SCREENING FOR DISEASE TOLERANCE USING RANDOM AMPLIFIED POLYMORPHIC DNA'S IN *HEVEA BRASILIENSIS*

DISSERTATION SUBMITTED TO

PERIYAR UNIVERSITY, SALEM

FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE IN BIOTECHNOLOGY

SUBMITTED BY

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THIRUCHENGODE - 637 205

CERTIFICATE

This is to certify that the work entitled 'Screening for Disease Tolerance Using Random Amplified Polymorphic DNA's in Hevea brasiliensis' submitted to Periyar University, Salem is a record of original work done by Miss. Tessy Joe during the period of the study from Jan 2002 to March 2002, under my guidance and supervision, for the award of the Degree of Master of Science in Biotechnology. I further certify that this research work has not previously formed the basis for the award of any other degree, diploma, associate ship, fellowship or other similar title to any candidate of any University.

15/3/02

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Certificate

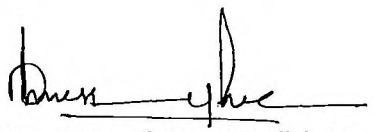
This is to certify that the dissertation entitled 'Screening for disease Tolerance Using Random Amplified Polymorphic DNA's in Hevea brasiliensis', submitted by Ms. Tessy Joe, Vivekanandha College of Arts & Science for Women, Thiruchengodu, was carried out at Biotechnology Division, Rubber Research Institute of India under my supervision. It is also certified that this work has not been presented for any other degree or diploma.

15/3/02

Dr. A. THULASEEDHARAN
Deputy Director (Biotechnology)

DECLARATION

I do hereby declare that the project work entitled 'Screening for Disease Tolerance Using Random Amplified Polymorphic DNA's in Hevea brasiliensis" submitted to the Periyar University, Salem for the award of the Degree of Master of Science in Biotechnology, is a record of original and independent research work done by me during Jan 2002 to March 2002, under the supervision and guidance of Dr. A. Thulaseedharan, Deputy Director, Biotechnology, Rubber Research Institute of India, Kottayam and it has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar tittle to any candidate of any university.



Signature of the candidate

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TESSY JOE

DEDICATED TO

MY PARENTS

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INTRODUCTION & REVIEW OF LITERATURE

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Hevea brasiliensis is the most important commercial source of natural rubber which is the most versatile industrial raw material of plant origin. Since the beginning of 20th Century natural rubber from *Hevea brasiliensis* (para rubber tree) has become the prime source of raw material for nearly 50.000 industrial products all over the world. Sir Henry Wickham was the main architect in introducing the Wickham genepool of *H. brasiliensis* to South East Asian countries from the Amazon river basin in Brazil (Lane 1953). The rubber plant was introduced in India towards the end of 19th Century by cultivating the seedling (Petch 1914, Dean 1987) on experimental basis and finally became the cynosure of plantation crops owing to its industrial dimensions and now India occupies the fourth place in the natural rubber production. *Hevea* is now commercially cultivated in Thailand, Indonesia, Malaysia, India, China, Sri Lanka, Colombia, Bolivia, Guyana, Peru, Venezuela etc. (Wycherley 1977).

The majority of rubber bearing plants belongs to a few plant families like Euphorbiaceae, Moraceae, Apocyanaceae and Asteraceae. Among these the genus *Hevea* of the Euphorbiaceae family accounts for almost 99% natural rubber production in the world. Of the ten species namely, *H. brasiliensis, H. guianesis, H. bethaminana, H. antida, H. camaryona, H.*

rigidifolis, H. spryceana, only *H. brasiliensis* is the economically viable source. Other minor sources of natural rubber include *Parthinium argentatum* (Guayule), *Manihot glaziroii, Castilla elastica, Ficus elastica, Cryptostegia grandflora, and Funtunia elastica*. Among these, only guayule rubber has gained some importance as it can withstand temperatures as low as –20°C, making its cultivation possible in countries like USA where cultivation of *Hevea* is difficult.

The latex, which is the cytoplasm of specialised cells called laticiferous cells, is obtained from mature trees through controlled wounding at the trunk region, a process called tapping. The rubber particles, a polymer of iosoprene units in the cis-configaration, are seen scattered in the latex. When the bark of the tree is tapped, the cytoplasmic contents of the laticifers are expelled in the form of latex. It is a milky substance, which upon coagulation and further processing yield rubber.

Various diseases, most of them are fungal in origin, that strikes through the rubber plantations are responsible for considerable loss in terms of latex yield in each year. Abnormal leaf fall caused by *Phytophthora,* powdery mildew by *Oidium hevea*, and leaf spot by *Cornyspora cassicola* are major diseases affecting the rubber tree. Abnormal leaf fall caused by *Phytophthora* is the most economically significant fungal disease in India.

Ever since it is first reported in the Palapilly Estates of Thrissur district in Kerala (McRae, 1918) in 1910, the abnormal leaf fall continues to be the most destructive disease of the rubber tree. Though the disease was reported from all the rubber growing countries severe incidence is observed mainly in South India.

