STUDIES ON GENETIC POLYMORPHISM IN *SANTALUM ALBUM* L.

THESIS

SUBMITTED TO

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

IN PARTIAL FULFILLMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN ENVIRONMENTAL BIOTECHNOLOGY

UNDER THE FACULTY OF

ENVIRONMENTAL STUDIES

T. B. SUMA M.Phil.

KERALA FOREST RESEARCH INSTITUTE
PEECHI, THRISSUR – 680 653, KERALA, INDIA.

JULY 2002

Kerala Forest Research Institute
(An Autonomous Institute under Government of Kerala)
Peechi - 680 653, Thrissur, Kerala, India.

Tel : +91 - (0)487 699 884 (Director)
699 037, 699 061 - 64
699 365
Fax : +91 (0)487 699 219
Email : kfri@kfri.org
Website : www.kfri.org

Dr. M. Balasundaran, M.Sc., Ph.D.
Scientist E1,
Scientist-in-Charge,
Division of Pathology.

CERTIFICATE

This is to certify that the thesis entitled '**STUDIES ON GENETIC POLYMORPHISM IN *SANTALUM ALBUM* L.**' embodies the result of original research work conducted by Ms. T. B. Suma (Reg. No. 1835), under my guidance and supervision. No part of the thesis has previously formed the basis for the award of any degree, diploma, associateship or other similar titles of this or other institute or any University or society.

15. 07. 2002

M. Balasundaran

Phone : + 91 - (O) 487 - 282365, 282037, 282061 - 64, Grams KEFORINST.
FAX + 91 - (O) 487 - 282249; email : kfri@kfri.org; web www.kfri.org

DECLARATION

I hereby declare that this thesis entitled '**STUDIES ON GENETIC POLYMORPHISM IN *SANTALUM ALBUM* L.**', submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environmental Biotechnology under the faculty of Environmental Studies of Cochin University of Science and Technology is my original work and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Peechi.
15. 07. 2002

T. B. Suma

ACKNOWLEDGEMENTS

I express my profound sense of gratitude and indebtedness to my research supervisor, Dr. M. Balasundaran for his valuable guidance, constant encouragement and relentless support throughout the course of the study and for the preparation of the manuscript.

My sincere thanks to Dr. K.S.S. Nair, former Director and Dr. J.K. Sharma, Director, KFRI, for their constant support and for permitting me to use the laboratory facilities of this institute.

I am greatly privileged to acknowledge my sincere thanks and indebtedness to Dr. E.M. Muralidharan, for his valuable and constructive suggestions and criticisms during the early stages of my research work. I express my deep sense of gratitude to Dr. Ammini Joseph, Professor, Department of Environmental Studies, Cochin University of Science and Technology, for her timely help, good will and valuable suggestions. I wish to place on record my heartfelt thanks to Dr. R.V. Varma, Chairman, Ph.D. programme committee, whose kind and encouraging words were always a source of inspiration to me.

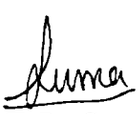
The assistance provided by the library staff, Kerala Forest Research Institute is gratefully acknowledged. My special appreciation to Dr. K.V. Sankaran, Dr. C. Mohanan, Dr. E.J. Maria Florence and Dr. S. Kumaraswamy for their encouragements and generous help. I extend my thanks to the officials

of the Forest Department of Kerala, Karnataka and Tamil Nadu for providing necessary help to collect sandal seeds from those states.

My sincere thanks to the Department of Biotechnology, Govt. of India, for a junior research fellowship and CSIR, Govt. of India, for a senior research fellowship.

Indeed I owe a lot to my friend Dr. Sunil Thomas for his untiring help, and moral encouragements. My profound thanks to Mr. P.V Pramod, Mr. Subash Kuriakose, Mr. K.H. Hussein, Mr. P.V. Santhoshkumar, Mr. C.M. Brijesh, Mr. M.A. Sreenivasan, Mr. N. Ratheesh, Ms. Binu. C. Nair and to all my friends in Pathology and Genetics Divisions for their timely help. I thankfully acknowledge Ms. C. Sunanda for her helpful suggestions in the statistical analysis and interpretation of the data. I am ever grateful to Shri. K.C. Joby for the pains he has taken to computerize my thesis with meticulous care.

