RAPD ANALYSIS TO ASSESS THE GENETIC STABILITY IN TISSUE CULTURE DERIVED BLACK PEPPER (*Piper nigrum* L.) PLANTS

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "**RAPD analysis to assess the genetic stability in tissue culture derived black pepper (*Piper nigrum* L.) plants.**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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We, the undersigned members of the Advisory Committee of **Mr. T.P. Haneesh Babu,** a candidate for the degree of **Master of Science in Horticulture** with major in **Plantation Crops and Spices**, agree that the thesis entitled "**RAPD analysis to assess the genetic stability in tissue culture derived black pepper (*Piper nigrum* L.) plants.**" may be submitted by Mr. T.P. Haneesh Babu, in partial fulfilment of the requirements for the degree.

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T.P. Haneesh Babu

Dedicated to my beloved

Parents

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LIST OF ABREVIATIONS

CPBMB	- Centre for Plant Biotechnology and Molecular Biology
Cs	- Cytosins
CTAB	= Cetyl Trimethyl Ammonium Bromide
DNA	= Deoxy ribo nucleic acid
dNTPs	= Deoxy nuclcotide triphosphates
E	= Enzyme
EDTA	= Ethylene Diamine Tetra Acetic Acid
Gs	- Guanins
KAU	- Kerala Agricultural University
N	- dNTPs
OP	- Operon
P	- Primer
P_1	- Panniyur - 1
P_2	- Panniyur - 2
P_3	= Panniyur = 3
P_4	= Panniyur = 4
PCR	= Polymerase Chain Reaction
PVP	= Polyvinyl Pyrolidone
Px	= Callus derived source plants of variety P_4
RAPD	= Random Amplified Polymorphic DNA
RFLP	= Restriction Fragment Length Polymorphism
RNA	- Ribonucleic Acid
RNase	- Ribonuclease
SDS	- Sodium Dodecyle Sulphate
TAE	- Tris Acetate - EDTA
TC	- Tissue Culture
TP	- Thermal Profile
TE	- Tris EDTA
UV	= Ultraviolet

INTRODUCTION

INTRODUCTION

Black pepper (*Piper nigrum* L.) is the most important and earliest known spice crop of India and is valued for its intrinsic qualities. It stands first among the spice crops and contributes 1/5th of the income from spice export. Though pepper is propagated by rooted cuttings from runner shoots, dearth of quality planting material is one of the serious problems that curtail black pepper production in the Country. The conventional propagules distributed all over the State are found infected with the soil-borne pathogens/nematodes. Distribution of disease free planting materials would be a novel idea especially when the pepper cultivation is severely hit by the phytophthora foot rot (quick wilt) disease. At present, plant tissue culture offers great potential for micropropagation through *in vitro* manipulation and this has been commercially adopted in various crop species.

The protocol for large scale *in vitro* multiplication of black pepper has been developed at the College of Horticulture, Vellanikkara. This would very well be utilised at commercial level provided the genetic stability of the regenerants is assured. Variation may occur among the tissue culture regenerants based on the source materials used, the routes followed for micropropagation, the subculture level etc.

The discovery of molecular markers and polymerase chain reaction has now made the DNA finger printing easier in crop plants. A wide variety of techniques to reveal the DNA sequence polymorphism have been developed in the past few years and several molecular markers like RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), microsatellites etc. have been utilised in various fields of phylogenic studies, genetic diversity analysis, forensic science, paternity determination, plant varietal characterization etc. Similar studies would also be used to characterise the tissue culture regenerants for their genetic stability. However, such studies have not been

initiated at Kerala Agricultural University (KAU) and reported so far from elsewhere in black pepper. Results of RAPD analysis are independent of environmental influences, tissue types etc. and provide greater resolution than the other techniques. The procedure is faster and easier than other molecular analysis.

The present study aims at standardisation of DNA isolation techniques and the protocol for RAPD analysis in black pepper, so as to assess the genetic stability of tissue culture regenerants, so that the technology could be commercially exploited for large scale *in vitro* multiplication of elite plant types.

2. REVIEW OF LITERATURE

Black pepper belongs to the family Piperaceae of the genus *Piper*. It is traditionally propagated by rooted cuttings from runner shoots.

2.1 Micropropagation in black pepper

In recent years, the *in vitro* response of black pepper has been studied and reported by various workers. Mathew and Rao (1984) have succeeded in producing multiple shoot buds by culturing excised shoot tips of pepper seedlings in MS medium. They have also reported successful callus production from seedling explants.

Rajmohan (1985), reported good callus growth from various explants of black pepper without further differentiation. The *in vitro* response of black pepper studied at the College of Horticulture, Vellanikkara, indicated the feasibility of *in vitro* cloning in black pepper and the use of specific antibiotics in reducing the systemic bacterial interference in cultures (Nazeem *et al.*, 1990; Louis *et al.*, 1994).

Philip *et al.* (1992) have succeeded in *in vitro* culture of shoot tips of black pepper at the University of Calicut, Kerala. The rate of proliferation recorded by them would allow the production of 15000 plants from a single shoot tip in an year.

Joseph *et al.* (1994) developed a viable protocol for mass multiplication of black pepper at the College of Horticulture, Kerala Agricultural University, Vellanikkara. They have also evaluated the *ex vitro* performance of the plantlets upto the flowering stage.

Bhat *et al.* (1995) have also reported the feasibility of plant regeneration from various explants in black pepper.

Joseph *et al.* (1996) succeeded in callus proliferation, somatic embryogenesis and germination of embryos from zygotic embryos of black pepper.

Though protocols have been developed by various workers for the *in vitro* culture of black pepper, its commercial feasibility could be ascertained only after evaluating the genetic stability of the regenerants. Reports on field evaluation of tissue culture derived plants are lacking in black pepper. Great variation in field performance of *in vitro* raised plants have been reported in crops like banana (Drew and Smith, 1990; Epsino *et al.* and Sheela, 1995) and cardamom (Sudarsan *et al.*, 1996 and Chandrappa *et al.*, 1996). Such studies are yet to be taken up in black pepper for utilising the *in vitro* technology for large scale multiplication of elite plants.

2.2 Molecular markers

Genetic studies over the past several years have led to the establishment of several different types of molecular marker which include biochemical markers like isozyme marker and DNA marker. Protein and DNA markers are together called molecular markers. Molecular marker based on genomic DNA have been reported to have great significance in finger printing individuals and in genetics and plant breeding studies.

One of the biochemical markers most widely exploited is proteins. Proteins extracted from different genotypes or tissue sources can be compared by the techniques of electrophoresis. The spectrum of proteins present in the extract can be separated and compared on the basis of size (molecular weight), charge and conformation. The proteins are then visualized by staining.

Another class of biochemical markers that has been investigated include various types of secondary metabolites.

Although these methods have proved to be very useful, they do not provide a complete picture of genetic stability. This is because they are based on the expressed products of genes. All these characters may not be expressed uniformly, as their expression may be related to environmental or physiological factors.

More recently, methods to analyse the genome at the DNA level have been developed. The advantages of these methods are greater resolution and uniformity of DNA in all cells. Several DNA markers have been developed in recent years based on the extensive variation at DNA level among natural populations. Most important among these markers are RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA).

2.2.1 RFLPs

Restriction Fragment Length Polymorphism relies on the specificity of enzymes called restriction endonucleases, which recognise specific base sequences (recognition sites) at which they bind and cut the DNA. This produces fragments of different lengths from the same stretch of genomic DNA of different strains or related species. These differences are detected as bands differing in mobilities on southern blots. A generalized procedure for RFLP is as follows:

Genomic DNA is extracted from several strains or related species. Each of these DNA is then digested with a selected restriction enzyme. The fragments in these digests are separated by gel electrophoresis, each digest being assigned a separate lane in the gel. The DNA in the gel are then denatured and transferred on to a suitable solid support like nitrocellulose membrane and the fragments are fixed to it by southern blotting. The fragments are then hybridized with appropriate radioactive single stranded DNA probes (southern hybridization) and the free probes are washed off. The bands with which the selected probe has hybridized are detected by autoradiography.

The lanes of different strains/related species are compared and RFLPs are detected as differential movement of a band on the lanes.

2.2.2 Polymerase Chain Reaction and RAPD

Kary Mullis discovered Polymerase Chain Reaction (PCR) in 1985 and since then PCR has revolutionised molecular biology studies. It is the amplification of a DNA fragment into millions of copies using a short DNA oligomer as primer. The two oligonucleotide primers flank the DNA fragment by hybridizing to opposite strands of the target DNA.

In PCR a reaction similar to DNA replication takes place, where the essential requirements of DNA replication i.e. the template strand, the four nucleotides primer in the form of deoxyoligonucleotide and DNA polymerase are supplied from outside. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequence and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the primers. Since the extension product of each primer can serve as a template for the other primer, each cycle doubles the amount of the DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, upto several millions in a few hours. This takes place in a thermal cycler in which the temperature levels for denaturation, annealing and extension are controlled automatically. By using the thermostable Taq DNA polymerase isolated from the thermophilic bacterium, *Thermus aquaticus* it has been possible to avoid inactivation of the polymerase enzyme during denaturation step. This has led to the automation of PCR by a variety of temperature-cycling devices.

RAPD is a modification of the basic PCR technique. Instead of using a pair of carefully designed and fairly long oligonucleotide primers to amplify a

specific target sequence, a single short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. The number of amplified products generated by PCR depends on the length of the primer and the size of the target genome, and is based on the probability that a given DNA sequence (complementary to that of the primer) will occur in the genome on opposite strands of the DNA, in opposite orientation within a distance readily amplifiable by PCR. The primers are generally of random sequence, based to contain at least 50 per cent Gs and Cs, and to lack internal inverted repeats. The products are easily separated by standard electrophoretic techniques and visualised by ultraviolet illumination of ethidium-bromide stained gels.

2.2.2.1 RAPD and species relationships

RAPD markers have been widely used for taxonomic and related studies. Demek *et al.* (1992) investigated the potential use of RAPDs for taxonomic studies using *Brassica, Sinapis* and *Rophanus* taxa. Analysis of the RAPD bands revealed the relationship between diploid and amphidiploid *Brassica* taxa. Results showed that the *Raphanus sativus* and *Sinapis alba* were distinct from the *Brassica* taxa.

Halward *et al.* (1992) reported the application of RAPD technique to cultivated peanut and related wild species to determine whether this approach would be feasible for the construction of a genetic linkage map in peanut or for systematic studies of the genus. No variation in banding pattern was observed among the cultivar and germplasm lines of *A. hypogenea*, whereas the wild *Arachis* species were uniquely identified with most primers tested. The bands were scored (+/-) in the wild species and the PAUP computer programme for phylogenetic analysis and the Hyper RFLP programme for genetic distance analysis were used to generate dendrograms. The two analysis produced nearly identical dendrograms of species relationships.

Dunemann *et al.* (1994) investigated the use of RAPD markers for taxonomic studies in *Malus*. Eighteen accessions of wild species and 27 apple cultivars were tested with 29 preselected primers. The analysis of the bands using unweighted pair group arithmatic average showed the relationships among the cultivars which was in agreement with the known lineage. A dendrogram generated for wild species gave relationships that were in accordance with the known phylogenetic information.

Ratnaparkhe *et al.* (1995) used RAPD markers for the identification of pegeon pea [*Cajanus cajan* (L.) Mill sp.] cultivars and its wild species. The results showed extremely high level of polymorphism among the wild species while little variation was detected within the accessions. The cultivars and wild species under study were distinguished with the help of different primers.

Lazaro and Aguinagalde (1998) carried out a study to evaluate the genetic diversity in 29 populations of wild taxa of the *Brassica oleracea* L. group and two cultivars, using RAPDs. The results were compared with the results of earlier study using isozymes. Genetic diversity in *Brassica* populations were estimated using the ANOVA programme.

Santalla *et al.* (1998) investigated the genetic diversity of 19 land races of the cultivated mungbean and three weedy and wild relatives including *Vigna mungo*, *V. luteola* and *V. radiata* var. *sublabata*, using RAPD technique. Polymorphic bands of 229 numbers were obtained and the analysis of the bands resulted in a dendrogram which separated the accessions studied into three main clusters.

2.2.2.2 RAPD for identification of cultivars/genotypes

RAPD assay has been used for the identification of cultivars and genotypes and for genetic finger printing and related studies in a variety of crop

species. Detection of DNA sequence polymorphism among closely related lines of common wheat (*Triticum aestivum*) has been reported by He *et al.* (1992). A high level of polymorphism was observed among a number of commercial varieties and breeding lines of wheat. Over 38 per cent of the 65 primers, used for PCR amplification, produced readily detectable and reproducible DNA polymorphism.

Fukuoka *et al.* (1992) have reported the use of RAPDs for the identification of rice accessions. Sixteen rice accessions were assayed with 28 primers which generated 116 polymorphic bands. All accessions were uniquely distinguished by at least one RAPD and clustered into three distinct groups which corresponded to *Japonica, Javanica* and *Indica*.

Collins and Symons (1993) detected polymorphism between the nuclear DNA of grape vine (*Vitis vinifera*) cultivars and clones. Unique finger prints of a number of cultivars were distinguished using either one or two primers.

Koller *et al.* (1993) differentiated eleven apple cultivars using RAPD markers obtained by PCR. A set of bands consistent in their presence or absence were choosen to create a differentiating band pattern. They have proposed a key by which one can differentiate apple cultivars using commercially available primers.

Wolf and Van Rijiu (1993) studied the genetic variation in Chrysanthemum (*Dendrauthema grandiflora*) using RAPDs. The results showed that the variation between cultivars was high and that the cultivars used could be distinguished from each other by using only two different primers. A family of cultivars, derived from one original cultivar by vegetative propagation, had identical fragment patterns.

Mori *et al.* (1993) characterised 36 potato cultivars released in Japan and three introduced cultivars by RAPD assay. The banding patterns were highly reproducible and identical using DNA samples obtained from different locations

and tissues. Five decamer primers amplified 15 polymorphic bands shared among the cultivars.

Coussirat (1994) have reported genetic diversity and varietal identification in *Nicotiana tabacum* by RAPD markers. Thirtytwo varieties were screened using 160 primers and most of the amplification products were constant for all the varieties, but nine primers gave 29 bands showing intervarietal polymorphism. These markers allowed differentiation of the varieties by calculation of genetic distance and cluster analysis.

Keil and Griffin (1994) assessed the reproducibility of the RAPD technique and its ability to discriminate between individual genotypes for verification of clonal identities in four separate studies, using samples of *Eucalyptus* supplied by several organisations. RAPD profiles unique to a genotype were generated reliably and simply and even closely related genotypes were distinguished.

N'goran *et al.* (1994) assessed the genetic diversity of 106 genotypes of *Theobroma cacao* L. using RFLP and RAPD methods. Both RFLP and RAPD bands separated the individuals into three distinct groups. Similar results were obtained with RFLPs and RAPDs with regard to the genetic structure of *Theobroma cacao* L.

Barcaccia (1994) tried to optimize PCR variables for RAPD analysis in the genus Medicago and the evaluation of the reproducibility of genome specific finger prints. The influence of type of thermal cycler, ramping temperatures, magnesium concentration, polymerase brand and primer performance on the optimal DNA yields and useful electrophoretic patterns was studied.

Gidoni *et al.* (1994) studied the feasibility of developing cultivar specific markers in commercial strawberries. Reproducible RAPD finger prints were

generated for eight cultivars, each containing at least one polymorphic DNA product. A combination of 10 polymorphic DNA products exhibited cultivar-specific patterns enabling the distinction between closely related varieties.

Virk *et al.* (1995) tested rice accessions obtained from the major world collection held at IRRI using RAPD technique to study the diversity within the large germplasm collection, at the University of Birmingham, UK. Optimisation of PCR technique by testing the several factors affecting reproducibility of RAPD amplification was carried out. Variation between rice accessions was observed with 18 of the 24 primers employed.

Karihaloo *et al.* (1995) carried out RAPD analysis on 52 accessions of *Solanum melongena* and related weedy forms. Twenty two primers amplified a total of 130 fragments. *S. melongena* exhibited 117 fragments, all of which were also present in wild forms. Wild forms displayed an additional 13 fragments not found in the cultivated *S. melongena*. The RAPD results were closely concordant with the result of an isozyme survey.

Bhat *et al.* (1995) carried out a study to identify the cultivars of *Musa* using RAPDs and RFLPs. Genomic DNA from 57 *Musa* cultivars were amplified with 60 random primers which generated 605 polymorphic products. Statistical analysis of the data grouped the cultivars into specific clusters depending upon their genomic similarities. The diploid ancestral species of cultivated banana and plantains, namely *M. acuminata* ssp. *Malaccensis* and *M. balbisiana* were farthest apart from each other in the phenogram. The edible fruit yielding cultivars with the genomic constitution of AA, AAA, AB, AAB, ABB and ABBB were grouped in different clusters according to overall genetic homologies. The result of RFLP analysis were comparable with that of RAPD analysis.

Lashermes *et al.* (1996) have successfully employed RAPD markers to analyse genetic diversity among cultivated and subspontaneous accessions of *Coffea arabica*. The narrow genetic base of commercial cultivars was confirmed by their study. On the other hand, a relatively large genetic diversity was observed within the germplasm collection. Results suggested an East-West differentiation in Ethiopia, the primary centre of diversification of *C. arabica*.

Hang *et al.* (1996) studied the DNA variation and genetic relationships among hulless barley accessions using RAPD at the National Small Grain Research Facility, Aberdeen, USA. Thirty six hullers barley accessions from North America, China, Turkey and Central Asia were analysed and 47 reproducible fragments were produced by amplification. Cluster analysis revealed that most of the accessions from North America belonged to one well-defined cluster. Those from China, Turkey, Central Asia and one cultivar from North America belonged to another group.

Samec and Nasinec (1996) used RAPD technique for the identification and classification of *Pisum sativum* L. genotypes. Genomic DNAs of 42 genotypes representing four wild and cultivated subspecies were used for the study. Amplification with eight decamer primers generated 149 polymorphic products. Each genotypes were clearly identified and separated from the others.

Mechado *et al.* (1996) carried a study in Brazil to evaluate polymorphism and genetic similarity between 39 Mediterranean mandarin genotypes, using RAPD. One hundred and eleven amplification products were identified using 21 random primers, cluster analysis revealed the low level of genetic variation between accessions of Mediterranean mandarins, whereas their hybrids with other citrus species showed greater genetic dissimilarity.

Demek *et al.* (1996) studied the genetic diversity of 28 potato genotypes using RAPD procedure. Twelve decamer primers yielded a total of 158 amplified fragments and analysis of the RAPD bands reflected the expected trends in relationships of the genotypes. However, there were important exceptions to this general trend and it appeared that the related varieties could be genetically different as varieties with no immediate relationships.

Ford and Taylor (1997) also reported the application of RAPD markers for potato cultivar identification. Of the 63 decamer primers screened, 51 produced 256 amplification product of which 33 were polymorphic between the cultivars assessed. Polymorphic bands were selected to produce cultivar specific markers to identify correctly suspect materials in commercial plantings.

Stavrakakis *et al.* (1997) used RAPD technique in order to identify and discriminate between eight cultivars of *Vitis vinifera*. Over 140 reproducible polymorphic fragments were generated by this method. Each grape cultivar showed a unique banding pattern for more than five of the primers used. The degree of genetic similarity was calculated and a dendrogram was constructed. The results showed that RAPD analysis a reliable and very useful method for the identification and genomic analysis of grape cultivars.

Iqbal *et al.* (1997) have used RAPD analysis to evaluate the genetic diversity of elite commercial cotton varieties. Twenty two varieties belonging to *Gossypium hirsutum* and one to *G. arboreum* were analysed with 50 random decamer primers. Forty nine primers detected polymorphism in all varieties tested giving 349 bands, cluster analysis showed that 17 varieties can be placed in two groups.

Duran *et al.* (1997) analysed 48 coconut types belonging to the East African Tall types by different DNA marker techniques including RAPDs,

microsatellite primed PCR and ISTR analysis. All three approaches detected large number of DNA polymorphism among the set of genotypes and allowed the identification of single genotypes by individual-specific finger prints. The cluster and principal co-ordinate analysis were done and the observed clustering and association of individuals corroborated the expectations based on the known geographical origin and parental relationships.

Varghese *et al.* (1997) evaluated the applicability of RAPD markers in the cultivated rubber tree, *Hevea*, using 43 primers in a set of 24 clones selected in different south-east Asian countries. Out of the total 220 fragments amplified 111 were polymorphic. The statistical analysis indicated the absence of a distinct geographical grouping because of the breeding history of *Hevea*.

2.2.2.3 RAPD and hybrids

RAPD technique has been used for the identification of hybrids and their parentage determination as well. Wang *et al.* (1994) proposed RAPD fingerprinting as a convenient tool for the identification, protection and parentage determination of plant hybrids. In their study, DNA from three families of rice plants selected in Northern China (each comprising the male sterile, the restorer, the hybrid F_1 and and the maintainer lines) was extracted and amplified by RAPD technique. The results obtained were useful for identification of each single plant line.

Truksa and Prochazka (1996) reported different banding pattern based on the DNA polymerase used for testing three lines of cucumber used for production of hybrid seeds. Low level of polymorphism was obtained which indicated that RAPD was not suitable for verifying the hybridity of seeds.

Tatineni *et al.* (1996) analysed genetic diversity of 16 near-homozygous elite cotton genotypes derived from interspecific hybridization. The study was carried out at the DNA level using RAPD procedure and at phenotypic level using

stable and highly heritable morphological characters. Analysis of the data from both procedures produced two clusters with one resembling *Gossypium hirsutum* and the other *G. barbadense*. Classification of all genotypes based on the two methods gave similar results.

Harvey and Botha (1996) evaluated two PCR based methodologies in the determination of DNA diversity between 20 commercial sugarcane hybrids and six 'outgroup' varieties of *S. spontaneum*, *S. officinarum* and hybrids from early in the geneology. First method involved RAPD while the second protocol utilized specific microsatellite and telomere sequences as primers. A total of 41 RAPD primers were screened across the varieties of which 15 were used in the calculation of DNA diversity. The RAPD data indicated that there had been a gradual decline of DNA diversity from the early interspecific hybrids to the commercial hybrids.

2.2.2.4 RAPDs for identification of somatic hybrids

One of the limiting factors for the efficient exploitation of protoplast fusion is the difficulty of unequivocally identifying nuclear hybrids. RAPDs have been used to characterise molecularly both interspecific and intraspecific somatic hybrids. Baird *et al.* (1992) proposed RAPDs for the identification of hybrids at an early stage following fusion in potato. Inter and intra-specific somatic hybrids of potato were characterised by using RAPD along with sexual hybrids.

Xu *et al.* (1993) used RAPD assay for the identification of somatic hybrids between *Solanum tuberosum* and *Solanum brevidens*. Somatic hybrids showed a combination of the parental banding pattern with four of the five primers surveyed. Whereas regenerants from one of the parents had a similar banding pattern as that of the parent.

RAPD analysis for the confirmation of somatic hybrids in the dihaploid breeding of potato (*Solanum tuberosum* L.) was reported by Takemori *et al.* (1994).

Polymorphism was easily detected even among closely related clones. All the bands of dihaploids were transmitted stably to the respective hybrids. Hybridity of all the fusion-derived regenerants of 32 fusion combinations were unequivocally confirmed.

Identification of somatic hybrids of tobacco was reported by Filippis *et al.* (1996) from Germany. The relationship between two parental species (*Nicotiana tabaccum* and *N. sustica* and six somatic hybrids produced as a result of fusion of vacuolated and evacuolated protoplasts were analysed. The procedure and protocols were applicable to species as well as to hybrids tested and just four primers gave enough polymorphic data.

2.2.2.5 RAPD and somaclones

There are several reports on the use of RAPD technique for assessing the genetic stability of tissue culture derived plants. It will be highly advantageous if we could identify the variants among the regenerants at an early stage. RAPD method seems to be a suitable method for this.