Outburst of this disease in Kerala is usually occurred with the onset of South West monsoon during June – September. Continuous spell for 6 – 9 days without intermittent sunshine is the ideal condition for spreading of the disease. Temperature ranges from 23 – 28°C and a relative humidity of above 90% are most congenial for the outbreak of the disease. Low range of temperature coupled with heavy humidity abets and aids the spread of this fungal disease. The last seasons resting spores of the fungus present in the decaying pods, leaves and twigs in the soil as well as trees starts to germinate in favorable conditions. The affected green pods are turned to dull gray colour with soaked lesions. The spreading of the enormous number of sporangia produced by the pathogen is assisted by the water particles blown by the wind and also by various insects (Edathil and Pillay, 1976). The infected fruits rarely produce viable seeds. On the leaves, petioles are affected first and later lesions may also appear on the leaflets, midrib and lamina. The affected leaves fall off with leaflet intact and while they are still

green in colour. If the condition remains favorable, heavy leaf fall occurs and the pathogen invades the leaf- bearing twigs and causes extensive die back.

Severe infection in young trees may causes retarded growth resulting in an extended period of immaturity. Extensive defoliation in mature plantations can cause yield loss of 20 – 40% if the trees were left unsprayed for the whole season (Ramakrishnan, 1960). Growth and vigour of the plants were also adversely affected. Other effects includes, increase in plugging index, reduction in dry rubber content and more weed growth (Jacob, 1989). Different species of *Phytophthora* are reported to be causing the leaf fall disease in various countries. In India, four species namely, *P. meadii, P. nicotianae, P. palmivora and P. botryosa* were isolated from various infected samples. However, the most common species in the traditional areas is *P. meadii.*

From the initial germplasm collections of 19th century, many promising clones with several desirable traits were evolved through conventional breeding programmes in the last century. Anyway, most of the high yielding clones widely cultivated in our country are susceptible to abnormal leaf fall disease. But some of the clones are showing certain degree of tolerance to this disease while some others are highly susceptible. A plant which is attacked to the same degree as other plants but which suffers less

damage (in terms of yield and qualities) as a result of attack is said to be tolerant.

All the cultivated clones of *Hevea* in Asia and Africa were actually originated from a few seeds collected from a specific locality of Amazon River basin. The genetic advance of *Hevea* through conventional breeding programmes have slow down in recent years due to its narrow genetic base. In a perennial crop like *Hevea* where immature phase itself lasts for around seven years, plant improvement through conventional breeding programmes is time consuming and labour intensive. Its long breeding and selection cycle and difficulties in raising the F_2 progeny makes the classical approaches for crop improvement rather difficult. On the other hand, molecular markers, which deal directly with DNA were reliable, free from environmental interactions and can be assayed at any stage of plant growth and practically with all types of tissue.

The characteristics of a species are ultimately determined by the nucleotide sequences of its DNA, the fundamental molecule of life, that makes it different from others. Even within the species there will be some differences in the DNA sequences in each individual. The sequence of DNA, which is unique for an individual plant or group of plants exhibiting special characters can be called as molecular genetic markers. This can be used as

a tool to screen variations within a population. Techniques which are particularly promising in assisting selection for desirable characters using molecular markers involves, Random Amplified Polymorphic DNAs (RAPD), Restriction Fragment Length polymorphisms (RFLP), microsatellites or simple sequence repeats (SSR), Amplified Fragment Length polymorphisms (AFLP) etc.

Molecular markers have provided a means for plant genetic mapping, improved selection in plant breeding programmes and for the cloning of genes whose products are unknown. Among the various molecular markers developed, RFLPs were the first reported one by Botstein et al, 1980 for human genome mapping and later they were adopted for plant genome mapping also (Weber, 1989). It is actually the differences obtained in the fragment size arising from restriction enzyme digestion of the DNA. These differences can efficiently detected by Southern hybridisation, by means of certain probes that can specifically bind with their complementary bases in the denatured DNA. Microsatellites are DNA sequences composed of a tandem repetition of simple short sequences occurring in the genome of many higher organisms (Rafalski and Tingley, 1993). They are usually 1 - 6 base pairs long and are ubiquitous in eukaryotes. In AFLP (Vos et al, 1995) PCR amplification were used in place of Southern for the detection of restriction fragments.

RAPD technique developed by Williams et al, (1990) relies on the differential enzymatic amplification of small DNA fragments using PCR with single oligonucleotide primers (usually 10-mers). Polymorphisms results from either chromosomal changes in the amplified regions or base changes that alter primer binding sites. RAPD has its advantages over RFLP as it requires only small amounts of DNA and polymorphisms can be visualised through agarose gel electrophoresis avoiding the need for Southern hybridisation using hazardous radio labeled materials. This technique is relatively simple is more rapid. The RAPD markers are usually dominant because polymophisms are detected as the presence / absence of bands.