I owe profound gratitude to my parents, brother, sister and in-laws, without their blessings, moral support and inspiring encouragement, I would not have come upto this level. I dedicate this thesis to my caring husband, for, without his selfless sacrifice, boundless patience and unflagging interest, this study would never have seen the light. And finally I thank ***GOD,*** *just for everything.*



T.B. Suma

CONTENTS

LIST OF TABLES

CHAPTER 3

CHAPTER 4

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

ABBREVIATIONS

µg	: microgram
µl	: micro litre
µM	: micro molar
2,4-D	: 2,4- Dichlorophenoxy acetic acid
ABA	: Abscisic acid
AFLP	Amplified fragment length polymorphism
ATP	: Adenine triphosphate
BAP	Benzyl aminopurine
bp	base pair
CAPS	Cleaved amplified polymorphic sequence
CH	Casein hydrolysate
CTAB	Cetyl trimethyl ammonium bromide
CTP	Cytosine triphosphate
DNA	: Deoxy ribonucleic acid
EDTA	: Ethylene diamine tetra acetate
EST	: Esterase
GPI	: Gluco phosphate isomerase
GTP	: Guanine triphosphate
HCI	: Hydrochloric acid
$HgCl_2$	Mercuric chloride
IAA	: Indole acetic acid
IBA	: Indole butyric acid
k Da	: kilo Dalton
KCl	Potassium chloride
mA	Milli ampere
MDH	Malate dehydrogenase
Mg Cl_2	Magnesium chloride

MS	: Murashige and Skoog
MTT	: Tetrazolium thiazolyl blue
NaCl	: Sodium chloride
NAD	: Nicotinamide adenine dinucleotide
NADP	: Nicotinamide adenine dinucleotide phosphate
NaOH	: Sodium hydroxide
NBT	: Nitro blue tetrazolium
ng	: nanogram
NTP	: Nucleotide triphosphate
OD	: Optical density
PAGE	: Poly acrylamide gel electrophoresis
PCR	: Polymerase chain reaction
PMS	Phenazonium methosulphate
PRX	: Peroxidase
PVP	: Polyvinyl pyrrolidone
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA
RFLP	: Restriction fragment length polymorphism
rpm	: revolutions per minute
SCAR	Sequence characterized amplified region
SDS	: Sodium dodecyl sulphate
SKDH	: Shikimate dehydrogenase
SSRs	: Simple sequence repeats
TBE	: Tris borate EDTA
TMV	: Tobacco mosaic virus
TTP	Thymine triphosphate
UPGMA	Unweighted pair group mean analysis

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. SANDAL

Santalum album L. occupies a prime position in Indian forestry and has been rated as the most precious and valuable tree (Srinivasan *et al.*, 1992; Kulkarni, 1995; Srimathi *et al.*, 1995; Radomiljac *et al.*, 1998). The scented heartwood of sandal, 'the East Indian sandalwood', yields the fragrant sandalwood oil by steam distillation (Rai and Kulkarni, 1986; Srinivasan *et al.*, 1992; Rao, 1995; Shankaranarayana *et al.*, 1995; Srimathi *et al.*, 1995). Sandalwood has been intimately associated with human civilization since time immemorial and is a part of Indian culture and heritage (Srinivasan *et al.*, 1992). The heartwood of sandal is estimated to be fetching approximately Rs. 9 lakhs per tonne in the international market (Ananthapadmanabha, 2000). Thirty- to sixty-year-old trees having a girth of 40-60 cm, generally have the best heartwood suitable for carving as well as for oil extraction (Shankaranarayana *et al.*, 1998). The oil is present in the heartwood of both the stem and root and hence the tree is invariably harvested by uprooting. Sandalwood oil is in great demand for manufacturing cosmetics, soaps and perfumes and for ayurvedic and allopathic systems of medicine (Srinivasan *et al.*, 1992; Tandon, 1995). A growing tree can put an increment of 1 kg of heartwood per year and attain a girth of 1-5 cm per year (Venkatesan, 1980; Rai, 1990). India exported around 2000 tonnes of wood and 100 tonnes of oil annually to various countries and accounted for 99 per cent of sandal oil produced in the world (Lakshmi Sita and Bhattacharya, 1998).