Oropeza *et al.* (1995) have characterised somaclonal variants resistant to sugarcane virus using RAPD markers. The somaclones were obtained from a susceptible cultivar through somatic embryogenesis by increasing the number of subcultures of embryogenic callus. The selected resistant subclones have maintained the resistance over seven years of testing in the field.

Rani *et al.* (1995) used RAPD markers to assess the genetic fidelity of 23 micropropagated plants of a single clone of *Populus deltoides*. Of the eleven primers used, five distinguished a total of 13 polymorphism common across six micropropagated plants. Apart from these six plants, the amplification products were monomorphic across all the micropropagated plants, the mother plant and four additional field grown control plants.

Sangwan *et al.* (1995) employed RAPD technique to investigate molecular differences and also to generate polymorphic DNA markers in regenerated cassava plants. Twenty primers were used to generate patterns from several regenerants of two cultivars.

Schneider *et al.* (1996) have employed RAPD technique to identify the somaclonal variants in grape vine (*Vitis vinifera*) regenerants. Fortyseven plants, each one regenerated from a single protoplast were analysed using sixty primers. Only one primer indicated somaclonal variation.

HuyBao *et al.* (1996) revealed molecular evidence for the occurrence of stable genomic changes in the transgenic plants by a study in Italy. RAPD approach was employed to analyse the microspore-derived embryogenic rice cells grown in suspension cultures, transgenic plants recovered from protoplasts produced from the cultured cells and self pollination progeny of the transgenic plants. Occurrence of somaclonal variation in the material used to produce protoplasts for gene transfer and the stability of the foreign gene in transgenic plants were established.

Damasco *et al.* (1996) reported detection of dwarf off types from micropropagated cavendish banana plants (*Musa* spp. AAA). Fifty seven normal and 59 dwarf plants generated through micropropagation of cutlivars New Guinea Cavendish and Williams were analysed using 60 random primers of which 19 gave polymorphism between normal and dwarf plants.

Mandal *et al.* (1996) tested seven somaclones of *Lathyrus sativus* having contrasting features and the parent cultivar for genetic identity using RAPD. Out of the 81 primers used, 24 revealed polymorphism. Differences were observed between somaclones and also between some of the somaclones and parent cultivar. It was not possible to identify a particular somaclone with a single primer and a combination of two or more primers was suggested.

Munthali *et al.* (1996) reported detection of somaclonal polymorphism using RAPD, the results of which was similar to a previously reported study employing isozyme and RFLP technologies. One hundred and twenty regenerants of beet and a parent plant were analysed with five primers and two polymorphisms were obtained. Thirty secondary regenerants were then tested and only single band polymorphism was obtained.

Angel *et al.* (1996) have analysed the stability of cassava plants stored under *in vitro* conditions. The study was carried out at CIAT to determine whether any DNA rearrangements resulting from *in vitro* storage under slow growth could be detected by molecular analysis in retrieved plants. RFLPs with homologous probes, RAPDs with twenty primers and DNA fingerprinting with the M-13 probe were tested to detect variation in cassava plants after 10 years of *in vitro* storage. The data obtained in this study support the stability of the cassava germplasm under *in vitro* storage conditions.

Walther *et al.* (1997) described the possibility of early detection of *in vitro* mutants using RAPD analysis. Four different types of somaclonal variants were identified and characterised in banana plants generated by meristem culture.

Piccioni *et al.* (1997) reported the estimation of somaclonal variation in alfalfa by RAPD fingerprinting. Plantlets obtained through enhanced release of axillary buds on a growth regulator-free medium was compared with those obtained through indirect somatic embryogenesis. No polymorphism was exhibited by plantlets derived by enhanced release of axillary buds whereas nine out of 39 plantlets regenerated by indirect somatic embryogenesis different from that of the donor plant for atleast one primer and one amplification product.

Kokaeva *et al.* (1997) have compared several lines and somaclonal variants of pea (*Pisum sativum*) using RAPD analysis. Data obtained revealed that

there was considerable divergence between lines originating from different cultivars and between somaclonal variants that originated from same cultivar.

Godwin *et al.* (1997) performed RAPD analysis among eight rice somaclonal families known to vary for specific characters and somaclonal families which were phenotypically normal. The parental cultivar was found to be homogeneous and homozygons and all but one of the RAPD loci. Polymorphism were reported at 28 of the 45 bands among the somaclonal families. All somaclonal material differed significantly from the parental material.

A report from Japan by Shoyama *et al.* (1997) shows confirmation of homogeneity among plantlets of ginseng regenerated through somatic embryogenesis by employing RAPD technique. Analysis using 21 primers gave monomorphic amplification products for all the plantlets of *Panax notoginseng*.

Hashmi *et al.* (1997) analysed peach [*Prunus persica* (L.) Batsch] regenerants from cv. Sunhigh and Redhaven. Of the 60 primers tried, 35 primers produced results with scorable bands and only 10 of them revealed polymorphism for regenerants of cv. Sunhigh and one revealed low level of polymorphism for regenerants of Redhaven.

Parani *et al.* (1997) reported RAPD fingerprinting in twenty micropropagated plants and the mother plant in *Piper longum*. The RAPD fragments were scored for presence/absence of bands to evaluate Jacard's similarity index. Further analysis of the data showed eighteen micropropagated plants forming a major cluster along with the mother plant. The other two plants could be regarded as somaclonal variants as they have shown less than 80 per cent similarity to the mother plant and other micropropagated plants.

Rout *et al.* (1998) used RAPD markers to evaluate the genetic stability of micropropagated plants of *Zingiber officinale* cv. W3S18. Fifteen primers were

used for the study and all the RAPD profiles from micropropagated plants were monomorphic and similar to those of field grown control plants.

Rival *et al.* (1998) conducted RAPD analysis in order to investigate the genetic fidelity of somatic embryogenesis-derived regenerants of oil palm (*Elaeis guinensis* Jacq.). Clonal variants were compared with the normal type using 387 primers. Of these, 73 primers enabled the identification of polymorphism between clones. No intraclonal variability and no difference between mother palms and regenerants could be identified.

A study by Khanuja *et al.* (1998) at the Central Institute of Medicinal and Aromatic Plants, Lucknow proved the utility of RAPD markers for rapid isolation of somaclones of altered genotypes in *Mentha arvensis*.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiments for the present study were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Vellanikkara during 1997-99. Experiments were conducted to standardize the DNA isolation technique, protocol for RAPD analysis in black pepper and to evaluate the genetic stability of tissue culture derived plants.

3.1 Materials used

3.1.1 Plant material

Rooted cuttings of black pepper varieties namely P-1, P-2, P-3, P-4 and Karimunda, maintained in pots in the glass house attached to the College of Horticulture, Vellanikkara were used for the standardization of DNA extraction and developing protocols for the RAPD analysis.

Tissue culture (TC) derived plants maintained in polybags in the glass house were used for the evaluation of genetic stability among the regenerants. The *in vitro* performance of the cultures and their post plant out performance are depicted in Plate 1. The TC derived plants at two stages of growth namely just hardened stage (2 months old) and just before field planting (6 months old) were evaluated for genetic stability. The mother plants of these regenerants, maintained in the field were assessed for ensuring clonal fidelity.

3.1.2 Chemical, glassware and plasticware

Most of the chemicals used for the study were of good quality (AR Grade) from various agencies with which College of Horticulture has rate contract agreement. Some other specific items were procured from outside agencies. Liquid nitrogen required for the study was obtained from Madras Oxyacetylene Company, Coimbatore. The Taq DNA polymerase enzyme supplied by Bangalore Genei Ltd.,

Plate 1a: In vitro performance of tissue culture regenerants of black pepper.

Plate 1b: Post-plantout performance of tissue culture regenerants.

Bangalore and Amersham, Germany was used for the study. The decamer primers were procured from Operon Technologies Inc., USA (Appendix I).

All the glassware used were obtained from Borosil India Ltd. and the plasticware of polypropylene were supplied by Merck India and Tarsons India Ltd.

3.1.3 Equipment and machinery

The equipment available the Centre for Plant Biotechnology and Molecular Biology and the Biochemistry Lab., College of Horticulture were used for the present study. The spectrophotometer, Spectronic Genesys-5 from Spectronic Instruments Inc., USA, the electrophoresis unit of Hoefer Pharmacia, the programmable thermal cycler *PTC-200* from *MJ Research, USA*, the transilluminator of Hero Lab, Germany and the gel documentation system of Alpha Infotech, USA were used for the study (Appendix II).

3.2 *Standardization of DNA isolation*

Quality of DNA is an important factor which influence the PCR reactions. DNA isolation methods suggested in three authentic reports viz., Dellaporta (1983), Doyle and Doyle (1987) and Rogers and Bendich (1994) were tried for the extraction of genomic DNA in black pepper.

Tender leaves were taken from the selected plants using sterile blades. The leaf samples were collected on ice and then wiped with cotton soaked in 70 per cent alcohol and immediately used for extraction.

3.2.1 Protocol-I

The method suggested by Dellaporta (1983) was slightly modified and tried for black pepper.

3.2.1.1 Reagents used

I Extraction buffer

100 mM Tris-HCl - pH8

50 mM EDTA - pH8

500 mM NaCl

10 mM β-Mercaptoethanol (added immediately before use)

II 20% SDS

III 5 M Potassium acetate

IV Resuspension Buffer-I

50 mM Tris-HCl - pH8

10 mM EDTA - pH8

V Resuspension Buffer-II

10 mM Tris-HCl – pH8

1 mM EDTA - pH8

VI 3M Sodium acetate or 10M Ammonium acetate

VII Isopropanol

VIII Ethanol 70% and 100%

3.2.1.2. Procedure

Tender leaf sample (1.5 g) was collected on ice, cut into pieces with a sterile blade and transferred to a pre-chilled mortar. Tissue was then frozen in liquid nitrogen and ground to a fine powder using a pestle. The powder was transferred to a 50 ml centrifuge tube kept on ice containing 7.5 ml extraction buffer and 20 μl of β-mercaptoethanol. 0.5 ml of 20 per cent SDS solution was added to the tube, mixed well and incubated at 65°C for 10 min. 2.5 ml of 5 M potassium acetate was added, vortexed and incubated the sample at 0°C for 20 minutes. The mixture was then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatent was filtered through a sterile muslin cloth into a clean 50 ml centrifuge tube containing 5 ml of isopropanol. The mixture was then incubated at -20°C for 30 minutes and after

incubation centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatent was then discarded and the DNA in the pellet form was saved and dried. The pellet was then dissoved in 0.4 ml of resuspension buffer-I, transferred to 1.5 ml eppendorff tube and centrifuged at 10000 rpm for 10 minutes. The supernatent was transferred to a clean eppendorff tube, mixed with equal volume of isopropanol:ammonium acetate mixture (3:1), centrifuged at 10,000 rpm for 3 minutes and the pellet was saved. The DNA in the pellet form was then washed first with 70 per cent alcohol and then with absolute alcohol. The pellet was air dried and redissolved in 0.3 ml of resuspension buffer-II.

3.2.1.3 Protocol-Ia

Protocol-I was modified in which β-mercaptoethanol was not added in the extraction buffer, and tried for extraction of DNA without making any change in the other reagents and procedure.

3.2.1.4 Protocol-Ib

Protocol-I was slightly modified in which the leaf tissue was not frozen before grinding the sample. This was done to study the effect of freezing the tissue before grinding. No other changes were made.

3.2.2 Protocol-II

The method suggested by Doyle and Doyle (1987) was slightly modified and tried for DNA isolation in black pepper.

3.2.2.1 Reagents

I Extraction Buffer (4x)

256 g sorbitol

48 g Tris

7.4 g EDTA disodium salt

Dissolved in 80 ml sterile Milli Q water; adjusted the pH to 7.5 with HCl, made up the volume to 1 litre. Added 3.8 g sodium metabisulfate (0.38%) prior to extraction.

II Lysis buffer

200 ml 1.0 M Tris - pH8

200 ml 0.25 M EDTA

200 ml sterile H_2O

20 g CTAB, stirred to dissolve, then added 400 ml of 5.0 M NaCl

III TE Buffer

10 mM Tris - pH8

1 mM EDTA, dissolved and made upto 100 ml, autoclaved and stored at room temperature

IV Iso-propanol

V Chloroform: isoamyl alcohol mixture (24:1, v/v)

VI 5% Sarcosin

VII Ethanol 100% and 70%

3.2.2.2 Procedure

One gram of the leaf sample was collected, ground in a pre-chilled mortar after freezing with liquid nitrogen using a pestle and 6 ml of 1X extraction buffer was added. The homogenate was then poured into a centrifuge tube (50 ml) containing 15 ml lysis buffer and 2.5 ml sarcosin. The content was mixed well by inversion. Equal volume of chloroform:isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion, and centrifuged at 10,000 rpm for 10 minutes at 4°C. The upper aqueous phase was pipetted out and transferred to a 50 ml centrifuge tube. Added 2/3 volume chilled isopropanol into the tube containing the aqueous phase. The contents were mixed by gently inverting until the DNA was precipitated. The DNA was pelletted by centrifuging at 10,000 rpm for 5 minutes at 4°C. The isopropanol was poured off, drained well and the pelletted DNA was

washed with 70 per cent alcohol and absolute alcohol. The pellete was then allowed to air dry and resuspended in 250 μl of TE buffer.

3.2.2.3 Protocol-IIa

Original protocol was slightly modified and β-mercaptoethanol was added to the extraction buffer. Other conditions were similar to that of protocol-II.

3.2.2.4 Protocol-IIb

Protocol II was modified to include a step in which leaf tissue was not frozen in liquid nitrogen before grinding. No other changes were effected.

3.2.3 Protocol-III

The method suggested by Rogers and Bendich (1994) was slightly modified and tried for DNA extraction in black pepper.

3.2.3.1 Reagents

I 2X CTAB buffer
2% CTAB (w/v)
100 mM Tris (pH8)
20 mM EDTA (pH8)
1.4 M NaCl
1% PVP

II 10% CTAB solution
10% CTAB (w/v)
0.7 M NaCl

III TE buffer
10 mM Tris (pH8)
1 mM EDTA (pH8)

IV Chloroform

Isoamyl alcohol (24:1 v/v)

V Isopropanol

VI Ethanol 70% and 100%

3.2.3.2 Procedure

One gram of leaf sample was ground in a pre-chilled mortar, using a pestle, in presence of liquid nitrogen and transferred to a sterile 50 ml centrifuge tube containing 5 ml hot 2X CTAB extraction buffer. The mixture was then incubated at 65°C for 15-20 minutes. Equal volume of chloroform:isoamyl alcohol mixture was added to the mixture, mixed gently by inversion and centrifuged at 10,000 rpm for 10 min at 4°C. The upper aqueous phase was pipetted out and transferred to another tube. To $1/10^{th}$ of the volume, 10 per cent CTAB was added to the tube and mixed gently by inverting the tube. Equal volume of chloroform:isoamyl alcohol mixture was added to the mixture, mixed gently to form an emulsion and centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was again collected carefully and transferred to another tube. 2/3 volume of chilled isopropanol was added and mixed gently until the DNA precipitated. The contents were centrifuged at 10,000 rpm for 5 min at 4°C to pellet the DNA. The isopropanol was poured off and the pelleted DNA was washed first with 70 per cent alcohol and then with absolute alcohol. The DNA pellet was then air dried and dissolved on 250 μl TE buffer.

3.2.3.3 Protocol-IIIa

Protocol-III was slightly modified and β-mercaptoethanol was added in the extraction buffer. No other change was made.

3.2.3.4 Protocol-IIIb

Original protocol was modified and the leaf tissue was not frozen in liquid nitrogen before grinding the tissue. Other conditions were same to that of protocol- III.

3.3 Purification of DNA

The DNA isolated will also contain RNA in it. To exclude the RNA, the sample has to be treated with RNase.

3.3.1 Preparation of RNase

The Ribonuclease A from Sigma, USA was used to prepare RNAse. One per cent solution was prepared by dissolving RNAse A in TE buffer at 100°C for 15 min. Solution was allowed to cool to room temperature, dispensed into aliquots and stored at -20°C.

3.3.2 Incubation of DNA with RNAse

The extracted DNA suspended in TE buffer (250 μl) was treated with 15 μl of RNAse solution and incubated at 37°C for 1 hour. After incubation, the sample was treated with equal volume of chloroform:isoamyl alcohol mixture. The top layer was saved using a micropipette and transferred to a sterile eppendorff tube, 2/3 volume of chilled isopropanol was added, mixed gently until the DNA was precipitated and centrifuged at 10,000 rpm in a microcentrifuge for 3 min at room temperature. The isopropanol was poured off and the DNA pellet washed first with 70 per cent alcohol and then with absolute alcohol. The DNA was then allowed to air dry, redissolved in 150 μl of TE buffer and stored at -30°C for further use.

3.4 Estimation of quality and quantity of DNA

The quality and quantity of isolated DNA was evaluated through electrophoresis and by spectrophotometry.

3.4.1 Electrophoresis of DNA samples

The purity of the genomic DNA isolated from black pepper leaves was tested by carrying out agarose gel electrophoresis.

3.4.1.1 Materials and equipment

1. Agarose
2. TAE Buffer (50X 1 litre)

   242 g Tris base

   100 ml 0.5 M EDTA (pH8)

   57.1 ml Glacial acetic acid. Mixed the content well and stored at room temperature.

3. Tracking dye

   Bromophenol blue - 0.5%

   Glycerol - 40 ml

   TAE Buffer - 50 ml

4. Ethidium bromide solution
5. Electrophoresis unit, power supply unit, casting tray and comb.

3.4.1.2 Procedure

Gel buffer (TAE 1X) was taken in a conical flask (100 ml for large gel and 30 ml for small). Agarose (1% for DNA and 1.4% for RAPD samples) was weighed, added to the flask, stirred and boiled with frequent stirring till the agarose dissolved completely. Ethidium bromide was added into the flask and it is allowed to cool to 65°C. The open end of the gel casting tray was sealed with cellotape and placed on a horizontal surface and the comb was placed properly on the tray. The dissolved agarose was poured gently into the tray. The gel was allowed to solidify for 30 minutes and then the comb was removed carefully. The gel was then placed in the electrophoresis unit with the well side directed towards cathode. 1X TAE buffer was added to cover the gel with a few mm of buffer. 10 μl of DNA sample

(15 μl in case of RAPD products) was pipetted out onto a parafilm and mixed well with 4 μl of loading dye. The samples were then loaded carefully into the well by using micropipette. Standard DNA molecular weight markers were also added in one well. The cathode and anode of the electrophoresis unit were then connected to the power supply and the gel was run at constant voltage (50 mA). The power supply was turned off when the loading dye moved to the required distance.(1 1/2 to 2 hours).

3.4.1.3 Gel documentation

The gel was taken from electrophoresis unit and viewed under UV light in a UV transilluminator. The ethidium bromide stain intercalates between the nitrogen bases of DNA and flouresce in orange colour under UV light. The image of the gel was monitered and stored in a gel documentation system (Alpha Imager-2000, Alpha Infotech, USA).

3.4.2 Spectrophotometric determination

The genomic DNA isolated and detected as pure through electrophoresis were further evaluated for its quantity using a spectrophotometer (Spectronic-Genesys 5). The samples were diluted 10 times using sterile water and the optical density was determined by reading the absorbance at two specific wave lengths viz., 260 nm and 280 nm. The 260/280 ratio was then calculated to check the purity. Pure DNA gives a ratio 1.8. The DNA in the sample was quantified as per the equation.

OD = 1 at 260 nm $\approx$ 50 μg/ml DNA

i.e., OD at 260 x 50 = μg of DNA/ml–(a). According to DNA dilution a x 2.5 $\approx$ quantity of DNA in μg g^{-1} leaf tissue.

3.5 Effect of leaf maturity on quality and quantity of DNA

To study the effect of leaf maturity on the quality and quantity of DNA extracted, leaf samples at three stages of maturity were tried with the best method of

extraction procedure selected. Mature, half mature and tender leaf samples of the variety P_4 were tried.

3.6 Random amplified polymorphic DNA (RAPD) analysis

RAPD is relatively a new technique in which a single short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a template DNA.

The number of amplified products in RAPD depends on the length of primer and the size of the target genome, and is based on the probability that a given DNA sequence (complementary to that of the primer) will occur in the genome on opposite strands of the DNA, in opposite orientation within a distance of readily amplifiable by PCR. The products were easily separated by standard electrophoretic techniques and visualised by ultraviolet illumination of ethidium bromide stained gels. The procedure described earlier (3.4) was followed for electrophoresis of the amplified DNA samples.

PCR amplification process involve repeated thermal cycles. The procedure reported by Demeke (1992) was attempted for the amplification of black pepper DNA. The cycles included,

a. DNA denaturation at 92°c

b. Annealing of primer to the template DNA at 37°C

c. Primer extension at 72°C catalysed by Taq. DNA polymerase enzyme

The reaction mixture consisted the following:

i. Template DNA

ii. dNTPs

iii. Primer

iv. 10X assay buffer with 15 mM $MgCl_2$

v. Taq DNA polymerase

vi. Sterile Milli-Q water to make up to the required volume

3.6.1 Standardization of reaction mixture

Different combinations of enzyme, primer, dNTPs and template DNA were tried to determine the optimum concentration of components for RAPD analysis in black pepper.

3.6.1.1 Optimization of enzyme, dNTPS and primer

Template DNA concentration was maintained at 50 ng per reaction mixture (25 μl) and different levels of the other components were tried.

The different levels of components were

Enzyme	dNTPs	Primer (OPP-8)
E_1 - 0.3 U	N_1 - 75 μM	P_1 – 5.0 p moles
E_2 - 0.5 U	N_2 - 100 μM	P_2 - 7.5 p moles
E_3 - 0.9 U	N_3 - 150 μM	P_3 – 10.0 p moles

All possible combinations of the three levels of primer, dNTPs and enzyme were tried and the best one was identified based on the amplification pattern.

The combinations tried were

1. $E_1N_1P_1$	10. $E_2N_1P_1$	19. $E_3N_1P_1$
2. $E_1N_2P_1$	11. $E_2N_2P_1$	20. $E_3N_2P_1$
3. $E_1N_3P_1$	12. $E_2N_3P_1$	21. $E_3N_3P_1$
4. $E_1N_1P_2$	13. $E_2N_1P_2$	22. $E_3N_1P_2$
5. $E_1N_1P_3$	14. $E_2N_1P_3$	23. $E_3N_1P_2$
6. $E_1N_2P_2$	15. $E_2N_2P_2$	24. $E_3N_2P_2$
7. $E_1N_2P_3$	16. $E_2N_2P_3$	25. $E_3N_2P_3$
8. $E_1N_3P_2$	17. $E_2N_3P_2$	26. $E_3N_3P_2$
9. $E_1N_3P_3$	18. $E_2N_3P_3$	27. $E_3N_3P_3$
		28. Control without primer
		29. Control without DNA

The reaction mixture was prepared as a master mix for the required number of reactions. The aliquots of the master mix was dispensed to 0.5 ml PCR tubes to which the template DNA and sterile water were added. To control samples were also run without primer in one and DNA in the other. The reaction mixtures were centrifuged in a microcentrifuge for mixing the components. The contents were overlaid with 25 μl mineral oil and the PCR tubes were loaded in a thermal cycler (PTC 200, MJ Research, USA).