The RAPD technique is based on the development of an *in vitro* procedure, called Polymerase Chain Reaction (PCR) for the primer directed enzymatic amplification of a specific DNA fragment (Saiki et al, 1988). The PCR technique relies on the capability of a thermostable DNA polymerase called *Taq* DNA polymerase, isolated from a thermophilic bacterium *Thermus aquaticus*, to remain active even at 96°C which is essential for the denaturation of template DNA. The reaction is based on the annealing and extension of two oligo nucleotide primers that flank the target regions in duplex DNA. After denaturation of the DNA, achieved by heating the reaction each primer hybridises to one of the two separated strands such that

extension for each 3' hydroxy end is directed towards the other. This extension was carried out by *Taq* DNA polymerase in presence of deoxynucleotide triphosphates. Thus each cycle of PCR involves three steps *i.e.* denaturation, annealing and extension. During each cycle the quantity of the amplified fragments get doubled.

RAPD studies have efficiently been done to investigate genetic relationship between and within populations (Gen-Lou Sun et al, 1999; Duarte et al, 1999; Bai et al, 1998), to evaluate the clones (Scheepers et al, 1997; Hicks, 1998), to study the inheritance and gene introgression (Frello et al, 1995; Garcia et al, 1995; Gomez et al, 1996) and to identify molecular markers for various characters like disease resistance (Nair et al, 1995; Borovkova et al, 1995; Salentijen et al, 1995; Castaheira et al, 1999).

In *Hevea* RAPD technique was previously used to study phylogenic relationship through mitochondrial DNA polymorphisms (Luo, 1995), clone identification, gene mapping and estimation of genetic relatedness (Seguin, 1995; Varghese, 1997). RAPD markers for Powdery mildew resistance was reported by Shoucai et al, (1994) and RAPD markers for Tapping Panel Dryness tolerance was reported by Thulaseedharan et al, (1997). Since *Hevea brasiliensis* is the only cultivated and high latex yielding rubber crop,

there is an urgent need to develop improved clones for various characters mainly disease resistance.

Hence the present study has been undertaken with the following objectives.

AIMS OF THE PRESENT STUDY

1. To optimise the conditions for generating RAPD profiles for different clones of *Hevea*.

2. To identify DNA markers related to Abnormal leaf fall disease in rubber tree.

MATERIALS & METHODS

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Germplasm

The clones used in the present study, their parents, geographic origin and disease status to abnormal leaf fall is listed in Table 1. All plant materials were obtained from the germplasm collection and nursery of the Rubber Research Institute of India.

Sample Collection

Young, healthy, uninfected leaf samples were selected. The harvested leaf samples were immediately stored in polybags and were packed in an icebox and transported to the laboratory where DNA was extracted immediately.

Genomic DNA Isolation

All the glasswares and plasticwares required for the procedure were washed thoroughly, dried overnight in an oven and autoclaved. Total cellular genomic DNA was isolated and purified from young leaf material of individual samples by the CTAB (hexadecyltrimethylammonium bromide) extraction procedure (Doyle, 1990). Genomic DNA was extracted from 2 g of young leaves. Leaves first washed in tap water were then wiped with sterile water and alcohol using sterile cotton. Leaves were ground to a very fine powder in liquid nitrogen in a mortar with pestle and transferred into 50 ml centrifuge

Table- 1 Clones used in the present study

Sl. No	Clone	Origin	Parents	Disease Status
1	RRII 105	India	Gl 1 x Tjir 1	tolerant
2	Gl 1	Malaysia	Primary clone	tolerant
3	BD 10	Indonesia	Primary clone	tolerant
4	RRII 33	India	Primary clone	tolerant
5	GT 1	Indonesia	Primary clone	tolerant
6	RRIM 600	Malaysia	PB 86 x Tjir 1	Susceptible
7	Tjir 1	Indonesia	Primary clone	Susceptible
8	PB 86	Malaysia	Primary clone	Susceptible
9	RRIM 701	Malaysia	44/553 x RRIM 501	Susceptible
10	PR 107	Indonesia	Primary clone	Susceptible

tubes. 20 ml of 2x CTAB buffer [(2% CTAB), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 0.1 M Tris-HCl (pH 8.0), 1% polyvinylpolypyrolidone (PVPP), 1% 2-mercaptoethanol] was added and incubated the extract in a water bath at 65°C for 30 min with occasional swirling. The suspension was mixed gently and spun at 8000 rpm to pellet the cell debris. The supernatant was transferred to a new centrifuge tube and mixed thoroughly with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 10,000 rpm for 10 min. The aqueous phase was carefully transferred to a fresh centrifuge tube where RNA was eliminated by treatment with DNase-free RNase and the solution was incubated at 37°C for 2 hrs. Then the inactivation of RNase and removal of proteins were done by treatment with proteinase K at 37°C for 1hr. Equal volume of chloroform:isoamyl alcohol (24:1) was added to the tubes and centrifuged for 10 min at 10,000 rpm at room temperature. The supernatant was transferred to a fresh tube and reextracted with an equal volume of chloroform and centrifuged (10,000 rpm, 10 min). Following centrifugation, the aqueous phase was removed to a clean tube and DNA was precipitated by the addition of 0.6 volume of ice cold isopropyl alcohol (100%). The tubes were kept in ice for 20 min. and the precipitated DNA was pelleted by centrifuging at 4°C (10,000 rpm for 10 min). The supernatant was discarded and the pellets were washed with 70% ethanol. It was mixed thoroughly and then spun at 8000 rpm for 5 min. The supernatant was decanted and the pellet was air dried for 20 min. by keeping