1.1.1. Distribution

The genus *Santalum* is tropical in distribution between 30° N and 40° S from India in the West to Juan Fernandez islands in the East and from Hawaiian Archipelago in the North to New Zealand in the South (Brennan and Merlin, 1993). The widely distributed and economically important genus *Santalum* consists of 29 species (Hewson and George, 1984) which are xylem tapping root hemi-parasites belonging to the family Santalaceae. The better known Indian sandal, *S. album* grows in a wide range of temperature and soil types in tropical and subtropical India, Srilanka and Indonesia (Nageswara Rao *et al.*, 1999). Four *Santalum* spp. namely *S. spicatum* (R.Br.) A.DC., *S. accuminatum* (R.Br.) A.DC., *S. murrayanum* (Mitchell) C. Gardn. and *S. lanceolatum* (R.Br.) are native to Western Australia (Sawyer and Jones, 2000). Among the *Santalum* spp., *S. album* has got the highest oil content (6-7%) while *S. spicatum* (2% oil content) and *S. lanceolatum* (3-5% oil content) yield poorly scented wood and low quality oil (McKinnell, 1990).

In India, *S. album* is found naturally distributed over 9600 km^2 (Srinivasan *et al.*, 1992; Radomiljac *et al.*, 1998) in different eco-climatic and edaphic conditions from Kerala in the South to Uttar Pradesh in the North (Jain *et al.*, 1998). Nearly 90 per cent of total area is in Karnataka (5245 km^2) and Tamil Nadu (3043 km^2) states. In Kerala, it is mainly distributed in the forest ranges of Marayoor, Kasargod, Wayanad and Thenmalai (Srimathi *et al.*, 1995). Sandal is also found in Andhra Pradesh (175 km^2), Maharashtra (84 km^2), Madhya Pradesh (33 km^2) and Orissa (25 km^2) (Jeeva *et al.*, 1998).

1.1.2. Regeneration

Sandal produces flowers and fruits twice a year (September/October and March/April) and is capable of regenerating itself profusely in the sandal tracts of open forests. Fresh seeds have a dormancy period of 2 months and retain their viability upto 12 months. The seeds normally take 4 to 8 weeks to germinate and the dispersal of seeds is usually by birds (Venkatesan, 1995). Sandal is managed in natural forests under physical rotation; dead and fallen trees are periodically extracted. Occurrence of fire, excessive grazing, hacking and encroachments often hamper the efficiency of natural regeneration (Venkatesan, 1981). Therefore, given the necessary protection, any area with a few scattered trees can be stocked with adequate natural regeneration. Artificial regeneration is achieved by dibbling of seeds in pits, sowing in mounds, trenching around mother trees for wounding the roots for obtaining root suckers and planting nursery-raised, vegetatively multiplied and tissue culture-raised seedlings (Rai and Kulkarni, 1986). Vegetative propagation is achieved through stem cutting, grafting, air layering or through suckers; but rooting is achieved only in 15-20 per cent of cuttings (Rao and Srimathi, 1976; Uniyal *et al.*, 1985; Balasundaran, 1998; Sanjaya *et al.*, 1998). As heart wood formation is genetically controlled, it is desirable to obtain seeds from genetically superior trees for developing a successful artificial regeneration programme and better productivity (Srinivasan *et al.*, 1992).

1.2. DEPLETION OF SANDAL FORESTS

In addition to regeneration problems, the depletion of sandal forests is mainly attributed to factors such as illicit felling and diseases. In India, sandalwood smuggling is very rampant and it is the major problem in all the states where sandal grows (Nageswara Rao *et al.*, 1999). Smuggling ultimately results in genetic erosion, because smugglers remove genetically superior trees and such populations tend to possess more and more genetically inferior trees (Venkatesan *et al.*, 1995). Uma Shaanker *et al.* (2000) reported a decline in the genetic diversity of natural population due to indiscriminate extraction of sandalwood. The annual production of sandalwood has dwindled from 4000 tonnes in 1965 1970 to merely 1000 tonnes during 1999 - 2000. The oil production has also reduced to 40 - 50 tonnes during 1999 2000 from a constant rate of 60 tonnes during 1981 1994 (Ananthapadmanabha, 2000).