3.6.1.2 Optimization of template DNA

Optimum concentration of template DNA was determined by trying its different levels and keeping the concentration of other components at the optimum level identified in the previous experiment i.e.,

Enzyme	- 0.5 U
Primer	- 10.0 p moles
dNTPs	- 150 μM
Assay Buffer	- 1X per 25 μl

The different levels of template DNA tried were

D_1 - 25 ng

D_2 - 50 ng

D_3 - 75 ng per 25 μl

3.6.2 Standardization of temperature profiles

Different thermal profiles were tried to find out the optimum thermal conditions for proper amplification of black pepper DNA. The details were as follows:

Step no.	Temperature (°C)	Duration (min)	Steps involved	No. of cycles
1. T.P.1				
1	94	3	Initial denaturation	1
2	94	1	Denaturation	
3	37	1	Annealing	→ 45
4	72	2	Extension	
2. T.P. 2				
1	94	1.5	Initial denaturation	1
2	94	1.0	Denaturation	
3	37	2.0	Anncaling	→ 40
4	72	2.0	Extension	
5	72	5.0	Final extension	1
3. T.P. 3				
1	92	1.0	Initial denaturation	1
2	92	1.0	Denaturation	
3	35	1.0	Annealing	→ 35
4	72	2.0	Extension	
5	72	5.0	Final extension	1
4. T.P. 4				
1	94	2	Initial Denaturation	1
2	92	2	Denaturation	
3	37	1	Annealing	→ 45
4	72	1.5	Extension	
5	72	10	Final Extension	1
5. T.P. 5				
1	94	1.5	Initial denaturation	1
2	92	1.0	Denaturation	
3	37	1.0	Annealing	→ 45
4	72	1.5	Extenstion	
5	72	10	Final extension	1
6. T.P.6				
1	93	2	Initial denaturation	1
2	92	1	Denaturation	
3	40	1	Annealing	→ 40
4	72	2	Extention	
5	72	10	Final extension	1
7. T.P. 7				
1	93	2	Initial denaturation	1
2	92	1	Initial denaturation	
3	37	1	Anealing	→ 45
4	72	2	Extension	
5	72	10	Final Extension	1

The PCR products were electrophoresed on 1.4 per cent agarose gel to observe the amplification pattern. The thermal cycle setting that gave best amplification was selected for further studies.

3.6.3 Screening of random primers for RAPD

The decamer primers obtained from 'Operon Technologies', USA, were used for the study. Twenty primers each under the three sets of OPE, OPF and OPP were tried with the best thermal cycle setting using the genomic DNA from variety Panniyur-1. The details of primers tested is given Table 1. Those primers that gave better amplification with 5 to 10 reproducible bands were selected and further utilized for varietal screening.

3.6.4 Screening of black pepper varieties by RAPD assay

Genomic DNA isolated from five different varieties, namely, Panniyur-1, Panniyur-2, Panniyur-3, Panniyur-4 and Karimunda were subjected to RAPD assay using the selected primers and reaction mixture under the best thermal cycle setting (the details of primer selected is given in Table 2). The PCR products were analysed by gel electrophoresis. The polymorphism observed among the varieties were documented and those primers that gave polymorphic bands were selected to evaluate clonal fidelity and genetic stability of tissue culture regenerants of black pepper.

3.7 Evaluation of genetic stability of tissue culture regenerants

Tissue culture derived plants at two stages of growth, namely just hardened and just before field planting were analysed by RAPD assay. Tissue culture regenerants from different mother plants of varieties P_1, P_2 and P_4 were also tested (Table 3). The DNA from these mother plants and tissue culture regenerants was extracted by the best method selected. The DNA was purified, subjected to quality and quantity tests and amplified using selected primers. Three primers viz.

Table 1. List of primers used for screening

Sl.no.	Primder code	Primer sequence
1	OPE-1	CCCAAGGTCC
2	OPE-2	GGTGCGGGAA
3	OPE-3	CCAGATGCAC
4	OPE-4	GTGACATGCC
5	OPE-5	GCAGGGAGGT
6	OPE-6	AAGACCCCTC
7	OPE-7	AGATGCAGCC
8	OPE-8	TCACCACGGT
9	OPE-9	CTTCACCCGA
10	OPE-10	CACCAGGTGA
11	OPE-11	GAGTCTCAGG
12	OPE-12	TTATCGCCCC
13	OPE-13	CCCGATTCGG
14	OPE-14	TGCGGCTGAG
15	OPE-15	ACGCACAACC
16	OPE-16	GGTGACTGTG
17	OPE-17	CTACTGCCGT
18	OPE-18	GGACTGCAGA
19	OPE-19	ACGGCGTATG
20	OPE-20	AACGGTGACC
21	OPF-1	ACGGATCCTG
22	OPF-2	GAGGATCCCT
23	OPF-3	CCTGATCACC
24	OPF-4	GGTGATCAGG
25	OPF-5	CCGAATTCCC
26	OPF-6	GGGAATTCGG
27	OPF-7	CCGATATCCC
28	OPF-8	GGGATATCGG
29	OPF-9	CCAAGCTTCC
30	OPF-10	GGAAGCTTGG
31	OPF-11	TTGGTACCCC
32	OPF-12	ACGGTACCAG
33	OPF-13	GGCTGCAGAA
34	OPF-14	TGCTGCAGGT
35	OPF-15	CCAGTACTCC
36	OPF-16	GGAGTACTGG
37	OPF-17	AACCCGGGAA
38	OPF-18	TTCCCGGGTT
39	OPF-19	CCTCTACACC
40	OPF-20	GGTCTAGAGG
41	OPP-1	GTAGCACTCC
42	OPP-2	TCGGCACGCA
43	OPP-3	CTGATACGCC
44	OPP-4	GTGTCTCAGG
45	OPP-5	CCCCGGTAAC
46	OPP-6	GTGGGCTGAC
47	OPP-7	GTCCATGCCA
48	OPP-8	ACATCGCCCA
49	OPP-9	GTGGTCCGCA

Contd.

Table 1..Continued

Sl.no.	Primder code	Primer sequence
50	OPP-10	TCCCGCCTAC
51	OPP-11	AACGCGTCGG
52	OPP-12	AAGGGCGAGT
53	OPP-13	GGAGTGCCTC
54	OPP-14	CCAGCCGACC
55	OPP-15	GGAAGCCAAC
56	OPP-16	CCAAGCTGCC
57	OPP-17	TGACCCGCCT
58	OPP-18	GGCTTGGCCT
59	OPP-19	GGGAAGGACA
60	OPP-20	GACCCTAGTC

Table 2. Primers selected for varietal screening

Sl.no.	Primer code	Primer sequence
1	OPE-15	ACGCACAACC
2	OPE-16	GGTGACTGTG
3	OPF-6	GGGAATTCCC
4	OPF-9	CCAAGCTTCC
5	OPP-1	GTAGCACTCC
6	OPP-7	GTCCATGCCA
7	OPP-8	ACATCGCCCA
8	OPP-12	AAGGGCGAGT
9	OPP-13	GGAGTGCCTC
10	OPP-14	CCAGCCGAAC

Table 3. List of mother plants and tissue culture regenerants

Sl.no.	Mother plant	No. of tissue culture plants taken
A. Just hardened (2 months old)		
I	Panniyur-1	
1	P_1-32	2
2	P_1-70	2
3	P_1-57	2
4	P_1-34	1
5	P_1-54	1
II	Panniyur-2	
1	K-10	4
2	K-14	3
3	K-17	3
III	Panniyur-4	
1	A-25	5
2	A-22	3
3	A-9	3
B. Ready to plant out (6 months old)		
I	Panniyur-1	
1	P_1-2	6
2	P_1-3	6
3	P_1-4	6
4	P_1-5	6
II	Panniyur-2	
1	P_2-2	6
2	P_2-4	6
3	P_2-5	6
III	Panniyur-4	
1	P4-5	6
2	Px-1	6
3	Px-2	6
4	Px-3	6
5	Px-4	6

OPP 1, OPP 8, OPP 14 were tried for the evaluation of tissue culture derived plants. The amplification products were electrophoresed on agarose gel (1.4%), the gel was viewed under UV illuminator and the image was documented.

RESULTS

4. RESULTS

The results of the experiments conducted on standardization of procedure for genomic DNA extraction in black pepper, standardization of RAPD assay and the evaluation of tissue culture regenerants using RAPD assay are given in this chapter.

4.1 Genomic DNA isolation in black pepper

4.1.1 Standardisation of protocol

Three different procedures reported earlier and their modifications were tried for the extraction of genomic DNA from black pepper. Results of experiments are presented in Table 4.

The quality of DNA isolated was assessed by agarose gel electrophoresis (Plate 2). The results showed that the quality of DNA was better when the tissue was ground in liquid nitrogen in all the three protocols. Among the three protocols tried, protocol-III gave good quality DNA as evidenced in Plate 2 (Table 4). A distinct and discrete band without any smear showed the quality of DNA. It was observed that protocol-IIIa was the best method in terms of quality and quantity of DNA.

In the representative samples shown in Table 4 the ratio of absorbance at 260/280 was between 1.8 and 2.0 in almost all cases except in protocol-Ib and protocol-IIb wherein leaf tissue was not frozen in liquid nitrogen before grinding. The quantity of the DNA extracted by the three protocols varied between 22 to 57 μg per one gram of leaf tissue. Higher recovery was obtained with protocol-IIIa wherein the quality was also good as indicated by the ratio (1.82). Repeated trials confirmed this result.

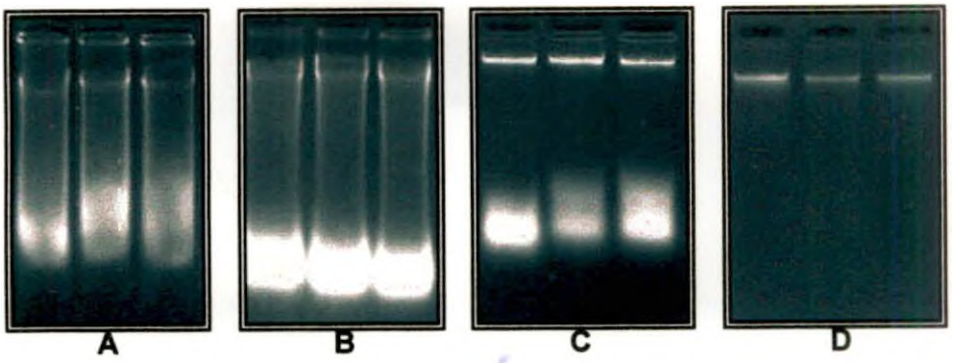

Plate 2. Quality of DNA isolated from black pepper through different protocols
A– Totally smeared DNA (protocol 1) ; B– Partially smeared DNA (protocol 2) ; C-- Intact DNA before RNase treatment (protocol 3 a) ; D– Intact DNA after RNase treatment.

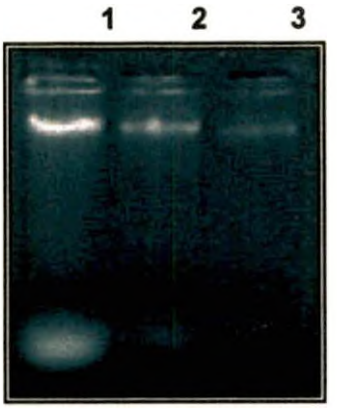

Plate 3. Effect of leaf maturity on quality and quantity of isolated DNA
Lane 1: DNA from tender leaf ; lane 2: DNA from half mature leaf and lane 3 : DNA from mature leaf

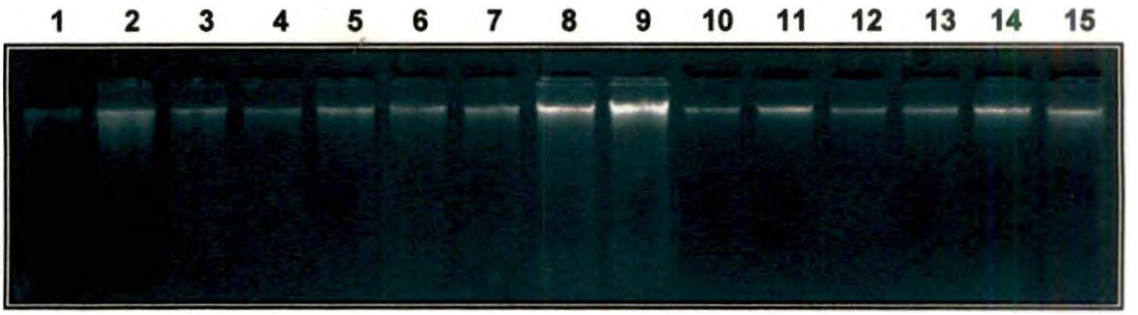

Plate 4. Quality of DNA isolated from TC derived black pepper (Protocol 3 a)

Table 4. Quantity and Quality of genomic DNA isolated using different methods

Sl. no.	Method of DNA isolation	Nature of bands	Absorbance		260/280 ratio	Quantity µg	Quality
			at 260 nm	at 280 nm			
1	Protocol I	Totally smeared	0.318	0.163	1.91	39.7	Poor
2	Protocol Ia	Totally smeared	0.459	0.232	1.97	57.3	Poor
3	Protocol Ib	Totally smeared	0.290	0.139	2.08	36.2	Poor
4	Protocol II	Totally smeared	0.449	0.229	1.96	56.1	Average
5	Protocol IIa	Partly smeared	0.335	0.175	1.91	41.8	Good
6	Protocol IIb	Partly smeared	0.177	0.100	1.77	22.1	Poor
7	Protocol III	Clear narrow	0.349	0.188	1.86	43.6	Good
8	Protocol IIIa	Clear narrow	0.405	0.223	1.82	50.6	Very good
9	Protocol IIIb	Partly smeared	0.286	0.150	1.91	35.7	Average

4.1.2 Effect of leaf maturity on recovery of genomic DNA

Effect of leaf maturity on the quality and quantity of DNA extracted was analysed by taking mature, half mature and tender leaf tissues. Though there was not much difference in the quality of DNA, the yield was more in case of tender leaves (Plate 3 and Table 5).

4.1.3 Isolation of DNA from TC derived black pepper plants

Genomic DNA was isolated from 15 tissue culture regenerants following the protocol IIIa. The protocol was found effective and the high interference of RNA was nullified by RNase treatment. The quality and quantity of the isolated DNA are presented in Table 6 and Plate 4. Absorbance ratio at 260/280 nm ranged between 1.8 and 1.9. The recovery of DNA was between 21 and 35 $\mu g_{-1} g^{-1}$. The electrophoretic profile showed clear narrow bands.

4.2 Standardization of RAPD technique

Standardization of RAPD technique in black pepper was carried out using genomic DNA from variety P_4. In the first step different levels of enzyme, dNTPs and primer were tried. In the second step the level of template DNA was standardized and in the third step the temperature profiles were standardised.

4.2.1 Standardization of reaction mixture

The results of the 27 combinations of different levels of enzyme (E), primer (P) and dNTPs (N) are given in Table 7 and Plate 5. Low level of enzyme (E_1 - 0.3 units) gave poor amplification at lower levels of primer or the dNTPs. However, banding pattern was improved when the amount of primer and dNTPs were increased. It was observed that perfect amplification was obtained with primer at P_3 (10 p moles) and dNTPs at N_3 (150 μM) levels with 0.3 units (E_1) enzyme (Treatment $E_1N_2P_3$ and $E_1N_3P_3$). Intensity of bands were less at lower concentration

Table 5. Effect of leaf maturity on the quality and quantity of genomic DNA isolated in black pepper

Sl. no.	Maturity of leaf	Absorbance		260/280 ratio	Quantity µg	Quality
		at 260 nm	at 280 nm			
1	Mature	0.198	0.107	1.85	24.7	Average
2	Half mature	0.300	0.163	1.84	37.5	Good
3	Tender	0.340	0.183	1.85	42.5	Very good

Table 6. Quality and quantity of DNA extracted by the best protocol selected

Sl.no.	Plant code	260 reading	280 reading	260/280 ratio	Quantity µg	Quality
1	Px.1	0.205	0.112	1.83	25.6	Good
2	Px 1.1	0.174	0.095	1.84	21.7	,,
3	PX1.2	0.234	0.126	1.85	29.2.	,,
4	Px.1.3	0.196	0.105	1.86	24.5	,,
5	Px.1.4	0.248	0.135	1.83	31.0	,,
6	Px.1.5	0.263	0.140	1.85	32.8	,,
7	Px.1.6	0.183	0.098	1.86	22.8	,,
8	Px.2	0.211	0.117	1.80	26.3	,,
9	Px.2.1	0.221	0.119	1.85	27.6	,,
10	Px.2.2	0.228	0.126	1.80	28.5	,,
11	Px.2.3	0.241	0.138	1.81	30.0	,,
12	Px.2.4	0.276	0.151	1.82	34.5	,,
13	Px.2.5	0.254	0.136	1.86	31.7	,,
14	Px.2.6	0.281	0.155	1.81	35.1	,,
15	Px.4	0.268	0.146	1.83	33.5	,,

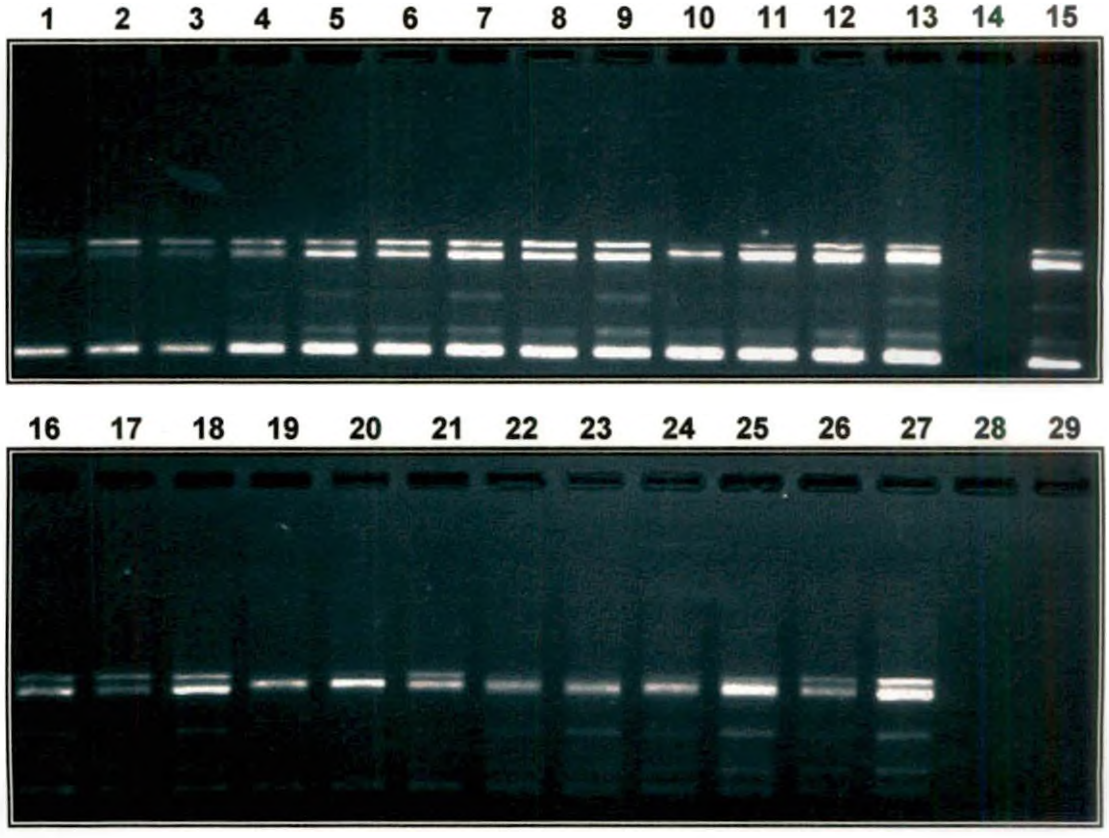

Plate 5. Optimisation of reaction mixture for RAPD assay in black pepper
Lane 1- 28: RAPD profile for treatments 1 to 27 (Table 7) ;
lane 29: Control (without template DNA)

Table 7. Amplification pattern of black pepper DNA at different levels of enzyme, dNTPs and primer

	Treatments	DNA		Band stability
		Amplification	No. of bands	
1	$E_1N_1P_1$	Poor	3	Unstable
2	$E_1N_2P_1$	Poor	3	Unstable
3	$E_1N_3P_1$	Poor	3	Unstable
4	$E_1N_1P_2$	Poor	5	Unstable
5	$E_1N_1P_3$	Average	5	Unstable
6	$E_1N_2P_2$	Average	5	Stable
7	$E_1N_2P_3$	Good	5	Stable
8	$E_1N_3P_2$	Average	4	Stable
9	$E_1N_3P_3$	Good	5	Stable
10	$E_2N_1P_1$	Poor	4	Unstable
11	$E_2N_2P_1$	Poor	4	Unstable
12	$E_2N_3P_1$	Average	4	Unstable
13	$E_2N_1P_2$	Average	5	Unstable
14	$E_2N_1P_3$	Nil	0	Stable
15	$E_2N_2P_2$	Good	5	Stable
16	$E_2N_2P_3$	Good	5	Stable
17	$E_2N_3P_2$	Good	4	Stable
18	$E_2N_3P_3$	Good	5	Unstable
19	$E_3N_1P_1$	Poor	2	Unstable
20	$E_3N_2P_1$	Poor	2	Unstable
21	$E_3N_3P_1$	Poor	3	Unstable
22	$E_3N_1P_2$	Poor	3	Stable
23	$E_3N_1P_3$	Average	5	Stable
24	$E_3N_2P_2$	Average	5	Stable
25	$E_3N_2P_3$	Average	5	Stable
26	$E_3N_3P_2$	Average	5	Stable
27	$E_3N_3P_3$	Good	5	Stable
28	Control without primer	Nil	0	Stable
29	Control without DNA	Nil	0	Stable

of enzyme, primer and dNTPs. Minimum number of bands observed was two and maximum was five.

Number of bands at higher levels of enzyme (E_2 - 0.5 units) were more or less same with all levels of primer and dNTPs. However, more stable bands were obtained with primer and dNTPs at P_2, P_3 and N_2, N_3 levels respectively. Here also the intensity and stability of bands were better with higher levels of primer and dNTPs.

When the enzyme level was further increased (E_3 - 0.9 units) amplification was poor with lower levels of primer and dNTPs. Better amplification was obtained with higher levels and the number of bands also increased with increase in concentration. Among the 27 combinations tried, the optimum combination of reaction mixture identified for black pepper was $E_1N_3P_3$. There was no amplification in the control samples tried.

When the different DNA concentrations were tried it was observed that there was not much difference in banding pattern and it was more or less identified. For further studies, 50 ng template DNA was taken in all cases.

