribution varies among species. Maize has 1 SNP per 60–120 bp (Ching *et al*., 2002), while humans have an estimated 1 SNP per 1,000 bp (Sachidanandam *et al*., 2001).

2.4.3.9. Inter Simple Sequence Repeat (ISSR) Markers

Among the molecular marker techniques available today, the PCR-based approaches are useful because of their simplicity and requirement for only small quantities of sample DNA. The choice of a molecular marker technique depends on its reproducibility and simplicity. ISSRs are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Amplification in the presence of non anchored primers also has been called microsatellite-primed PCR, or MP-PCR, (Meyer *et al*., 1993).

2.4.3.10. RAPD (Random Amplified Polymorphic DNA)

Welsh and McClelland (1991) developed a new PCR based genetic assay namely randomly amplified polymorphic DNA (RAPD). This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is

formed through PCR amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individual. They are dominant markers and hence have limitations in their use as markers for mapping, which can be overcome to some extent by selecting those markers that are linked in coupling (Williams *et al*., 1991).

Genetic variability in vetiver was initially studied by Kresovich et al. (1994). These authors investigated some selected accessions from the United States, employing molecular methods e.g. random amplified polymorphic DNA (RAPD technique) together with rigorous biometric analysis. Their data showed the feasibility and high degree of resolution of the method employed. RAPD patterns were very stable within clones.Non-fertile Huffmann and Boucard cultivars yielded essentially the same genotype, while three samples of the USDA PI 196257 accession (a seed introduction from North India) though morphologically similar were genetically distinct.

Deca-nucleotides are used as the primers for most RAPD analysis. The success of PCR is highly dependent on these small arbitrary oligo-nucleotides that hybridise onto the complementary DNA fragments. These short nucleotides function in pairs (a forward primer and a reverse one), and are used to amplify a set of DNA fragments (Lexa *et al*., 2001).

Adams(2000) used Random Amplified Polymorphic DNAs (RAPDs) to examine accessions of vetiver and related taxa from its region of origin and around the world. It appears only one *V. zizanioides* genotype, 'Sunshine', accounts for almost all germplasm utilized outside South Asia. Additional RAPD analyses revealed that several other non-fertile accessions are distinct genotypes.

Kresovich *et al*. (1994) reported on clonal variation of vetiver using RAPDs. They found RAPD patterns were very stable within clones and were able to distinguish between 'Huffman' and 'Boucard' plants, and the USDA PI 196257 introductions.

Dong *et al* (2004) studied 13 ecotypes of vetiver grass from 8 countries, the genetic relationships of them were analyzed by means of RAPD molecular makers. The results showed that a total of 220 reproducible RAPD fragments were produced using 18 random primers. 186 fragments (84.55% of the total observed) were polymorphic, which indicated that there were very high genetic diversity and conspicuous genetic differentiation within 13 ecotypes of vetiver grass.

The genomic relationships of Thai vetiver and related taxa have been intensively studied by two different techniques such as Single-Strand Conformational Polymorphism And Random Amplified Polymorphic DNA Technique. RAPD provided a simple, quick and reliable alternative to identify genetic variation (Srifah, 1999).

2.4.3.11. MSAP (Methylation Sensitive Amplification Polymorphism)

DNA methylation, especially methylation of cytosine in eukaryotic organisms, has received considerable attention in recent years. In animals and human beings, numerous studies suggest that DNA methylation has both epigenetic and mutagenic effect on various cellular activities such as differential gene expression, cell differentiation, chromatin inactivation, genomic imprinting and carcinogenesis (Gonzalgo and Jones, 1997). In vetiver, no literatue are reported on MSAP and hence in this chapter review on the MSAP of other crops are included.

DNA methylation variation has been hypothesized as an underlying mechanism of tissue culture induced mutagenesis due to the high frequency of quantitative phenotypic variation, the activation of transposable elements, heterochromatin-induced chromosome-breakage events, and the high frequency of sequence change (Selker and Stevens, 1985).

In higher plants, DNA methylation plays a role in gene expression (Meyer *et al*., 1994; Ulian *et al*., 1996; Rossi *et al*., 1997; Bergman and Mostoslavsky, 1998) as actively transcribed sequences are more often less methylated than the promoters and certain coding regions of silent genes (Finnegan *et al*., 1993). DNA methylation plays an essential role in regulating plant development. Recent research has demonstrated that DNA methylation plays an integral role in timing of flowering and in endosperm development in Arabidopsis (Finnegan *et al*., 2000). Significant differences in the level of cytosine have been observed among various tissue types in some plant species such as tomato (Messeguer *et al*., 1991), maize (Lund *et al*., 1995) and rice (Joel and Zhang, 2001).

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

In the past, nucleotide modified by methylation was not considered to be part of primary nucleotide sequence of an individual. However, since DNA methylation occurs at defined target sequences and not all target sites are methylated, it represents a potentially important form of polymorphism. In this way, epigenetic information systems, like DNA methylation, could generate epigenetic variation that had never been considered as the cause of phenotypic variation (Tsaftaris and Polidoros, 2000). DNA methylation, in addition to being the cause of epigenetic variation, is also cause of mutation and generation of genetic variation.

Methylated cytosine frequently deaminates to thiamine, thus 5mCytosines are hot spots for mutations, providing an interconnection between epigenetic and genetic variation. Dhar *et al*. (1990) studied the tissue specific adenine methylation in rice and also reported the differential methylation status of specific repeat sequences in rice during the transition from embryo to shoot without any external inducing factor. To obtain evidence for the possible involvement of DNA methylation, Tsaftaris and

Polidoros (1993) studied the genome activity and total DNA methylation in maize and found they are negatively correlated. Although there has been tremendous progress in genome research, mapping and molecular genetic studies of rice, little has been done on methylation status of the rice genome.

Xiong *et al*. (1999) assessed the extent and pattern of cytosine methylation in rice genome using the Methylation Sensitive Amplification Polymorphism (MSAP) in seedling and flag leaf tissues of an elite hybrid in China *viz*., Shanyou 63 and its parental lines. They observed a small proportion of the sites were found to be differentially methylated and suggested the utility of MSAP technique in studying the possible role of methylation in heterosis.

Tsaftaris *et al*. (1999) studied the pattern of methylation in certain sites of DNA in maize parental inbreds and their hybrids and reported that the hybrids in general are less methylated than parental inbreds. These findings supported the hypothesis made by several researchers that systematic selfing for isolation of inbreds leading to inbreeding depression lead to a gradual accumulation of more methylated sites which then could be released and/or repatterned when the selfed lines are crossed to generate hybrids (Tsaftaris and Polidoros, 2000).

Joel and Zhang (2001) studied the direction of methylation and its effect on heterosis in 27 crosses obtained in diallel fashion. They observed that the frequency of demethylation was higher at seedling stage and decreased with a remarkable increase in hyper-methylation in later stages and this change in the direction of methylation was found to be significantly correlated with heterosis.

Wang *et al*. (2004) studied the DNA methylation polymorphism in a set of elite cultivars and its possible contribution to inter-cultivar differential gene expression. They showed the association between transcriptional activity of mis-match repair (MMR) gene and its CpG methylation patterns. Takata *et al*. (2005) studied DNA methylation polymorphism in nine strains of *Oryza sativa* and *O*. *rufipogon* and

identified epi-markers (polymorphism of methylation state) and linked such polymorphism to phenotypic variation.

Parallel with the long-standing interest in the functional role of DNA methylation, there has been a series of developments in the methods used for detecting DNA methylation. There is currently a wide range of methods designed to yield quantitative and qualitative information on genomic DNA methylation. While the earliest approaches were concentrated on the study of overall levels of methylcytosines based on HPLC (Fraga *et al*., 2000) and *in situ* hybridization (Monpellier *et al*., 1994), the recent efforts have focused on the study of methylation status of specific DNA sequences.

The most widely used methods for studying DNA methylation patterns of specific regions of DNA with no base modifications are based on the use of methylation-sensitive and insensitive restriction endonucleases (Cedar *et al*., 1979). The most common isoschizomers used are the *Hpa*II/*Msp*I pair. Once DNA has been digested with restriction endonucleases, identification of methylation status of a particular sequence can be accomplished by Southern blot hybridization or PCR procedures. Because of its ease and amenability for high throughput routine screening, PCR based amplification and gel separation have become popular.

Reyna-Lopez *et al*. (1997) first demonstrated the MSAP technique in fungi and was later used by various workers in plants (Takata *et al*., 2005). In addition, novel methods designed to search for new methyl cytosine hotspots have yielded further data without requiring prior knowledge of the DNA sequence

Thanananta *et al* (2006) has adapted Methylation-sensitive amplified polymorphism (MSAP) technique to screen a photoperiod responsive gene of rice. They found MSAP as a very effective tool to reveal restriction fragment polymorphism

Zhong *et al* (2009) conducted a study to assess the DNA methylation alteration induced by salt stress by using MSAP. They suggested that the changes in DNA methylation can be considered either a simple indirect effect of salt stress or a precise defensive mechanism for regulating the gene expression.

MSAP is a powerful tool for genotypic characterisation of somatic embryo derived grapevines. DNA methylation variation in somaclones compared to mother clones were found. The detection of the same polymorphic bands in numerous somaclones of different cultivars suggests the possibility of hot spots of DNA methylation variation (Schellenbaum *et al*, 2008)**.**

Kadam, A.R (2012) report methylation as an important factor which contributed to variation in micropropagated banana plants. MSAP technique was used to detect cytosine methylation using 4 primer combinations and it was found that MSAP assay could detect variation in methylation pateern in micropropagated banana plants.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Induction of somaclones in vetiver (*Chrysopogon zizanioides* L. Roberty)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, during the period from 2010 to 2012 with the objective of inducing somaclonal variation in vetiver through callus culture and the analysis of variation using morphological, molecular and biochemical markers. Description of the materials used and the methodology adopted in the study has been furnished in this chapter.

3.1 MATERIALS

3.1.1 Planting Material

The south Indian vetiver accession ODV-23 was collected from Aromatic and Medicinal Plant Research Station, Odakkali and planted in the CPBMP garden (Plate 1).

3.1.2 Explant

The explant material collected was the unopened inflorescence from the variety ODV 23 from Aromatic and Medicinal Plant Research Station, Odakkali (Plate 2).

3.1.3 Chemicals, Glassware and Plastic Wares

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

Plate 1. Vetiver plant in CPBMB garden

3.1.4 Equipment and Machinery

The present research work was carried out using the Molecular Biology and Tissue Culture facilities and equipment items available at CPBMB, College of Horticulture. Quantification of DNA was done by NanoDrop^{R} ND-1000 spectrophotometer. The PCR was done in the thermal cycler of model Eppendorf Master Cycler (Eppendorf, USA) and the horizontal gel electrophoresis system (BIO-RAD, USA) was used for agarose gel electrophoresis. Gel Doc- BIO-RAD was used for imaging and documenting the agarose gel. The details are given in Annexure I.

3.2 Methods

3.2.1 Preparation of culture media

Standard procedures (Gambog and Shyluk, 1981) were followed for the preparation of plant tissue culture media. Stock solutions of major and minor nutrients were prepared and stored in pre-cleaned amber coloured bottles in refrigerated conditions.

A clean steel vessel rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel. For preparing media with the full strength ,20 ml was pipetted from 50X stocks and 10 ml from 100X stocks. A small volume of distilled water was added to it and later on, required quantities of sucrose and inositol were added and dissolved in it. The desired volume was made up by adding distilled water. The pH of the medium was adjusted to 5.7 using 0.1N NaOH.

For making the solid media, agar was added at 0.70 per cent (w/v) concentration after adjusting the pH. The medium was stirred and heated to melt the agar and was poured when hot into culture vessels and which were plugged with non-absorbent cotton. Test tubes and jam bottles were used as culture vessels. Vessels

containing media were sterilized in an autoclave at 121°C in 15psi for 20 min. The medium was allowed to cool to room temperature and stored in the culture room until used.

3.1.5. Carbon source

Sucrose was used as the main carbon source (3.0 per cent) in this study

3.1.6 Growth regulators

Auxins (2,4-D, IAA) and cytokinins (BA, Kinetin) were incorporated in the media at various stages of culture for callus induction, regeneration, multiplication and rooting.

3.1.7 Transfer Area and aseptic manipulations

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants, subsequent sub culturing, regeneration of shoots and induction of roots studies were carried out under the hood of a laminar air flow cabinet fitted with UV lamp.

3.1.8 **Culture room**

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

3**.2 Standardization of media**

For standardization of callus induction, the following 28 different growth regulator combinations (Table 1) were tried with basal MS media (Details of composition are given in Anexure II).

Table 1. Growth regulator combinations for callus induction

Sl. No	2,4-D (mgl^{-1})	BA (mgl^{-1})	Kinetin (mgl^{-1})
1.	0.25	0	0
2.	0	0.25	0
3.	0	0	0.25
4.	0.25	0.25	0
5.	0.25	0	0.25
6.	0	0.25	0.25
7.	0.25	0.25	0.25
8.	0.5	0	0
9.	0	0.5	0
10.	0	0	0.5
11.	0.5	0.5	0
12.	0.5	0	0.5
13.	0	0.5	0.5
14.	0.5	0.5	0.5
15.	1.0	0	0
16.	0	1.0	0
17.	0	0	1.0
18.	1.0	1.0	0
19.	1.0	0	1.0
20.	0	1.0	1.0
21.	1.0	1.0	1.0
22.	1.5	0	0
23.	0	1.5	0
24.	0	0	1.5
25.	1.5	1.5	0

1. The inflorescence

2. The part used for inoculation

Plate 2. Inflorescence and its parts used for inoculation

26.	**1.5**	**0**	**1.5**
27.	**0**	**1.5**	**1.5**
28.	**1.5**	**1.5**	**1.5**

3.2.2 Inoculation of explants to the media

The unopened inflorescences were collected from Aromatic and Medicinal Plants Research Station Odakkali. They were cut into small pieces without removing the sheath and were first washed with liquid soap for 5 minutes to remove surface adhered particles of dirt. Then the segments washed using tap water to remove the soap traces. Further, surface sterilization was carried out under the hood of laminar airflow cabinet. The segments were treated with 70 per cent alcohol for 1 minute. The inflorescence segments were then treated with 0.1 per cent $HgCl_2$ for 30 seconds followed by 4 times rinsing with distilled water. The inflorescence segments were then blotted dry using sterile blotting paper and the sheath was cut opened and were inoculated into the test tubes. The test tubes were then placed in the dark culture room for two days and then transferred to light rooms for callus induction.

Callus Index (CI) is computed by multiplying per cent cultures initiating callus with growth score (G), which was assessed by visual rating (poor=1, medium=2, good=3, and profuse=4).

3.2.3 Sub culturing of callus

For inducing more variation and to increase the callus mass, sub culturing upto 4 times was done in the respective callus induction media

3.2.4 Regeneration of shoots from callus

Various concentrations of BA were used for shoot regeneration from inflorescence derived callus are given in Table 2.

Table 2. Growth regulator combinations for shoot regeneration

Sl.No.	BA (mgl^{-1})
1.	1.0
2.	2.0
3.	3.0
4.	4.0
5.	5.0

3.2.5 Rooting of shoots

Various concentrations IAA as mentioned below were used for induction of roots in regenerated shoots.

Table 3. Growth regulator combination for root induction

Sl.No.	IAA (mgl^{-1})
1.	0.5
2.	1.0
3.	1.5
4.	2.0
5.	2.5

3.2.6 Hardening and transplanting of plantlets

Hardening was carried out in paper pots filled with sterile sand. They were kept in poly house conditions. The hardened plants were transplanted to earthen pots filled with potting mixture and then kept in poly house conditions.

3.3. STANDARDISATION OF GENOMIC DNA ISOLATION

The procedures reported by Doyle and Doyle (1987) for the extraction of nucleic acids were used for the isolation of genomic DNA. The young leaves from

healthy plants were collected early in the morning and were frozen in liquid nitrogen and stored at -80 ºC and later used for the genomic DNA isolation.

3.3 DNA Isolation (CTAB method)

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

1. 2 X CTAB Extraction buffer (2x)
2. 10 per cent CTAB solution
3. TE buffer
4. Isopropanol
5. Chloroform: Isoamyl alcohol (24:1, v/v)
6. Ethanol 100 and 70 per cent

3.3.2 Protocol:

Young and tender leaf tissue (1g) was weighed and ground in liquid nitrogen using mortar and pestle along with 50 µl of ß- mercaptoethanol and a pinch of Poly Vinyl Pyrrolidone (PVP). The sample was ground into fine powder using excess liquid nitrogen and 4ml of extraction buffer (2x) and the powder was transferred to a sterile 50 ml centrifuge tube containing 3 ml of pre-warmed extraction buffer (total 7 ml). The homogenate was incubated for 30 minutes at 65 ºC with intermittent mixing. Equal volumes of chloroform and isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was collected, 0.6 volume of chilled isopropanol was added and incubated at -20 ºC for 30 minutes. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4 ºC. The pellet was collected and washed first with 5mM ammonium acetate, 70 per cent alcohol and later with 100 per cent alcohol. It was air dried for 30 minutes at room temperature and dissolved in 100 µl of TE buffer.