in an inverted position over a sheet of blotting paper. The air-dried DNA pellet was resuspended in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). Samples were stored in 1.5 ml microfuge tubes at 4°C for short term or at -20°C for long term storage.

The quality of the DNA isolated was checked using agarose gel electrophoresis. One µl each loading buffer (0.25% bromophenol blue, 30% glycerol in TE buffer pH- 8) was added to 2 µl of DNA and the samples were loaded to 0.8% agarose gels prepared in 0.5X TBE (Tris-Borate-EDTA) buffer (Sambrook et al, 1989). Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 50V for nearly 4 hrs or until the bromophenol dye front has migrated to the bottom of the gel. The molecular standard used was the λDNA double digested with EcoR I and Hind III restriction enzymes. Staining was carried out with 0.5 µg/ml ethidium bromide. The gels were visualised in a UV transilluminator and were photographed under UV light. The concentration in each sample was spectrophotometrically measured and appropriate dilutions were made accordingly.

RAPD analyses

Prior to RAPD analysis, the DNA samples were diluted so as to get 10 ng DNA/ µl of the sample. Initially one DNA sample was used to screen 30

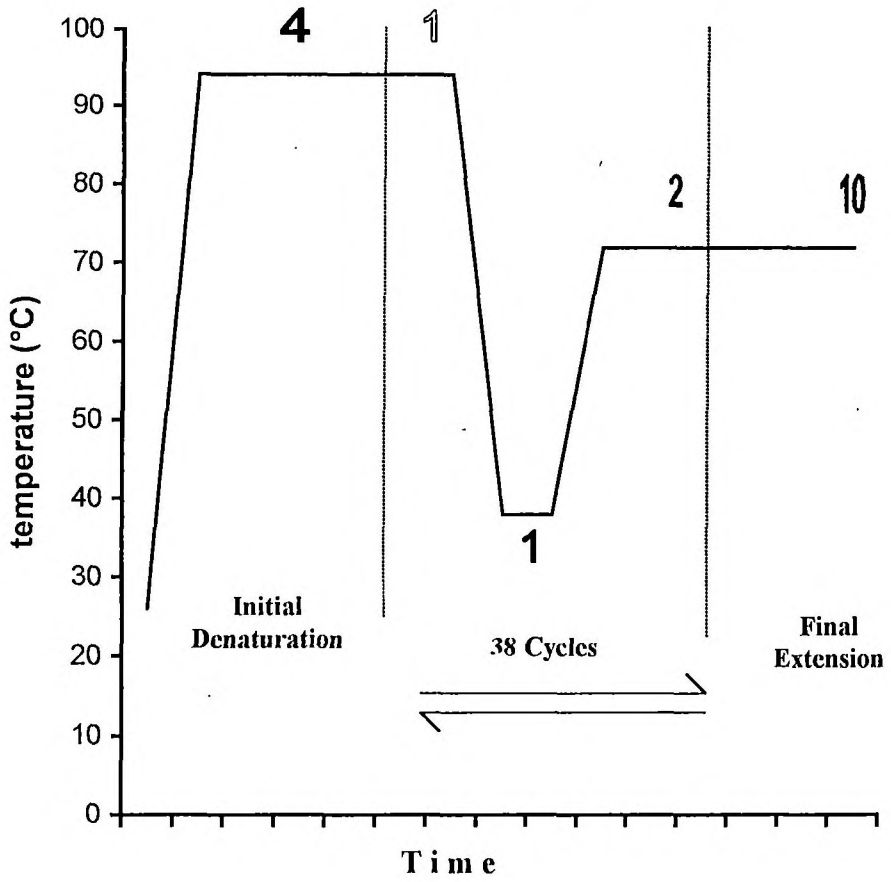
random oligonucleotide primers. Later, the primers which are giving clear, reproducible banding patterns were used to screen all the individual clones.

Williams et al. (1993) discusses the importance of the optimal concentrations of the components of the RAPD assay in the amplification. A series of assays were carried out to evaluate the optimum template DNA concentrations for PCR amplifications. DNA concentrations ranging from 5 – 50 ng were tried in the present experiment. For determining the optimum concentration $MgCl_2$ in the PCR reaction a series of reactions were performed with varying concentration of $MgCl_2$ (ranging from 0.5 – 2.5 mM). Like that the effect of enzyme concentration was also investigated. Different units of Taq DNA polymerase (0.1, 0.3, 0.5, 0.75 and 1) were tried to determine the ideal concentration.