4.2.2 Standardization of temperature profile

Table 8 shows the results of amplification of genomic DNA of variety P_4 using the primer OPP 8 and OPP 12 for the seven temperature profiles tried at selected levels of dNTPs and enzyme. With the primer OPP 8 the thermal profile TP_4 gave the best amplification (Plate 6), while TP_3 gave average quality amplification and it was poor with the other cycles tried. The number of bands for TP_1, TP_2, TP_3, TP_4 and TP_5 cycles observed were three while it was two in others. Amplification products obtained in all the cycles except TP_1 and TP_2 were found stable in repeated experiments. The amplification pattern with the primer OPP 12

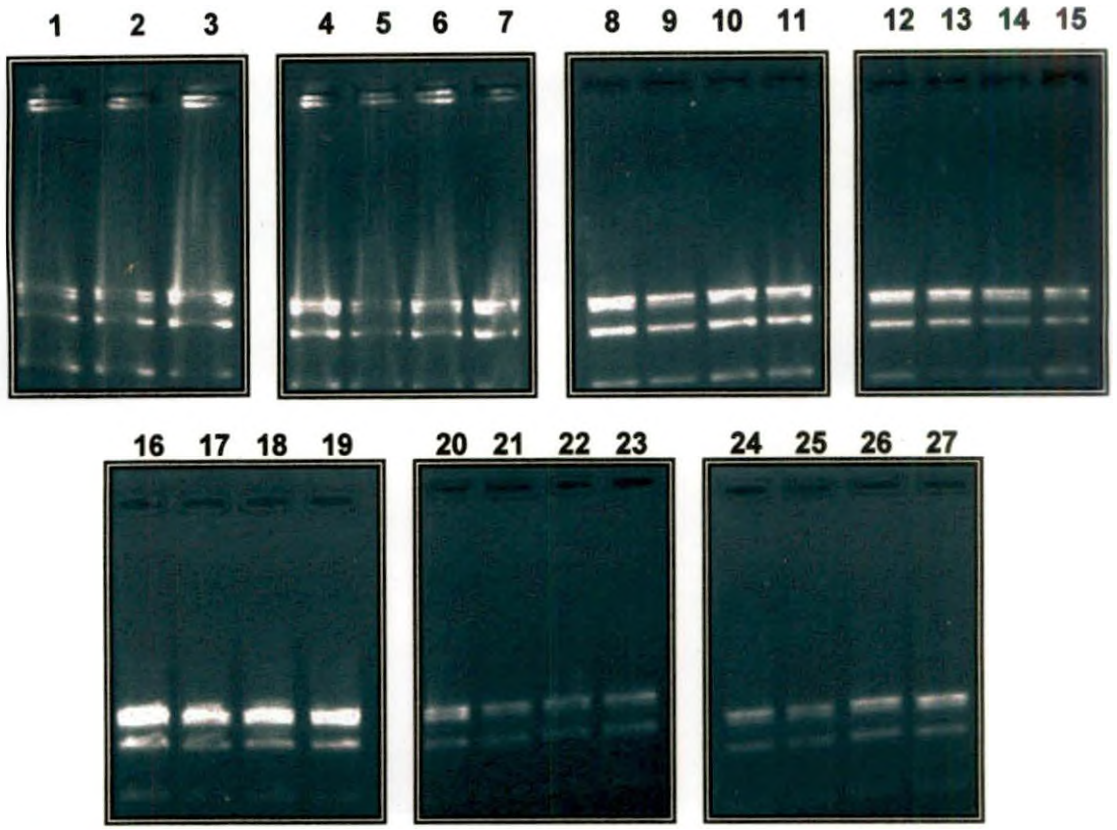

Plate 6. Amplification pattern of black pepper DNA at different thermal settings with primer OPP 8 and selected reaction mixture (Table 8)
Lane 1– 3: TC 1; lane 4– 7: TC 2 ; lane 8– 11: TC 3; lane 12-15: TC 4 ; lane 16– 19: TC 5; lane 20-23: TC 6; lane 24– 27: TC 7

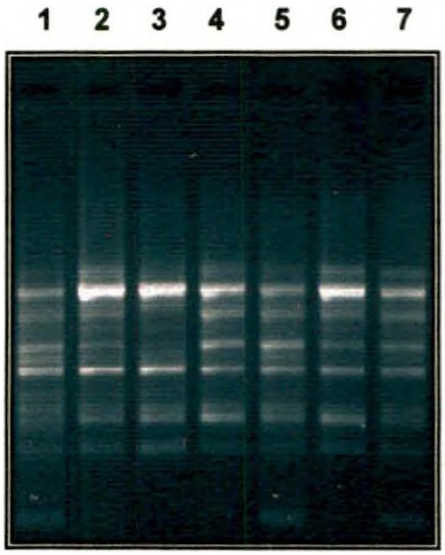

Plate 7. Amplification pattern of DNA at different thermal settings with primer OPP 12 and selected reaction mixture (table 8)
Lane 1: TC1; lane 2: TC2; lane 3: TC3; lane 4: TC4; lane 5: TC 5; lane 6: TC 6; lane 7: TC 7

Table 8. Effect of different temperature profile on the amplification pattern of black pepper

Primer:OPP –8, Reaction mixture – $E_1N_3P_3$

Sl.no.	Temperature profiles	Amplification	No. of bands	Stability
1	TP1	Poor	3	Unstable
2	TP2	Poor	3	Unstable
3	TP3	Average	3	Unstable
4	TP4	Good	3	Stable
5	TP5	Average	3	Unstable
6	TP6	Average	2	Unstable
7	TP7	Average	2	Unstable

Primer:OPP-12, Reaction mixture – $E_1N_3P_3$

Sl.No.	Temperature profiles	Amplification	No. of bands	Stability
1	TP1	Average	5	Unstable
2	TP2	Average	5	Unstable
3	TP3	Poor	4	Unstable
4	TP4	Good	6	Stable
5	TP5	Good	5	Unstable
6	TP6	Average	6	Unstable
7	TP7	Average	4	Unstable

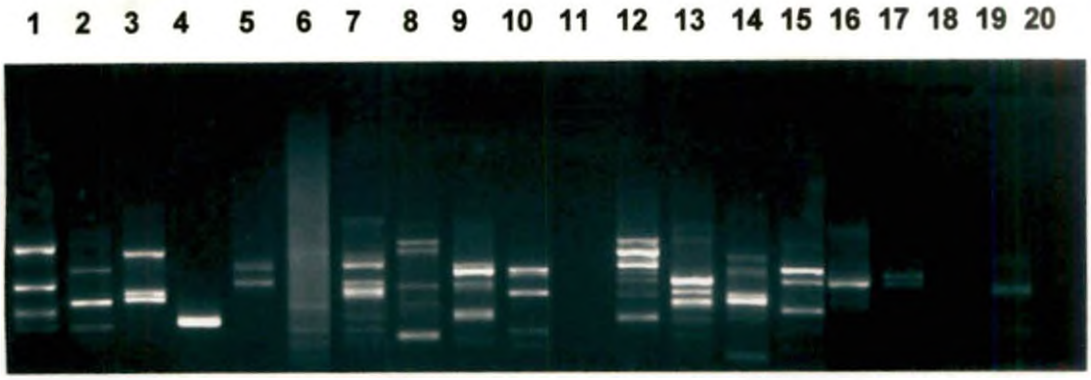

Plate 8. Amplification of black pepper genomic DNA using different decamer primers in OPP series
Lane 1-20 : OPP 1 to OPP 20 respectively

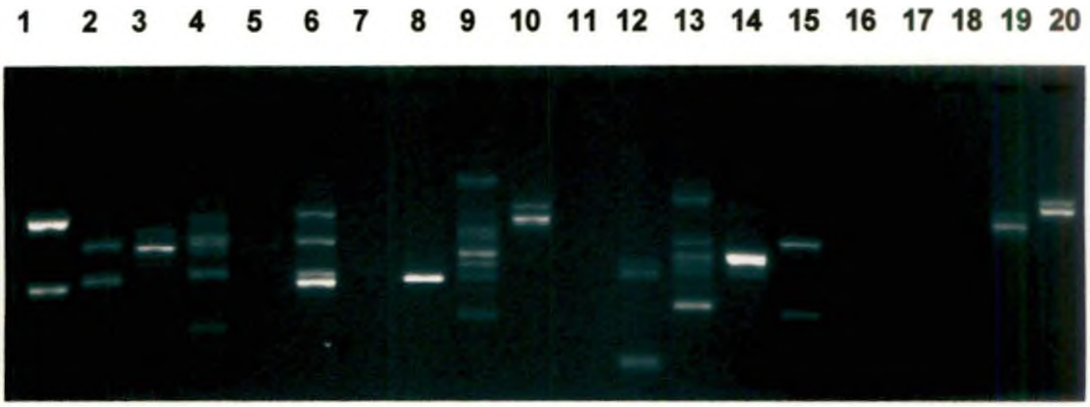

Plate 9. Amplification of black pepper genomic DNA using different decamer primers in OPF series
Lane 1-20: OPF 1 to OPF 20 respectively

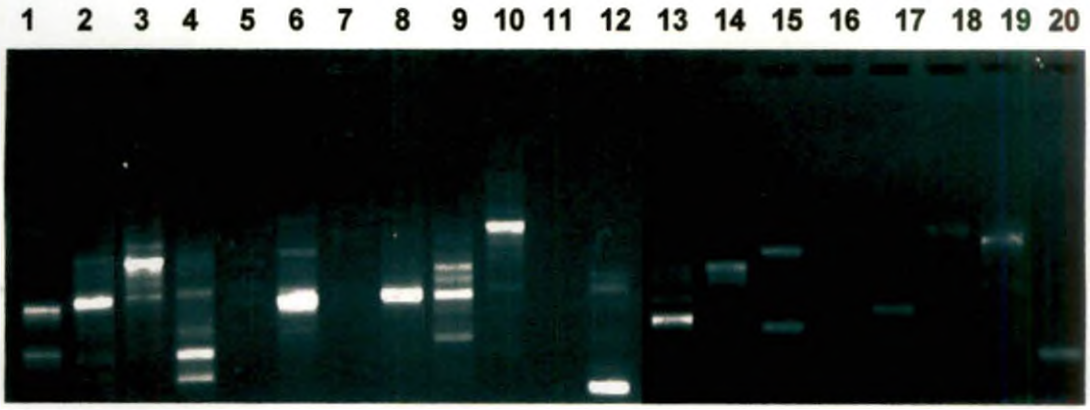

Plate 10. Amplification pattern of black pepper genomic DNA using different decamer primers in OPE series
Lane 1-20 : OPE 1 to OPE 20 respectively

with seven thermal cycle settings is given in Plate 6 and Table 8. The treatment TP_4 gave the best amplification with this primer also (Plate 6 and 7).

4.2.3 Screening of random primers

Sixty random primers were screened with the selected reaction mixture and thermal settings.

4.2.3.1 OPP series

The results of screening of 20 primers of OPP series are presented in Table 9 and Plate 8. Out of the twenty primers screened, six gave good amplification. The number of bands ranged between 0 and 7. Those that gave good amplification were OPP 1, OPP 7, OPP 8, OPP 12, OPP 13 and OPP 14. Number of amplification products were also more for these primers. OPP 1 produced four bands, OPP 7, 8 and 13 produced six bands and OPP 12 and 14 gave seven bands each. A few other primers gave an amplification of average quality and some others with poor quality amplification or no amplification at all. Repeated tests gave similar results. OPP 1, 7, 8, 12, 13 and 14 were selected for further studies.

4.2.3.2 OPF series

Table 10 shows the results of the screening with 20 primers in OPF series. Four primers viz., OPF 6, 9, 12 and 13 gave good amplification with 4, 8, 4 and 7 bands respectively. OPF 1, 5, 8, 11, 15, 16, 17, 18 and 20 gave poor amplification of average quality. Number of bands varied from zero to eight. Subsequent trials gave the same results. OPF 6 and 9 were selected due to better stability for further analysis (Plate 9).

4.2.3.3 OPE series

Table 11 shows the results of screening of 20 primers of OPE series. Out of these, OPE 15 and 16 gave good amplification with five and four number of bands respectively. Number of bands among the primers tested varied from zero to

Table 9. Amplification pattern of black pepper genomic DNA with different decamer primers under OPP series at selected temperature profile and reaction mixture

Primer code	Primer sequence	No. of bands	Quality of amplification
OPP-1	GTAGCACTCC	4	Good
OPP-2	TCGGCACGCA	3	Average
OPP-3	CTGATACGCC	3	Average
OPP-4	GTGTCTCAGG	1	Poor
OPP-5	CCCCGGTAAC	2	Poor
OPP-6	GTGGGCTGAC	4	Average
OPP-7	GTCCATGCCA	6	Good
OPP-8	ACATCGCCCA	6	Good
OPP-9	GTGGTCCGCA	5	Average
OPP-10	TCCCGCCTAC	3	Poor
OPP-11	AACGCGTCGG	0	Poor
OPP-12	AAGGGCGAGT	7	Good
OPP-13	GGAGTGCCTC	6	Good
OPP-14	CCAGCCGACC	7	Good
OPP-15	GGAAGCCAAC	3	Poor
OPP-16	CCAAGCTGCC	4	Average
OPP-17	TGACCCGCCT	3	Poor
OPP-18	GGCTTGGCCT	0	Poor
OPP-19	GGGAAGGACA	2	Poor
OPP-20	GACCCTAGTC	0	Poor

Table 10. Amplification pattern of black pepper genomic DNA with different decamer primers under OPF series at selected temperature profile and reaction mixture

Primer code	Primer sequence	No. of bands	Quality of amplification
OPF-1	ACGGATCCTG	2	Poor
OPF-2	GAGGATCCCT	2	Poor
OPF-3	CCTGATCACC	2	Average
OPF-4	GGTGATCAGG	5	Average
OPF-5	CCGAATTCCC	0	Poor
OPF-6	GGGAATTCGG	4	Good
OPF-7	CCGATATCCC	0	Poor
OPF-8	GGGATATCGG	1	Poor
OPF-9	CCAAGCTTCC	7	Good
OPF-10	GGAAGCTTGG	2	Average
OPF-11	TTGGTACCCC	0	Poor
OPF-12	ACGGTACCAG	3	Average
OPF-13	GGCTGCAGAA	6	Average
OPF-14	TGCTGCAGGT	2	Average
OPF-15	CCAGTACTCC	2	Average
OPF-16	GGAGTACTGG	0	Poor
OPF-17	AACCCGGGAA	0	Poor
OPF-18	TTCCCGGGTT	0	Poor
OPF-19	CCTCTACACC	2	Average
OPF-20	GGTCTAGAGG	2	Average

Table 11. Amplification pattern of black pepper genomic DNA with different decamer primers under OPE series at selected temperature profile and reaction mixture

Primer code	Primer sequence	No. of bands	Quality of amplification
OPE-1	CCCAAGGTCC	2	Poor
OPE-2	GGTGCGGGAA	3	Poor
OPE-3	CCAGATGCAC	4	Poor
OPE-4	GTGACATGCC	5	Average
OPE-5	GCAGGGAGGT	0	Poor
OPE-6	AAGACCCCTC	4	Poor
OPE-7	AGATGCAGCC	0	Poor
OPE-8	TCACCACGGT	1	Poor
OPE-9	CTTCACCCGA	5	Average
OPE-10	CACCAGGTGA	3	Poor
OPE-11	GAGTCTCAGG	0	Average
OPE-12	TTATCGCCCC	3	Poor
OPE-13	CCCGATTCGG	2	Poor
OPE-14	TGCGGCTGAG	2	Poor
OPE-15	ACGCACAACC	2	Good
OPE-16	GGTGACTGTG	0	Good
OPE-17	CTACTGCCGT	1	Poor
OPE-18	GGACTGCAGA	1	Poor
OPE-19	ACGGCGTATG	1	Average
OPE-20	AACGGTGACC	1	Average

five. A few primers gave average quality amplification (OPE 4, 9, 11, 19, 20) and in others the quality of amplification was poor. OPE 15 and 16 were selected for further analysis (Plate 10).

The primers for further analysis were tested based on the number of bands, quality of amplification and stability of expression. Those primers which gave more distinct banding pattern with good quality amplification and reproducibility were selected for further analysis.

4.3 Screening of black pepper varieties by selected primers

The genomic DNA extracted were amplified using the selected primers at selected thermal cycles. Table 12 shows the results of screening of five black pepper varieties using primers selected from the initial screening. In the case of primer OPP-1 two bands were common in all the five varieties viz., Panniyur-1, 2, 3, 4 and Karimunda. The variety Karimunda showed polymorphism with the absence of one band shared by all the Panniyur varieties and expression of an additional band absent in all the Panniyur varieties. The primer OPP 1 also gave polymorphism for variety P_2 and P_3 with the expression of an additional band of high molecular weight (Plate 11, Fig.1).

Amplification with primer OPP 7 resulted in four giving five amplification products common to all the five varieties. The varieties P_1 and P_2 gave uniform pattern whereas P_3 gave polymorphism without expressing any one of the bands. The variety P_4 gave the minimum number of amplifications while the variety Karimunda was unique with eight bands in which three were polymorphic to the variety P_4 (Plate 11, Fig.2).

Primer OPP 8 gave an RAPD profile in which three bands were monomorphic to all the five varieties. Three additional bands were observed for P_3 and the same was shared by P_4 and Karimunda. The variety P_4 expressed five

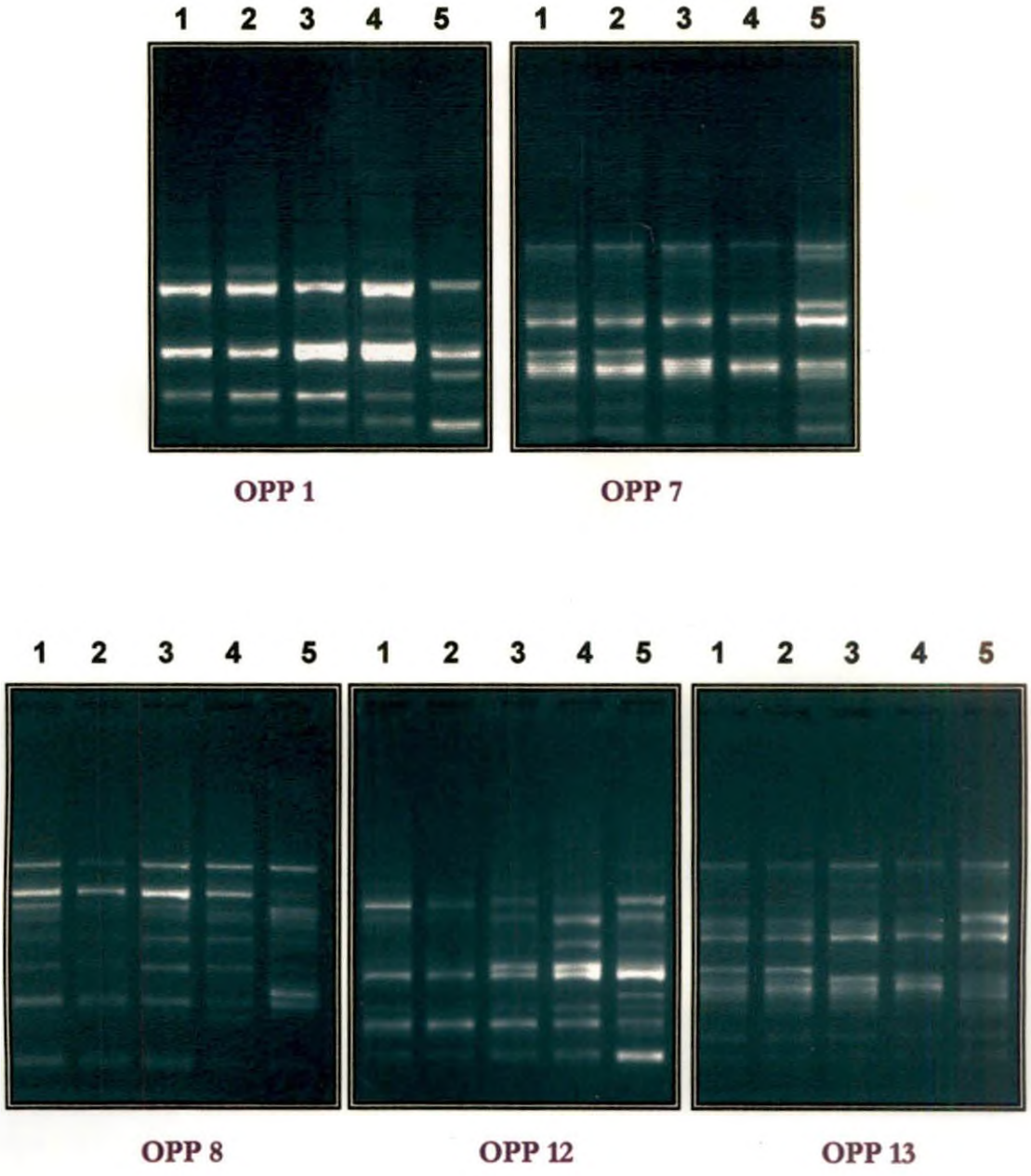

Plate 11. RAPD profile of black pepper varieties using selected primers

Lane 1 : Panniyur 1; lane 2 : Panniyur 2; lane 3 : Panniyur 3,
lane 4 : Panniyur 4 ; lane 5 : Karimunda

polymorphic bands of which three were shared both with P_3 and Karimunda. Two additional bands were found shared only with Karimunda. The variety Karimunda was unique with six polymorphic bands of which one was specific for the variety (Plate 11, Fig.3).

Primer OPP 12 gave amplification products with seven bands common in all the varieties. However, varieties P-1 and P-2 gave an additional band. The variety Karimunda also showed uniqueness with the expression of two additional bands when compared with all other varieties. However, the amplification pattern with this primer was not clear (Table 12, Fig.4).

All the Panniyur varieties (P_1, P_2, P_3 and P_4) gave six monomorphic bands for the primer OPP 13 (Plate 11). However, the intensity of banding differed among the varieties. The variety Karimunda was distinct with the presence of one clear polymorphic band (Plate 11). Another band of relatively low molecular weight was not found expressed in this variety (Plate 11, Fig.5).

Primer OPP 14 produced an amplification pattern with seven bands in P_1 and Karimunda. Six monomorphic bands were observed in P_2, P_3 and P_4 (Table 12).

Primer OPE 16 produced amplification products with five monomorphic bands and two polymorphic bands. Panniyur varieties showed six bands shared among all of them while two polymorphic bands were observed in Karimunda (Table 12).

Primer OPF 6, 9 and OPE 15 produced only monomorphic bands. OPF 9 and OPF 6 produced four monomorphic bands while OPE 15 expressed six bands. However, the banding pattern expressed by OPF and OPE series were not clear and distinct.

Table 12. Screening of black pepper varieties by RAPD assay

Primer	Variety	Amplification products	No. of monomorphic bands	No. of polymorphic bands
OPP-1	Panniyur-1	3	2	1
	2	5	2	3
	3	6	2	4
	4	6	2	4
	Karimunda	4	2	2
OPP-7	Panniyur-1	7	4	3
	2	7	4	3
	3	5	4	1
	4	4	4	Nil
	Karimunda	8	4	4
OPP-8	Panniyur-1	5	3	2
	2	3	3	Nil
	3	6	3	3
	4	8	3	5
	Karimunda	9	3	6
OPP-12	Panniyur-1	8	7	1
	2	8	7	1
	3	7	7	Nil
	4	7	7	Nil
	Karimunda	9	7	2
OPP-13	Panniyur-1	8	6	2
	2	6	6	Nil
	3	8	6	2
	4	8	6	2
	Karimunda	7	6	1
OPP-14	Panniyur-1	7	6	1
	2	6	6	Nil
	3	6	6	Nil
	4	6	6	Nil
	Karimunda	7	6	1
OPE-16	Panniyur-1	6	5	1
	2	6	5	1
	3	6	5	1
	4	6	5	1
	Karimunda	7	5	2
OPF-6	Panniyur-1	4	4	Nil
	2	4	4	Nil
	3	4	4	Nil
	4	4	4	Nil
	Karimunda	4	4	Nil

Contd.