3.4 DNA Purification

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

3.4.1 Reagents

1. Phenol : chloroform mixture (1:1,v/v)
2. Chilled isopropanol
3. 70% Ethanol
4. TE buffer
5. Chloroform: Isoamyl alcohol (24:1,v/v)
6. 1.0 % RNase

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

RNase solution (2 µl) was added to 100 µl DNA sample and incubated at 37 ^{0}C in dry bath (GeNei, Thermocon) for 1 hour. The volume was made up to 250 µl with distilled water and equal volumes of phenol: chloroform (1:1) mixture was added. This was then centrifuged at 12,000 rpm for 10 minutes at 4 ^{0}C. The aqueous phase was collected in a fresh micro centrifuge tube and added equal volumes of chloroform: isoamyl alcohol (24:1). Again it was centrifuged at 12,000 rpm for 10 minutes at 4 ^{0}C. The above two steps were repeated and finally DNA was precipitated from the aqueous phase with 0.6 volume of chilled isopropanol. The mixture was then incubated at -20 ^{0}C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4 ^{0}C. The pellet of DNA obtained was washed with 70 per cent ethanol. The pellets were air dried and dissolved in 100 µl TE buffer.

3.4.2 Assessing the quality of DNA by electrophoresis

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

Materials for agarose gel electrophoresis

1) Agarose (SRL, Low EEO)
2) 50X TAE buffer (pH 8.0)
3) 6X Loading/ Tracking dye
4) Electrophoresis unit, power pack (BIO-RAD), casting tray, comb
5) Ethidium bromide solution (0.5 µg/ml)
6) UV transilluminator (Wealtec)
7) Gel documentation and analysis system (BIO-RAD)

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

1X TAE buffer was prepared from the 50 X TAE stock solutions. Agarose (0.9 per cent) was weighed and dissolved in TAE buffer by boiling. Then ethidium bromide was added at a concentration of 0.5 µg ml^{-1} and mixed well. The open end of gel casting tray was sealed with a cellotape and kept on a horizontal levelled surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 20-25 minutes after which the comb was removed carefully. The tray was kept in the electrophoresis unit with the well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (4µl) along with the tracking dye (1µl) was loaded into the wells using a micropipette carefully. λDNA/*Eco*RI+*Hind*III double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100 V) and current (50 A). The power was turned off when the tracking dye reached 2/3rd length of the gel.

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

3.4.3. Assessing the quality and quantity of DNA by NanoDrop method

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

1OD at 260 nm = 50 µg DNA/ml

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

3.5. Screening for somaclones

3.5.1. RAPD (Random Amplified Polymorphic DNA) ASSAY

The good quality genomic DNA isolated from the leaves of the somaclones by CTAB method were subjected to RAPD assay as per the procedure reported by Williams *et al*. (1990). Random decamer primers supplied by 'Operon Technologies' USA with good resolving power were used for amplification of DNA. Decamer primers for RAPD assay were selected after an initial primer screening.

3.5.1.2. Screening of Random Primers for RAPD Analysis

The basic RAPD procedure suggested by Venkatachalam *et al*. (2008) was used for the primers. Fourty decamer primers in the series OPS, RN, and OPA, were screened with the genomic DNA of mother plant.. Genomic DNA at the concentration of 40 ng was subjected to amplification using selected random primers.

PCR reactions were carried out in an Eppendorf Master Cycler (Eppendorf, USA). Each reaction tube contained 100 ng template DNA, 2.5 mM $MgCl_2$, 100 µM of dNTPs mixture, 1x *Taq* buffer, 100 pM of decanucliotide primer and 1 unit of *Taq* DNA polymerase made up to a final volume of 20 µl. A master mix of all reagents for the required number of reactions was prepared first and aliquots were dispended into PCR tubes followed by addition of template DNA in each tube, and one tube without template DNA as control.

Composition of the reaction mixture for PCR (20.0 µl)

Genomic DNA (40 ng)	2.0 µl
1X *Taq* buffer	2.0 µl
10mM dNTP mix	1.0 µl
2.5 mM $MgCl_2$	1.6 µl
Decamer primer(100 pM)	1.0 µl
1 U *Taq* DNA polymerase	0.3 µl
Autoclaved distilled water	12.1 µl
Total volume	= 20.0 µl

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

Step 1: 94^{o}C for 5 min. - Initial denaturation

Step 2: 94°C for 1 min. - Denaturation
Step 3: 37°C for 1 min. - Annealing } 35 cycles
Step 4: 72°C for 2 min. - Extension
Step 5: 72°C for 10 min. - Final extension
Step 6: 4°C for 10 min. - Cooling of samples

The amplified products were run on 1.4 per cent agarose gel using 1x TAE buffer stained with ethidium bromide along with marker (λDNA/*Hind*III+*Eco*RI double digest). The profile was visualised under UV transilluminator and documented using gel documentation BIO-RAD system. The documented RAPD profiles were carefully examined for polymorphism. Number of bands produced by each primer was counted and tabulated. Those primers, which gave good amplification with more than four bands were selected for further studies.

3.5.3 Methylation-sensitive amplification polymorphism (MSAP) assay

The method was adapted from Reyna-Lopez *et al*. (1997) who modified the protocol for AFLP described by Vos *et al*. (1995) to incorporate the use of methylation-sensitive restriction enzymes. The modified protocol involved the use of the isoschizomers, *Hpa*II and *Msp*I in place of *Mse*I as the frequent cutter while the rare cutter *Eco*RI was unchanged. Both *Hpa*II and *Msp*I recognize the same tetranucleotide sequence (5'-CCGG-3'), but display differential sensitivity to DNA methylation. *Hpa*II is inactive when any of the two cytosines is fully methylated (both strands methylated), but cuts the hemi-methylated 5'-CCGG-3'; whereas, *Msp*I cuts when an internal cytosine is methylated (5'-C5mCGG-3') but not when external cytosine is methylated (5'-5mCCGG-3'). The MSAP assay is described here under the following heads *viz*., restriction digestion, adapter ligation, pre-amplification, selective amplification and polyacrylamide gel separation followed by silver staining.

3.5.3.1 Restriction digestion of genomic DNA

Two sets of digestion reactions, with *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I, were carried out simultaneously. The genomic DNA and the reagents were taken in a centrifuge tube as mentioned in the Table 4. Then it was mixed gently and centrifuged. The mixture was incubated for 5 hours at 37^0C. The restriction was then inactivated at 65^0C for 15 minutes and then placed on ice.

Table 4. Reagents for Restriction Digestion of genomic DNA

Sl.No.	**Component**	**Tube I**	**Tube II**
1	Genomic DNA	1 µl	1 µl
2	10 x buffer B	5 µl	5 µl
3	BSA (10 µg/ µl)	0.5 µl	0.5 µl
4	Enzyme	Eco R1 +MSP - 2 µl	Eco R1+HPA - 2 µl
5	Distilled water	41.5 µl	41.5 µl
Total		50 µl	50 µl

3.5.3.2 Adapter ligation

The adapter and primer for the 'less-frequent cutter' enzyme *Eco*RI is the same as that used in a standard AFLP analysis as described by Vos *et al*. (1995). The adapters and primers for the isoschizomers *Hpa*II and *Msp*I were modified in order to be compatible with their recognition sequence 5'-CCGG-3'. The adapters were prepared by mixing equimolar amounts of the two DNA strands, incubating the mixture at 95 ºC for 5 min and then cooling it down to room temperature. An adapter ligation solution containing 0.5 pM *Eco*RI adapter, 5 pM *Hpa*II – *Msp*I adapter, 1 mM ATP in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM DTT, 25 µg/ml BSA and 10 U T4 DNA ligase was prepared. The digested DNA fragments from the two reactions were ligated separately with adapter/ligation solution by incubating at ^{230C} for 5 hours. The

ligation mixture was diluted in a 1:10 ratio with sterile water and used as template in the pre-amplification. The quantity of different reagents used are given in Table 5.

5. Reagents used for Adapter Ligation

Sl.No.	Component	Tube I	Tube II
1	1 X T_4 DNA Ligase Buffer	2.0 µl	2.0 µl
2	10 V T_4 DNA Ligase	3.0 µl	3.0 µl
3	5 p mol Eco R1 Adapter	2.5 µl	2.5 µl
4	5 p mol MSP I HPA II adapter	2.5 µl	2.5 µl

3.5.3.3 Pre-amplification PCR

Preamplification was conductcd by using 5 µl of the above diluted ligation product with *Eco*RI + 'A' (one nucleotide) and *Hpa*II/*Msp*I primers in a volume of 50 µl containing 1X PCR buffer, 0.1 mM each dNTP, 5 pM of each primer and 1 U Taq polymerase. The details of reagents used and its quantity is given in Table 6.

Table 6. Details of reagents used for pre-amplification

Sl.No.	Component	Tube I	Tube II
1	Ligated Product	5 µl	5 µl
2	Eco R1 + 0 primer	3 µl	3 µl
3	MSP I + 0 primer	3 µl	0
4	HPA II + 0 primer	0	3 µl
5	$MgCl_2$	3 µl	3 µl
6	dNTP	2 µl	2 µl
7	Taq Polymerase	1 µl	1 µl

8	Taq Buffe B	5 µl	5 µl
9	Distilled Water	28 µl	28 µl
Total		50 µl	50 µl

The PCR reaction involved 20cycles of 94^0 C for 1 minute, 56^0 C for for 1 minute, 72^0 C for 2 min with a final extension at 600 C for 30 min. After completion, 10 µl of product was loaded on 2 per cent agarose gel and the presence of low molecular weight smear indicated successful amplification. The pre-amplified products were then diluted 50 times its volume with sterile water and further used as template for the selective amplification.

3.5.3.4 Selective amplification PCR

Using 3 primer combinations selective amplifications were conducted in volumes of 20 µl containing 5 µl of the diluted pre-amplified product as template, 1X PCR buffer, 1.5 mM $MgCl_2$, 5 pM each of *Eco*RI and *Hpa*II/*Msp*I primers and 1 U Taq polymerase. The PCR amplification reactions were performed using the touch-down cycles as described in the original AFLP protocol (Vos *et al*., 1995). The deatails are given in Table 7.

Table 7. Details of reagents and their quantity for selective amplification

Sl.No.	**Component**	**Tube I**	**Tube II**
1	Diluted sample	5.0 µl	5.0 µl
2	Eco R1 + 2 primer	4.0 µl	4.0 µl
3	MSP I + primer	2.0 µl	-
4	HPA II + primer	-	2.0 µl
5	$MgCl_2$	1.5 µl	1.5 µl
6	dNTP	1.0 µl	1.0 µl

7	Taq Polymerase	2.0 µl	2.0 µl
8	1 X PCR Buffer	2.0 µl	2 µl
9	Distilled Water	2.5 µl	2.5 µl
Total		**20 µl**	**20 µl**

The PCR reaction steps and temperature profile are as follows.

- 1 Initial denaturation 94^0C for 2 min
- 2 Denaturation 94^0C for 30 s
- 3 Primer annealing 65^0C for 30 s
- 4 Primer extension 72^0C for 1.5 min
- 5 No. of cycles Cycle to step 2 for 10 times
- 6 Denaturation 94^0C for 30 s
- 7 Primer annealing 56^0C for 30 s
- 8 Primer extension 72^0C for 2 min
- 9 No. of cycles Cycle to step 6 for 30 times
- 10 Final extension 60^0C for 20 min

3.5.3.5 Polyacrylamide gel electrophoresis of PCR products

The amplified products were separated using Sequi-Gen® GT gel system (BioRad, USA). The laboratory protocol for polyacrylamide gel electrophoresis is described here.

Step 1: Preparation of the inner plate (notch plate)

The plate was wiped with surgical spirit using tissue paper. 250 µl of Repel silane (Dimethyldichlorosilane, Pharmacia Biotech, USA) in 750 µl of the acetoethanol was applied and spread evenly with a tissue paper and allowed to dry for 5 min.

Step 2: Preparation of the outer plate

About 3 µl of Bind silane (g-methacryloxypropyl trimethoxysilane, Sigma, USA) in 1 ml acetoethanol was applied on the plate and spread uniformly with tissue paper and allowed to dry for 5 min.

Step 3: Assembling and pouring the gel

A pair of 0.4 mm spacers was placed along the vertical edges of the notched plate above which the outer plate, with Bind silane-coated surface facing the spacers, was placed. The glass plates and the spacers were held in position using lever clamps and this assembly was fitted above the precision caster base and kept horizontally. Flat-side of the 48 well shark-tooth comb was placed between the two plates in such a way that no air bubble is trapped. 80 µl of TEMED (N,N,N',N'-tetramethylethylene diamine, Sigma, USA) and 800 µl of 10% ammonium per sulphate was added to 80 ml of 6% acrylamide solution and the gel solution was injected steadily from the bottom with the help of syringe, till it reached the top. The gel was allowed to polymerise for 20 min.

Step 4: Setting up of gel assembly and Pre-run

The gel-sandwich was fixed to the electrophoresis apparatus with 1X TBE in the lower and upper buffer chambers. The comb was carefully removed and the well surface was rinsed with the buffer using a syringe. The gel was set to run at 80 W, 2000 V maximum, 50mA setting for about 45 min to 1 h to achieve a gel surface temperature of 50 °C.

Step 5: Sample preparation

About 5 µl of formamide-loading dye was added to the PCR product and the samples along with molecular weight markers were denatured at 99 °C for 4 min using

thermocycler (Perkin Elmer 9700, USA). The samples were immediately chilled by placing them on ice.

Step 6: Loading of samples and running the gel

After the pre-run, the well area was rinsed to clean the crystallized urea with a syringe and the shark tooth comb was carefully inserted in to the well area till the point where the tips of the teeth just touch the gel surface. Six micro litre of denatured sample was loaded quickly in order to avoid the drop in the gel surface temperature. Then, the gel was set to run at 100 W, 3000 V maximum, and 50 mA while maintaining a constant gel temperature of 500 C. When the xylene-cyanol dye front has run about 2/3 of the gel length, the unit was stopped and the buffer was drained. The plate was removed from the apparatus for staining.

3.5.3.6 Silver staining

Silver staining of polyacrylamide gel was followed as described by Panaud *et al*. (1996). The gel plate was kept in a tray and 2 l of 10 per cent acetic acid solution (fixing solution) was poured and incubated for 20 min on a rotary shaker. Then the fixing solution was removed and the gel was washed twice in fresh double distilled water by keeping it on a shaker for 2 min each. The water was poured out and 2 l of silver staining solution was added and shaken gently for 20 min. The silver stain was poured off and the gel was washed briefly in double distilled water for 5-10 s. The gel was quickly drained and the chilled developer solution was added and shaken gently until the bands developed to optimum intensity. The developer solution was poured off and the gel was incubated in fixing solution for 10 min in a shaker. The gel was washed in double distilled water for 10 min and air dried overnight. The gel was scanned using a scanner and documented. The gels were kept above the illuminator and scored.

3.5.3.7 Data analysis and interpretation

3.5.3.7.1 Scoring and calculation of methylation levels

The scoring of differential methylation pattern was based on the presence and absence of bands, assuming each band represented a restriction site of CCGG. Since *Hpa*II is sensitive to full methylation (methylation of both strands) of either cytosine residue at the recognition site and *Msp*I is sensitive only to methylation at the external cytosine, a band detected in the *Eco*RI + *Msp*I digest but not in the *Eco* I + *Hpa*II was referred as resulting from an internal methylation. Conversely, a band detected in *Eco*RI + *Hpa*II digest but not in the *Eco*RI + *Msp*I digest was referred to be the result of a hemi methylation. The number of internal and hemi methylation sites were counted in each genotype, and expressed in percentage against the total number of bands detected (Joel and Zhang, 2001). The methylation percentage was calculated as follows.