1.3. DISEASE AND PEST PROBLEMS

The major disease that causes mortality of sandal trees is the spike disease caused by phytoplasma (Mukerji and Bhasin, 1986; Ghosh *et al.*, 1992; Srinivasan *et al.*, 1992; Srimathi *et al.*, 1995; Thomas, 2000). The disease is prevalent in three of the major sandal growing states in India namely Karnataka, Tamil Nadu and Kerala (Rangaswamy, 1995). The leaves and internodes of the affected trees show extreme reduction in size, and the shoots resemble spike inflorescence. The death of infected trees in the entire affected area within 1-2 years is characteristic of the disease (Ghosh *et al.*, 1992). In addition to spike disease, other diseases associated

with sandal include leaf spot diseases caused by different fungal pathogens such as *Ascochyta santali, Ganoderma applanatum, Ganoderma lucidum, Macrophomina phaseoli* and *Asterina congesta* (Srinivasan *et al.*, 1992). Many insect species are also known to attack sandal, but only very few are serious pests which adversely affect the growth of the plants. They include defoliators, sap suckers, stem borers and termites. Of these, the sap suckers belonging to the family Coccidae cause die back, poor growth, reduction of flowering and fruiting, and sometimes even mortality to plants in nurseries, plantations and reserve forests (Remadevi *et al.*, 1998).

1.4. GENETIC DIVERSITY OF SANDAL POPULATIONS

Genetic diversity forms the base of the biodiversity hierarchy (Namkoong *et al.*, 1996). The amount and distribution of population genetic diversity (population genetic structure) is the most fundamental piece of information required for proper genetic management of species (Brown, 1978). Genetic diversity in *S. album* is assumed to be imperiled, owing to the dysgenic selection and wanton felling perpetrated by smugglers and due to the destructive spike disease (Muthana, 1995). Fragmentation of natural sandal population due to excessive human encroachment and land use changes also contributed to the decline of species diversity (Nageswara Rao *et al.*, 2001).

Sandal is a highly polymorphic species (Kulkarni, 1995). Morphological studies showed that the trees vary significantly in leaf length and breadth, colour of heartwood and in the oil content (Bagchi and Veerendra, 1987; Kushalappa, 1983;

Kulkarni, 1995). A study of anatomical characteristics viz. xylem cell diameter, epidermal thickness, cortex width and number of vascular bundles has shown that there are genotypic differences in sandal populations (Veerendra and Bagchi, 1986). A priori information on genetic variation/diversity of sandal will assist in valid sampling to capture most of the genetic variation present in natural populations and can hasten progress of both conservation and tree improvement programmes.

1.5. MICROPROPAGATION IN SANDAL GENETIC IMPROVEMENT

Tree improvement in sandal aims mainly at evolving trees that can yield more heartwood and oil in a short span of time, coupled with spike disease resistance (Venkatesh and Kedarnath, 1963; Srimathi *et al.*, 1977, 1980; Kulkarni *et al.*, 1998). Concerted efforts have to be initiated for the preservation of existing sandal populations and the development of techniques for rapid multiplication of superior disease evaded trees. Tissue culture or micropropagation technique is an important method of mass multiplication of superior plants to obtain true-to-type plantlets.

Somatic embryogenesis in forest trees is reported to be one of the most efficient method of micropropagation and has got the advantage of producing several plantlets within a limited period to meet the ever increasing demand in reforestation activities (Thorpe, 1995). There is also the possibility of long term storage of somatic embryos, which aids in genetic conservation of rare genotypes. Despite these advantages, somatic embryogenesis in forest trees are often met with

developmental anomalies during somatic embryo induction, maturation, conversion and final establishment in the field (Tang, 2001).

In *S. album,* though somatic embryogenesis is reported to be the efficient method of *in vitro* propagation, the high per cent of abnormal embryos and poor rate of conversion of somatic embryos to plantlets hinder the efficient utilization of the technique (Ilah *et al.*, 2002). Though, culture conditions required for somatic embryogenesis in sandal are well defined, the molecular and biochemical events underlying the phenomenon of somatic embryogenesis are ill defined (Bapat and Rao, 1984; 1998).

1.6. OBJECTIVES OF THE STUDY

Loss of natural sandal populations due to illicit felling, forest encroachment and spike disease have an adverse effect on genetic diversity of the species. To initiate any genetic improvement programme in sandal, a precise understanding of the population genetic diversity structure is essential. The concern over the loss of genetic variability in sandal is particularly critical, as there is hardly any information regarding the diversity status of the natural populations.