Table 12. Continued

Primer	Variety	Amplification products	No. of monomorphic bands	No. of polymorphic bands
OPF-9	Panniyur-1	4	4	Nil
	2	4	4	Nil
	3	4	4	Nil
	4	4	4	Nil
	Karimunda	4	4	Nil
OPE-15	Panniyur-1	6	6	Nil
	2	6	6	Nil
	3	6	6	Nil
	4	6	6	Nil
	Karimunda	6	6	Nil

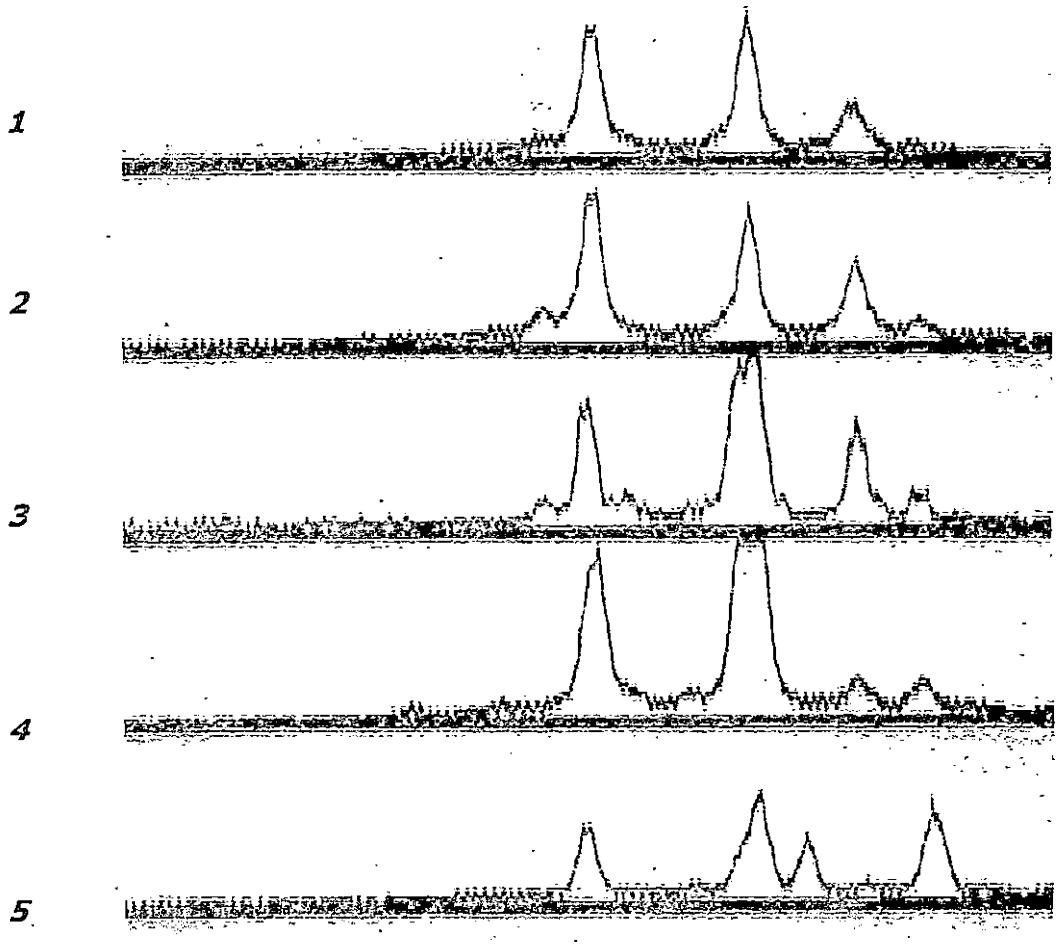

Figure 1. Graphical reprasentation of RAPD profile of different varieties of black pepper with primer OPP 1

- 1, 2, 3, 4, 5 represent varieties Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4 and Karimunda respectively.
- Peaks represent intensity of bands in the RAPD profile

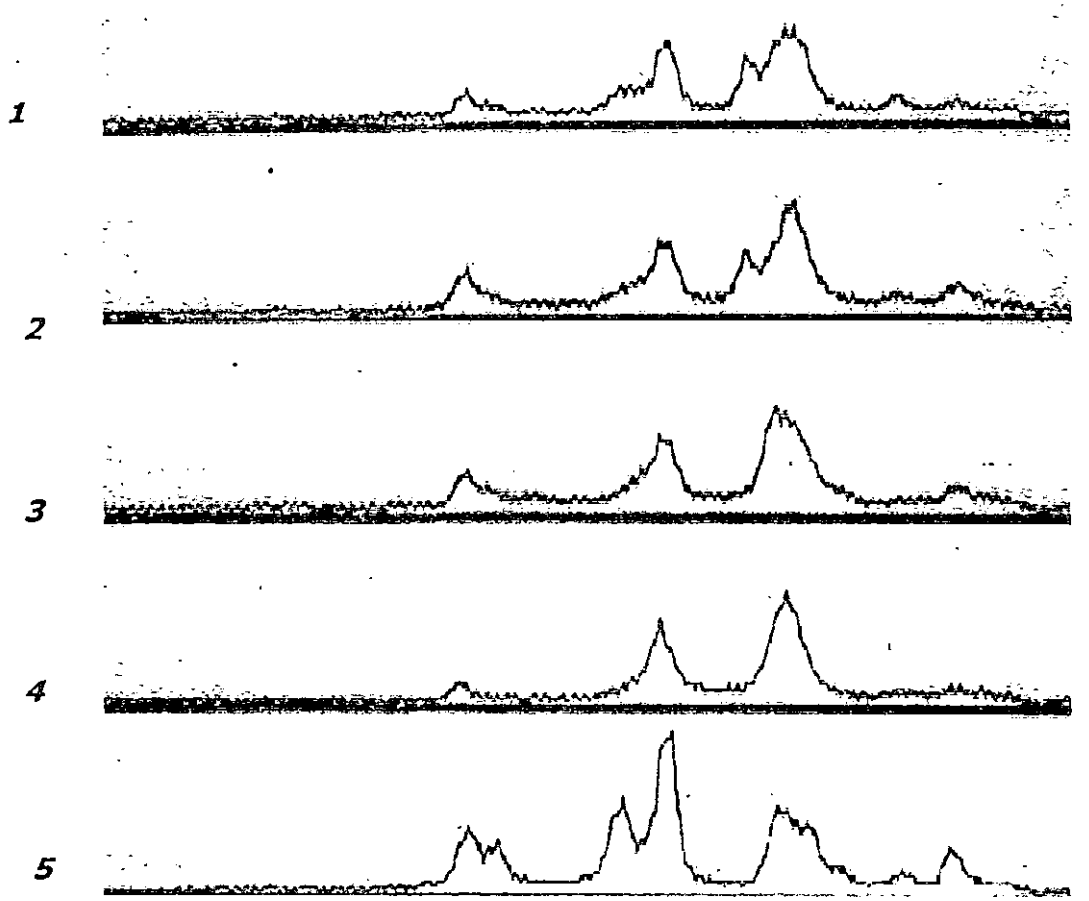

Figure 2. Graphical reprasentation of RAPD profile of different varieties of black pepper using primer OPP 7

- 1, 2, 3, 4, 5 represent varieties Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4 and Karimunda respectively.
- Peaks represent intensity of bands in the RAPD profile

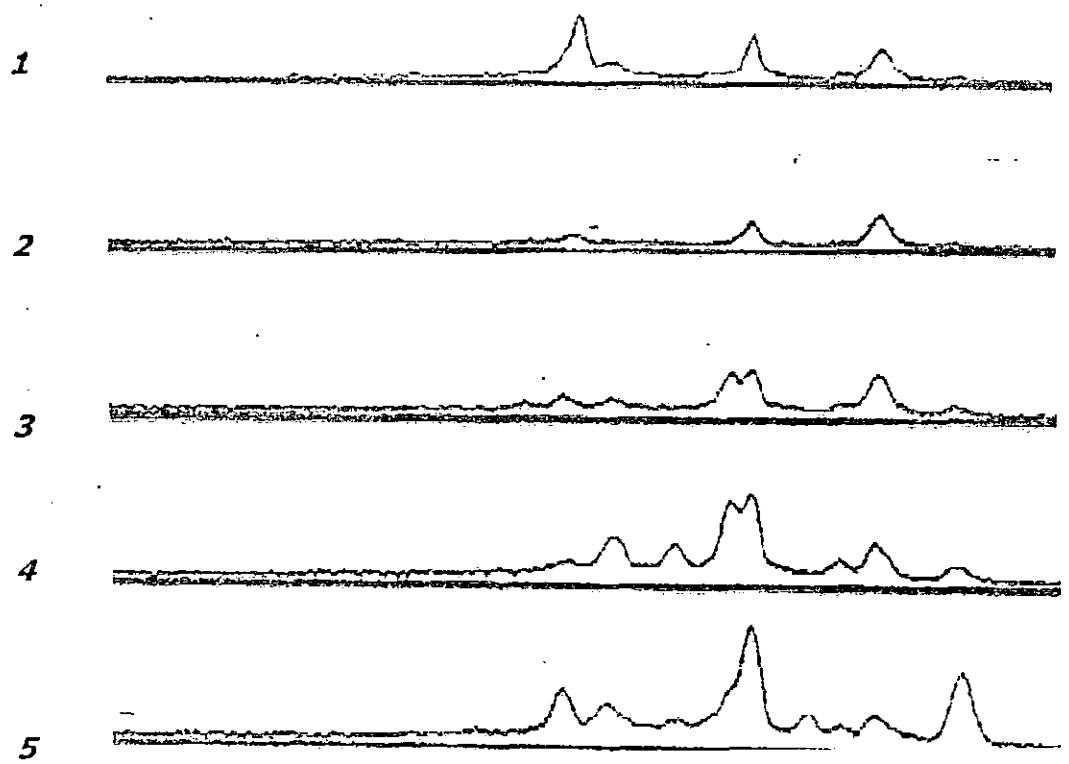

Figure 3 . Graphical reprasentation of RAPD profile of different varieties of black pepper using primer OPP 8.

- 1, 2, 3, 4, 5 represent varieties Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4 and Karimunda respectively.
- Peaks represent intensity of bands in the RAPD profile

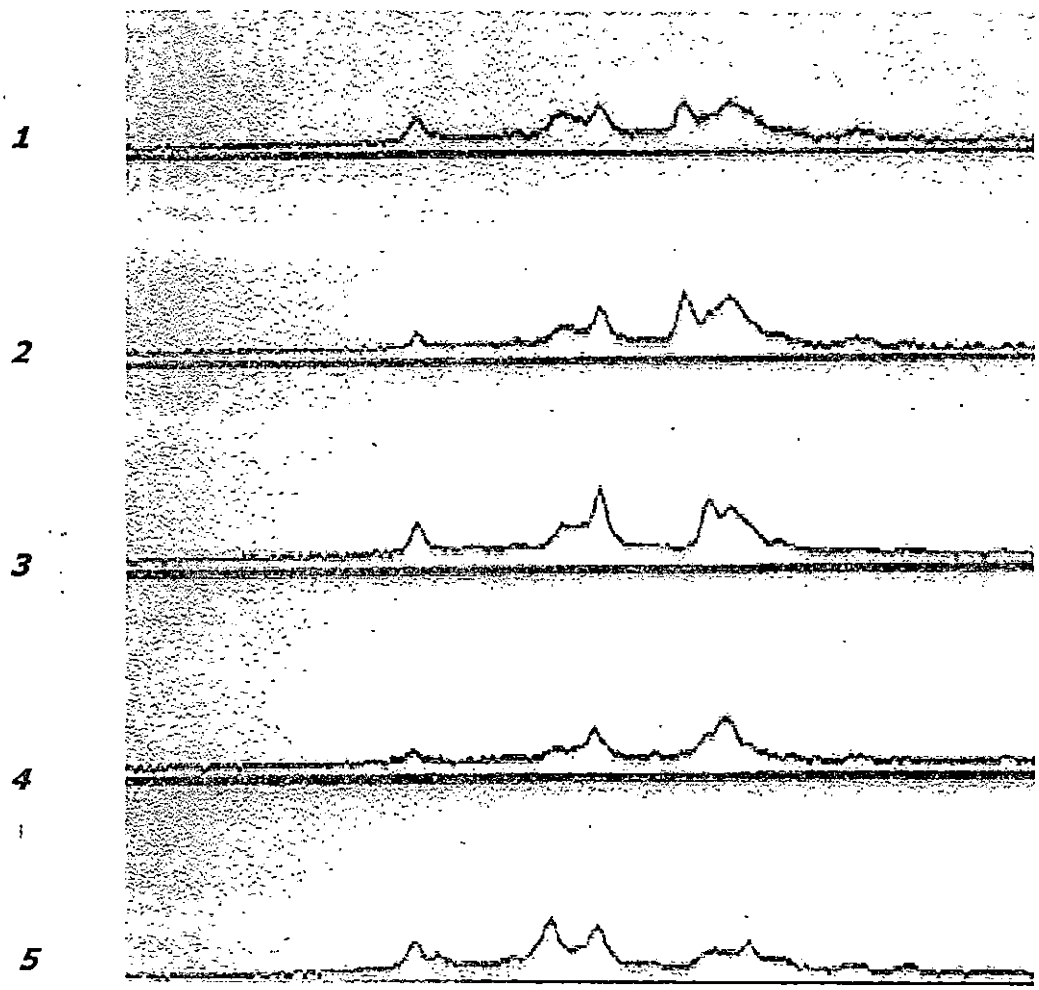

Figure 4. Graphical reprasentation of RAPD profile of defferent varieties of black pepper using primer OPP 12

- 1, 2, 3, 4, 5 represent varieties Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4 and Karimunda respectively.
- Peaks represent intensity of bands in the RAPD profile

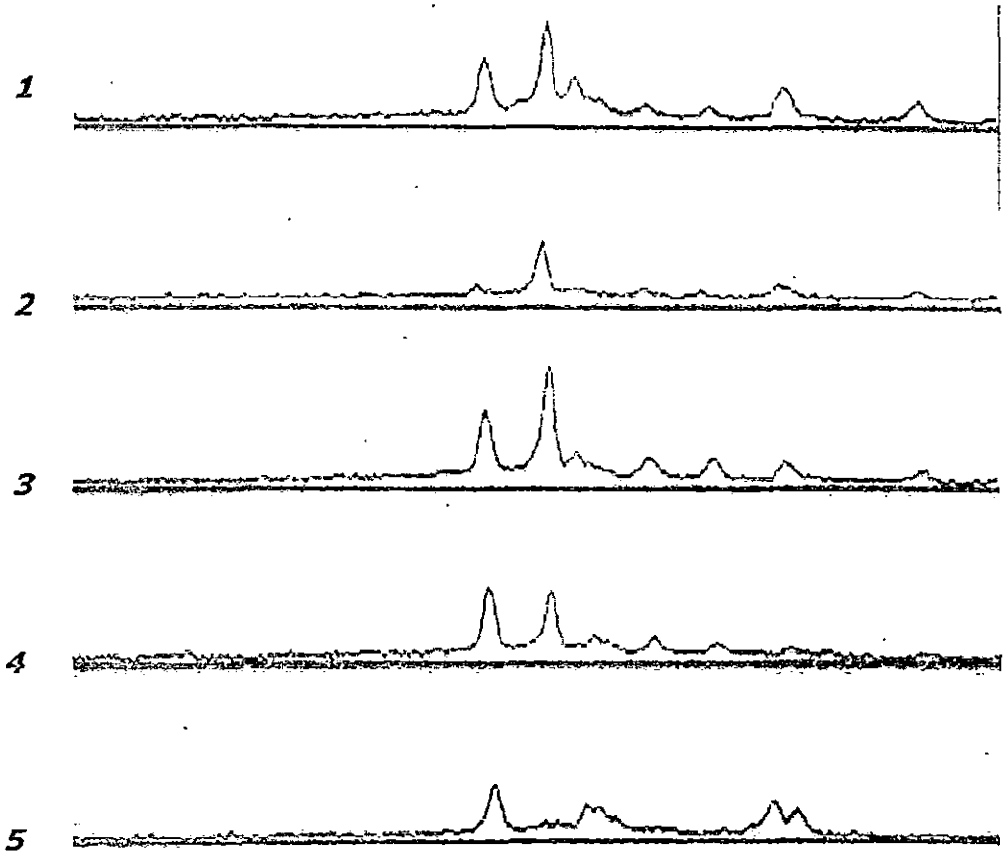

Figure 5. Graphical reprasentation of RAPD profile of defferent varieties of black pepper using primer OPP 13

- 1, 2, 3, 4, 5 represent varieties Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4 and Karimunda respectively.
- Peaks represent intensity of bands in the RAPD profile

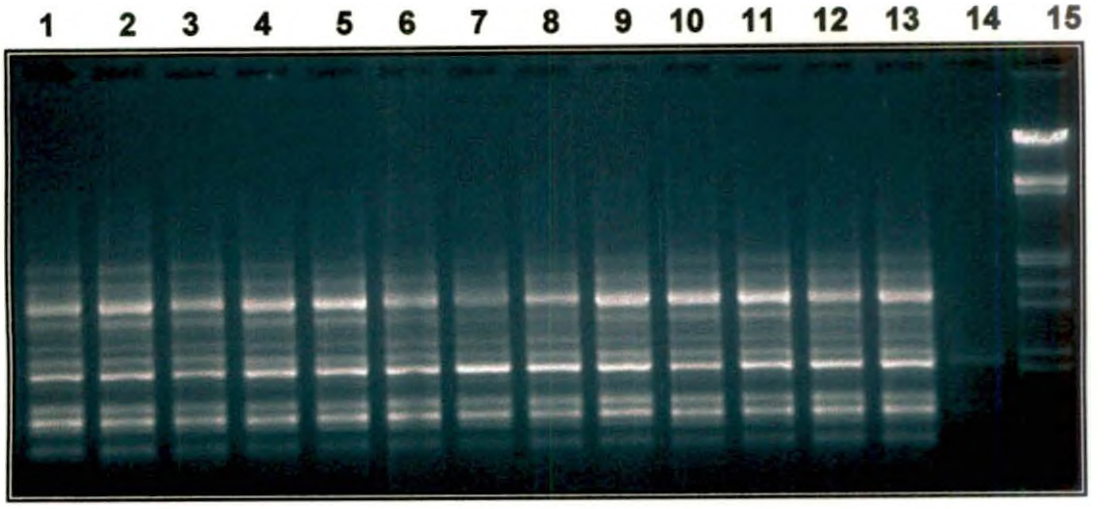

Plate 12. RAPD profile for the TC regenerants from different source plants (P_1- 2 and P_1-3) of variety Panniyur 1 with Primer OPP 1

Lane 1 : Mother plant (P_1- 2) ; lane 2-7: TC plants (P_1-2) ;
lane 8 : Mother plant (P_1- 3); lane 9-14 : TC plants (P_1-3) ;
lane 15: Marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

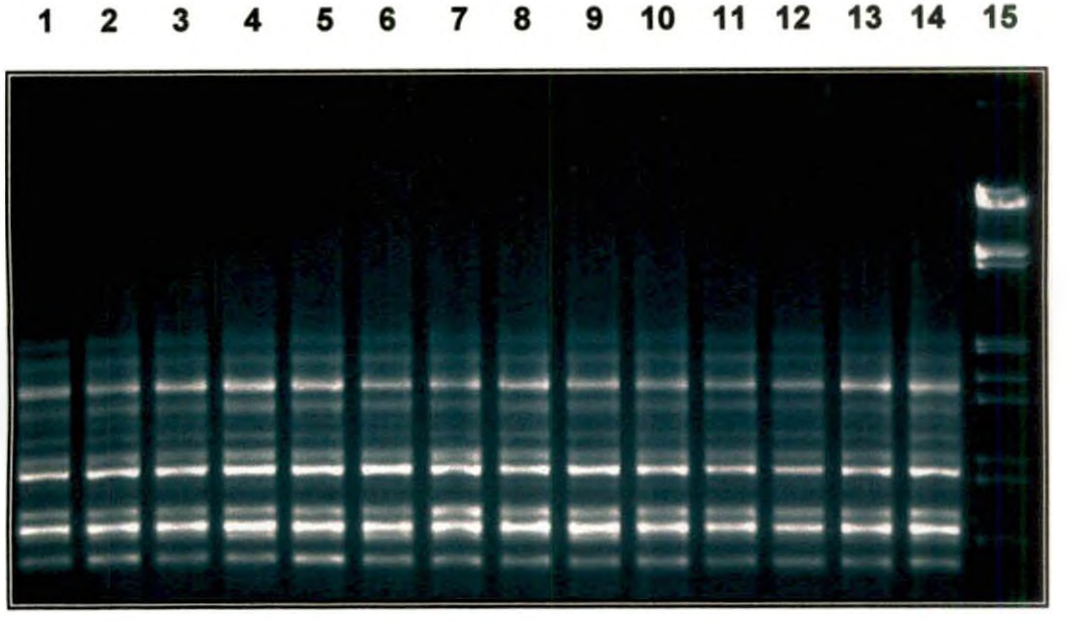

Plate 13. RAPD profile for the TC regenerants from different source plants (P_1-4 and P_1-5) of variety Panniyur 1 with Primer OPP 1

Lane 1 : Mother plant (P_1- 4) ; lane 2-7 : TC plants (P_1-4) ;
lane 8 : Mother plant (P_1- 5) ; lane 9-14 : TC plants (P_1-5) ;
lane 15 : Marker DNA (λ DNA *Eco* R1/*Hind III* digest)

4.4 Evaluation of genetic stability of tissue culture derived plants

4.4.1 Primer OPP 1

The results of RAPD analysis of tissue cutlure regenerants (6 month old) using the primer OPP 1 is presented in Table 13. The tissue culture regenerants of variety P_1 showed no variation among them and also with the mother plant. The RAPD profile showed a monomorphic banding pattern with clear bands common in the mother plants as well as their regenerants (Plate 12, 13).

Mother plants of variety P_2 and their tissue culture derivatives showed the same trend with no variation among them. The monomorphic banding pattern exhibited by the RAPD profile of these plantlets included six common bands in P_2-2 and P_2-3 and seven bands in P_2-5 samples. The TC regenerants showed uniform banding pattern and were similar to the mother plants. Regenerants of P_4-5 also showed a monomorphic banding pattern. The RAPD profiles for the TC regenerants of varieties P_2 and P_4 are presented in Plates 14 and 15.

In the regenerants of Px series, there was no polymorphism between mother plants and also among the regenerants. In the source plants Px-1 and Px-2 and among their TC regenerants, there was eight monomorphic bands (Table 13). The source plants Px-3 and Px-4 and their TC regenerants also showed no variation with nine monomorphic bands expressed at varying intensities in all the samples (Plate 16 and 17).

Table 14 shows the banding pattern among the regenerants of two month old stage using primer OPP 1. There was no variation among regenerants of varieties P_1, P_2 and P_4. Five mother plants of variety P_1 and their eight regenerants each showed monomorphic bands with eleven bands among all the samples (Plate 18). Among the ten tissue culture plantlets of variety P_2, derived from three mother plants, there were six bands in common. Monomorphic pattern was observed among the eleven tissue culture plantlets of P_4 variety derived from three mother plants.

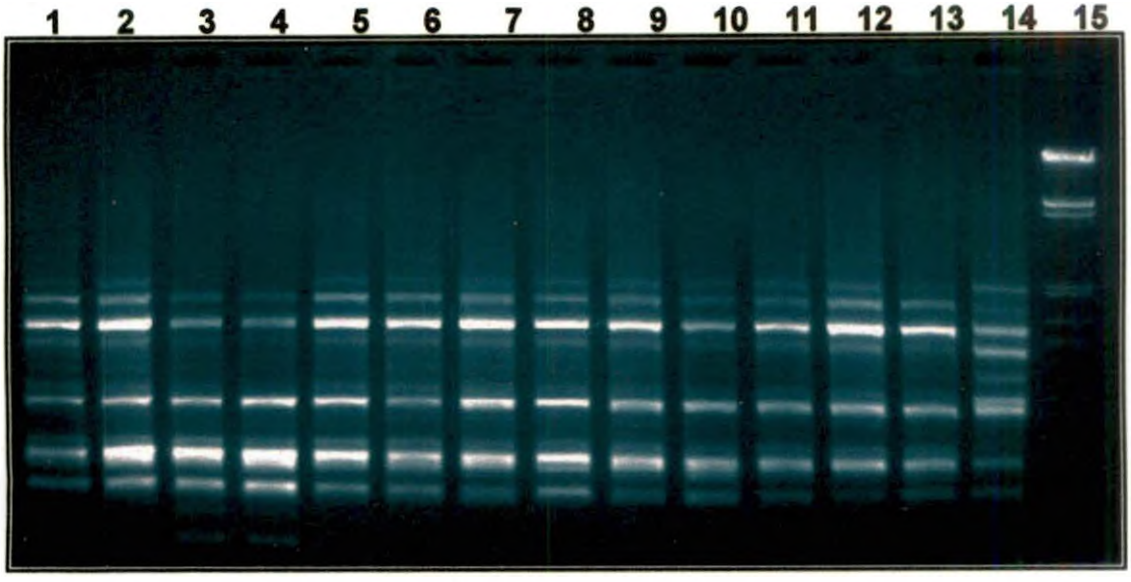

Plate 14 . RAPD profile for the TC regenerants from different source plants (P_2-2 and P_2-3) of variety Panniyur 2 with Primer OPP 1

Lane 1: Mother plant (P_2- 2) ; lane 2-7 : TC plants (P_2-2) ; lane 8: Mother plant (P_2- 3) ; lane 9-14 : TC plants (P_2-3) ; lane 15 marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

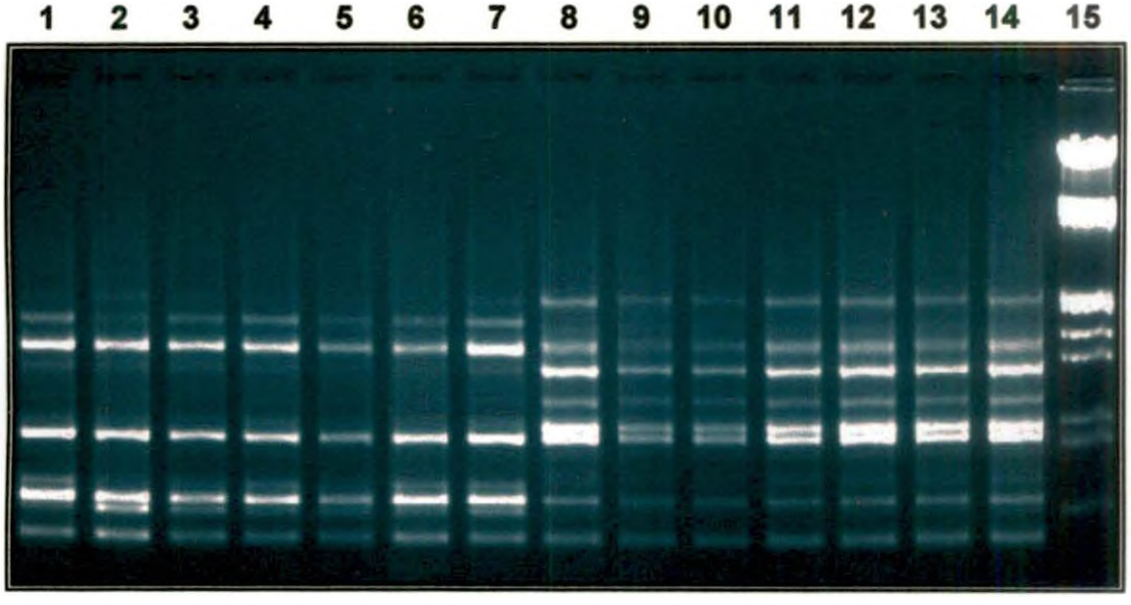

Plate 15. RAPD profile for the TC regenerants from different source plants (P_2-5 and P_4-5) of variety Panniyur 2 & Panniyur 4 with Primer OPP 1.