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

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

$$\text{Percent total methylation (\%TM)} = \%HM + \%IM$$

3.6. Biochemical analysis

3.6.1. Solvent extraction of vetiver roots

Plants for chemical analyses were uprooted carefully and the shoot portion was cut and removed. The tuft of roots was cleaned by washing with a gentle stream of running water to remove soil and other adhering particles taking care that the finer roots were not lost. The cleaned roots were dried under shade for 3 to 4 days, turning them over at regular intervals until dry. The dry roots were cut into pieces of about 2.5 cm in length using a knife and ground to fine powder in a grinder. The ground samples were stored in glass bottles until analysis.

The roots were extracted with solvent to collect the oil and resinous components in them. One to two grams of the sample powder was soaked with 100ml acetone for 24 hours in a round bottom flask, stirring the contents intermittently. The contents were then refluxed for half an hour. The extract was collected by decantation. The residue was reextracted in a similar manner with 100 ml hexane. The acetone and hexane extracts were bulked and the total extractable matter was recovered by distilling off the solvent at reduced pressure in a rotary vacuum evaporator. The yield of solvent extractable was recorded.

Oleoresin obtained was dissolved in 10 times it weight of acetone, centrifuged for 5 min. at 2500 rpm and conserved for gas chromatographic analysis.

3.6.2. Gas chromatography

Gas chromatography was performed using a Master GC equipment (M/s. DANI, Italy). The chromatographic conditions are given below.

a. Column: DB-5 (WCOT capillary column)
b. Length of column : 30 m
c. Column diameter: 0.25 mm
d. Coating thickness: 0.25 µ
e. Injection volume: 1 µl
f. Split ratio: 0-100 (selected as per sample)
g. Injector temperature : 240^{o}C

h. Detector type: Flame ionisation detector

i. Detector temperature : 250 ^{o}C

j. Column temperature and nitrogen flow rates:

Time (min)	Temperature (^{o}C)	Nitrogen flow rate (ml/min)
0	90	1.0
60	270	1.0
65	270	1.2
75	270	2.0

k. Flow rate of hydrogen : 40 ml/min

l. Flow rate of air : 250 ml/min

m. Instrument control and Data analysis: were undertaken using CLARITY software (M/s. DANI, Italy).

Results

4. RESULTS

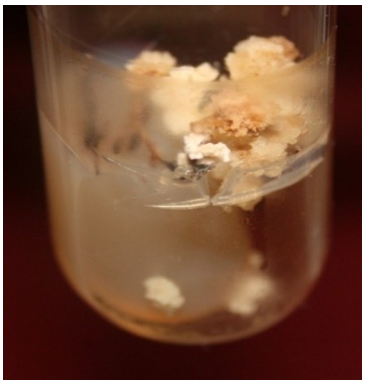
The results of the investigations conducted on "Induction of somaclones in *Chrysopogon zizanioides* L Roberty" undertaken during the period from 2010 to 2012 at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara are presented in this chapter under the following different subheadings.

1. Screening of media for callus induction
2. Screening of media for regeneration
3. Screening of media for root induction
4. Hardening and transplanting
5. Isolation of Genomic DNA
6. Quantification of Genomic DNA
7. RAPD assay
8. MSAP assay
9. Biochemical analysis

4.1. Screening of media for callus induction

The explants were screened for callus induction in 28 different media combinations. The basal media used was full MS. Out of this, seven growth regulator combinations responded with profuse callus formation. The calli formed were friable in nature and off white in colour (Plate 3). The number of days taken for callus induction and the callus index varied according to the growth regulator combinations. The results obtained with respect to callus induction are given in Table 8.

The best media recorded for callus induction as per the study was 1.5mgl^{-1} 2,4-D, which was having a callus index of 200.



0.5 mg/l 2,4-D

0.5 mg/l 2,4-D+KINETIN

1 mg/l 2,4-D

1 mg/l 2,4-D+KINETIN

1 mg/l 2,4-D+KINETIN+BA

1.5 mg/l 2,4-D

1.5 mg/l 2,4-D+KINETIN

Plate 3. Callus induction in different growth regulator combinations.

Table 8. Response of inflorescence explants to callus induction in media with various growth regulator combinations

Basal media used : Full MS

Treatment No.	Days taken for callus initiation	Contamination (%)	Nature of callus	Cultures initiating callus(%)	Average Callus score	Callus index
1	-	16.6	-	0.0	0	0.0
2	-	22.2	-	0.0	0	0.0
3	-	22.2	-	0.0	0	0.0
4	-	11.1	-	0.0	0	0.0
5	-	33.3	-	0.0	0	0.0
6	-	5.5	-	0.0	0	0.0
7	-	0.0	-	0.0	0	0.0
8	19	11.1	Compact	72.2	1.92	138.88
9	-	16.6	-	0.0	0	0.0
10	-	0.0	-	0.0	0	0.0
11	20	16.6	Compact	55.5	1.7	94.40
12	-	33.3	-	0.0	0	0.0
13	-	33.3	-	0.0	0	0.0
14	-	11.1	-	0.0	0	0.0
15	14	16.6	Compact	61.1	1.36	83.33
16	-	11.6	-	0.0	0.0	0.0

17	-	5.5	-	0.0	0.0	0.0
18	15	16.6	Compact	50	1.22	122.00
19	-	22.2	-	0.0	0.0	0.0
20	-	22.2	-	0.0	0.0	0.0
21	14	16.6	Compact	77.7	1.71	133.00
22	19	0.0	Compact	83.3	2.4	200.00
23	-	33.3	-	0.0	0.0	0.0
24	-	16.6	-	0.0	0.0	0.0
25	19	5.5	Compact	61.1	2.09	127.70
26	-	5.5	-	0.0	0.0	0.0
27	-	22.2	-	0.0	0.0	0.0
28	-	5.5	-	0.0	0.0	0.0

4.2. Screening of media for regeneration

Various concentrations of the growth regulator BA with full MS were used for regenerating shoots from the subcultured callus tissue. The response of calli to regeneration was different for different concentrations of growth regulators (Plate 4). The nature of regenerants with respect to leaf shape and growth pattern was also different according to the media. The details are given in Table 9.

It is noticed that as the growth regulator combination increases, the number of shoots regenerated also increases. More number of shoots were observed in medium with BA concentration of 5.0 mgl^{-1}.

1mg/l BA

2 mg/l BA

3 mg/l BA

4 mg/l BA

5 mg/l BA

Plate 4. Response of callus to different regeneration media

Table 9. Response of various callus cultures to regeneration under various concentrations of growth regulators

Basal media used : Full MS

Growth regulator combination -BA (mgl^{-1})	Days taken for regeneration	No.of shoots regenerated		Length of regenerated shoots	
		3 weeks	8 weeks	3 weeks (cm)	8 weeks (cm)
1	14	2.0	6.0	0.8	4.6
2	12	2.0	7.0	0.76	5.1
3	14	4.0	11.0	1.1	5.2
4	13	3.0	14.0	0.73	4.9
5	11	4.0	12.0	1.6	5.6

4.3. Screening of media for root induction

Various concentrations of IAA with full MS were used for the induction of rooting in the regenerants (Plate 5). The nature of roots, no.of days taken for rooting are varied with varying doses of IAA used. As the concentration increased the regenerants become more curly in nature. The details are given in Table 10.

It was noticed that as the growth regulator combination increases, the number of roots induced also increase. More number of roots were observed in medium with IAA concentration of 2.5 mgl^{-1}.

0.5mg/l IAA

1.0 mg/l IAA

1.5mg/l IAA

2 mg/l IAA

2.5 mg/l IAA

Plate 5. Response of regenerants for different rooting media

Table 10. Response of regenerants to root induction under various growth regulator concentrations

Basal media used : Full MS

Growth regulator combination (mgl^{-1})	Days taken for root initiation	Length of roots (cm)		No.of roots	
		2 weeks old	4 weeks old	2 weeks old	4 weeks old
0.5 IAA	11	0.2	0.8	1	4
1.0 IAA	10	0.4	1.1	1	3
1.5 IAA	11	0.4	1.3	2	5
2.0 IAA	9	0.3	1.2	2	4
2.5 IAA	9	0.2	1.4	2	6

4.4. Hardening .

Hardening of rooted plantlets were done in cups filled with sand as well as in cups filled with potting mixture in poly house (Plate 6). The hardening was done under poly house conditions. Only plants hardened in cups filled with sand survived.

After hardening, the somaclones were classified based on the growth regulator combination used for callusing. A total of seven groups were marked for further analysis. The details of hardening and classification are given in Table 11.

Table 11. Details of hardening of the somaclones

Plant Group	**Treatment No.**	**No.of leaves (One month old)**	**Height (One month old) (cm)**	**Survival (%)**
SC 1	8	9	11.5	33.0

7 days after hardening

30 days after hardening

Plate 6. Somaclones after hardening

SC 2	11	7	9 .0	40.0
SC 3	15	11	12 .0	42.8
SC 4	18	9	7.8	37.5
SC 5	21	7	9.5	60 .0
SC 6	22	10	9.0	44 .0
SC 7	25	11	10.5	50 .0

4.5. Transplanting

The hardened plantlets are transplanted to the pots filled with potting mixture (Plate 7). The growth parameters observed in transplanted plantlets are presented in the Table 12. Three plants per clone are maintained at CPBMB.

Table 12. The response of somaclones upto 6 months after planting

Plant Group	***Height (cm)***				***No.of leaves***			
	7 days	***14 days***	***1 month***	***6 months***	***7 days***	***14 days***	***1 month***	***6 months***
SC 1	*15*	*17*	*26*	*94*	*8*	*10*	*16*	*74*
SC 2	*13*	*14*	*22*	*105*	*5*	*5*	*11*	*96*
SC 3	*10*	*12*	*25*	*103*	*8*	*10*	*17*	*107*
SC 4	*9*	*9.5*	*15*	*81*	*5*	*4*	*12*	*66*
SC 5	*16*	*19*	*27*	*109*	*14*	*17*	*22*	*108*
SC 6	*16*	*17*	*25*	*114*	*12*	*14*	*19*	*106*
SC 7	*9.5*	*11.5*	*18*	*91*	*8*	*8*	*16*	*71*

Plate 7. The response of somaclones to transplanting at 6 months stage

Morphological observation shows that there is variation in the height and number of leaves produced. The growth parameters are best for somaclone number 6 and poor for somaclone number 4.

4.6. Standardisation of DNA Isolation

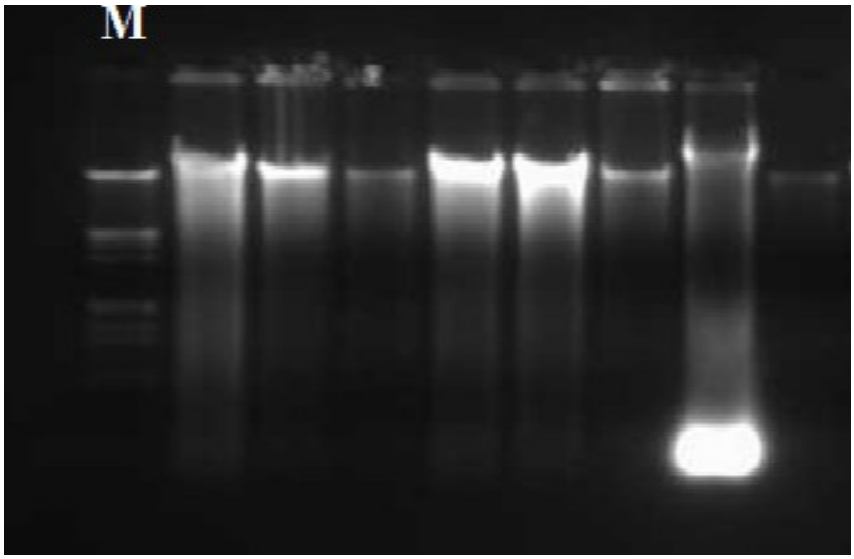
The CTAB method (Doyle and Doyle, 1987) was used for the extraction of genomic DNA from vetiver and the details are shown in Plate 8. The tender leaves were the best for DNA isolation with respect to yield and purity. The quality of DNA isolated using the aforesaid protocols was assessed using agarose gel electrophoresis. Good quality genomic DNA was obtained using CTAB method without using 10 per cent CTAB. Treatment with RNAse gave more intact DNA.

4.7. Quantification of DNA

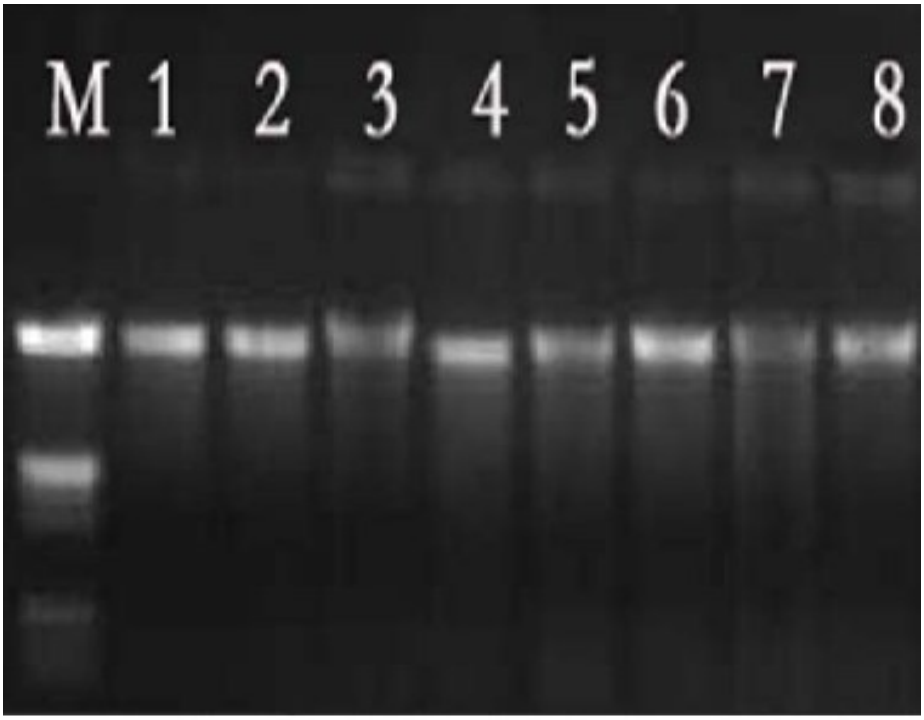
The quality and quantity of isolated DNA were analysed by both electrophoresis and NanoDrop^{R} ND-1000 spectrophotometer. In both the cases, intact clear bands indicated that non-degraded DNA was obtained. The ratio of absorbance ranged between 1.8- 2.0 which indicated that the quality of DNA was good (Table 13). The DNA after appropriate dilutions was used for further studies

Table 13. Quality and quantity of genomic DNA isolated from somaclones

Sr. No.	Somaclones	Quantity (ng/ml)	Absorbance 260/280	Quality
1	SC 1	1333.35	1.92	Good
2	SC 2	1754.52	1.88	Good
3	SC 3	1045. 33	1.84	Good
4	SC 4	1344.21	1.89	Good
5	SC 5	936.20	1.90	Good
6	SC 6	1253.50	1.87	Good
7	SC 7	954.20	1.93	Good



Quality of DNA isolated from different Somaclones by CTAB method



Intact DNA after treatment with RNase A

M – Marker, 1 - Mother plant, 2 – SC 1, 3 - SC 2, 4 – SC 3 , 5 – SC-4, 6 – SC 5, 7 – SC 6, 8 – SC 7

Plate 8. Standardisation of DNA isolation from somaclones

4.8. RAPD ANALYSIS

The DNA of seven somaclones and the mother plant was subjected to RAPD analysis. It was screened with random primers and then with selected primers and finally the analysis of results was carried out using NTSYS software.

4.8.1 Screening of primers for RAPD analysis .

The preliminary screening of primers was done with the mother plant and 10 primers belonging to different RAPD primer series viz., OPS, OPA and S were selected. The selected 10 primers were S 07, S 8, S 10, S 15, S 16, S 18, S 19, OPS 125, OPS 127, and OPS 141. The deatails of screening has given in Table 14.