Identifying fast growing, disease resistant, oil rich sandal trees through breeding and their mass multiplication for afforestation are the best method for ensuring sustainable supply of superior sandalwood. The healthy sandal trees existing in heavily spike diseased area can be used as a promising starting point for any such breeding programme (Venkatesh, 1978). So far, no genetic information is available regarding the resistant nature of spike disease evaded trees left in heavily

infected patches. The high rate of depletion of the superior trees in South Indian sandal reserves due to illegal felling and spike disease has necessitated an urgent need for conservation of the surviving trees.

Widespread occurrence of spike disease in Marayoor forest reserve was reported in 1981 (Ghosh and Balasundaran, 1995). Because of the high density of trees and varying intensity of spike disease, Marayoor sandal population was found to be ideal for experimental studies in sandal (Ghosh *et al.*, 1985). Fifteen trees of reserve 51 of Marayoor range had been selected as candidate plus trees for growth and spike disease evasion (Fig.1.1.) (Balasundaran, 1998). These trees have been selected for mass multiplication through tissue culture technique.

The major objectives of the present study are:

1. Assessment of genetic diversity within and between South Indian sandal populations using isozyme and RAPD markers.
2. Protein, Isozyme and RAPD marker analysis in spike diseased and disease evaded sandal trees in Marayoor.
3. Tissue culture of spike disease evaded sandal trees in Marayoor and study of protein, isozyme and RAPD profiles during the ontogeny of somatic embryos.

Figure 1.1. A spike disease evaded sandal tree in Marayoor reserve

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Forests are the green gold of a country and the harbingers of economic prosperity and ecological stability. They are the biologically most diverse terrestrial ecosystems within which, trees are central to the habitat and environment of other plant and animal species. Tropical forests are characterized by a great diversity of tree species. In India, there are only 75 million ha of forest area which works out to 19.27 per cent of the geographical area and is far below the requirement of 33 per cent (ICFRE, 2000).

This natural forest genetic resource is being wantonly plundered, the motivation invariably being greed rather than need (Douglas *et al.*, 1982). The need for augmenting country's forest wealth has got immense importance in this context. Because of the intense human pressure on these resources, many of the economically important forest tree species are highly fragmented with incredibly low number of individuals and are rendered rare, endangered or threatened with extinction (FAO, 1999; Annamalai, 1999). This situation has demanded an imperative need for conservation of vital genetic resources. Critical information on the population status and an accurate knowledge about the spatial distribution pattern of genetic diversity are essential to arrive at informed decisions in planning effective conservation strategies and genetic improvement programmes (Boshier and Young, 2000).

2.1. GENETIC DIVERSITY IN FOREST RESOURCE CONSERVATION

Genetic diversity is characterized by differences in composition or frequency of genes or alleles among individuals in a species or population. Understanding the current diversity status of forest genetic resources is essential before efficient forest gene conservation programme can be designed (Namkoong *et al.*, 1996). A population or species which has many individuals, but little genetic variation among them, will be more susceptible to pressures towards extinction than with genetically diverse species or population. A number of economically important forest tree species are under threat due to human pressures and the extent of reduction in population size provides an indication of the extent of loss of diversity (Boyle, 2001). The advent of molecular marker techniques, bioinformatics and the use of geographical information system (GIS) could help to develop better methods to survey, sample and assess the genetic diversity (Rao and Koskela, 2001). An array of population genetic studies had indicated that genetic diversity is structured at various levels of ecosystem in a kind of genetic architecture, knowledge of which is important in developing *in situ* conservation strategies (Libby and Critchfield, 1988).

2.2. POPULATION GENETIC PRINCIPLES

Population genetics is the quantitative study of the amount and distribution of genetic variation/diversity in populations, and the dynamics of the underlying processes. Description of population genetic structure and its dynamics are based on the analysis of allele and genotype frequencies in sampled populations with

simply inherited Mendelian traits (Frankel, 1983). The factors that affect allelic frequencies in a random-mating population are random drift (chance fluctuations), mutation, natural selection and migration. The estimate of allele frequencies at a locus from knowledge of genotype frequencies is forthright under the assumptions of Hardy-Weinberg principle (Hardy, 1908; Weinberg, 1908) which is the foundation for all population genetic investigations.