Lane 1: Mother plant (P_2-5) ; lane 2-7 : TC plants (P_2-5) ; lane 8 : Mother plant (P_4- 5) ; lane 9-14 : TC plants (P_4-5) ; lane 15, marker DNA (λ DNA *Eco* R1/ *Hind* III digest).

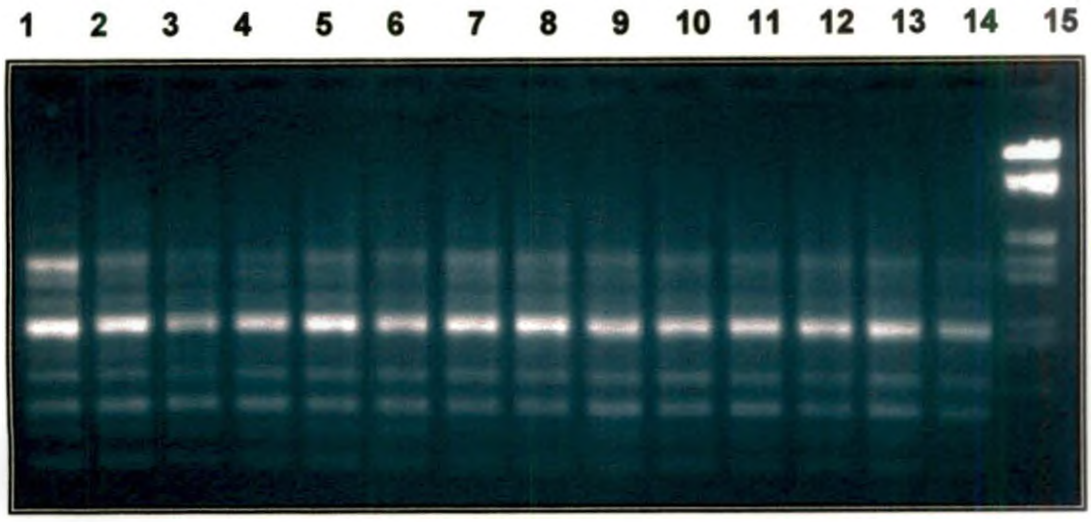

Plate 16 . RAPD profile for the TC regenerants from the callus derived source plants (Px-1 & Px– 2) of variety Panniyur 4 with Primer OPP 1

Lane 1 : Mother plant (Px-1); lane 2-7 : TC plants (Px-1) ; lane 8 : Mother plant (Px – 2); lane 9-14 : TC plants (Px-2) ; lane 15 : marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

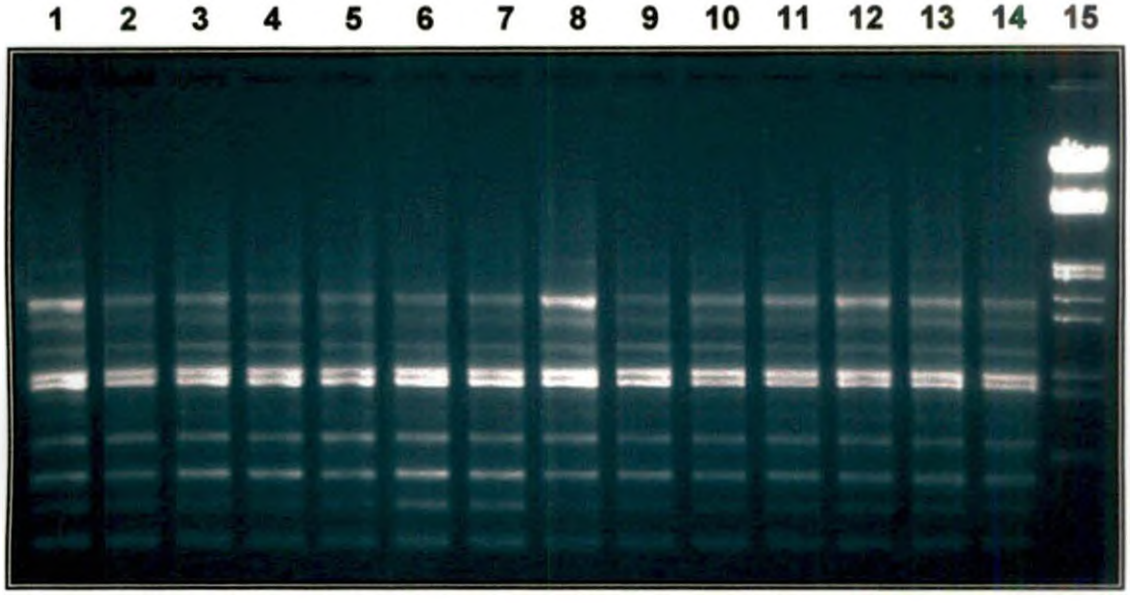

Plate 17. RAPD profile for the TC regenerants from the callus derived source plants (Px- 3 & Px– 4) of variety Panniyur 4 with Primer OPP 1

Lane 1 : Mother plant (Px-3) ; lane 2-7 : TC plants (Px-3) ; lane 8: Mother plant (Px – 4) ; lane 9-14 : TC plants (Px-4) ; lane 15 : marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

Table 13. RAPD profile for the TC regenerants (6 months old) of different black pepper varieties using Primer OPP-1.

Source plant, no.	No. of TC plants analysed	Maximum no. of band per sample	No. of monomorphic bands	No. of polymorphic bands
$P_1 2$	6	11	11	0
$P_1 3$	6	11	11	0
$P_1 4$	6	11	11	0
$P_1 5$	6	11	11	0
$P_2 2$	6	6	6	0
$P_2 3$	6	6	6	0
$P_2 5$	6	7	7	0
$P_4 5$	6	8	8	0
$P_x 1$	6	8	8	0
$P_x 2$	6	8	8	0
$P_x 3$	6	9	9	0
$P_x 4$	6	9	9	0

Table 14. RAPD profile for the TC regenerants (2 months old) of different black pepper varieties using Primer OPP-1

Primer OPP-1

Variety	No. of TC plants analysed	Maximum no. of band per sample	No. of monomorphic bands	No. of polymorphic bands
P_1	8	11	11	0
P_2	10	6	6	0
P_4	11	8	8	0

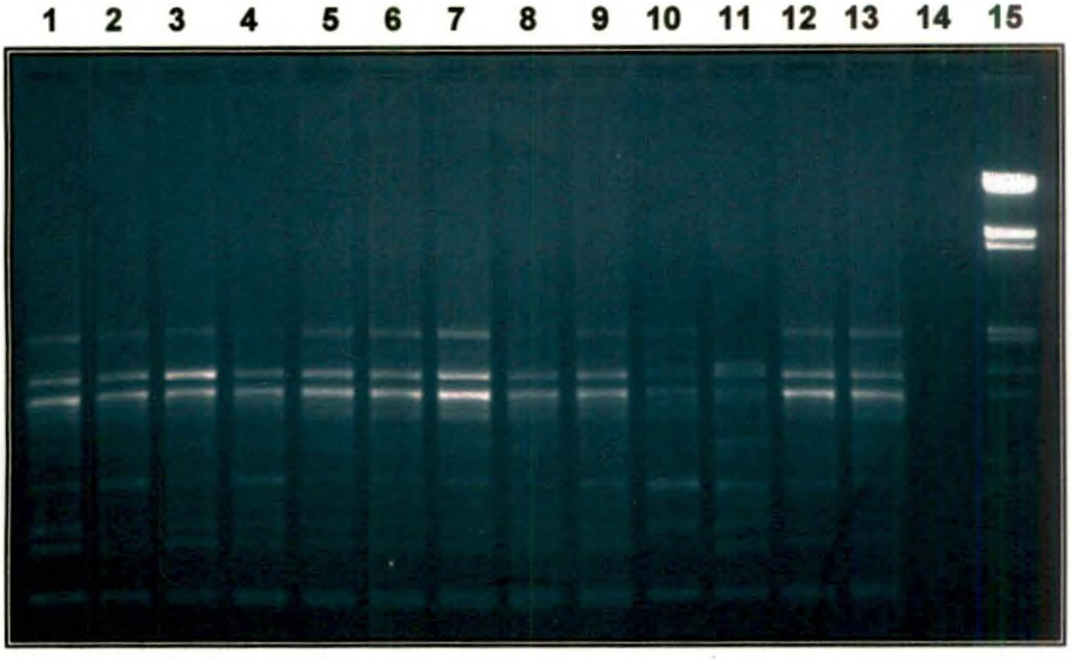

Plate 18. RAPD profile for the TC regenerants from different source plants (P_1-4 and P_1-5) of variety Panniyur 1with Primer OPP 8

Lane 1 : Mother plant (P_1- 4) ; lane 2-7 : TC plants (P_1-4) ; lane 8 : Mother plant (P_1- 5); lane 9-14 : TC plants(P_1-5) ; lane 15 : Marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

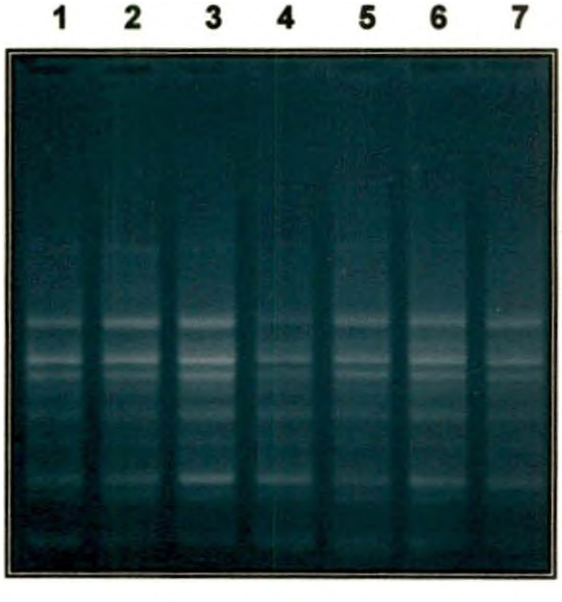

Plate 19. RAPD profile for the TC regenerants of variety Panniyur 2 with Primer OPP 8

Lane 1 : Mother plant (P_2-2) ; lane 2-7 : TC plants

4.4.2 Primer OPP 8

Table 15 reveals the results of analysis of tissue culture plantlets (6 month old) using primer OPP 8. There was no variation in the RAPD profile among the regenerants.

In the case of P_1, there was eight similar bands among the regenerants and their mother plants. The four mother plants viz., P_1-2, P_1-3, P_1-4 and P_1-5 and their respective derivatives showed a similar banding pattern with eight monomorphic bands (Plate 18).

Tissue culture regenerants of variety P_2 showed no variation with their corresponding mother plants. The plantlets derived from three mother plants viz., P_2-2, P_2-3 and P_2-5 showed monomorphic pattern with eight common bands (Plate 19 and 20) when amplified with OPP 8.

The variety P_4-5 and its regenerants also showed no variation and expressed twelve bands shared among all the samples (Plate 21).

The RAPD profile for the TC regenerants from callus derived source plants also showed monomorphic pattern with nine common bands shared among the plantlets derived from the mother plants of Px series (Plate 22, 23).

The results of analysis of plantlets at two month old stage using primer OPP 8 is given in Table 16. Results showed no variation among the regenerants and their mother plants. In the varieties P_1 and P_2, the regenerants expressed eight monomorphic bands and in P_4 samples twelve bands were observed (Plate 24, 25).

4.4.3 Primer OPP 14

Table 17 shows the pattern of amplification among the regenerants (six month old) using the primer OPP 14.

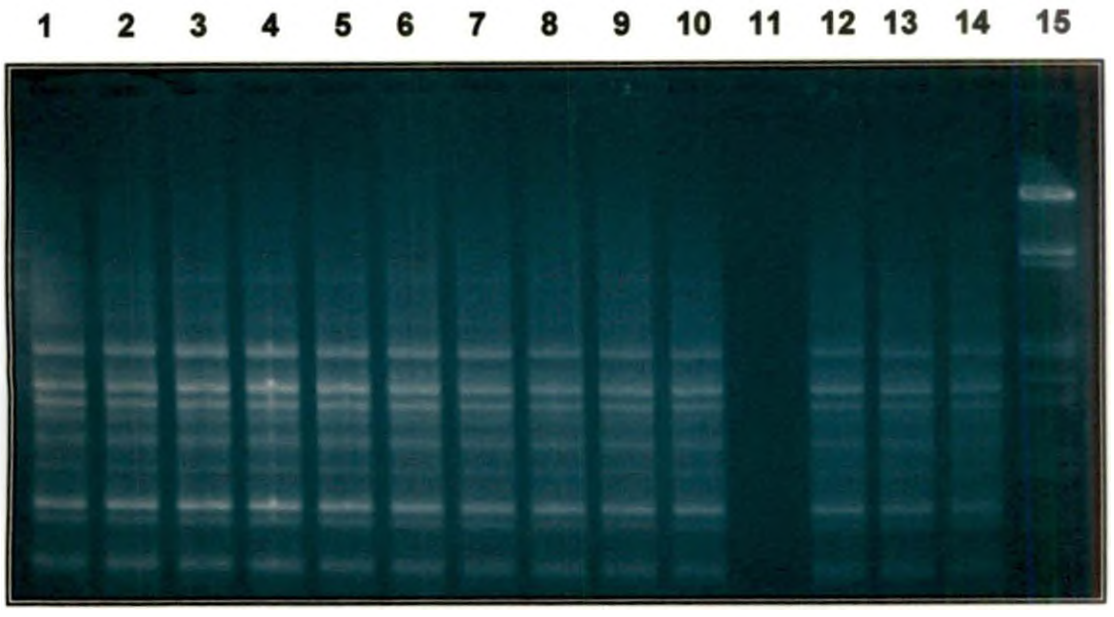

Plate 20. RAPD profile for the TC regenerants from the source plants of (P2-5 & P2-3) variety Panniyur 2 with Primer OPP 8.

Lane 1 : Mother plant (P_2-5) ; lane 2-7 : TC plants (P_2-5) ; lane 8 : Mother plant (P_2- 3) ; lane 9-14 : TC plants (P_2-3) ; lane 15 : Marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

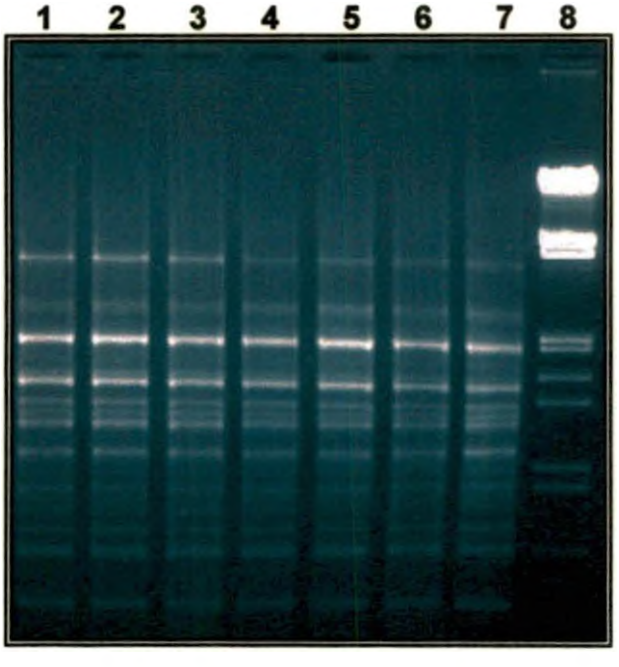

Plate 21. RAPD profile for the TC regenerants of variety Panniyur 4 with Primer OPP 8.

Lane 1 : Mother plant (P_4-5) ; lane 2-7 : TC plants

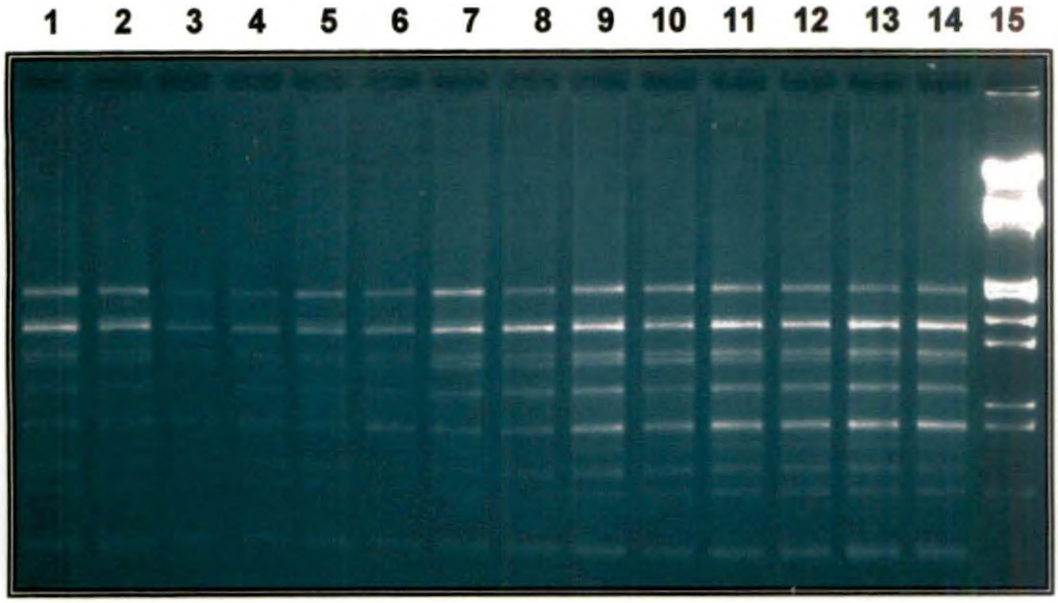

Plate 22. RAPD profile for the TC regenerants from the callus derived source plants (Px-1 & Px–2) of variety Panniyur 4 with Primer OPP 8

Lane 1 : Mother plant (Px-1) ; lane 2-7 : TC plants (Px-1) ; lane 8 : Mother plant (Px - 2) ; lane 9-14 : TC plants (Px-2) ; lane 15 : Marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

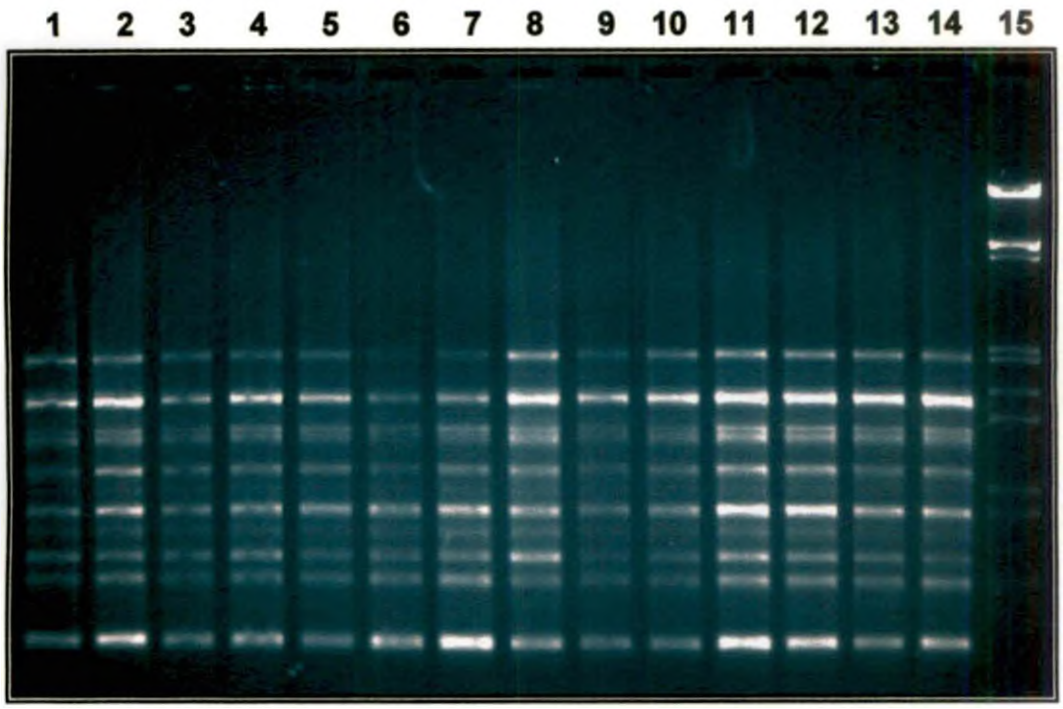

Plate 23. RAPD profile for the TC regenerants from the callus derived source plants (Px-3 & Px–4) of variety Panniyur 4 with Primer OPP 8

Lane 1 : Mother plant (Px- 3) ; lane2-7 : TC plants (Px-3) ; lane 8 : Mother plant (Px-4), lane 9-14 : TC plants (Px-4) ; Lane 15 : DNA (λ DNA *Eco* R1/ *Hind* III digest)

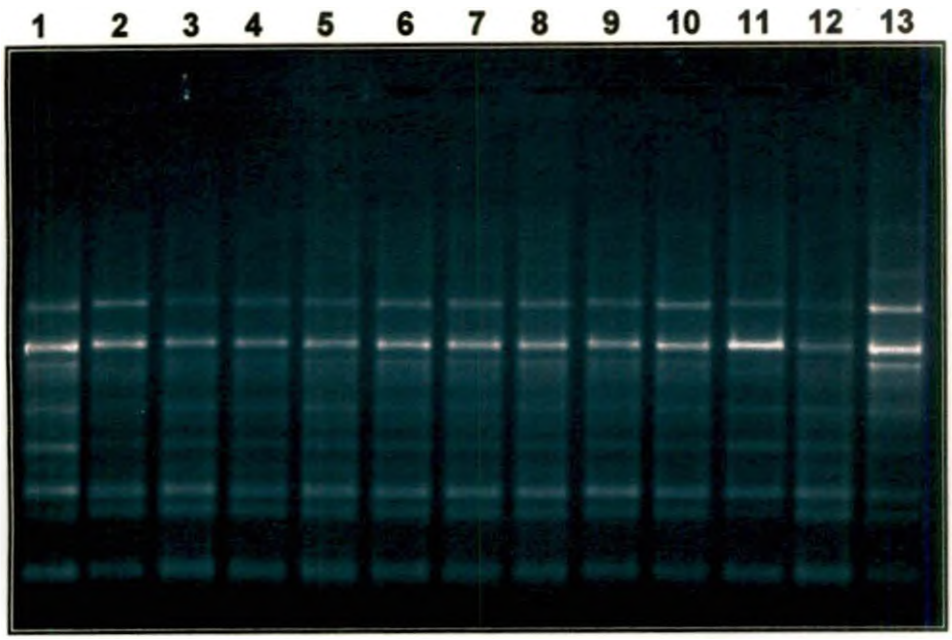

Plate 24. RAPD profile for the TC regenerants (2 month old) from the source plants of variety Panniyur 2 with Primer OPP 8

Lane 1-3 : Mother plants ; lane 4-13 : TC plants.