Table 14. Details of screening for RAPD primers

Sl. No	Primer	Nucleotide sequence (5'–3')	Amplification pattern	Primer selected or not
1	S 1	GTT TCG CTC C	Average	Not selected
2	S 2	TGA TCC CTG G	Average	Not selected
3	S 3	CAT CCC CCT G	Poor	Not selected
4	S 4	GGA CTG GAG T	Average	Not selected
5	S 5	TGC GCC CTT C	Poor	Not selected
6	S 6	TGC TCT GCC C	Average	Not selected
7	S 7	GGT GAC GCA G	Good	**Selected**
8	S 8	GTC CAC ACG G	Good	**Selected**
9	S 9	TGG GGG ACT	No amplification	Not selected
10	S 10	CTG CTG GGA C	Good	**Selected**

11	S 11	GTA GAC CCG T	Poor	Not selected
12	S 12	CCT TGA CGC A	Poor	Not selected
13	S 13	TTC CCC CGC T	Poor	Not selected
14	S 14	TCC GCT CTG G	Poor	Not selected
15	S 15	GGA GGG TGT T	Good	**Selected**
16	S 16	TTA GCC CGG A	Good	**Selected**
17	S 17	AGG GAA CGAG	Poor	Not selected
18	S 18	CCA CAG CAG T	Good	**Selected**
19	S 19	ACC CCC GAA G	Good	**Selected**
20	S 20	CAG GCC CTT C	Poor	Not selected
21	OPA 1	CAG GCC CTT C	Poor	Not selected
22	OPA 2	TGC CGA GCT G	Average	Not selected
23	OPA 3	AGT CAG CCA C	Poor	Not selected
24	OPA 4	AAT CGG GCT G	Average	Not selected
25	OPA 5	AGG GGT CTT G	Good	Not selected
26	OPA 6	GGT CCC TGA C	Poor	Not selected
27	OPA 7	GAA ACG GGT G	Poor	Not selected
28	OPA 8	GTG ACG TAG G	Poor	Not selected
29	OPA 9	GGG TAA CGC C	Poor	Not selected
30	OPA 10	GTG ATC GCA G	Poor	Not selected
31	OPS 101	GGT CGG AGA A	No amplification	Not selected

32	OPS 103	AGA CGT CCA	Poor	Not selected
33	OPS 107	CTG CAT CGT G	Average	Not selected
34	OPS 109	TGT AGC TGG G	No amplification	Not selected
35	OPS 115	AAT GGC GCA G	No amplification	Not selected
36	OPS 125	CCG AAT TCC C	Good	**Selected**
37	OPS 126	GGG AAT TCG G	Poor	Not selected
38	OPS 127	CCG ATA TCC C	Good	**Selected**
39	OPS 141	CCC AAG GTC C	Good	**Selected**
40	OPS 145	TCA GGG AGG T	No amplification	Not selected

4.8.2. Amplification with the selected primers

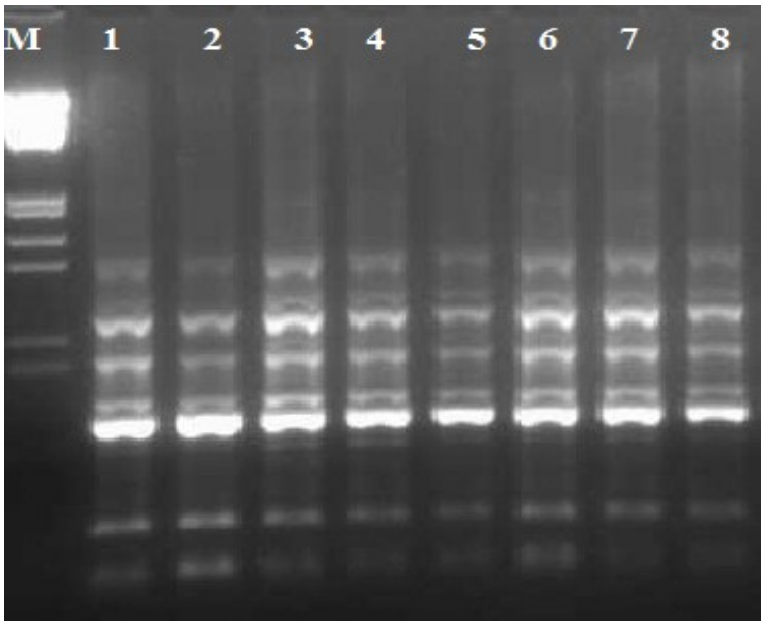
A total of 10 primers were screened for detection of polymorphism among the seven somaclones and the mother plant and polymorphic bands were obtained with all the primers. Number of bands produced by the primers varied from 5 to 14.

S 07

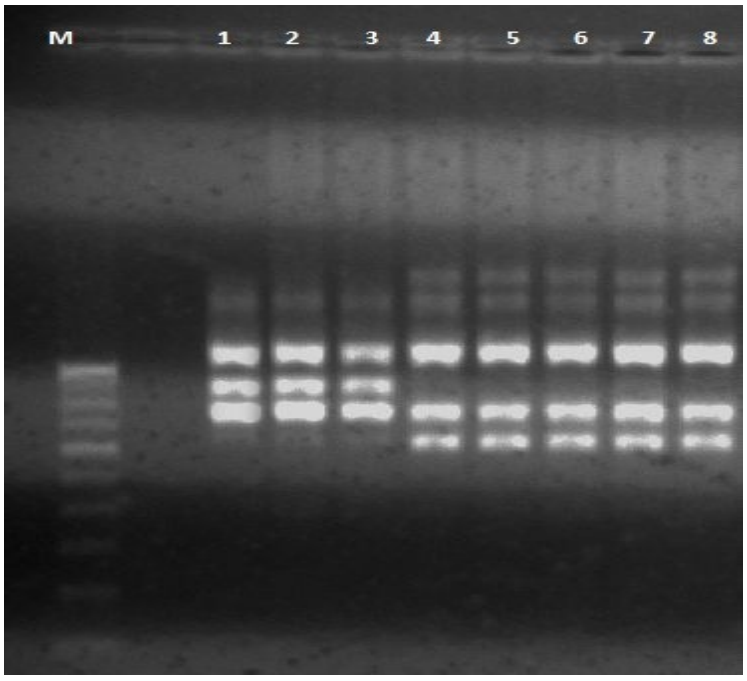
Eight amplicons were observed on the agarose gel for the DNA amplified with the primer S 07 (Plate 9). One band was polymorphic and the rest monomorphic among the genotypes. Two bands were clear and distinct. This primer was able to differentiate the vetiver somaclones.

S 08

A total of five amplicons were obtained after DNA amplification with the primer S 08. Two bands were polymorphic and the rest were monomorphic. The pattern of amplification is shown in Plate 9.



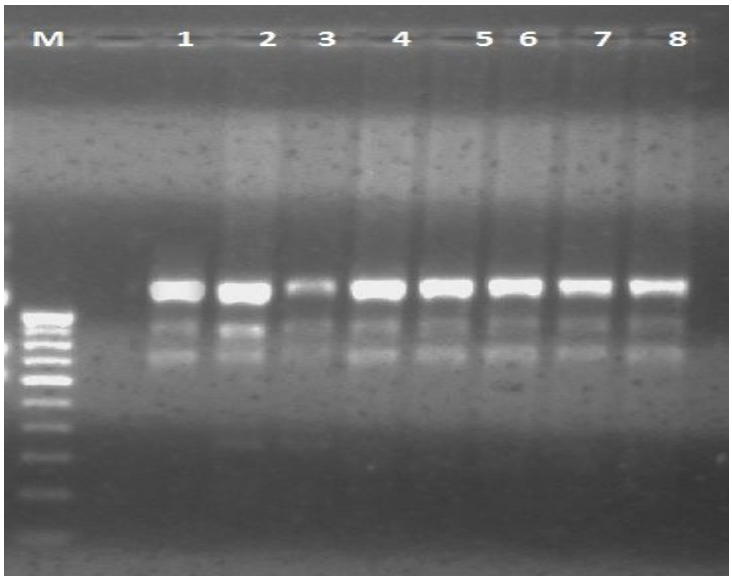
S 07



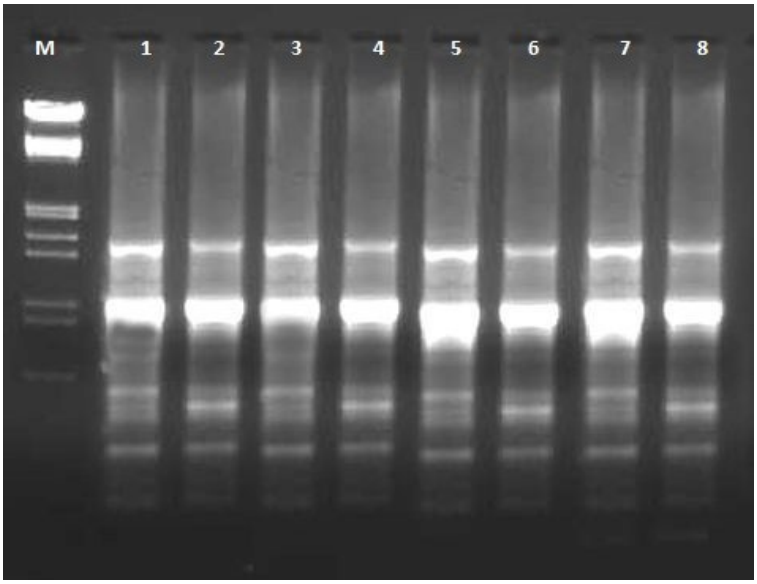
S 8

M – Marker, 1 - Mother plant, 2 – SC 1, 3 - SC 2, 4 – SC 3 , 5 – SC-4, 6 – SC 5, 7 – SC 6, 8 – SC 7

Plate 9. RAPD profiles of somaclones with primers S 7 and S 8



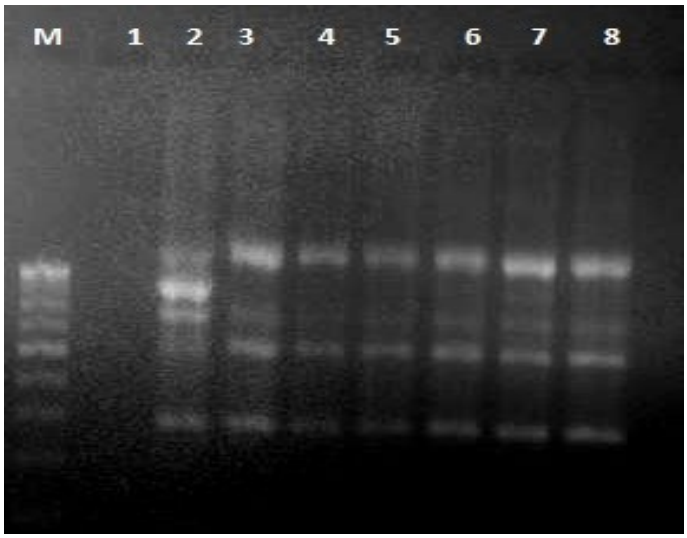
S 10



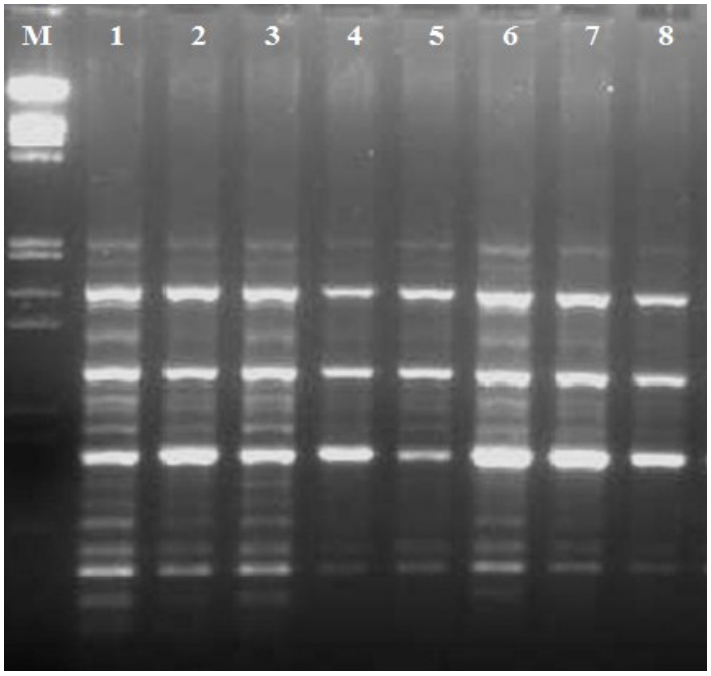
S 15

M – Marker, 1 - Mother plant, 2 – SC 1, 3 - SC 2, 4 – SC 3 , 5 – SC-4, 6 – SC 5, 7 – SC 6, 8 – SC 7

Plate10. RAPD profiles of somaclones with primers S 10 and S 15



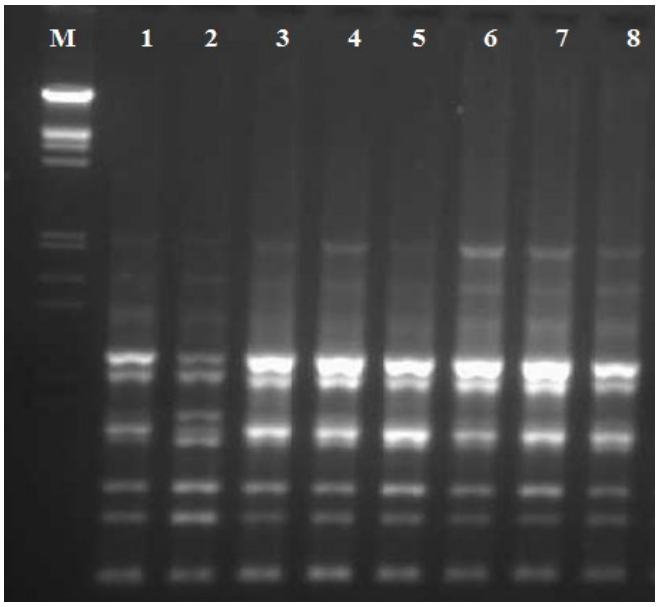
S 16



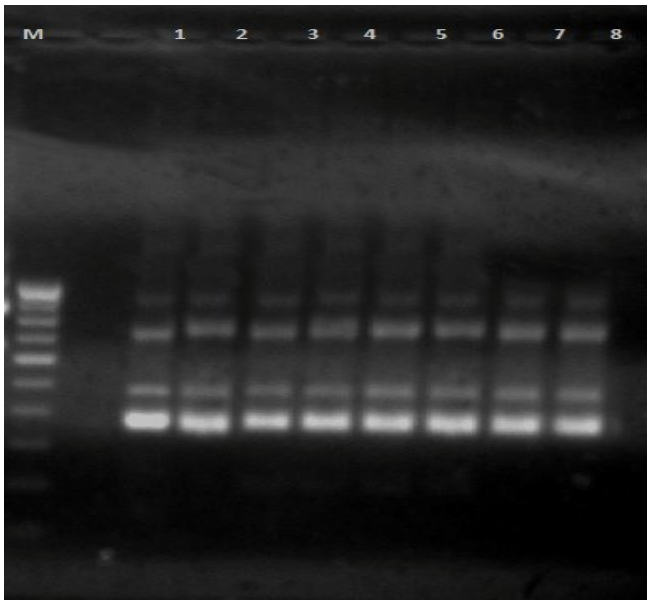
S 18

M – Marker, 1 - Mother plant, 2 – SC 1, 3 - SC 2, 4 – SC 3 , 5 – SC-4, 6 – SC 5, 7 – SC 6, 8 – SC 7

Plate 11. RAPD profiles of somaclones with primers S 16 and S 18



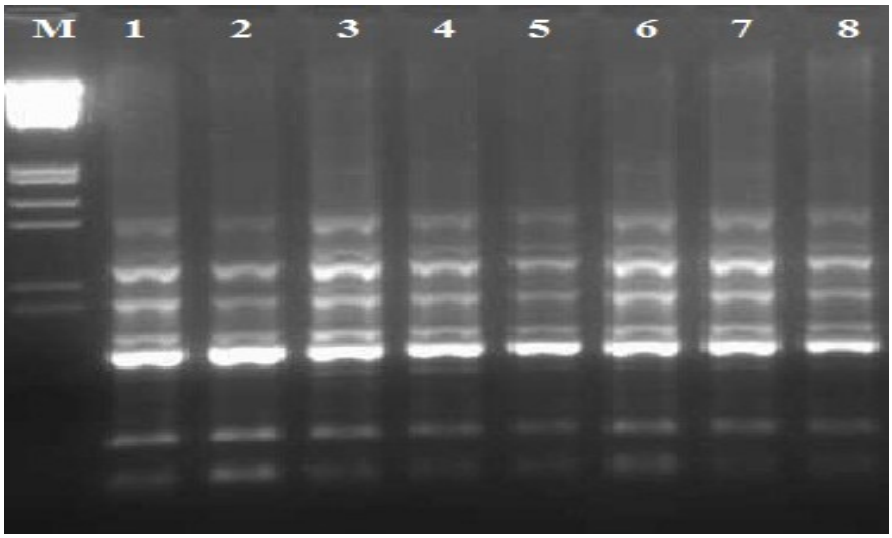
S 19



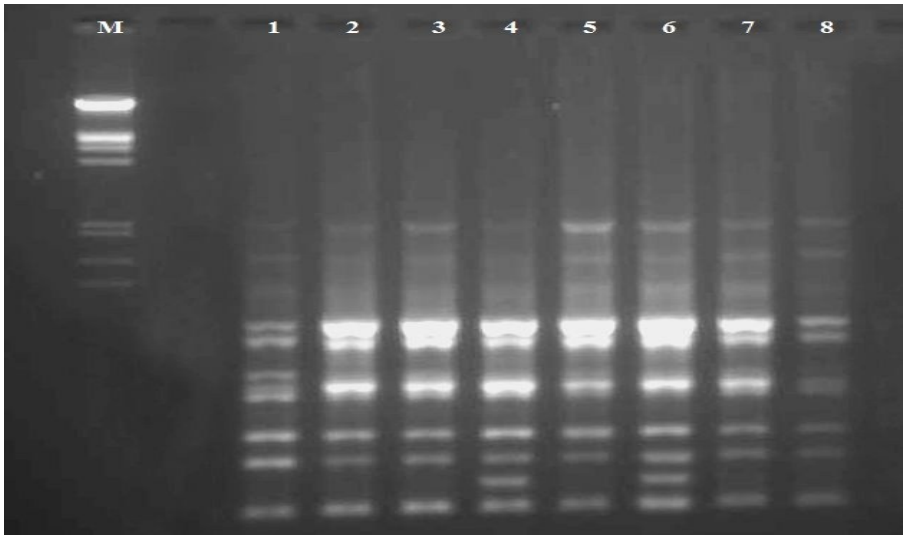
OPS 125

M – Marker, 1 - Mother plant, 2 – SC 1, 3 - SC 2, 4 – SC 3 , 5 – SC-4, 6 – SC 5, 7 – SC 6, 8 – SC 7

Plate 12. RAPD profiles of somaclones with primers S 19 and OPS 125



OPS 127



OPS 141

M – Marker, 1 - Mother plant, 2 – SC 1, 3 - SC 2, 4 – SC 3 , 5 – SC-4, 6 – SC 5, 7 – SC 6, 8 – SC 7

Plate 13. RAPD profiles of somaclones with primers OPS 127 and S 141

S 10

A total of three amplicons were obtained after DNA amplification with the primers S 10. The pattern of amplification is shown in Plate 10. The amplicons obtained with this primer were almost monomorphic.