The principle states that in a large random mating population, the allele and genotype frequencies will remain constant from generation to generation when there is no mutation, migration and natural selection. A population is said to be in Hardy-Weinberg equilibrium (HWE) when frequencies of three genotypes viz. homozygous dominant (AA), heterozygous (Aa) and homozygous recessive (aa) at a diallelic locus (dominant and recessive) are p^2, 2pq and q^2 respectively such that $p^2 + 2pq + q^2 = 1$, where the allele frequencies p (A) and q (a) can be calculated from genotype frequencies (Yeh, 2000). Deviations from HWE can arise from several factors, including population subdivision, Wahlund's principle (increase of homozygotes at the expense of heterozygotes - an inbreeding like effect), migration from outside, non-random mating, sampling of siblings, sex specific differences in allele frequencies, presence of null alleles and selection. It is always important to determine whether there is significant deviations from HWE in the survey of genetic diversity in natural populations (Yeh, 2000).

The deviations of genotype frequencies from HWE can be analysed by three parameters namely F_{IS} (inbreeding coefficient within population), F_{ST} (inbreeding

coefficient over the total populations) and F_{IT} (reduction in inbreeding coefficients due to differences between populations). If all the populations are in HWE, F_{IS} will be zero and F_{IT} will be equal to F_{ST}. F_{IS} and F_{IT} may also be positive or negative, indicating heterozygote deficit or excess. F_{ST} is always positive and is a measure of the extent of genetic differentiation among populations (Wright, 1951). Coefficient of genetic differentiation, G_{ST}, as proposed by Nei (1973) is identical to F_{ST}. Genetic distance (D) is a measure of the amount of genetic divergence between populations and is useful for genetic grouping of the populations (Nei, 1978).

To characterize population genetic structure, the following parameters that describe and quantify the genetic and geographic variation patterns are usually investigated viz. polymorphisms (P) to describe what proportion of gene loci are variable, average number of alleles per locus (A), average heterozygosity (h) to describe what proportion of all gene loci are heterozygous and the level of among-population differentiation (G_{ST}) (Brown, 1989). Forest trees have been exposed to various geographical distributions and extreme life history characteristics such as long life cycle, greater opportunity for accumulation of mutations and exposure to stresses. Thus, to develop and implement effective genetic improvement and conservation strategies in forest trees, it is necessary to integrate the information drawn from the above mentioned population genetic diversity parameters.

2.3. TOOLS TO STUDY POPULATION GENETIC DIVERSITY

With the rediscovery of Mendel's work by Correns, De vries and Schermark, the twentieth century has witnessed an indisputable revolution in the field of genetics

(Sturtevant, 1965). The use of genetic markers in plant breeding dates back to the beginning of the century, when Bateson and Punnet (1905) discovered the genetic linkage between gene controlling flower petal colour and shape of pollen grain. The use of markers in the practice of plant breeding was set by Sax in 1923, who explored the association between a qualitative character (seed coat colour), a marker and a quantitative character (seed size) for indirect selection in *Phaseolus vulgaris* (Sax, 1923). Prior to 1960's, markers used in plant genetics and breeding experiments were those derived from genes controlling discrete phenotypes for easy visual identification (morphological markers) such as dwarfism, chlorophyll deficiencies, and flower, seed or leaf colour and morphology through provenance and progeny trials. But these morphological markers are limited in number, expressed only at the whole plant level, greatly influenced by environment and have only a low per cent of polymorphism (Grattapaglia *et al.*, 1992). This picture began to change in the 1960's with the development of molecular markers based on isozyme polymorphism (Lewontin and Hubby, 1966) and they continue to provide simple and inexpensive method of obtaining genetic information. Since the classical population genetic studies of natural populations of *Drosophila pseudoobscura* using isozymes by Lewontin and Hubby (1966), techniques of isozyme analysis have been applied to a large number of other organisms to answer the most diverse genetic questions.

Besides isozymes, a range of DNA based molecular markers such as Restriction Fragment Length Polymorphisms (RFLPs), Simple Sequence Repeats