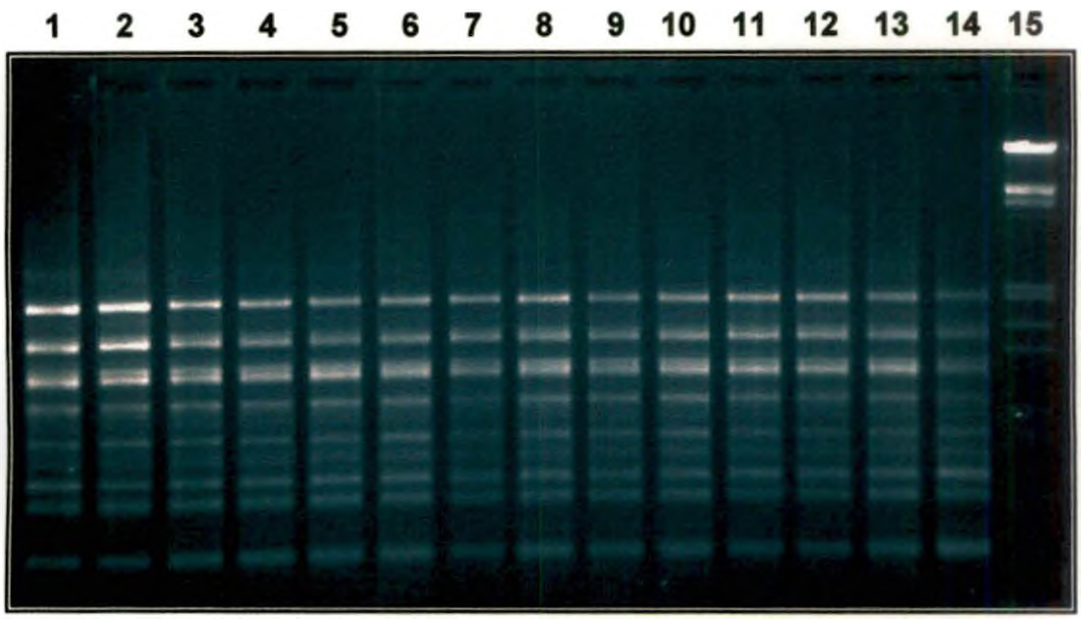

Plate 25. RAPD profile for the TC regenerants (2 month old) from the source plant of variety Panniyur 4 with Primer OPP 8

Lane 1-3 : Mother plants; lane 4-14 : TC plants ; lane 15: Marker DNA (λ DNA *EcoRI/Hind* III digest).

Table 15. RAPD profile for the TC regenerants (6 months old) of different black pepper varieties using Primer OPP-8

Mother plant	No. of TC plants analysed	Maximum no. of band per sample	No. of monomorphic bands	No. of polymorphic bands
$P_1 2$	6	8	8	0
$P_1 3$	6	8	8	0
$P_1 4$	6	8	8	0
$P_1 5$	5	8	8	0
$P_2 2$	6	8	8	0
$P_2 3$	6	8	8	0
$P_2 5$	5	8	8	0
$P_4 5$	6	12	12	0
$P_x 1$	6	9	9	0
$P_x 2$	6	9	9	0
$P_x 3$	6	9	9	0
$P_x 4$	6	9	9	0

Table 16. RAPD profile for the TC regenerants (2 months old) of different black pepper varieties using Primer OPP-8

Variety	No. of TC plants analysed	Maximum no. of band per sample	No. of monomorphic bands	No. of polymorphic bands
P_1	8	8	8	0
P_2	10	8	8	0
P_4	11	12	12	0

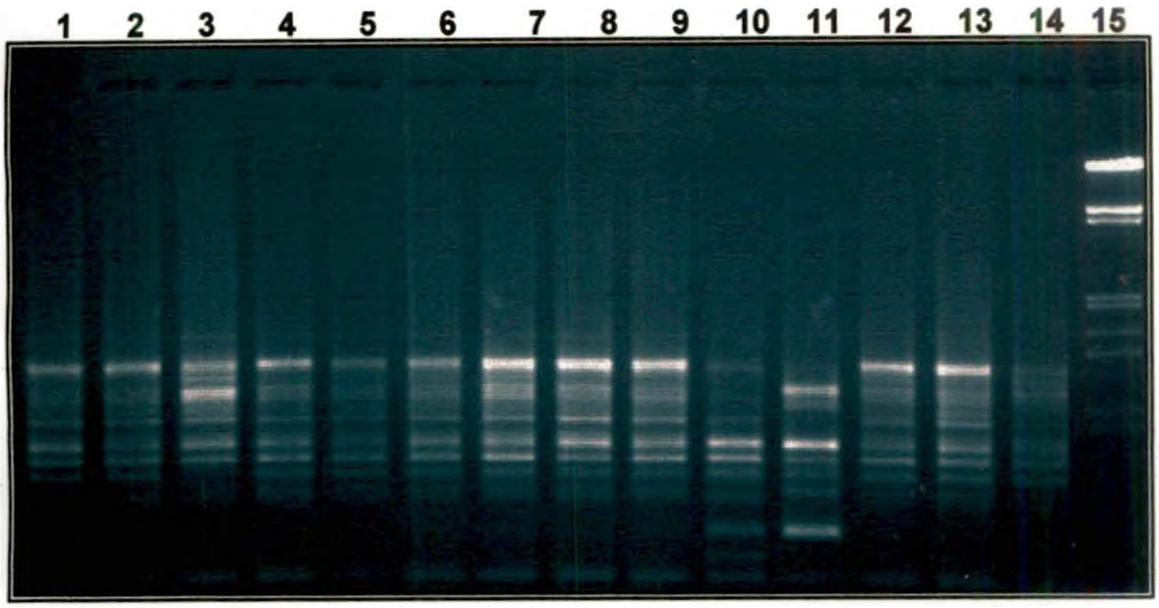

Plate 26. RAPD profile for the TC regenerants from the different source plants (P_1-4 & P_1-5) of variety Panniyur 1 with Primer OPP 14

Lane 1 : Mother plant (P_1-4) ; lane 2-7 : TC plants (P_1-4) ; lane 8 : Mother Plant (P_1-5); lane 9-14 : TC plants (P_1-5) ; lane 15 : marker DNA (λ DNA *Eco* R1/*Hind* III digest)

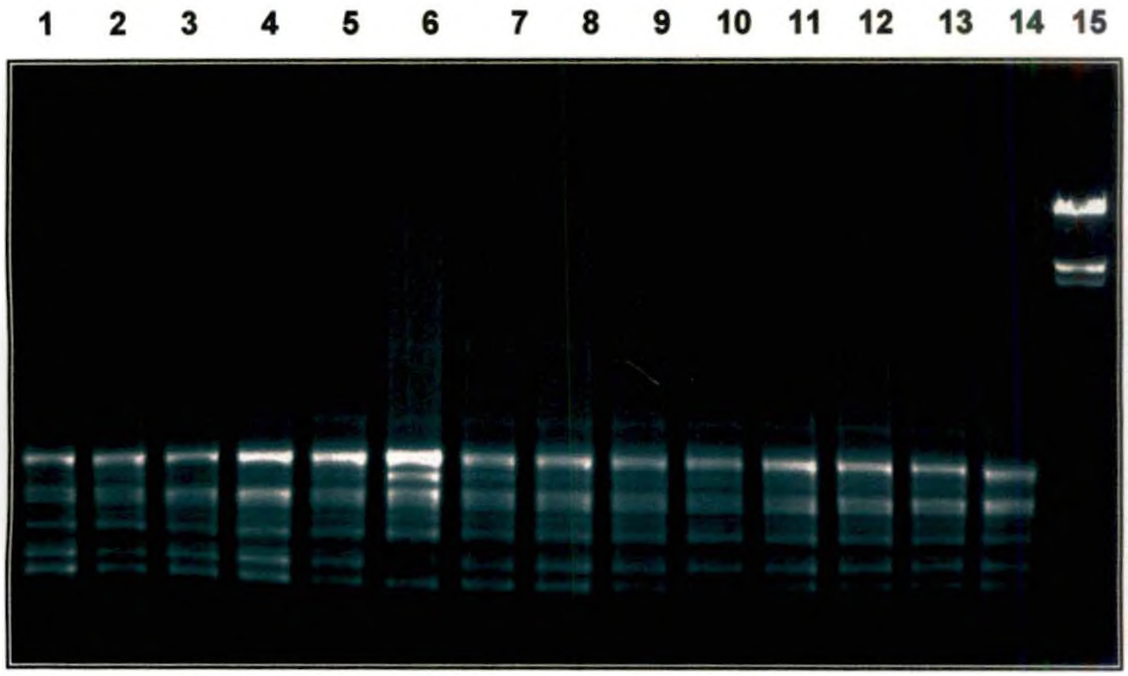

Plate 27. RAPD profile for the TC regenerants (6 month old) from the defferent source plant (P_2-2& P_2-3) of variety Panniyur 2 with Primer OPP 14

Lane 1 : Mother plant (P_2-2) ; lane 2-7 : TC plants (P_2-2) ; lane 8 : Mother Plant (P_2-3) ; lane 9-14 : TC plants (P_2-3) ; lane 15 : Marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

Among the P_1 regenerants there was slight variation in banding pattern observed in two regenerants out of the twenty four regenerants tested. All the regenerants from the mother plants P_1-2, P_1-3 and P_1-4 showed monomorphic banding pattern (Table 17). However, two of the regenerants from P_1-5 showed slight polymorphism with respect to two non-specific bands (Plate 26). Seven bands were observed in the RAPD profile for the tissue culture regenerants from the mother plant P_1-5. Expression of one of the bands was little more in two regenerants out of the six tested. Among the regenerants of mother plants P_1-2, P_1-3 and P_1-4 there was no such signs of variation.

Regenerants of mother plants of P_2 variety were similar to their respective mother plants as revealed by the amplification pattern. The monomorphic pattern showed six bands common to all the samples (Plate 27, Table 17).

The regenerants of mother plants P_4-5 also showed no variation among themselves and with the mother plant (Plate 28). Amplification profile showed seven monomorphic bands.

The regenerants from the source plants of Px series showed no variation among themselves and with their respective mother plants. The monomorphic pattern showed seven bands shared among all the samples.

Among the two month old TC regenerants, there was no variation between the regenerants and with their respective mother plant. The monomorphic pattern of amplification expressed six bands in P_1 and seven bands each in P_2 and P_4 regenerants (Table 18, Plates 29, 30).

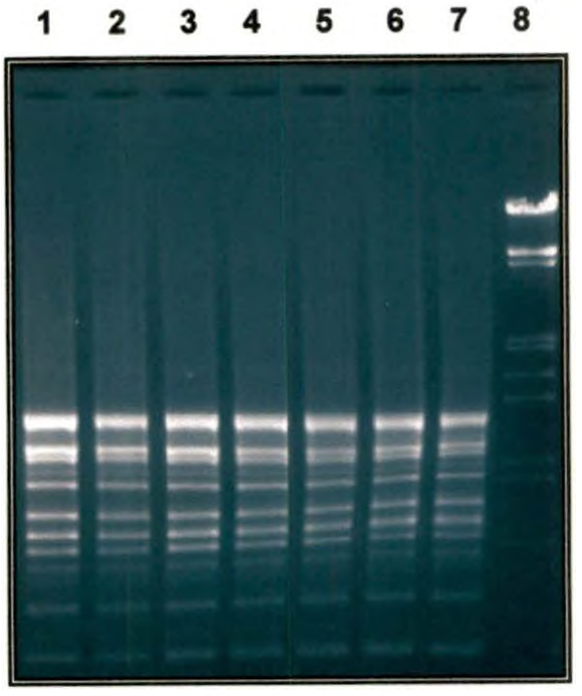

Plate 28. RAPD profile for the TC regenerants (6 month old) of variety Panniyur 4 with Primer OPP 14

Lane 1 : Mother plant ; lane 2-7 : TC plants ; lane 8: Marker DNA (λ DNA *Eco* R1/ *Hind* III digest)

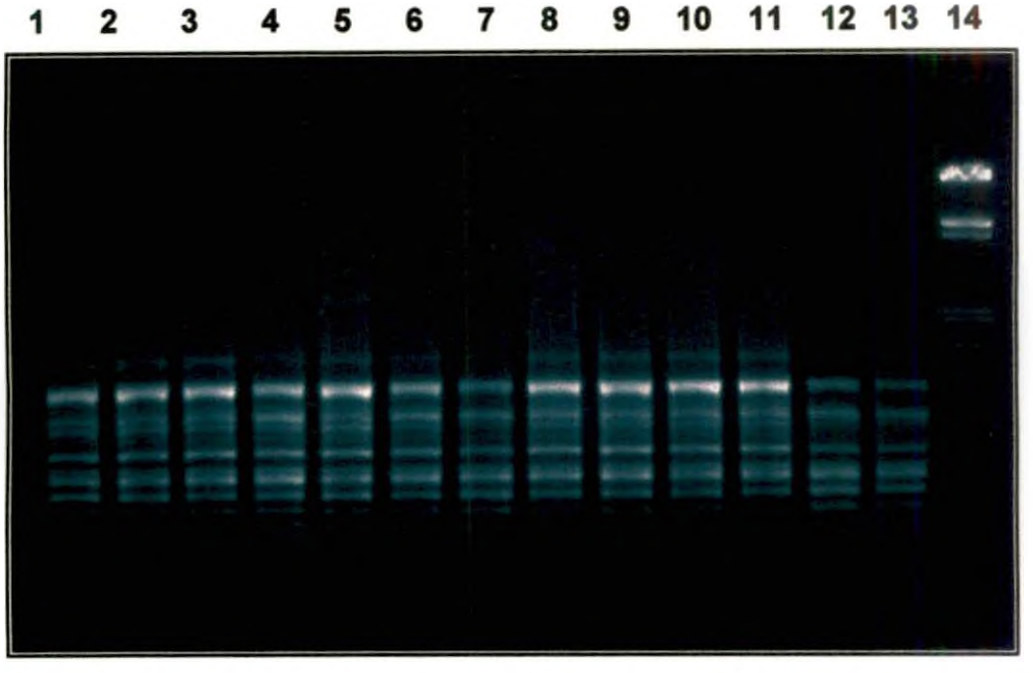

Plate 29. RAPD profile for the TC regenerants (2 month old) of variety Panniyur 1 with Primer OPP 14

Lane 1-4 : Mother plants; lane 5-13 : TC plants ; lane 14 : marker DNA (λ DNA *Eco* R1/*Hind* III digest)

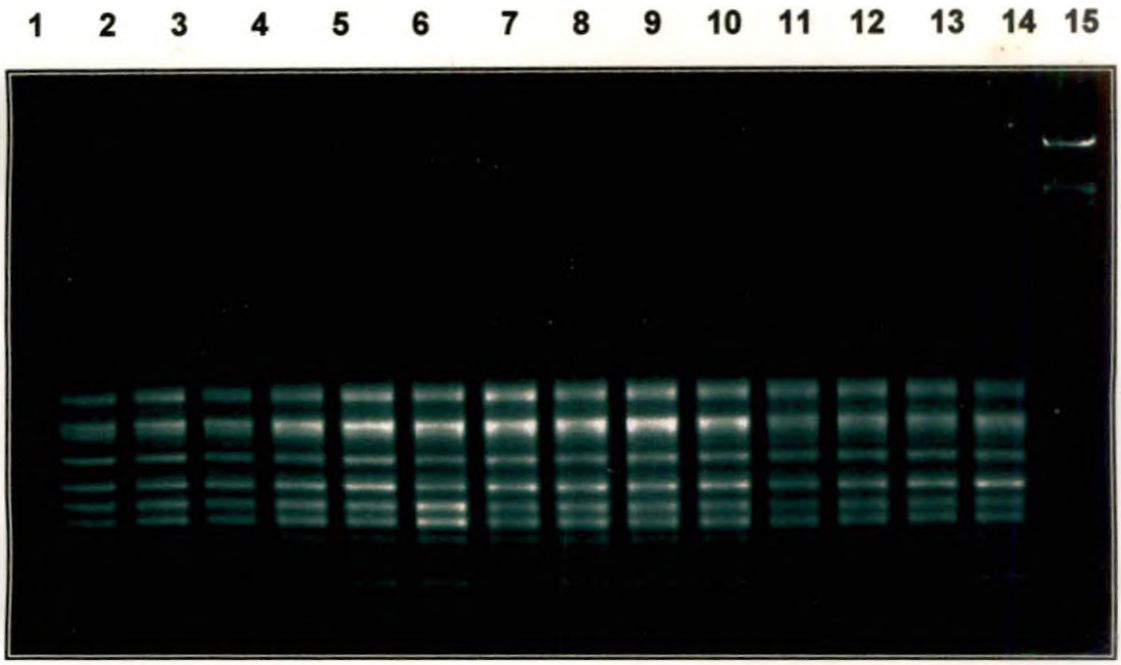

Plate 30. RAPD profile for the TC regenerants (2 month old) of variety Panniyur 4 with Primer OPP 14

Lane 1-4 : Mother plants; lane 5-13 : TC plants ; lane 15 : Marker DNA (λ DNA *Eco* R1/*Hind* III digest)

Table 17. RAPD profile for the TC regenerants (6 months old) of different black pepper varieties using Primer OPP-14

Mother plant	No. of TC plants analysed	Maximum no. of band per sample	No. of monomorphic bands	No. of polymorphic bands
$P_1$2	6	7	7	0
$P_1$3	6	7	7	0
$P_1$4	6	7	7	0
$P_1$5	6	7	5	2
$P_2$2	6	6	6	0
$P_2$3	6	6	6	0
$P_2$5	6	6	6	0
$P_4$5	6	7	7	0
P_x1	6	7	7	0
P_x2	6	7	7	0
P_x3	6	7	7	0
P_x4	5	7	7	0

Table 18. RAPD profile for the TC regenerants (2 months old) of different black pepper varieties using Primer OPP-14

Variety	No. of TC plants analysed	Maximum no. of band per sample	No. of monomorphic bands	No. of polymorphic bands
P_1	8	6	6	0
P_2	10	7	7	0
P_4	11	7	7	0

DISCUSSION

5. DISCUSSION

Tissue culture has emerged as the most ideal technique for the large scale multiplication of commercially important crops. It has replaced conventional methods of propagation in many of the horticultural plants and forest species. However, variability expressed among tissue culture regenerants is being viewed seriously as a disadvantage, especially when true to type plants are required. Various reports on this aspect confirm the presence of variation at genetic level among the micro-propagated plants (Drew and Smith, 1990; Sheela, 1995; Sudarsan *et al.*, 1996 and Chandrappa *et al.*, 1996).

Genetic variability present among cultured cells, plants derived from such cells or progeny of such plants is called somatic variation. Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits. Somaclonal variation may arise due to any of the events at molecular level such as changes in chromosome number and/or structure, gene mutation, alteration in gene expression, gene amplification, mitotic crossing over, transposable element activation and rearrangements in cytoplasmic genes (Singh, 1998).

Till recently, morphological traits and the keys developed upon them formed the basis for characterisation of cultivars and varieties. This was felt as inadequate as it failed to provide reliable information at genetic level. Inadequacy of morphological and biochemical traits in genotype identification resulted in the exploitation of molecular markers.

Novel techniques such as RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) have been utilised widely to characterise tissue culture regenerants for their genetic stability. RAPD technique is relatively fast, easy to perform, comparatively cheap and free from environmental influences, tissue types etc.

Black pepper is one of the most important spice crops of India. Though the crop is mainly propagated by rooted cuttings, scarcity of runner shoots in productive vines limit the availability of quality planting material and dearth of quality planting materials is one of the major factors that limits productivity of black pepper in the country.

The protocol for large scale multiplication of black pepper has been standardised at various centres (Mathew and Rao, 1984; Nazeem *et al.*, 1990; Philip *et al.*, 1992; Joseph *et al.*, 1994). The variants among the regenerants if detected in the early stages of development, will be highly advantageous in the distribution of true to type plants to the farming community. The viability of the protocol for large scale multiplication of the species is also to be confirmed.

The present study was an attempt to characterise the tissue culture regenerants using molecular marker, so as to ensure the viability of the protocol developed in our institute. Since this was the first attempt in black pepper, all the basic techniques had to be standardised. The study included standardisation of genomic DNA isolation in black pepper, standardisation of RAPD technique and analysis of tissue culture regenerants of black pepper using RAPD technique to assess their genetic stability. The results of these experiments conducted are discussed in this chapter.

5.1 Standardisation of procedure for isolation of DNA

Basically three reported procedures were tested for their suitability and were later modified slightly to improve efficiency.

The quality of DNA was estimated based on electrophoretic pattern of DNA bands on agarose gel after ethidium bromide staining and based on absorbance at 260 and 280 nm. For all the three methods and their modifications tried, the 260/280 ratio was between 1.8 to 2.0 except in protocol Ib (Table 4) and protocol IIb where the tissue was not ground in liquid nitrogen. In protocol I, grinding of tissue

was done after freezing in liquid N2 and there was no chloroform extraction involved. Chances of damage to nucleic acid was very less when the tissue was ground in liquid N2 which was done to break the cell wall in order to release the cellular constituents. Such a finding has also been reported by earlier workers (He *et al.*, 1992; Dunemann *et al.*, 1994; Virk *et al.*, 1995, Demeke *et al.*, 1996).

In all the three basic methods, good quality DNA was obtained when it was slightly modified by freezing the tissue with liquid N2 before grinding and using *β*-mercapto ethanol while grinding. Since black pepper contains high quantity of phenolic compounds, use of antioxidants like *β*-mercapto ethanol, PVP (Poly Vinyl Pyrolidone) and sodium metabisulphate have great significance. In the original protocols, it was *β*-mercapto ethanol in protocol I, sodium metabisulphate in protocol II and PVP in protocol III that reduced the phenolic interference. However, modifications were tried in protocol IIa with *β*-mercapto ethanol instead of sodium metabisulphate and in protocol IIIa with a combination of PVP and *β*-mercapto ethanol. The results were encouraging (Table 4, Plate 2). Quality of DNA was good when *β*-mercapto ethanol was used in the grinding step.

The quality and quantity of DNA samples were the best with protocol IIIa as shown by the data and distinct nature of electrophoretic bands (Table 4, Plate 4). Two levels of chloroform extraction enabled to get a pigment free sample of DNA. However, there was slight deterioration in the quality of DNA after the RNase treatment. This may be due to the further handling of DNA samples in the additional steps involved for removing RNA. Rogers and Bendich (1994) observed that there is chance of shearing of the DNA molecule during chloroform: iso-amyl alcohol extraction, as vigorous mixing is necessary for effective removal of proteins. This might have caused slight shearing of DNA in such samples.

Rajaseger *et al.* (1997) reported extraction of DNA using modified Honda and Hirai method which yielded brown coloured DNA, due to the presence of phenolic compounds. When they tried the method suggested by Murray and

Thompson (1980) with grinding the tissue in liquid N2 and using PVP and β-mercapto ethanol as antioxidants, browning was less compared to the earlier method. In our study the DNA pellet obtained was creamy white even though pepper is a crop with high content of polyphenols. This may be due to the use of a combination of PVP and β-mercapto ethanol. Fritz (1990) observed that it is difficult to obtain high quality DNA from most tropical tree species. Rogers and Bendich (1994) observed that some DNA samples would be brown due to the intervention of polyphenol oxidase and suggested that PVP and β-mercapto ethanol were equally effective in preventing phenolic degradation. Nesbit *et al.* (1995) has also reported similar findings in eucalyptus.