S 15

Five amplicons were obtained after DNA amplification with the primer S 15. The pattern of amplification is shown in Plate 10. The amplicons obtained with this primer were almost monomorphic while a single band was found to be polymorphic.

S 16

A total of four amplicons were obtained after DNA amplification with the primer S 16. The pattern of amplification is shown in Plate 11. One of the bands was polymorphic and was clear and distinct. The rest were monomorphic bands.

S 18

A total of eleven amplicons were obtained after DNA amplification with the primer S 18. The pattern of amplification is shown in Plate 11. The three amplicons obtained with this primer were clear and distinct. Four of the bands were polymorphic and rest shows monomorphic pattern.

S 19

Ten amplicons were obtained after DNA amplification with the primer S 19. The pattern of amplification is shown in Plate 12. The amplicons obtained with this primer were almost monomorphic while a single band was found to be polymorphic.

OPS 125

A total of four amplicons were obtained after DNA amplification with the primer OPS 125. The pattern of amplification is shown in Plate 12. The amplicons obtained with this primer were monomorphic.

OPS 127

A total of seven amplicons were obtained after DNA amplification with the primer OPS 127. The pattern of amplification is shown in Plate 13. The amplicons obtained with this primer were almost monomorphic.

OPS 141

A total of eight amplicons were obtained after DNA amplification with the primer OPS 141. The pattern of amplification is shown in Plate 13. The amplicons obtained with this primer were almost monomorphic while a single band was found to be polymorphic very distinctly.

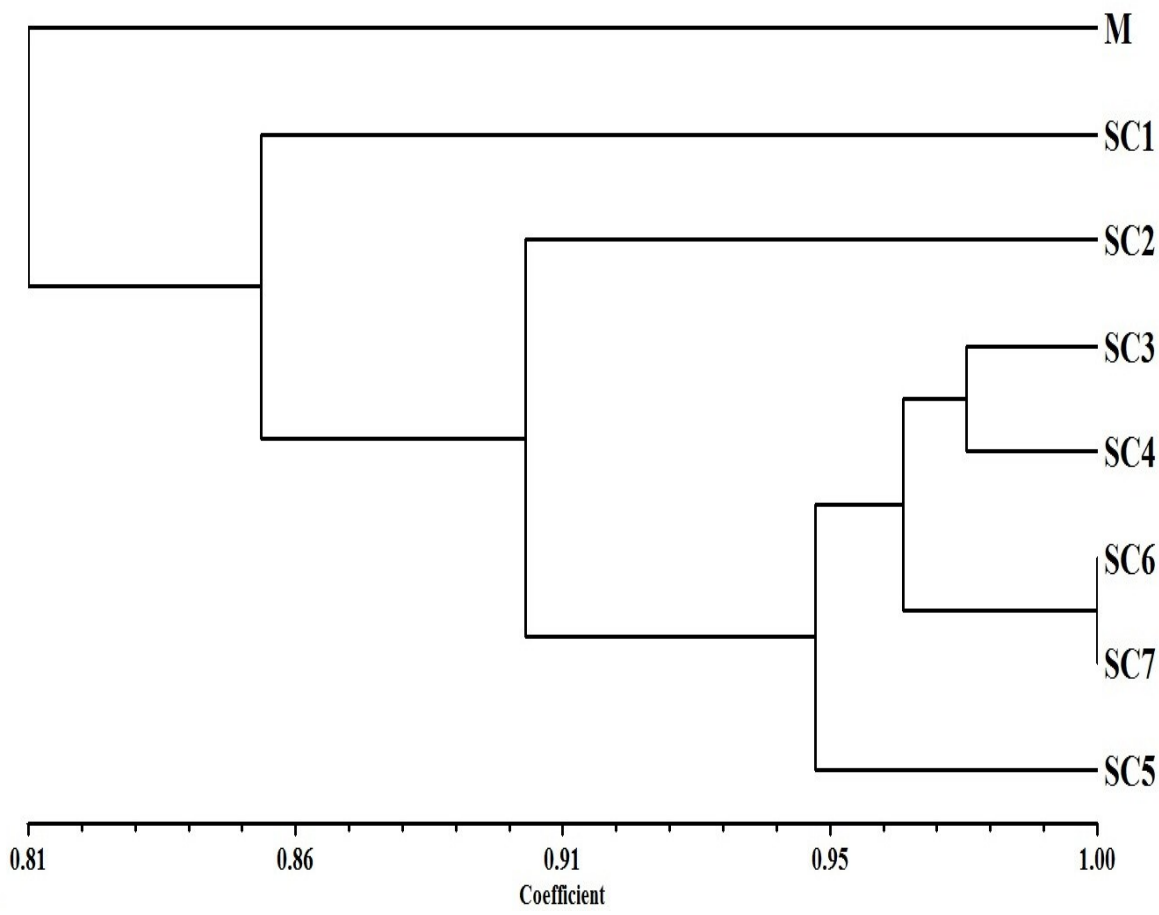
4.8.3. RAPD Data Analysis

Reproducible, well resolved fragments were scored manually and each scorable band was scored as presence (1) or absence (0). The number of scorable markers produced per primer ranged from 5 to 14. Data analysis was done using the software NTSYS pc version 2.02i.

NTSYS pc version 2.02i

A genetic variability matrix was constructed using the Jaccard's coefficients. Based on the proximity matrix obtained from Jaccard's coefficient, sequential agglomerative hierarchical non-overlapping (SAHN) clustering was done using unweighted pair group (Fig.1). Based on this the somaclones were divided into 4 clusters (Table 15)

As per the NTSYS analysis, there is a maximum variation of 19 per cent between the mother plant and the somaclones SC 6 and SC 7. The least variation noticed is for the somaclone SC 1 (4%) with the mother plant.



M - Mother plant, SC 1 - 0.5 mg/l 2,4-D, SC 2 - 0.5 mg/l 2,4-D+KINETIN, SC 3 - 1 mg/l 2,4-D , SC 4 - 1 mg/l 2,4-D+KINETIN, SC 5 - 1 mg/l 2,4-D+KINETIN+BA, SC 6 – 1.5 mg/l 2,4-D, SC 7 – 1.5 mg/l 2,4-D+KINETIN

Fig. 1. Dendrogram of RAPD primers

Table 15. Clustering of the somaclones on the basis of NTSYS analysis.

Cluster No	No of plant groups in each cluster	Plant groups
Cluster I	1	Mother plant
Cluster II	1	SC 1
Cluster III	1	SC 2
Cluster IV	5	SC 3, SC 4, SC 5, SC 6 and SC 7

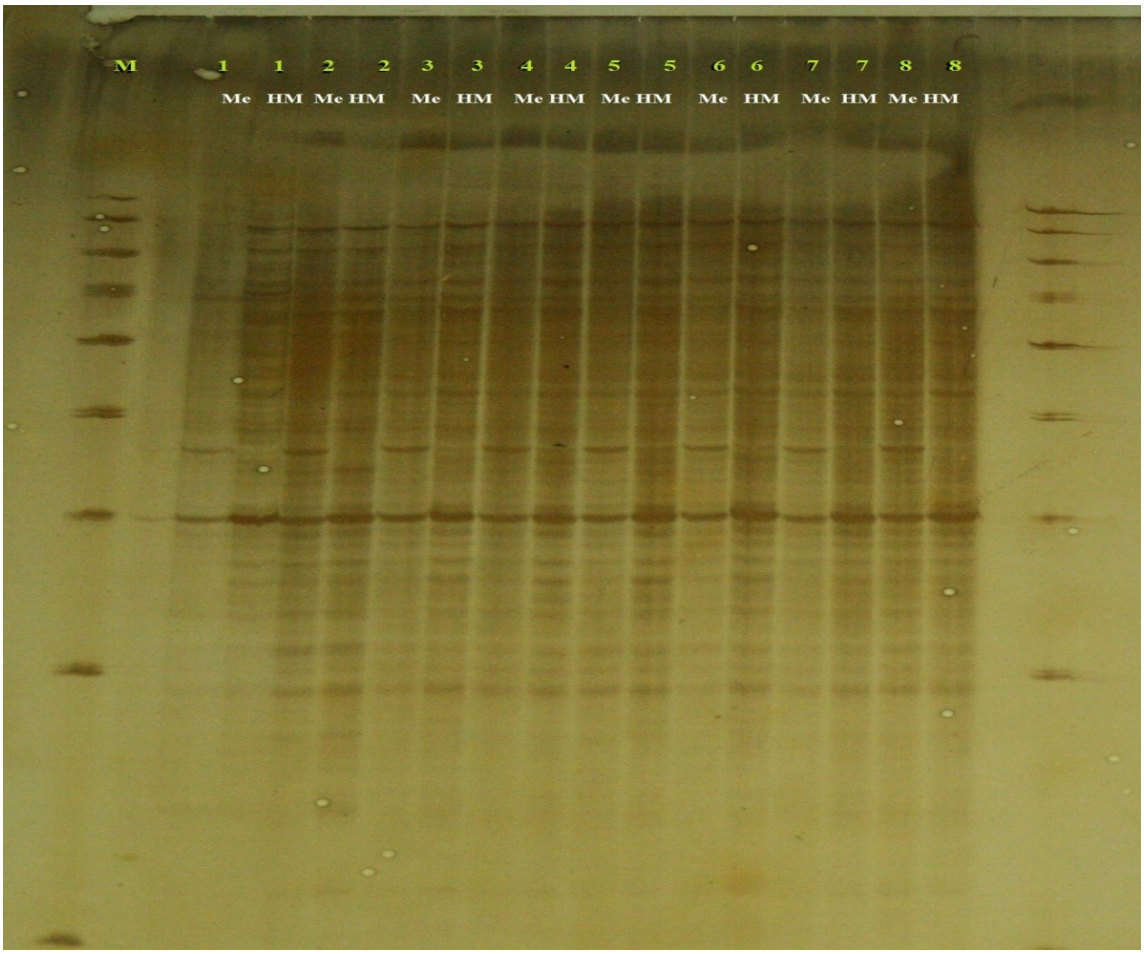
4.9. MSAP (Methylation Sensitive Amplification polymorphism)

The somaclones and their mother plants were used for molecular analysis to study the levels, patterns and directions of DNA methylation. The data were analysed for per cent hemi methylation, internal methylation and total methylation. The results obtained are presented in this section. MSAP was performed with the primers as listed below and all of them give good amplification (Plate 15).

- E+AT+MH+CA
- E+AC+MH+ATG
- E+AAC+HM+CAC
- E+AAC+HM+AGG

4.9.1. Analysis for MSAP

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



M – Marker, 1 - Mother plant, 2 – SC 1, 3 - SC 2, 4 – SC 3 , 5 – SC-4, 6 – SC 5, 7 – SC 6, 8 – SC 7

Me – Methylated

HM – Hemi-methylated

→ Indicating polymorphic bands

Plate 14. MSAP profiles of somaclones with primers OPS 127 and S 141

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

.

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

Table 16. Methylation in mother plant and different somaclones

Sl.No	Plant Group	Per cent hemi methylation (%)	Per cent internal full methylation (%)	Total Methylation (%)	Variation from mother plant
1	Mother plant	6.85	6.40	13.25	00.00
2	SC 1	6.93	5.63	12.55	0.70
3	SC 2	5.63	6.65	12.28	0.98
4	SC 3	5.5	6.20	11.70	1.55
5	SC 4	5.58	4.93	10.50	2.75
6	SC 5	6.38	10.63	17.00	4.25
7	SC 6	5.18	4.93	10.10	3.15
8	SC 7	7.28	4.40	11.68	1.75

The total methylation per cent varied from 10.10 to 17 among the somaclones and mother plant (Table 16). The somaclone No. 5 has shown more variation when compared with the mother plant and the Somacole No. 1 has shown least variation with the mother plant (Fig.2). Total methylation also was noticed in Somaclone No.5.

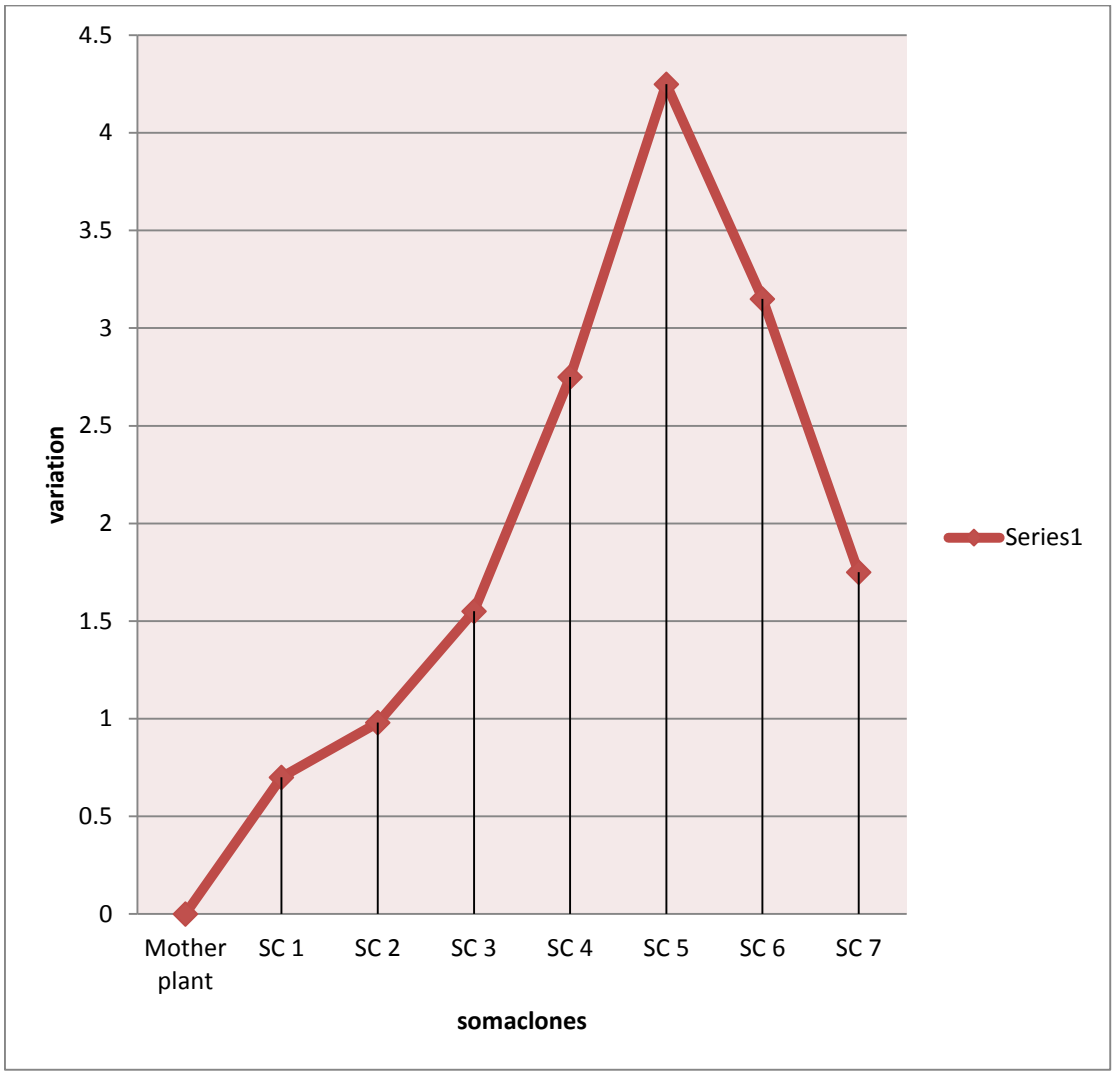


Fig.2. Graphical representation of the variation in methylation of somaclones

4.10. Biochemical Analysis (GC)

As the oil glands will be matured completely in vetiver plant at the stage of 18 months only, pure oil extraction is not possible with somaclones of 6 months age. Hence, the available oil along with the resinous matter (oleoresin) are extracted from the somaclones

4.10.1. Yield of solvent extractable matter

Exhaustive extraction of plant material in tandem with a polar solvent like acetone and a non polar solvent like hexane will yield an extract of all the secondary metabolites. In an essential oil crop like vetiver, such an extract will comprise mostly of the terpenoids viz., essential oil and the resins.