Random decamer primers were purchased from Operon Technologies Inc., Alameda, CA, USA and were used as single primers for the amplification of RAPD sequences. The PCR amplification reactions were performed under conditions similar to those used by Williams et al. (1990). Amplifications were carried out in a 20µl reaction volume, which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5mM $MgCl_2$, 1 mM dNTPs (dATP, dGTP, dCTP and dTTP), 0.5 unit of Taq DNA Polymerase enzyme, 25 ng of template DNA and 250 nM of RAPD primer. The reaction mixture was

overlaid with two drops of mineral oil in order to avoid evaporation. The amplifications were carried out in a Perkin-Elmer DNA Thermal Cycler 480, USA. All reaction mixtures were prepared as master mixes for each primer to minimize measurement deviation that is more pronounced in small volume pipetting. Tubes containing all the reaction components except for the DNA template were included as a control for each primer used. The PCR amplification profile consisted of a first cycle at 94°C for 4 min. followed by 38 cycles at 94°C for 1 min., 38°C for 1 min., and 72°C for 2 min. After the cycles a final extension step was given for 10 min. at 72°C (Chart- 1). On completion of the cycles, samples were refrigerated at 4°C before electrophoresis. 70µl chloroform was added to the PCR tubes to ease the recovery of the samples. 5µl loading buffer was added to each tube and the RAPD fragments were size fractionated in 1.5% agarose gels. The gels were stained in an ethidium bromide solution for 20 min. in darkness followed by a water rinse for 10 min to enhance the contrast and then photographed. The reproducibility of the amplification products was tested at least twice for each experiment.

Chart-1
Schematic Representation of the PCR Reaction



RESULTS & DISCUSSION

RESULTS & DISCUSSION

The RAPD technique is very useful in variety identification, detecting gene introgression and genetic diversity assessment within a species. The use of molecular markers should enhance the ability of breeders to tag the desirable traits like disease resistance. The main disadvantage of the RAPD is the sensitivity of DNA amplification to PCR reaction conditions. Anyone of the following factors influences the RAPD profiles: concentrations of template DNA, *Taq* DNA polymerase, primer, and Mg^{2+} as well as temperature and duration for denaturation, annealing and elongation (Devos and Gale 1992; Muralidharan and Wakeland 1993; Park and Kohel 1994; Zhang et al. 1996). Only after these factors are optimized, the reproducibility of RAPD, especially for the major bands, is acceptable (Joshi and Nguge 1993; Wei and Wang 1994). A high reproducibility of RAPD patterns is a prime requirement for the use of the RAPD technology in detection of polymorphisms. Different parameters affecting successful amplification and reproducibility were studied.

Successful PCR amplification of DNA of different clones with random primers was standerdised in *Hevea* (Fig- 2- 5).

Factors Influencing PCR Reactions and RAPD Products

Extensive care must be taken during all the operations involved in the PCR reaction. The RAPD profiles were greatly influenced by various factors like template DNA quality and concentration, *Taq* DNA concentration, Mg^{2+} concentration, primer sequences etc.

Purity and Concentration of the Template DNA

Campos et al. (1994) carried out approximately 120 assays to obtain the optimum DNA concentration for getting clear RAPD profiles in lotus species. Muralidharan and Wakeland (1993) reported that the template DNA concentrations evidently affected the RAPD products. In *Hevea*, Low et al. (1996) has been attempted to develop RAPD marker following the procedure of Williams et al. (1990). They obtained fruitful polymorphisms, but reproducibility of these results was difficult. Further they explained that reproducible polymorphism were obtained after modifications in the experimental procedure.

In this study genomic DNA was isolated according to CTAB extraction procedure in good quality and concentration (Fig- 1). Since, *Hevea* leaves had high phenolic contents and other secondary compounds, it was necessary to purify the DNA to get a good amplification in PCR. In the present study, the DNA was extracted with CTAB buffer and re-extracted

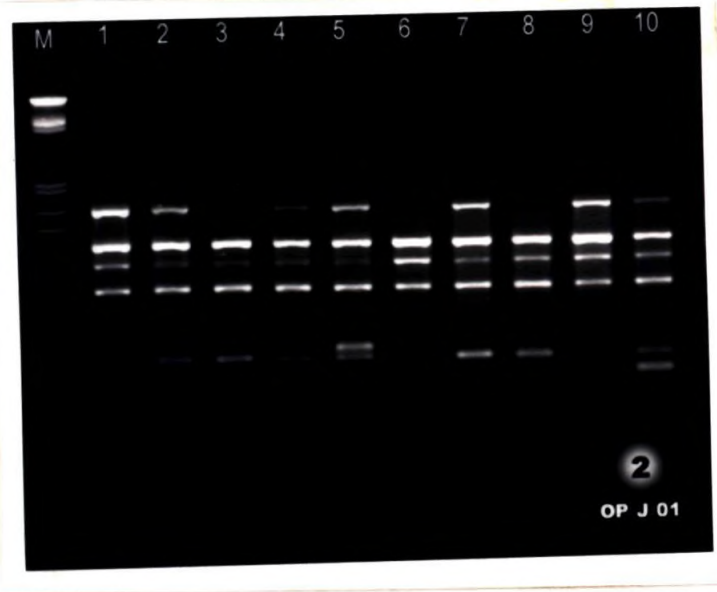
Fig. 1 Agarose gel electrophoresis of the genomic DNA isolated from leaf tissues of *Hevea*