The chemical components in the extraction mixture would have also contributed greatly for the isolation of nucleic acid. The cell membrane must be disrupted to release the nucleic acids into the buffer. This is achieved by detergents like SDS (Protocol I), sarcosin and CTAB (Protocol II and III). The released DNA has to be protected from endogenous nucleases. Detergents and EDTA (a chelating agent) binds with magnesium ions, a cofactor for most nucleases. Chloroform will remove the pigments, denature and separate the proteins from DNA. Tris HCl and sorbitol maintain the osmotic pressure.

In the present study, it was observed that the time between thawing of the frozen, pulverized tissue and its exposure to the extraction buffer should be minimised to avoid nucleolytic degradation of DNA. Another consideration associated with isolation of DNA from plants, algae and fungi is the enzyme inhibiting polysacharides. The use of CTAB and NaCl facilitate the separation of polysacharides based on their differential solubilities (Rogers and Bendich, 1994).

When the effect of maturity of leaf on the quality and quantity of isolated DNA was analysed, it was observed that the quality and quantity of DNA was the best with tender leaf tissue as compared to mature and half mature leaf samples (Plate 3). Tender leaves contain actively dividing cells with lesser intensity of extra

nuclear materials like proteins, carbohydrate, oils, fats and other metabolites that interfere with the isolation of nucleic acids.

5.2 Standardisation of RAPD technique

Random amplified polymorphic DNA (RAPD) analysis utilizes single arbitrary sequences of 6 to 10 base oligonucleotides as primers to amplify discrete fragments of DNA in a polymerase chain reaction (Williams *et al.*, 1990). This marker system has been used in many different applications involving the detection of DNA sequence polymorphism, isolation of markers linked to various traits, varietal characterisation and parentage analysis. In black pepper since we have no information on genetic sequence, RAPD is the best technique as it relies on universal sets of primers.

5.2.1 Standardisation of reaction mixture

The establishment of optimal conditions for RAPD analysis is difficult due to the sensitivity of the PCR system and the number of experimental variables. Therefore, it is necessary that the reaction conditions be standardised to obtain the amplifications of random genomic sequences in a reproducible way.

In the present study different levels of primer, dNTPs and enzyme were tried to optimize the amplification pattern. Each of these three constituents were tried at three levels and all possible combinations of these levels were tested. Poor amplification was observed when the concentration of primer and dNTPs was low (P_1 and N_1 levels) at lower level of enzyme (E_1). Better amplification was obtained when the concentration of primer and dNTPs were increased to P_3 and N_3 levels even at lower levels of enzyme. The amplification pattern was not found improved at higher levels of enzyme and hence the lower level was identified as the optimum one (Table 7, Plate 5).

Among the 27 combinations tried, the optimum reaction mixture selected was $E_1N_3P_3$. This combination was selected based on the number of bands, nature of bands upon agarose gel electrophoresis and the repeatability of banding pattern.

Optimum concentration of template DNA was determined by trying its different levels at 25, 50 and 75 mg. The other components were kept at the optimum level identified in the previous experiment. From this experiment it was observed that there was not much difference in banding pattern and it was more or less identical. No variation was tried for magnesium concentration since the buffer used contained 15 mM $MgCl_2$. The success of DNA amplification greatly depend on the optimum concentration of enzyme and substrates provided. Differential response at different levels of enzyme, primer and dNTPs has been reported by various earlier workers (Virk *et al.* 1995 and Hallden *et al.* 1996).

5.2.2 Standardisation of temperature profile

Among the seven temperature profiles tried, one gave good amplification (TP-4) and was found stable in repeated experiments. Other cycles tried gave unstable or nondistinct banding pattern. Repeated trials using different primers confirmed the efficiency of thermal cycle TP-4 for amplification of black pepper genomic DNA with the selected reaction mixture (Table 8, Plate 6, 7).

Due to the sensitivity of the PCR system and the number of experimental variables, the amplification of random genomic sequences in a reproducible way was only possible with rigorously standardised conditions. Several factors such as type of thermal cycles, ramping temperature, concentration of Mg ion, polymerase brand and primer performance appeared to influence optimal amplification of DNA to give useful electrophoretic pattern (Barcaccia, 1994).

The reaction mixture and thermal settings have been reported to vary with species. The amplification condition developed by Williams *et al.* (1990) for soybean DNA produced a smear of unspecific DNA fragments rather than a set of

discrete amplification products in tomato (Klein- Lankhorst *et al.*, 1991). A fivefold increase in the concentration of template DNA, a twofold increase in primer concentration, together with the application of temperature ramp in the PCR cycle, gave an amplification of discrete genotype specific fragments. Difference in purity between various template and primer preparations on difference in the thermal profile generated by respective PCR machines have been suggested as the reason for the lack of success with the earlier conditions. Virk *et al.*, (1995) also studied the effect of different factors on the final results of RAPD. Hallden *et al.* (1996) observed that minor changes in reaction conditions can significantly alter the number and intensity of amplification products and the reproducibility be difficult to maintain. Penner *et al.* (1993) reported difficulties in obtaining identical banding patterns from the same set of primers and materials among different laboratories. They also observed that there was no difference in band intensity when different concentrations of template DNA were tried.

In our study, an optimum concentration of different components was selected along with a temperature profile for the amplification of genomic DNA of black pepper.

5.2.3 Screening of random primers

A total of sixty decamer primers, twenty each in OPP, OPE and OPF series were screened initially. The primers for further analysis were selected based on the number of bands, quality of amplification and stability of expression.

The decamer primers supplied from Operon Technologies Inc., USA was used for the study. Many workers have reported the use of Operon primer which is available in large numbers in a ready to use manner (He *et al.*, 1992; Xu *et al.*, 1993; N'goran *et al.*, 1994; Gidoni *et al.*, 1994; Rani *et al.*, 1995; Mandal *et al.*, 1996).

Among the OPP series, six primers gave good amplification in the present study. Maximum number of bands produced were seven (OPP-12, OPP-14) and

minimum number was zero (OPP-11, 18, 20). The six primers that gave good amplification were selected for further analysis. For other primers of OPP series, the amplification was average or poor (Plate 8).

Among the OPF series, two primers gave good amplification. OPF-6 and OPF-9 were selected for further analysis due to the quality of banding (Plate 9).

Among the OPE series, two primers OPE-15 and OPE-16 produced good amplification. Other primers gave poor or average quality amplification (Plate 10).

Many reports reveal that there can be difference in banding pattern when different sources of enzymes are used (Virk *et al.*, 1995). Barcaccia (1994) observed similar but not identical banding pattern by using enzyme obtained from different sources prepared from *Thermus aquaticus*. He stressed the need for using the same DNA polymerase in all the experiments in order to generate comparable results. In our studies when the enzyme obtained from GIBCO BRL was used in the initial stages for screening purpose, number of bands were less (Plate 11). However, when the enzyme obtained from GENEI, Bangalore was used for analysis of tissue culture plants, number of bands increased (Plate 13).

Fukuoka *et al.* (1992) observed that a set of samples sometimes reduced or increased slightly all band intensities with different PCRs. Most bands are reproducible, but high molecular weight (> 2.2 kbp) was not reproducible, which were minor and varied in intensities. He *et al.* (1992) observed little or no polymorphism on agarose gel with a few minor bands in many cases which were not always reproducible and not reliable as markers. This arose from non-specific amplification i.e. when primer template homology is not perfect.

Difference in intensities of RAPD products is due to primers annealing to genomic loci which are mismatched resulting in reduced priming and extension efficiencies (Gidoni *et al.*, 1994).

Importance of applying the same protocol to ensure reproducibility has been highlighted by Virk *et al.* (1995). They also observed that certain primers produce more reliable banding pattern when used at higher concentration, probably because genomic DNA possesses an unusually high frequency of annealing sites for these primers so that their effective concentration for amplification is lowered in the reaction tube.

5.2.4 Screening of black pepper varieties by selected primers

The primers selected from the primary screening were used for screening of five black pepper varieties, viz., P-1, P-2, P-3, P-4 and Karimunda. P-1 is a hybrid between Uthirankotta and Cheriyakaniyakadan. P-2 is a selection from the open pollinated progeny of cv. Balancotta, while Panniyur-3 is a direct sister of P-1. P-4 is a selection from Kuthiravally (Edison *et al.*, 1991) and Karimunda is one of the most popular cultivars in Kerala. Thus these varieties have different genetic background and were screened with the ten selected primers.

Primer OPP-1 produced polymorphism in Karimunda with the absence of one band shared by all others and the expression of an additional band absent in all the Panniyur varieties. An additional band was found expressed in P-1 and P-2 (Plate 11).

Primer OPP-7 produced four monomorphic bands. Varieties P-1 and P-2 gave identical pattern and P-3 showed absence of a band. P-4 gave minimum number of bands while Karimunda showed eight bands of which these were absent in P-4. However, repeatability of the banding pattern was a problem with this primer as there was some feable and non-distinct bands expressed frequently (Plate 11).

Amplification with primer OPP-8 showed three monomorphic bands with three additional bands for P-3 which was shared by P-4 and Karimunda. P-4 showed four polymorphic bands and Karimunda showed six (Plate 11, Fig.3). Primer OPP-8

was selected for further analysis of TC plants as it showed stability of banding pattern in repeated experiments and better quality of bands.

Primer OPP-12 showed seven monomorphic bands. P-1 and 2 gave one additional band each. The variety Karimunda also showed uniquencss with two additional bands. However, the amplification pattern with this primer was not clear and distinct (Plate 11, Fig.4).

For the primer OPP-13 there was six monomorphic bands. The intensity of banding differed among the varieties. Karimunda showed an additional band and another one was found absent in this variety (Plate 11, Fig.5).

With OPP-14 there was five monomorphic bands shared among the five varieties. Karimunda expressed two additional bands, but one band common to Panniyur varieties was missing. P-1 also showed one additional band for this primer.

Amplification with primer OPE-16 showed polymorphism with five monomorphic bands and two polymorphic bands for Karimunda. One polymorphic band was common to all Panniyur varieties.

Primers OPF 6, 9 and OPE-15 showed no polymorphism with four monomorphic bands for OPF 6 and 9 and six bands for OPE-15 (Table 12).

Primers OPP-1, OPP-8 and OPP-14 were selected for further analysis of TC plants, based on the quality of amplification and stability of banding pattern in repeated experiments.

Polymorphism with RAPD analysis has been reported in many plants. Halward *et al.* (1992) successfully applied this technique to characterise cultivated and wild peanut. Ratnaparkhe *et al.* (1995) observed high level of polymorphism among wild species of pigeon pea with little variation among the cultivars. Genetic diversity of *Brassica* populations was estimated by Lazaro and Aguinagalde (1998)

using RAPD technique. Many other reports reveal the extensive use of RAPD technique for cultivars/genotype screening (Fukuoka *et al.*, 1992; Koller *et al.*, 1993; Gidoni *et al.*, 1994; Bhat *et al.*, 1995; Verghese *et al.*, 1997; Darokar *et al.*, 1999; Rani *et al.*, 1999; Sharany *et al.*, 1999; Rajagopal *et al.*, 1999).

In our screening of five varieties of black pepper with ten primers, in order to select primers for the analysis of tissue culture regenerants, seven primers showed polymorphism. However, there was a tendency among the four Panniyur varieties to be monomorphic. Three primers were selected out of the seven for evaluating the tissue culture regenerants.

5.2.5 Evaluation of genetic stability of TC plants

The six-month old plants when tested with primer OPP-1 showed no polymorphism. The tissue culture regenerants of variety Panniyur-1 showed no variation among and also with the mother plant. The monomorphic banding pattern showed by these plants when analysed by OPP-1 showed that the plantlets were identical to their mother plants (Plates 12, 13).

Variety P-2 and their tissue culture derivatives showed no sign of variation when tested with the primer OPP-1. The monomorphic banding pattern showed by these plantlets included six common bands in P_2-2 and P_2-3 and seven bands in P_2-5. Regenerants of P_4-5 also showed a monomorphic pattern when tested with the primer OPP-1, thus showing the genetic similarity among the mother plants and regenerants.

Among the regenerants of Px series, the banding pattern was monomorphic. Px-1 and Px-2 regenerants showed eight bands whereas Px-3 and Px-4 showed nine bands. There is more chance of variation among the callus derived source plants and their regenerants. However, the monomorphic banding pattern showed by the regenerants reveal the suitability of the tissue culture protocol followed for black pepper. The source plants of Px series were callus regenerants

and the results indicated that the bud cultures derived from such callus regenerants are quite uniform (Plates 16, 17).

There was no variation among the two-month-old plantlets when analysed with primer OPP-1. An identical banding pattern was shared among the regenerants and mother plants.

A similar trend was observed when the plantlets were analysed with primer OPP-8. The four Panniyur-1 mother plants viz., P_1-2, P_1-3, P_1-4 and P_1-5 and their respective derivatives showed eight monomorphic bands. In the case of variety Panniyur-2 also a monomorphic pattern was observed. A similar pattern was shown by the regenerants of mother plant P_4-5 with 12 bands. The regenerants of Px series showed no variation among them and with the mother plants. The banding pattern showed nine monomorphic bands (Plates 22, 23). Two month old plantlets also showed a monomorphic pattern when tested with OPP-8 with similar banding pattern corresponding to that of the six month old plants (Plates 24, 25).

Thus the TC regenerants showed no variation with respect to primer OPP-8. This confirms the genetic identity and similarity of the regenerants with their mother plants. This suggests that the tissue culture protocol followed has not led to any significant genetic change among the regenerants.

When the six month old plantlets were analysed with primer OPP-14, there was polymorphism in two plantlets. Seven bands were observed in the RAPD profile for the TC regenerants from the mother plants P_1-5. Expression of one of the band was little more in two regenerants out of the six tested. Among the regenerants of P_1-2, P_1-3 and P_1-4 there was no variation (Plate 26). Regenerants of mother plant P_2 and P_4 showed monomorphic pattern with six and seven common bands respectively. In the RAPD profile of regenerants of Px series also there was no variation expressed. Two month old regenerants also expressed their genetic stability with this primer (Plates 29, 30).

The TC regenerants when analysed with primer OPP-14 showed no significant variation. The polymorphism showed by two regenerants of mother plant P_1-5 was with respect to two nonspecific bands, feable in expression. The results of the evaluation was not subjected to any statistical analysis since there was no detectable variation noticed among the regenerants.

Thus the genetic stability and clonal fidelity of tissue culture derived black pepper plants were confirmed with RAPD assay. One of the most crucial concerns in *in vitro* propagation is to retain genomic integrity of the micropropagated plants. Numerous reports indicate that somaclonal variation in *in vitro* derived plants is not uncommon (Drew and Smith, 1990; Sheela, 1995; Sudarsan *et al.*, 1996; Chandrappa *et al.*, 1996). Somaclonal variation which is a welcome source of variation in crop breeding, is unwanted when direct regenerants have to be used or in the regeneration of genetically transformed plants.

In the present study, a comprehensive variation was absent ensuring the genetic fidelity of regenerants. Such reports of no polymorphism when the regenerants were analysed with molecular markers are abundant. Sangwan *et al.* (1995) reported the genetic stability and clonal fidelity of tissue culture regenerants of cassava plants. A study by Angel *et al.* (1996) using RFLP and RAPD supports the stability of cassava germplasm under the *in vitro* storage conditions.

Rani *et al.* (1999) evaluated micropropagated plants of eucalyptus and populus using RAPD. Out of the 175 plantlets, 11 were found polymorphic and others monomorphic.

Different kinds of chemical stresses influence the genetic make up of the regenerants. Piccioni *et al.* (1997) reported the estimation of somaclonal variation in alfalfa using RAPD. No variation was detected among plantlets derived by enhanced release of axillary buds, whereas nine out of 39 plantlets regenerated by indirect somatic embryogenesis differed from that of the donor plant.

In our studies we had tested bud culture derived plants from both normal pepper and callus derived mother plants.

Shoyama *et al.* (1997) reported confirmation of homogeneity among plantlets of ginseng regenerated through somatic embryogenesis, by using RAPD analysis. Genetic stability of micropropagated plants of ginger was revealed by Rout *et al.* (1998) by RAPD analysis.

Several reports suggest the suitability of RAPD analysis for detection of somaclonal variation [Rani *et al.* (1995), Filippis *et al.* (1996), Schneider *et al.* (1996), Mandal *et al.* (1996), Munthali *et al.* (1996), Khanuja *et al.* (1998)].

Parani *et al.* (1997) reported detection of somaclonal variation using RAPD analysis in *Piper longum*, a related species of black pepper. In our studies there was no conclusive evidence of variation when analysed with three primers. However, further analysis with more number of primers would help to conclusively establish the stability of regenerants.

Gidoni *et al.* (1994) have reported the appearance of RAPD products as often not consistent. Using the RAPD approach for cultivar identification, therefore, requires substantial screening of primers and selection of those primers that under certain PCR conditions, generate reproducible polymorphic products. Only consistent and reproducible products should be considered for varietal identification. Such an approach would be quite useful in varietal identification of black pepper also. The data generated in the present study could be very well utilised for fingerprinting the black pepper varieties.

Competition for priming sites is suggested as a determining factor of the outcome of RAPD. Amplification is probably initiated at many sites, but only a subject of all possible products are detected as visible bands after amplification (Hallden *et al.*, 1996).

In conclusion, the results of the study highlight the fact that the method described by Rogers and Bendich (1994) was the best method for isolation of DNA from black pepper. The best thermal cycle was identified as denaturation at 92°C for two minutes, annealing at 37°C for one minute and extension at 72°C for 1.5 minutes. The RAPD analysis confirmed uniformity among the TC regenerants and ensured their genetic fidelity of regenerants derived through bud culture. The protocol developed seems to be a viable one. The care taken in selection of explants (nodal segments) use of lower levels of growth regulators (1 mg l^{-1} each of IAA and BA), limited subculture cycles and lack of an intervening callus phase might have reduced the variability among the TC regenerants.

SUMMARY

6. SUMMARY

As part of the Post Graduate programme in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara experiments were conducted at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 1997-1999, to standardise the method of DNA isolation and the protocol for RAPD analysis in black pepper so as to assess the genetic stability and clonal fidelity of tissue culture derived black pepper plants.

Three methods reported earlier and their modifications were tried for DNA isolation. Modifications were tried to find out the effect of grinding the tissue in liquid N_2 and use of β-mercapto ethanol as antioxidant. Effect of leaf maturity on the quality and quantity of DNA was also assessed taking leaf samples from mature, half mature and tender leaves. The results showed that the method suggested by Rogers and Bendich (1994) was the best method with respect to quality and quantity of DNA. Grinding the tissue in liquid N_2 and use of β-mercapto ethanol was found to be effective. Tender leaves gave good recovery of quality DNA.

For the standardisation of RAPD technique, different levels and their combinations of dNTPs, primers and enzyme, each at three levels were tried. The effect of template DNA concentration was also tested. The results showed that the enzyme at 0.3 unit, the dNTPs at 150 µM and primer at 10 pmoles was the optimum combination. Concentrations of template DNA tried was found not influencing the amplification pattern and the concentration selected was 50 mg.

Seven temperature profiles were tried to select an optimum thermal cycle for the RAPD analysis of black pepper. Reactions were carried out at the concentration of components already optimised. Thermal profile 4 (Denaturation at 92°C for 2 min., annealing at 37°C for 1 min. and extension at 72°C for 1.5 min.) was found to be the best one with respect to the quality of amplification pattern.

Sixty random primers were screened to select primers which would give good quality amplification. Ten primers were selected for further analysis based on the nature of amplification. These ten primers were used to screen five varieties of black pepper (Panniyur-1, 2, 3, 4 and Karimunda) in order to select few primers to be used for the analysis of TC plants. Seven primers showed polymorphism and three primers were (OPP-1, OPP-8 and OPP-14) selected based on the stability of banding pattern.

Tissue culture regenerants from varieties P_1, P_2 and P_4 at two-month-old and six-month-old stage were subjected to RAPD analysis using the three primers selected. The TC regenerants from callus derived mother plants were also screened with the selected primers.

Monomorphic banding pattern was observed for the TC regenerants when compared with their respective source plants. Uniformity was confirmed at both stages of development studied. Thus the genetic stability and clonal fidelity was ensured for the tissue culture regenerants and the viability of the protocol was confirmed.

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*Originals not seen

APPENDIX I

Specific chemicals used	Firm
Cetyl trimethyl ammonium bromide (CTAB)	- E Merck, Germany
Socium dodysil sulphate (SDS)	- Sigma, USA
β mercapto ethanol	- E Merck, Germany
Ethidium bromide	- Sigma, USA
Agarose	- Genei, Bangalore
Taq DNA polymerase	- Genei, Bangalore
PCR reagents (10x Buffer, dNTPs)	- Genei, Bangalore
Decamer primers	- 'Operon', USA
Bromophenol blue	- Sigma, USA
Liquid nitrogen	- Madras Oxyaceletene Company, Coimbatore
Mineral oil	- Sigma, USA
Tris base	- Sigma, USA
DNA Mol. wt. Marker	- Genei, Bangalore
EDTA	- Sigma, USA

APPENDIX II

Equipment used for the study	Firm
1. Spectrophotometer	- Spectronic® Genesys-5 Spectronic Instruments Inc., USA
2. Refrigerated high speed centrifuge	- Kubota, Japan
3. Water purification system	- Millipore, Germany
4. Deep freezer	- Sanyo, Japan
5. Electronic Balance	- Sarturius
6. Laminar flow	- Kirloskar, India
7. Cytocentrifuge	- Spinwin
8. Electrophoresis system	- Hoefer, USA Biotech, Madras Genei, Bangalore
9. Thermal cycler	- Peltire PTC 200 M.J. Research, USA
10. Transilluminator	- Herolab, Germany
11. Documentation system	- Alpha Infotech, USA
12. Ice flaking machine	- Ice matics

RAPD ANALYSIS TO ASSESS THE GENETIC STABILITY IN TISSUE CULTURE DERIVED BLACK PEPPER (*Piper nigrum* L.) PLANTS

By

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ABSTRACT OF A THESIS

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Faculty of Agriculture

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ABSTRACT

As part of the Post Graduate programme in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara experiments were conducted at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 1997 to 1999; to standardise the method of DNA isolation and the protocol for RAPD analysis in black pepper so as to assess the genetic stability and clonal fidelity of tissue culture derived black pepper plants.

Three methods described by Dellaporta *et al.* (1983); Doyle and Doyle (1987) and Rogers and Bendich (1994) were tried. Modification of these methods were tried to find out the effect of grinding the tissue in liquid N_2 and use of β-mercapto ethanol. The method suggested by Rogers and Bendich (1994) was found better in terms of yield and quality of DNA. Grinding with liquid N_2 and use of β-mercapto ethanol was found effective. Tender leaves were found to be the best source for recovery of quality DNA.

Different levels and possible combinations of dNTPs, primer and enzyme were tried to standardise optimum levels of reaction components for RAPD analysis of black pepper. Best thermal cycle was identified for the amplification of black pepper genomic DNA. Different concentration of template DNA tried was found not influencing the amplification pattern.

Sixty decamer primers were screened for amplification of black pepper genomic DNA. Ten primers selected for good amplification were used to screen five varieties of black pepper. Three primers, which showed polymorphism and stability of amplification, were used for analysis of TC plants.

Tissue culture regenerants derived by bud culture were subjected to RAPD analysis using three primers (OPP-1, OPP-8, OPP-14). All the regenerants

studied gave a uniform RAPD profile except in two regenerants where there was difference in expression of two non-distinct bands.

The present study was effective in optimizing the protocol for RAPD analysis in black pepper and is the first of its kind reported in this valuable spice crop. The primers identified for varietal screening and the RAPD profile developed for the five important varieties can be utilised for fingerprinting of these varieties. The results also ensure the genetic stability and clonal fidelity of the TC plants and the suitability of tissue culture protocol for commercialisation.