The yield of solvent extractable matter of the seven somaclones in comparison to that of the mother plant is given in table 17.

Table 17. Oleoresin yield of mother plant and somaclones

Sl. No.	Plant Group	Oleoresin yield (%)
1	Mother plant	1.89
2	SC 1	1.71
3	SC2	2.14
4	SC3	1.6
5	SC4	2.67
6	SC5	2.22
7	SC6	1.94
8	SC 7	0.30

4.10.2. Gas chromatographic analysis of solvent extracts

Gas chromatography reveals a spectrum of the volatile components in a sample. Chromatographic conditions remaining the same, the retention time of a peak signifies the identity of the corresponding component and the area under the peak denotes its abundance in mass. Hence gas chromatographic fingerprinting technique can be used for qualitative comparison of the volatile components in samples.

The gas chromatographic traces (fingerprints) of the mother plant and the somaclones are presented in fig.2 a to d.

The procedure was standardised using Hyati vetiver oil (TMV Gardens). The chromatographic profiles varied for all the somaclones and the mother plant (Fig.3). The retention time in the X-axis of the graph denotes the identity of the compound and the area under the peak represents the total percentage of the particular compound in the sample. The number of component varied from 29 to 86. More number of components was obtained for SC 6 and the least number for SC 2.

Table 18. Quantity of the component at different peaks for somaclones

Sl.No.	Plant Group	Area (quantity) (%)				
		At 24.8	At 27.4	At 29.5	At 30.6	At 31.5
1	Mother plant	3.0	4.4	1.9	25.4	23.6
2	SC 1	2.5	0.9	8.4	13.3	1.0
3	SC2	3.0	1.1	4.7	3.7	0.0
4	SC3	0.4	0.0	1.0	2.7	1.3
5	SC4	0	0.0	6.2	12.8	0.0
6	SC5	0	0.3	2.2	2.5	1.2
7	SC6	0	0.6	1.1	2.0	2.0
8	SC 7	3.9	1.0	8.9	6.9	0.4

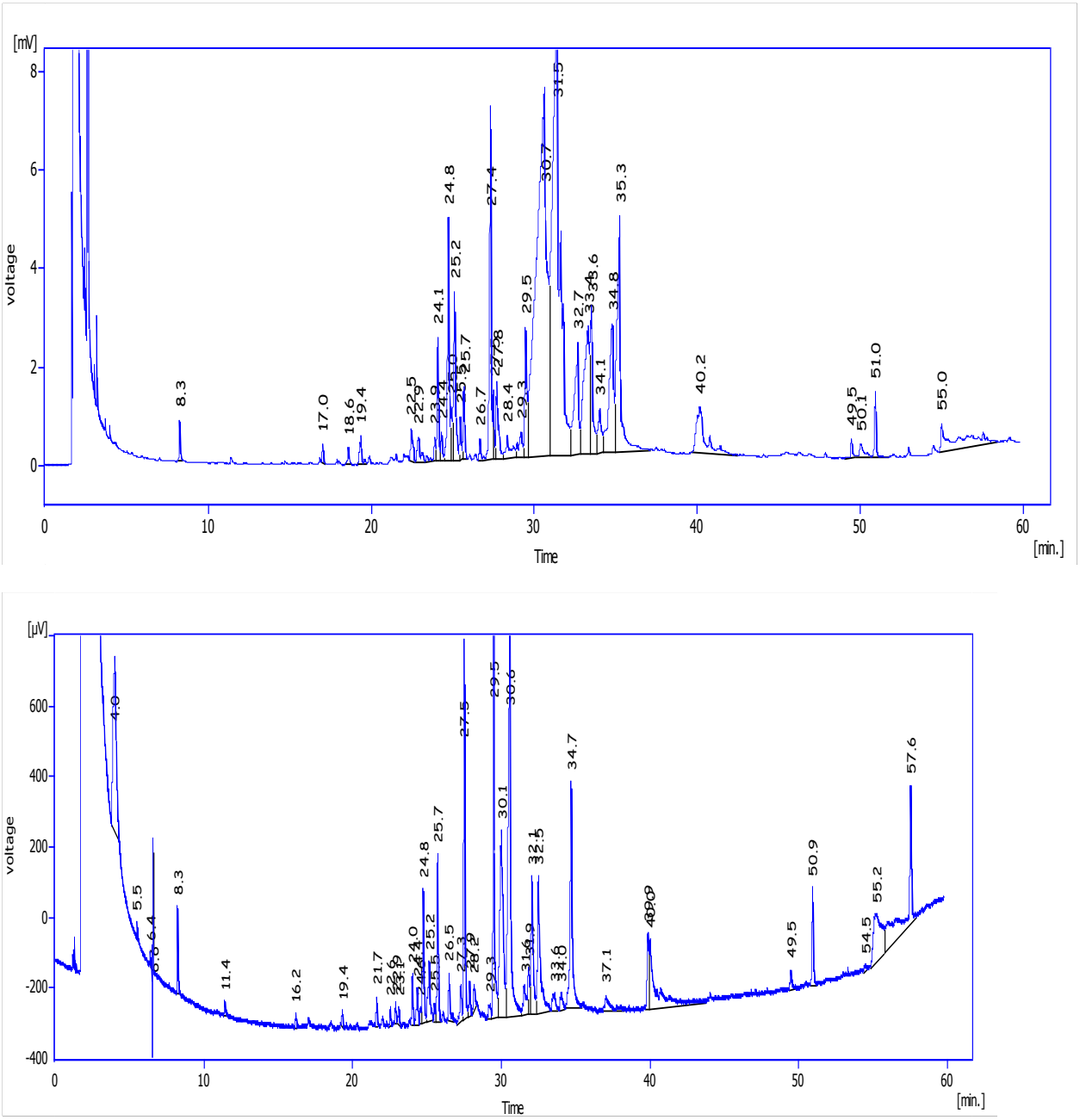
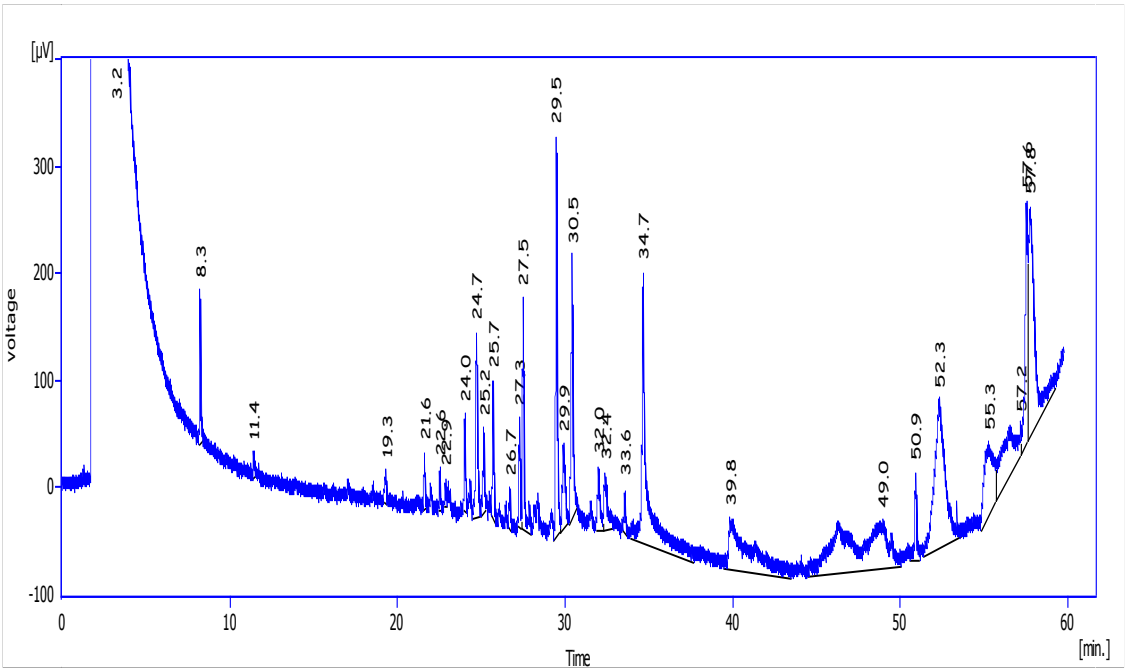


Fig. 3a. Gas chromatographic profiles of oil from Mother plant and somaclone I



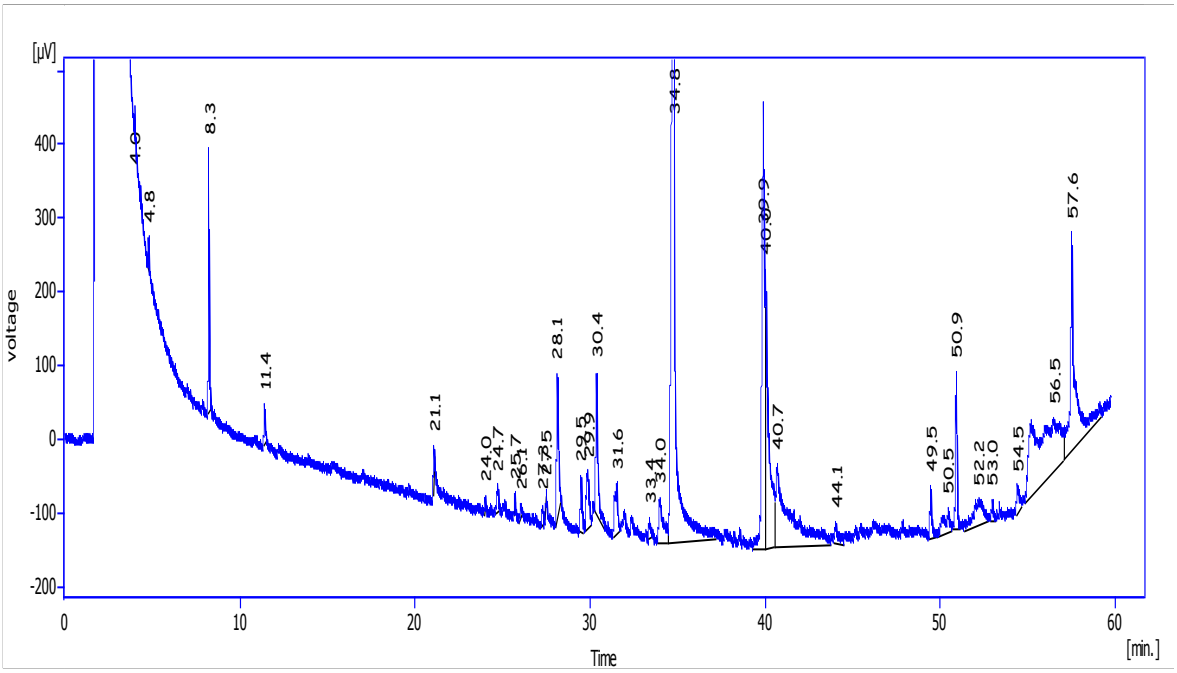
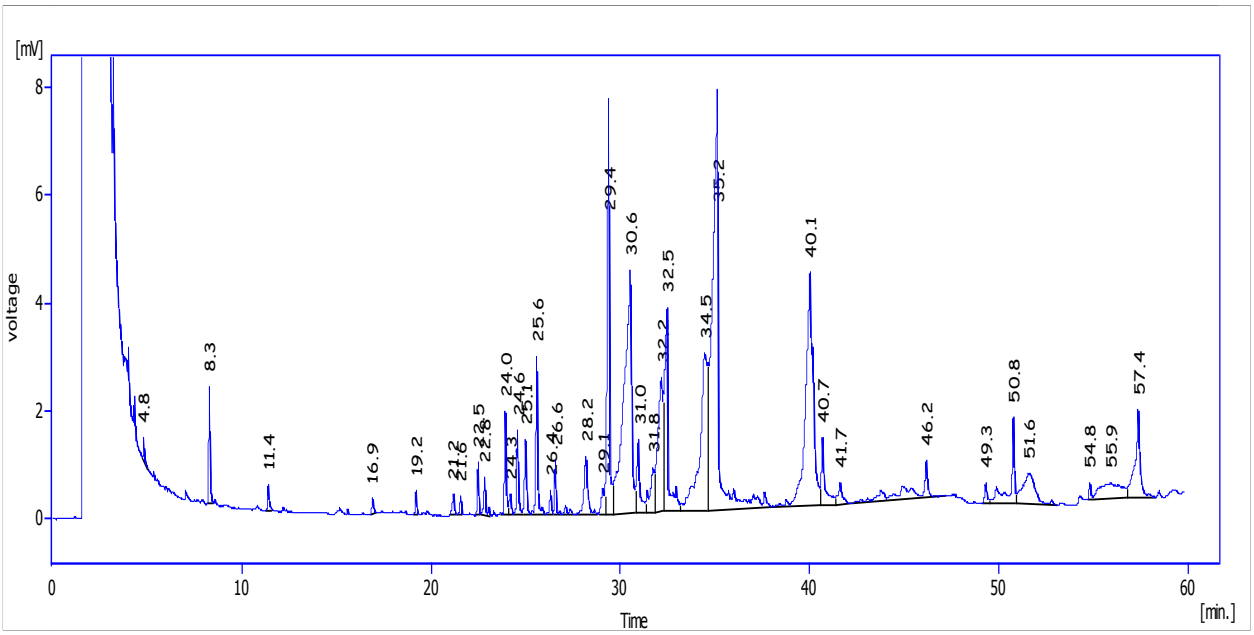