Lane 1 - DNA marker; Lanes 2- 5 DNA from different samples

Fig. 2 RAPD analysis of 10 different clones of *Hevea* using OP J01 primer

Lane 1 - DNA marker
Lane 2 – RRII 105
3 – GI 1
4 – BD 10
5 – RRII 33
6 – GT 1
7 – RRIM 600
8 – Tjir 1
9 – PB 86
10 – RRIM 701
11 – PR 107

Fig. 1



twice with phenol:chloroform to eliminate all other contaminating components and macromolecules other than deoxyribonucleic acids. The extracted DNA was precipitated with ethanol and sodium acetate and used for PCR amplification after air drying and dissolved the pellet in TE buffer. Concentration of the DNA was also critical factor to get better PCR amplification. If the template DNA was treated with RNase at the time of extraction, the electrophoretic profiles of RAPD products became clearer and most of smear backgrounds disappeared. To optimize the DNA concentration, the DNA samples with concentrations ranging from 5 – 50 ng were tried. In the present study if the DNA concentration was more than 20 ng per reaction, bands became faint and smeared lanes will appear on electrophoresis. Finally 10 ng/reaction, of concentration was selected and used for further RAPD amplification of genomic DNA. Similar results were also reported by Scheepers et al. (1997) in *Picea abies*.

Effect of *Taq* DNA Polymerase Concentration

Another important factor is *Taq* DNA polymerase concentration. In the present experiment, assays were carried out to obtain the optimum Taq DNA polymerase concentration. Different concentrations viz., 0.1, 0.3, 0.5 and 1.0 unit/reaction *Taq* DNA polymerase were tried for PCR amplification. Among the various concentrations used, it can be observed that the concentrations of 0.1 and 0.3 unit gave bands but not scorable and found irreproducible. Similar

results were also recorded by Zhang et al. (1996) in *Aegilops geniculata*. Increasing the concentration from 0.3 unit to 0.5 unit increased the intensity of bands. Amplified RAPD profiles with 0.5 unit were more reproducible than those amplified with 0.3 unit. This result was generally consistent for all of the samples and therefore the concentration of 0.5 unit was finally selected for the PCR amplification analysis. The present result was consistent with previous report of Campos et al. (1994). Muralidharan and Wakeland (1993) and Park and Kohel (1994) pointed out that there was no satisfactory explanation on this phenomenon yet.

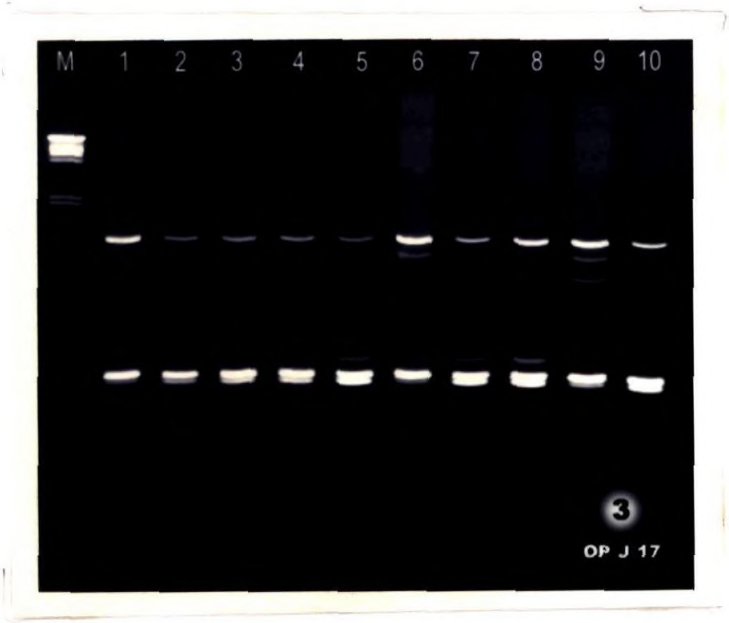
Effect of Mg^{2+} Concentration

The presence of $MgCl_2$ in the reaction buffer was also very critical factor to produce good PCR amplification. In the present experiment concentrations ranging from 0.5 - 2.5 mM) were tested. At least 1mM concentration was found to be necessary for the amplification and 1.5 mM was found to be the optimum for better DNA amplification without any streaking. When raising the concentration of $MgCl_2$ in the reaction mixture from 1.5 to 2.5 mM, the yield of RAPD products was decreased and smeared lanes appearing in the electrophoretic profile pattern. This may be due to the inhibition of *Taq* DNA polymerase activity by excess of Mg^{2+} concentration. In subsequent tests, it was decided to use 1.5 mM Mg^{2+}