(SSRs), Randomly Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs) and many deviations from these markers are currently available for direct visualization of variation at the DNA level and are being increasingly used in population and evolutionary genetic studies (Amaral, 2001; Wickneswary, 2001). DNA markers provide a more ample coverage of the genome and the results are not influenced by spatial and temporal effects of genes and environment (Wickneswary *et al.*, 1996). These markers can be employed for quantifying genetic diversity of commercially important and threatened forest tree species and for the subsequent management of the genepool (Namkoong *et al.*, 1996; Grattapaglia *et al.*, 1997). Following the discovery of PCR process by which segments of DNA can be amplified using a thermostable DNA polymerase (Mullis and Faloona, 1987), RAPD was introduced by Williams *et al.* in 1990 (Williams *et al.*, 1990) using short decamer primers of arbitrary sequences to direct the PCR, eliminating the need for prior sequence information. The use of the technique was demonstrated to study DNA polymorphisms, which were inherited as genetic markers. These DNA markers were used for genetic variability studies, gene mapping, linkage studies and clonal and varietal finger printing (Hedrick, 1992). RAPD markers can be effectively used to determine the specificity in plant-pathogen interaction and to identify markers linked to a resistant gene of interest within a short time (Naqvi *et al.*, 1995; Kuginuki *et al.*, 1997).

2.3.1. Isozymes

Isozymes constitute a group of multiple molecular forms of the same enzyme that exists in a species as a result of more than one gene encoding the enzymes (Moss, 1982; Yeh, 1989). These enzymes can be separated by gel electrophoresis. Isozyme techniques have been used to estimate the level and structure of genetic variability in natural populations, to study gene flow, hybridization, species dispersion, QTL mapping, varietal identification, germplasm evaluation as well as to carry out phylogenetic analyses (Tanksley and Orton, 1982; Soltis and Soltis, 1989; May, 1994; Mitton, 1998). The availability of 20-30 Mendelian loci per species enabled estimates of genetic variation/diversity to be made based on isozymes (Conkle *et al.*, 1982).

2.3.1.1. Historical aspects

Coolidge (1939) made the first effort to characterize proteins by electrophoretic mobility through the separation of serum albumin and globulin using a semi-porous medium. Using paper electrophoresis, Durrum (1950) separated five individual protein components of serum. The beginning of isozyme epoch was by the introduction of starch gel as a supporting medium by Smithies (1955). Hunter and Markert (1957) first reported the multiple molecular forms of enzymes in mammals. Markert and Moller (1959) coined and defined the term isozymes as 'structurally different forms of an enzyme with identical or nearly identical function'. In higher plants, peroxidase was initially used to study variation among individuals because of the simplicity of its detection method (Hosoya, 1960; Mc

Cune, 1961). In subsequent investigations, many other enzyme systems were found to consist of multiple molecular forms (Feret and Bergmann, 1976; Gottileb, 1977).

Schwartz (1960) provided the first genetic basis for alkaline phosphatase variation in maize endosperm. Schwartz *et al.* (1965) first demonstrated the existence of both multiple enzyme loci and multiple alleles at single locus in maize. The usefulness of these biochemical traits in population genetic studies as gene markers was first recognized by Lewontin and Hubby (1966) in Drosophila and by Harris (1966) in his survey of human populations. In higher plants, isozymes were first used as genetic marker in population surveys and breeding programs by Brown and Allard (1969) and Marshall and Allard (1969).

Because of their existence in isoforms, isozymes show Mendelian inheritance, codominant expression and complete penetrance (Wendel and Weeden, 1989). They are also direct products of genes and therefore closely represent variation at the DNA level (Hubby and Lewontin, 1966). Isozymes can be easily and inexpensively separated by electrophoresis and can be visualised as the coloured product of a chemical reaction catalyzed by the specific enzyme (Conkle *et al.*, 1982).

The separation of complex mixture of enzymes and other proteins was not satisfactorily performed before the introduction of zone electrophoresis in starch gel as supporting medium (Smithies, 1955). Because of the 'molecular' sieving effect of starch gel, the macromolecules could be separated both by charge as well as by size and shape. With the introduction of PAGE (Ornstein, 1964; Chrampach

and Rodbard, 1971) the resolution of enzyme and protein mixture was improved considerably. Although, PAGE provides maximum resolving power and superior zymograms for the analysis of large number of individuals for many enzymes, because of the complexity of gel preparation and toxic nature of the material used, starch gel electrophoresis is performed still in the field of evolutionary biology and systematics (Bergmann and Hattemer, 1998).

2.3.1.2. Applications in tree species

The first reports of the use of isozyme technique in the field of forestry research appeared in 1970 (Van Lear and Smith, 1970; Mc Mullan and Ebell, 1970). Isozymes are utilized all over the world as genetic marker mainly for the identification of clones, hybrids and varieties of forest species, early prediction of quality characteristics of forest tree species, quantitative trait loci mapping, linkage studies, mating patterns and for the population genetic analysis of forest trees in breeding studies (Conkle *et al.*, 1982; Tanksley and Orton, 1982; Soltis and Soltis, 1989; Adams *et al.,* 1992; Mandal and Gibson, 1998).