Fig. 3b. Gas chromatographic profiles of oil from somaclone II and III



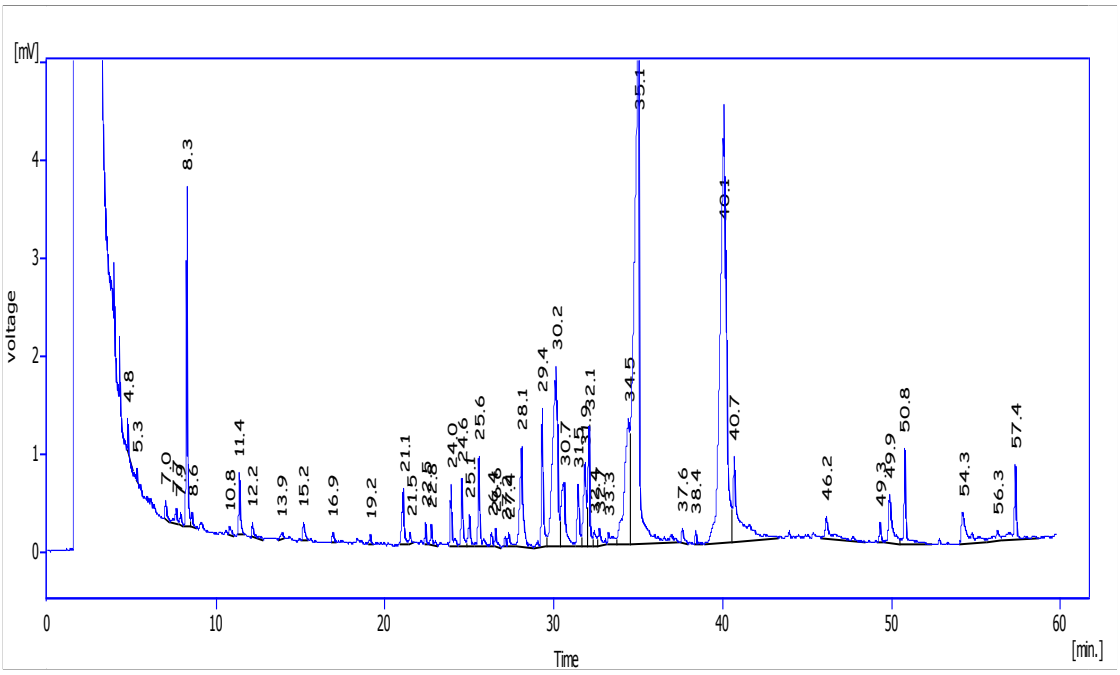


Fig. 3c. Gas chromatographic profiles of oil from somaclone IV and V

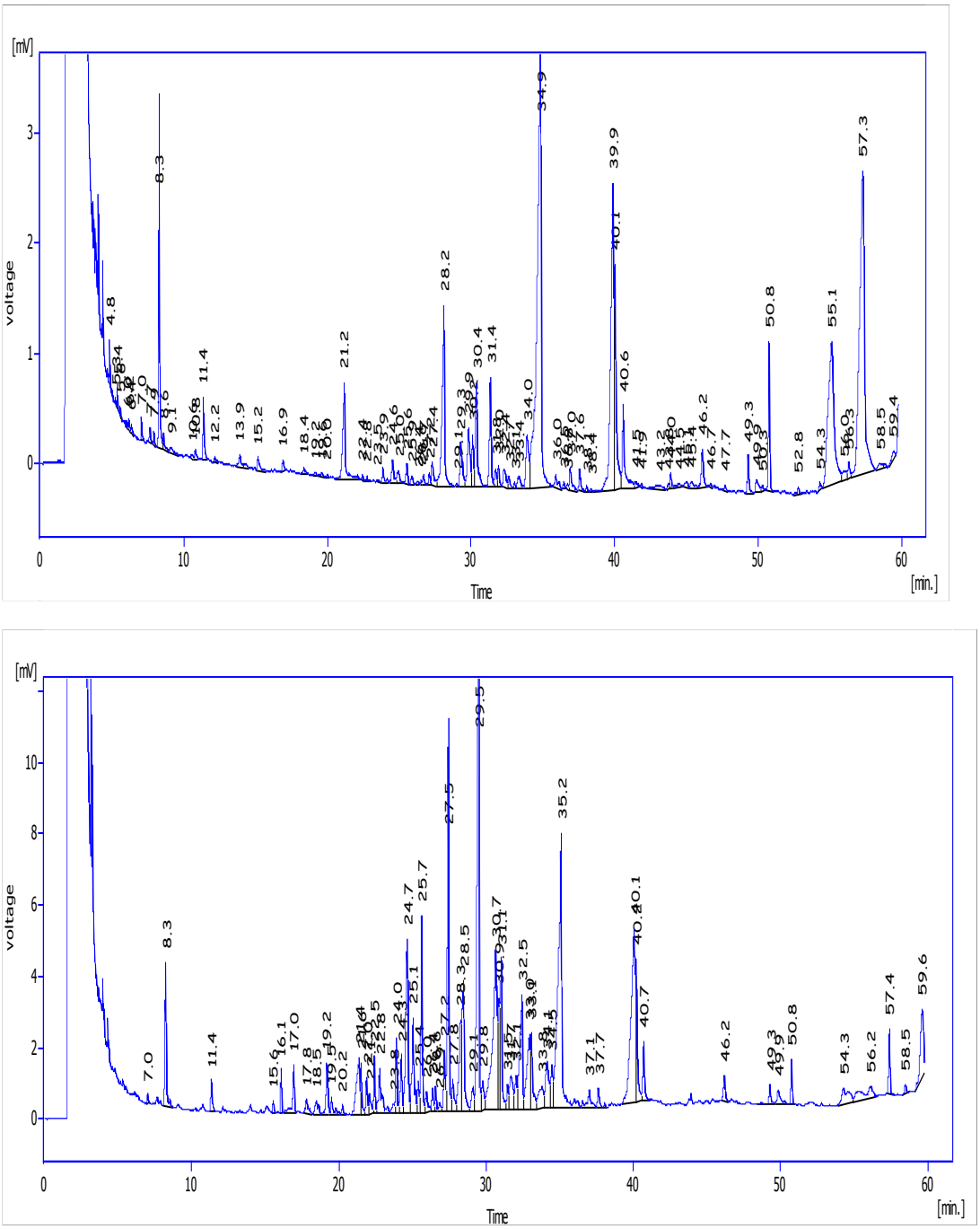


Fig. 3d. Gas chromatographic profiles of oil from somaclone VI and VII

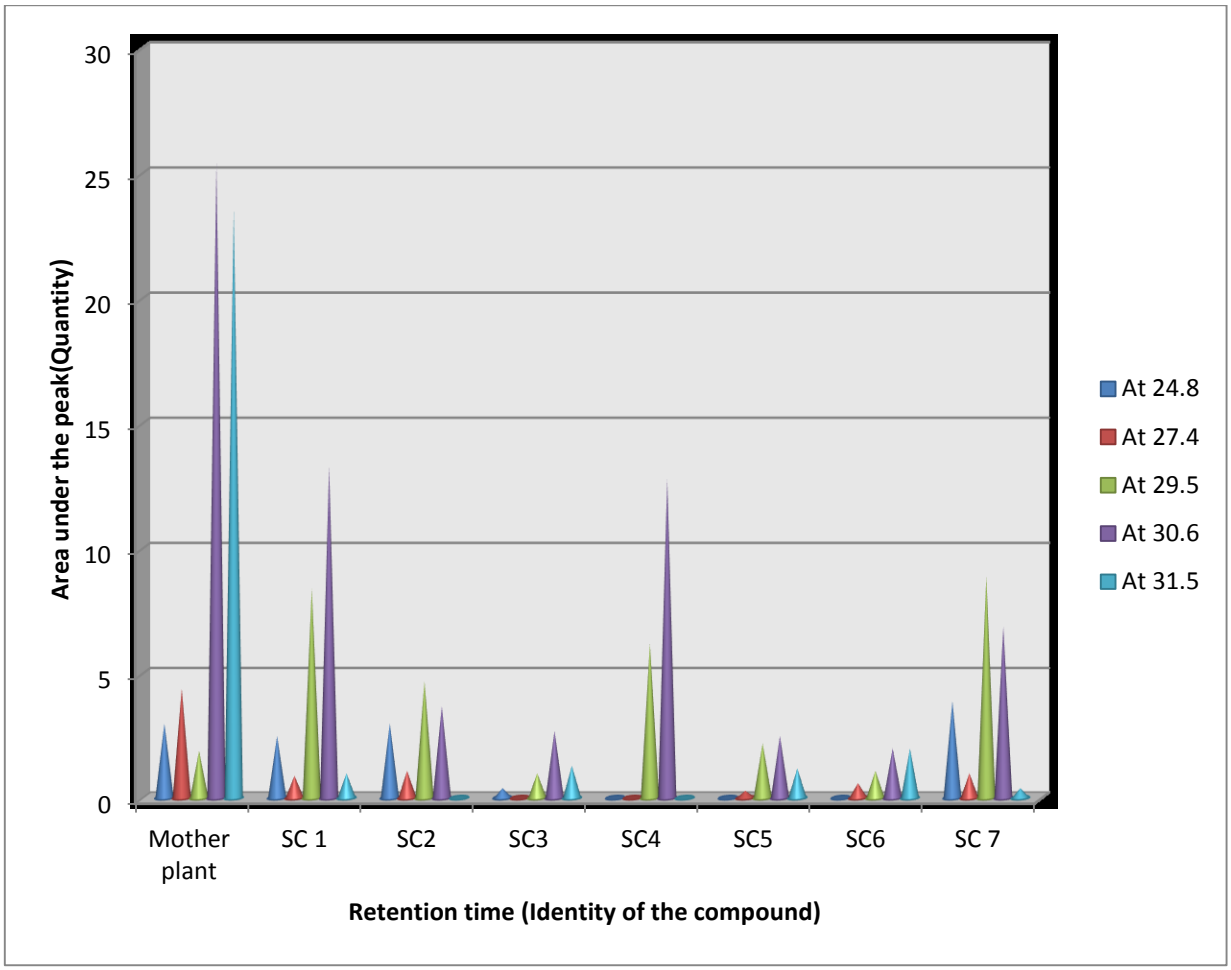


Fig.4. Variation in components for somaclones at selected peaks in gas chromatography

The graphical representation of the data is given in Fig.4. It shows that out of the five peaks selected, four of them are found to be abundant in mother plant and less in somaclones, while, a component at the peak 30.6 is less in mother plant and highest in SC -7. The component at the peak 24.8 is absent in three somaclones such as SC-4, SC-5 and SC-6. The component at 27.4 is absent in SC-3 and SC-4. The component at 31.5 is absent in SC-2 and SC-4. This shows that there is variation that exists between the mother plant and somaclones and also in between the somaclones.

Discussion

5. DISCUSSION

Vetiver is an omni-useful plant, almost all parts of which are used in one or more ways having direct as well as multifarious cultural and industrial applications. It is a gift of India to the modern world, and finds its greatest use in modern perfume creations. The roots are one of the most important organizational systems that make vetiver a miracle grass for its multifarious applications in soil and water conservation, soil health, and raw material for root handicrafts, environmental and perfumery products.

Vetiver is generally propagated by vegetative slips and so have little scope for genetic recombination and improvement. This has resulted in degradation of germplasm with respect to herbage yield, oil content, oil quality etc (Hussain, 1982). The possibility of developing superior strains of these grasses through the induction of somaclonal variation from high-frequency plantlet regeneration from callus cultures has been considered as an attractive option (Mathur *et al*, 1989)

In this context, the present study would help in creation of somaclonal variants in vetiver (*Chrysopogon zizanioides*), which could possibly lead to higher oil yield in this crop.

The results obtained in the study on "Induction of somaclones in vetiver [*Chrysopogon zizanioides*(L.)Roberty] are discussed in this chapter.

5.1. Screening of media for callus induction

Callus induction was obtained in full MS media with growth regulator combinations of 2,4-D, BA and Kinetin. Out of 28 media combinations used, the callus could be initiated in 7 combinations. Of these, three were various concentrations of 2,4-D alone and the other three were the combinations of 2,4-D and kinetin together and the seventh treatment with a combination of 2,4-D, BA and kinetin. The concentrations varied from .5 mg/l to 1.5 mg/l. The number of days taken for callus

induction varied from 14 to 20 days. The percentage of contamination observed varied from 0 to 33 per cent

The result was more or less in conformity with the findings of Sreenath *et al* (1994) who obtained callus from vetiver inflorescence in full MS with growth regulator combinations of varying levels of 2,4-D, BA and kinetin.

5.2. Screening of media for regeneration

The regeneration was obtained in MS media with BA at 1 to 5 mg/l concentration. It took 11 to 13 days for regeneration from the calli. The contamination per cent varied from 0 to 11. The nature of callus regeneration with respect to growth varied according to the concentration of BA used. More crowded growth was observed at higher concentrations.

Keshavachandran *et al* (1996) also reported that BA among the cytokinins induced the highest per cent of regeneration in vetiver which is in conformity with the present result.

5.3. Screening of media for rooting

Various concentrations of IAA in full MS were used for the induction of rooting in the regenerants. It took 8 to 11 days for initiation of rooting. An average of 16 per cent contamination was noticed. The length of the root varied from 0.2 to 1.4 cm at one month age according to the growth regulator concentration.

Sreenath *et al* (1994) also reported that use of IAA for root induction in vetiver which allows optimal rooting for transferring to soil conditions.

5.4. Hardening

Hardening of rooted plantlets was done in cups filled with sand and also in cups filled with potting mixture. Only the plants hardened in cups filled with sand

survived. The survival percentage varied from 33 to 60. Sreenath *et al* (1994) reported that 50 days old plantlets survived well under hardening

5.5. Transplanting

The hardened plants were transplanted to the pots filled with potting mixture. In 6 months old plants, there was variation in growth parameters. The height varied from 81 cm to 114 cm and the number of leaves varied from 66 to 109. The best growth was observed for somaclone number 6 and poor for somaclone number 4.

Joshi and Rao (2009) reported a range of morphological variation between the callus regenerated rice plants including the plant height and number of leaves.

5.6. Standardisation of DNA Isolation

Good quality of genomic DNA is a requirement for RAPD and MSAP assay. DNA was isolated from the tender leaves of the 7 somaclones as it contains fewer amounts of polysaccharides, phenols, tannins and secondary metabolites compared to mature leaves. The CTAB method (Doyle and Doyle, 1987) and its modification by avoiding 10 per cent CTAB were used for the extraction of nucleic acids. More quantity of DNA was obtained using the modified CTAB method. The quantity of DNA varied from 936 ng to 1754.52 ng and the absorbance ranged from 1.84 to 1.93

Adams (1999) reported that extraction using the hot CTAB protocol (Doyle and Doyle 1987) with the addition of 1 per cent (w/v) PVP in yield of good quality DNA.. Most vetiver accessions yielded larger molecular weight DNA and greater yields when the tissue was ground in liquid nitrogen and then placed in hot CTAB.

The homogenisation, pulverisation and uniformity of grinding of plant tissue were essential during DNA extraction. Excess liquid nitrogen was used for the homogenisation of the leaf tissue. Liquid nitrogen helps in maintaining the frozen tissue, preventing nucleic acid degradation and effect of secondary metabolites and

ensures a better mechanical disruption of tissue (Hernandez and Oyarzum, 2006). The problem of polyphenols was overcome by the addition of ß- mercaptoethanol and Poly Vinyl Pyrrolidone (PVP) along with the extraction buffer. ß- mercaptoethanol disrupts the protein disulphide bond and is thereby capable of initiating protein degradation. Poly Vinyl Pyrrolidone (PVP) removes polyphenols and inhibits co-precipitation of polysaccharides which resulted in good quality DNA. This was confirmed by De la Cruze *et al.* (1997) and Matasyoh *et al.* (2008).

The detergent present in the extraction buffer, CTAB, helps in the release of nucleic acids into the buffer after disruption of the cell membrane. The released DNA is protected from the action of DNase enzyme by EDTA present in the extraction buffer. It is a chelating agent, which efficiently blocks Mg^{2+}, the major cofactor of DNase enzyme. The DNA isolated by CTAB method was freed from chlorophyll by using the Chloroform: Isoamyl alcohol which aids in the separation of organic mixture and aqueous phase of the DNA isolation. EDTA was also a major component of TE buffer in which the DNA is dissolved and stored. RNase treatment was given in order to remove RNA contamination from the isolated DNA samples as was reported by Sambrook *et al.* (1989).

5.7. RAPD ASSAY

The RAPD technique was developed by Williams *et al*. (1991). This technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary oligonucleotide primers. In RAPD markers, polymorphism results from changes in either the sequence of the primer binding site (which prevents stable association with the primer) or from mutations like insertion, deletion, inversion etc. (which alter the size or prevent the successful amplification of a target DNA). Usually RAPD markers are dominant in nature because polymorphisms are detected as the presence or absence of bands.

5.7.1. Standardisation of RAPD

Initially, PCR conditions optimised for RAPD were standardized with various quantities of template DNA (20, 25 and 50 ng), dNTPs (100 mM) and $MgCl_2$ (1.5, 2 and 3 mM). Later, RAPD amplifications were performed routinely using PCR mixture (25 µl) which contained 100 ng of genomic DNA as template, 200 µM PCR buffer, 200 lM dNTPS, 1 unit (U) of *Taq* DNA polymerase and 1 µM of each primer. The concentration of $MgCl_2$ in the reaction mixture also influences the amplification pattern. A high Mg^{2+} concentration enhances the stability of primer-template interactions and also overcomes the RNA contamination in PCR (Welsh and McClelland, 1991).