Fig. 3 RAPD analysis of 10 different clones of *Hevea* using OP J17 primer

Lane 1 - DNA marker
Lane 2 – RRII 105
3 – GI 1
4 – BD 10
5 – RRII 33
6 – GT 1
7 – RRIM 600
8 – Tjir 1
9 – PB 86
10 – RRIM 701
11 – PR 107

Fig. 4 RAPD analysis of 10 different clones of *Hevea* using OP I 11 primer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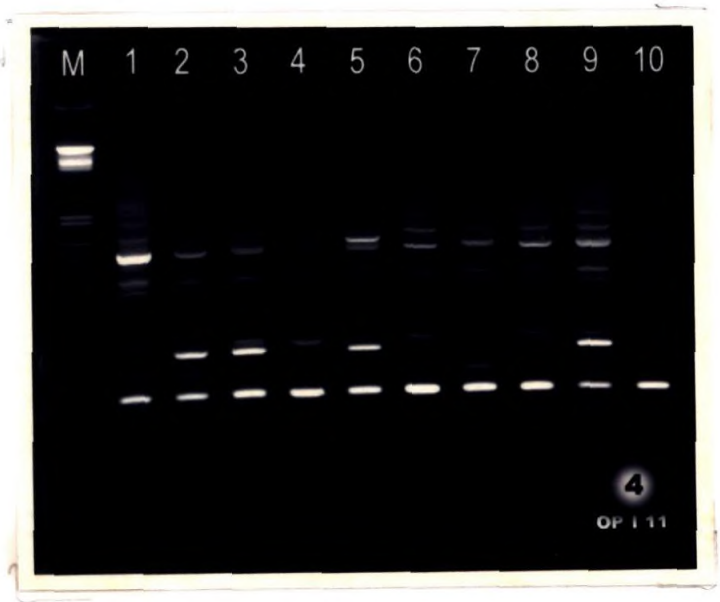
3
OP J 17


M 1 2 3 4 5 6 7 8 9 10
4
OP I 11

concentration for PCR amplification to detect polymorphism. Similar observations were also made by Zhang et al. (1996).

Another most important factor is primers, which influenced much on RAPD polymorphisms. Initially we screened 30 primers where 11primers were found suitable for the development of readable RAPD profiles. The other primers either gave no amplification or poor amplification with streaking. Muralidharan and Wakeland (1993) found that primer concentration greatly affected the PCR amplification. Campos et al. (1994) carried out 9 series of assays with various primers to get clear RAPD products in lotus. They observed that few primers failed to amplify and some primers produced smaller or larger number of bands. . Of the 76 primers screened by Yamagishi (1995) to identify informative primers in *lilium*, only 18 primers were informative, 11 primers produced a smear pattern and the remainder failed to amplify. Mailer et al. (1994) tested 100 primers, only 22 of these showed evidence of polymorphisms and 70 produced some product. Only 6 primers were ultimately selected for further PCR amplifications and other polymorphic primers produced patterns which were either poor and difficult to detect or not reproducible. These results are in agreement with our present experiment. Bai et al. (1998) used 329 primers for RAPD amplification, only 73 primers gave polymorphic primers which resulted in very clear RAPD products were selected for further screening of plants. A total of 60 random

primers were screened by Low et al. (1996) in *Hevea* and only three of these were able to elicit polymorphisms. Recently, Ghislain et al. (1999) suggested three criteria for primer selection: reproducibility, number of polymorphic fragment per assay and levels of polymorphism detected in a specific population. Therefore, identification of best primers for PCR amplification is one of the pre-requisite to apply RAPD marker technology for crop improvement programmes.

Development RAPD Marker for Abnormal Leaf Fall Disease

The RAPD technology has been applied already in several tree species including *Hevea* for molecular studies. Development of molecular markers for Powdery mildew disease resistance (Shoucai, 1994) and Tapping Panel dryness tolerance (Thulaseedharan, 1997) were reported in *Hevea* earlier. No attempt has been made to develop a DNA marker for abnormal leaf fall disease so far. The molecular tools most commonly used for germplasm studies in *Hevea* are isozymes, seed storage proteins, RFLPs etc. Although, informative and practical, the use of isozyme and protein markers have been limited by the number of loci that can easily be detected (Skroch et al. 1992; Zhang et al. 1996). The use of RFLPs for characterization of germplasm resources has been limited by the cost factor. Both isozymes and RFLPs are limited by the low level of polymorphism among cultivars of some crops, on the other hand, the RAPD technique can

generate polymorphic data more efficiently with less expense. Most importantly, the application of RAPDs does not need any prior knowledge of genomic nucleotide sequences (Williams et al. 1990).