The concentration of template DNA also influences the PCR reaction. In the present study, three concentrations of template DNA were tried and genomic DNA at the concentration of 50 ng produced good amplification using selected random primers while lower concentrations produced faint bands. Similar results were observed by Adams(1999).

5.7.2 Screening with RAPD Primers

Forty decamer primers in the series OPS, S, and OPA were screened with genomic DNA of the mother plant as template. Details of primers used for screening are given in Table 14. Dong *et al* (2004), Adams (2000), and Kresovich *et al* (1994) have used different random primers.

In OPS series primers, OPS 125, OPS 127, and OPS 141 showed good amplification with the DNA of the mother plant. In S series, S 7, S 8,S 10, S 15, S 16, S 18, and S 19 gave amplification. A total of 10 primers were selected after the screening.

Dong *et al* (2004) has reported good amplication with OPS series primers in vetiver.

5.7.3 Amplification with Selected Primers

A total 10 primers were screened for detection of polymorphism among the 7 somaclones and the mother plant. A total of 92 bands were obtained. Number of bands produced by the primers varied from 3 (OPS 125) to 11 (S 18). The average number of bands was 9.2 and average percentage of polymorphism was 7.4.

Kresovich et al. (1994) reported that RAPD patterns were very stable within clones of vetiver using different primers. Williams *et al*. (1990) used different random sequence primers to assess the quality and frequency of the polymorphism in corn, soybean and *Neurospora crassa.* Adams(2000) used Random Amplified Polymorphic DNAs (RAPDs) to examine accessions of vetiver and related taxa from its region of origin and around the world. Dong *et al* (2004) also supported this by studying 13 ecotypes of vetiver grass from 8 countries, the genetic relationships of them were analyzed by means of RAPD molecular makers. The results showed that a total of 220 reproducible RAPD fragments were produced using 18 random primers.

5.7.4. Data analysis for RAPD

A genetic variability matrix was constructed using the Jaccard's coefficients. Based on the proximity matrix obtained from Jaccard's coefficient, sequential agglomerative hierarchical non-overlapping (SAHN) clustering was done using unweighted pair group (Fig.1). Based on this, the somaclones were divided into 4 clusters (Table 15). As per the NTSYS analysis, the somaclones 1, 2 and the mother plant were dissimilar with each other and the rest of the somaclones.The fifth somaclone was somewhat similar to 3rd, 4th, 6th and 7th ones. Somaclones 6 and 7 were 100 percent similar. Similarity also exist between somaclones 3 and 4. The maximum variation observed was 19 percent between mother plant and SC 6 and SC 7. The least variation (4%) with mother plant was shown by SC 1.

Srifah *et al* (1999) used NTSYS for analysing RAPD results of vetiver with various primers and developed the phylogenetic tree showing relationship between various accessions.

5.8. MSAP analysis

In the present study, a MSAP technique was used to detect cytosine methylation in the genome of somaclones. The results showed that this technique was highly efficient for large scale detection of cytosine methylation as suggested by Xiong *et al*. (1999). A distinct advantage of this approach compared to other methods is the ease of recovery of the differentially amplified fragments from the gel which facilitates direct identification of the methylated sequences in the genome. However, it should be pointed out that the detection of cytosine methylation is restricted to the recognition site of the isoschizomers, i.e., 5'-CCGG.

5.8.1. Cytosine methylation status in vetiver genome

An attempt was made to find the extent of cytosine methylation in vetiver genome using the seven somaclones and the mother plant with 4 primer combinations. The results showed that cytosine methylation varied from 10.1 per cent to 17 per cent. Joel and Zhang (2001a) reported that the overall methylation level was 24.24 per cent using 35 genotypes with 9 primer combinations in rice. However, the proportion of cytosine residues that were methylated varied widely, ranging from about 6 per cent in Arabidopsis (Kakutani *et al*., 1999) to as much as 33 per cent in rye (Thomas and Sherratt, 1956). Gruenbaum *et al*. (1981) reported that in general 20 to 30 per cent of all cytosines may be methylated in plants. Thus, the finding of the extent of methylation in vetiver genome in the present study is in accordance with the earlier reports

5.8.2. Epigenetic variation

Another important observation was the existence of inter-individual variation for both internal and hemi-methylation in all the somaclones and mother plant which reveals the presence of variability for methylation level in vetiver somacoles

(Plate.14). Such a variation beyond DNA sequence variation is referred as epigenetic variability in the form of DNA methylation. Joel and Zhang (2001) and Wang *et al*. (2004) reported the presence of natural epigenetic variation based on differential methylation states in rice genome.

The observed differential methylation levels obtained for these genotypes will differentiate the genotypes under study. Wang *et al*. (2004) observed genotype specific DNA methylation patterns and referred them as epigenetic markers or epi-markers and described it as potential class of markers to differentiate genetically closely related cultivars. He reported that these epigenetic markers can potentially be used to generate methylation fingerprints of closely related genotypes.

5.9. Biochemical Analysis

The somaclones identified were further screened biochemically through the GC analysis of extracted oleoresin.

5.9.1. Extraction of oleoresin from Vetiver

The oil along with resinous material extraction was mediated through reflection with solvent extraction method. Highly volatile acetone and less volatile hexane were used as solvents. The oleoresin was light yellow in colour. The content varied from 0.3 (SC 7) to 2.67 (SC 4) percentage.

Saraswathy *et al* (2011) reported a good yield of bright yellowish coloured essential oil from the *in vitro* regenerated long morphotype when compared to the short morphotype of vetiver with light yellow color, along with the control plants. The long and short morphotypes yielded 2.1 and 1.9 per cent of oil, respectively, whereas the control plant yielded 1.8 per cent.

5.9.2. GC Analysis

GC Analysis was carried out using DB-5 column. The chromatographic profiles varied for all the somaclones and the mother plant (Fig.2). The retention time

in the X-axis of the graph denotes the identity of the compound and the area under the peak represents the total percentage of the particular compound in the sample. The number of component varied from 29 to 86. More number of components was obtained for SC 6 and the least number for SC 2.

Marriot *et* al (2001) reported the GC×GC technique for the analysis of overall volatile constituents in the vetiver root essential oils. They also used the DB-5 system for analysis. They obtained 42 components in the chromatogram.

The graphical representation of the data has been given in Fig.3. It shows that out of the five peaks selected, four of them found to be abundant in the mother plant and less in somaclones, while, a component at the peak 30.6 was less in the mother plant and highest in SC -7. The component at the peak 24.8 was absent in three somaclones such as SC-4, SC-5 and SC-6. The component at 27.4 was absent in SC-3 and SC-4. The component at 31.5 was absent in SC-2 and SC-4. This shows that there is a consider amount of variation existing between the mother plant and somaclones and also in between the somaclones.

Here, the components and peaks showed a huge difference between the mother plant and somaclones and the reason could be that the mother plant is well established under normal soil conditions and whereas the somaclones were in green house conditions. The variation among the somaclones could be due to the variation in growth regulator combinations used.

The variation created in the *in vitro* regenerated plants could be due to the culture conditions and culture type which could alter the content and composition of essential oil (Scragg, 1997). Related studies in plant cell cultures by Spreitzer *et al* (1997) also supported that the i*n vitro* conditions provided for the culture triggers the production of secondary metabolites there by favoring greater essential oil production and accumulation in different somaclones.

Summary

6. SUMMARY

Vetiver(*Chrysopogon zizanioides)* is a perennial aromatic grass belonging to the family Poaceae, native to India. The roots yield an oil which is widely used in perfumes. In India as well as the world market, the demand for vetiver oil is steadily increasing due to its unique odour, for which it is used in both flavour and fragrance industries. The oil cannot be made synthetically and is used for the base-note in perfumes.

Flowering occur in the South Indian types of vetiver grown in Kerala, but do not set seed. Hence genetic improvement of this crop is constrained. In this context induction of somaclonal variation through *in vitro* callus mediated regeneration is an option available for crop improvement. Somaclones have been created in various crops through this method. Callus will be initiated and plantlets regenerated, which will be screened and characterised phenotypically, biochemically and by using molecular markers.

Hence the present study on "Induction of somaclones in vetiver [*Chrysopogon zizanioides*(L.)Roberty] was undertaken during the period from 2010 to 2012 at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara. The explants collected from AMPRS, Odakkali was used for the study. The salient features of the studies are summarized below.

- Callus induction in vetver was induced in full MS media growth regulator combinations of 0.5mg/l 2,4-D, 0.5mg/l 2,4-D+KIN, 1mg/l 2,4-D, 1mg/l 2,4-D+KIN, 1mg/l 2,4-D+KIN +BA, 1.5mg/l 2,4-D, 1.5mg/l 2,4-D+KIN. The callus formed were friable in nature. The best media as per the study was MS with growth regulator combination of 1.5mg/l 2,4-D with a callus index of 200.
- Callus mediated regeneration in vetiver could be induced by using full MS media with growth regulator BA. The growth rate of regenerants varied according to the concentration of the growth regulator

- Root induction in vetiver regenerants could be induced in full MS media supplemented with IAA. The number of days taken for rooting and length of the root varied according to the concentration of growth regulator.
- Sand could be used as a medium for hardening where as the potting mixture was not suitable for the purpose. Better hardening will occur in green house conditions.
- One month after hardening, the plantlets could be planted in pots containing potting mixture for further growth
- The somaclone number six has shown the best growth with maximum number of leaves and maximum height among the somaclones and the somaclone number four showed poor morphological growth

- The CTAB protocol suggested by Doyle and Doyle (1987) was found to be the best for the isolation of genomic DNA from tender leaves of vetiver
- The RNA contamination was completely removed through RNAse- a treatment, which resulted in DNA with no impurities and suitable for RAPD and MSAP analysis.
- The protocol for RAPD and MSAP assay in vetiver was standardised. The results showed that the 40ng genomic DNA, 40 ng template DNA, 2.5 mM $MgCl_2$, 100 µM of dNTPs mixture, 1x *Taq* buffer, 20 pM of decanucliotide primer and 1 unit of *Taq* DNA polymerase made up to a final volume of 20 µl was suitable for RAPD.
- Forty random primers from different RAPD primer kits were screened and 10 of them showing good amplification were selected for RAPD profiling of the vetiver mother plant and the seven somaclones
- A total of 92 RAPDs were generated of which average percentage of polymorphism was 7.4.
- The scored data based on RAPD banding was used to construct a dendrogram using the NTSYS pc (ver. 2.02i). In the dendrogram, all the somaclones and the mother plant were grouped into 4 clusters. The first cluster included the mother plant. The second cluster included SC 1. The third singleton cluster included SC 2. Rest of the somaclones came under cluster 4. The maximum variation observed was 19 per cent

between the mother plant and somaclones SC 6 and SC 7, and the least variation was four per cent, between the mother plant and SC 1.

- The RAPD assay confirmed the existence of considerable variation (19%) at the DNA level in the somaclones obtained and also with the mother plant.
- MSAP analysis was done with 250ng of template DNA for each reaction
- MSAP analysis was done with four primers. The results showed that cytosine methylation varied from 10.10 per cent to 17 per cent. That also revealed that there was variation can be induced through callus mediated regeneration.
- The oleoresin extraction was done using solvent extraction method by reflexion and condensation. Highly volatile acetone and less volatile hexane were used for extraction.

The oleoresin was light yellow in colour. The oil content varied from 0.3 to 2.67 percentage. GC Analysis was carried out using DB-5 column. The chromatographic profiles varied for all the somaclones and the mother plant. More number of components was obtained for SC 6 and the least number for SC 2. Comparing the particular peaks obtained in the chromatographic profile, the somaclones have shown considerable variation with the mother plant and also between themselves

By interpreting the above mentioned analyses it is clear that induction of somaclonal variation is possible through callus mediated regeneration in vetiver grass, which may help in further studies for obtaining vetiver varieties with desirable qualities.Further analyses need to be carried out at a later stage of growth of the somaclones to confirm the variations.

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Appendices

ANNEXURE I

Details of laboratory equipment used for the study

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

ANNEXURE II

Composition of plant tissue culture media (MS)

Constituent	**Murashige and Skoog (1962) –MS (mg/l)**
$MgSO_4.7H_2O$	370
$CaCl_2.2\ H_2O$	440
KNO_3	1900
NH_4NO_3	1650
$K\ H_2PO_4$	170
$FeSO_4.7\ H_2O$	27.8
$MnSO_4.4\ H_2O$	22.3
KI	0.83
$CoCl_2.6\ H_2O$	0.025
$ZnSO_4.7\ H_2O$	8.6
$CuSO_4.5\ H_2O$	0.025
H_3BO_3	6.2
$Na_2MoO_4.\ 2H_2O$	0.25
EDTA disodium salt	37.3
m-inositol	100
Thiamine	0.1
Pyridoxine	0.5
Nicotinic acid	0.5
Glycine	2
Sucrose	30000

ANNEXURE III

CTAB method of DNA Isolation (Rogers and Bendich, 1994)

Reagents:

(a) 2X CTAB Extraction Buffer

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

Stock. I - CTAB (10%, w/v)
Stock. II - 1M Tris Buffer (pH 8)
Stock.III - 0.5M EDTA (pH 8)
Stock. IV - 4M NaCl

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

Stock .I - 12ml

Stock. II - 6 ml
Stock.III - 2.4ml
Stock .IV - 21ml
Distilled water- 18.6ml
Total - 60ml

(b) 10% CTAB Solution

10% CTAB (w/v)
0.7M NaCl

(c) TE Buffer

10mM Tris (pH 8)
1mM EDTA (pH 8)

ANNEXURE IV

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

242g Tris base
57.1ml glacial acetic acid
100ml 0.5 EDTA (pH 8.0)

2. Loading Dye (6X)0.25% bromophenol blue

0.25% xylene cyanol
30% glycerol in water

3. Ethidium bromide

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

Abstract

Induction of somaclones in vetiver

[*Chrysopogon zizanioides*(L.)Roberty]

By

RESMI S K
(2010-11-146)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement

for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

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Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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2013

ABSTRACT

The present investigation on "Induction of somaclones in vetiver "[*Chrysopogon zizanioides*(L.)Roberty]" was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2010-2012 with an aim to induce variation in vetiver through callus mediated culture and evaluation of the same using morphological, molecular and biochemical markers.

The explants collected from AMPRS, Odakkali was used for the study. Callus formation was induced in full MS media with growth regulator combinations of 0.5mgl^{-1} 2,4-D, 0.5mgl^{-1} 2,4-D+0.5mgl^{-1} KIN, 1.0mgl^{-1} 2,4-D, 1mgl-1 2,4-D+1.0mgl^{-1} KIN, 1.0mgl^{-1} 2,4-D+1.0 mgl^{-1} KIN +1.0 mgl^{-1} BA, 1.5mg^{l-}1 2,4-D and 1.5mgl^{-1} 2,4-D+1.5mgl^{-1} KIN. The regeneration and rooting were done in full MS media with various combinations of BA and IAA respectively. Hardening of the rooted plantlets were done in paper cups filled with sand under poly house. The hardened plantlets were established into pots containing potting mixture.

Standardisation of DNA extraction was done with the CTAB method. Optimum PCR conditions for RAPD were standardised with various quantities of DNA, dNTPs, $MgCl_2$, primers and *Taq* polymerase. Initially 40 RAPD primers were screened against genomic DNA of the mother plant for their ability to amplify DNA fragments. Of these, 10 RAPD primers were selected for further detailed RAPD profiling. All selected primers produced robust amplification patterns. The PCR products obtained were separated on agarose gel stained with ethidium bromide. A total of 92 RAPDs were generated of which average percentage of polymorphism was 7.4. The scored data based on RAPD banding was used to construct a dendrogram using NTSYS pc (ver. 2.02i). The RAPD assay confirmed the existence of considerable variation at the DNA level in the somaclones obtained..

MSAP analysis was done with 250 ng of template DNA for each reaction with four primers. The results showed that cytosine methylation varied from 10.1 per cent to 17.0 per cent. It also revealed that there was considerable variation between the somaclones

The oleoresin extraction was done using solvent extraction method by reflexion and condensation. Highly volatile acetone and less volatile hexane were used for extraction. The oleoresin obtained was light yellow in colour. The content varied from 0.3 (SC 7) to 2.67 (SC 4) percentage. GC Analysis was carried out using DB-5 column. The chromatographic profiles varied for all the somaclones and the mother plant. More number of components was obtained for SC 6 and the least number for SC 2. SC 4 has shown more variation from the mother plant.

In all the analysis, there is variation reported between the mother palnt and somaclones and also in between the somaclones. The oil extraction can be done further at 18 months of age of somaclones and the GC analysis result obtaining can be correlate with the molecular analysis and morphological analysis.