Initially 30 random 10-mers were used to find out the primers which are giving good amplification. Of which 11 primers gave good amplification with easily detectable, well-resolved bands which were reproducible over repeated runs with sufficient intensity to determine the presence or absence in samples with same relative band intensity. These 11 primers were used to screen all the 10 clones, including 5 tolerant and 5 susceptible clones. Total number of bands produced by each primer varied from 7 – 19. The size of the bands ranged from 300 – 3,500 base pairs. Other primers generated either streaking patterns or were failed to amplify. Among these 11primers one primer namely, OPJ18 has produced enough polymorphisms between tolerant and susceptible clones (Fig. 6). Two polymorphic bands with a molecular size of 900 and 830 bp obtained with primer OP J18 has of particular importance. Except in the case of clone GI 1 these two bands are present in all tolerant clones. But in the case of susceptible clones, only one of these bands is present. The 900 bp band was present in susceptible clones RRIM 600 and PR 107, while the 830 bp band was observed in Tjir 1, PB 86 and RRIM 701. Any interaction between the genes representing these two DNA sequences and whether this interaction can be correlated with the

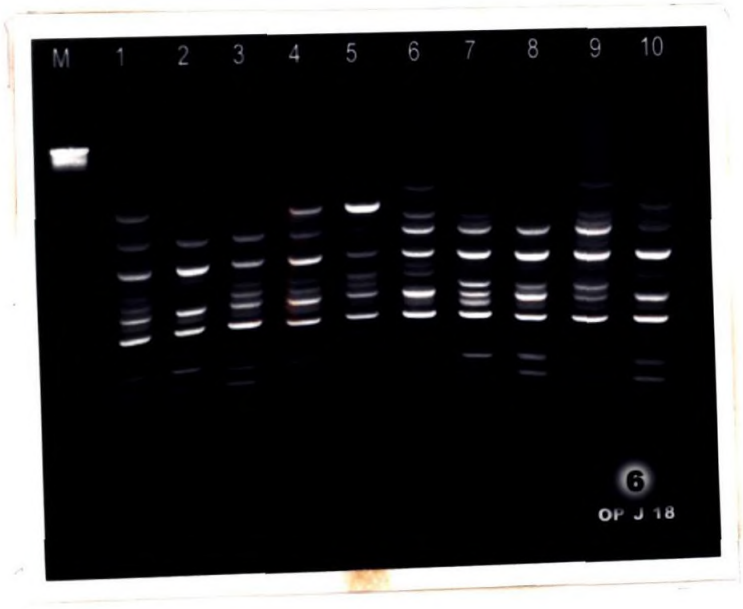
Fig. 5 RAPD analysis of 10 different clones of *Hevea* using OP I 16 primer

Lane 1 - DNA marker
Lane 2 – RRII 105
3 – GI 1
4 – BD 10
5 – RRII 33
6 – GT 1
7 – RRIM 600
8 – Tjir 1
9 – PB 86
10 – RRIM 701
11 – PR 107

Fig. 6 RAPD analysis of 10 different clones of *Hevea* using OP J 18 primer showing 900 and 830 bp bands in tolerant clones except GI 1

Lane 2- 6- tolerant clones; Lane 7- 11 susceptible clones


M 1 2 3 4 5 6 7 8 9 10
5
OP I 16


M 1 2 3 4 5 6 7 8 9 10
6
OP J 18

tolerance to the abnormal leaf fall disease in *Hevea* is to be investigated further. For that purpose these to fragments has to be isolated and cloned. The sequencing of these cloned fragments might give some insight in to the actual role of these fragments in tolerance.

Summary & Conclusion

The genetic improvement in *Hevea* through conventional breeding rpogrammes has been slowing down in recent years due to the narrow genetic base and long breeding cycle. Development molecular techniques will be a viable alternative for crop improvement. As it can be detected at any stages of plant growth and as it is not environmentally regulated they hold major advantages over conventional practices like selection of parents based on phenotype. Among various techniques for generating molecular markers, RAPD is the simplest one that can detect polymorphisms more rapidly and efficiently with minor quantities of template DNA. This technique is based on the enzymatic amplification of random DNA sequences using arbitrarily chosen oligonucleotide primers. In the present study PCR conditions and concentrations of various components of the PCR reaction were optimized for the successful and reproducible amplification of DNA from different clones of *Hevea*. Attempt has also been made to identify an RAPD marker linked with abnormal leaf fall disease, which is the most destructive disease of rubber plantations in India. RAPD analyses were carried out with five most tolerant and 5 highly susceptible clones using 11 oligonucleotide primers, which are giving good, reproducible amplifications. Two fragments with molecular size of 900 bp and 830 bp obtained with OP J18 primer is present in four of the five tolerant clones tested. But in the case of susceptible clones only any one of this band is present. To correlate the interaction between these two bands and abnormal leaf fall disease tolerance, cloning and sequencing of these RAPD products has to be done.

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