2.3.1.2.1. Genetic fidelity

Adams (1983) used isozymes as an efficient marker to detect labeling errors in Douglas fir seed orchard and to distinguish between trembling aspen and big tooth aspen (Cheliak and Pitel, 1984). Bergmann (1987) reported isozyme markers as an efficient tool to discriminate multi clonal aspen cultivars. Isozyme analysis was carried out to identify clones of *Tilia* (Maurer and Tabel, 1995). Cabrita *et al.* (2001) used isozyme marker system to test the fidelity of *Ficus carica* clones.

Several authors have reported the utility of isozyme phenotypes for identifying cultivars and determining genetic variation in apple (Bournival and Korban, 1987), red raspberry (Cousineau *et al.*, 1993), chestnut (Huang *et al.*, 1994) and pawpaw cultivars (Huang and Layne, 1997).

2.3.1.2.2. Linkage mapping

Isozymes have also been used as genetic markers to study linkage of economically important quantitative characters in forest tree species. Reviewing the limitations of molecular marker aided selection in forest tree breeding, Strauss *et al.* (1992) suggested that the breeding cycle could be shortened appreciably if selections in controlled mating could be based on one or more isozyme markers linked to desirable quantitative trait. The genetic mapping of *Eucalyptus* has been carried out using isozyme markers by Byrne *et al.* (1995). Isozymes were used as markers in linkage studies in *Pinus taeda* (Adams and Joly, 1980), *Picea mariana* (Boyle and Morgenstein, 1985), *Pinus strobus* L. (Echt and Nelson, 1997), *Pyrus communis* (Chevreau *et al.*, 1997), *Prunus avium* (Boskovic *et al.*, 1997) and *Populus nigra* (Benettka *et al.*, 1999).

2.3.1.2.3. Mating system

Patterns of mating vary with the reproductive biology and spatial structure of a species and combine to influence levels and dynamics of genetic diversity. Isozyme markers have proven to be an effective method in determining the mating system which in turn will help to understand the distribution of genetic variation between individuals and gene flow between and within populations (Boshier and

Billingham, 2000). Study of mating system and genetic differentiation using isozyme markers has been carried out in *Pinus sylvestris* (Longauer *et al.*, 1992), *Hevea brasiliensis* (Paiva *et al.*, 1994), *Acacia nilotica* (Mandal *et al.*, 1994), *Juglans nigra* (Rink *et al.*, 1994), *Tectona grandis* (Kertadikara and Prat, 1995), *Eucalyptus urophylla* (House and Bell, 1994) and *Pinus cembra* (Lewandowski and Burczyk, 2000).

The polycross-mating scheme is considered as one of the most efficient methods for estimating the general combining ability in tree breeding programs. The assumption of equal or differential male reproductive success in the polycross mating has been studied with isozyme markers in *Pinus radiata* (Moran and Griffin, 1985), *Picea abies* (Cheliak *et al.*, 1987), *Pinus taeda* (Wiselogel and Van Buijtenen, 1988) and *Pinus sylvestris* (Rogers and Boyle, 1991). In determining supplemental mass pollination also, isozymes have been used as an ideal marker in loblolly pines (Adams, 1992) and in Douglas fir (El-Kassaby *et al.*, 1993).

2.3.1.2.4. Genetic diversity/variation

Threats to diversity occur at many levels of biological organization from genes to species to ecosystems (Millar and Marshal, 1991). Knowledge of genetic variation/diversity of a species and its structure is important for adopting effective genetic conservation strategy. Studies of isozymes could provide insightful information on the genetic architecture of stands and populations throughout the natural distribution of a species (Mandal and Gibson, 1998). Isozymes have been used as a powerful tool in the analysis of genetic variation/diversity in *Magnolia*

(Qiu and Parks, 1994), *Tectona* (Kjaer *et al.*, 1996), *Quercus suber* (Elena-Rossello and Cabrera, 1996; Toumi and Lumaret, 1998; Jimenez *et al.*, 1999), wild cherry (Marriette *et al.*, 1997), *Azadirachta indica* (Kundu, 1999), loblolly pine (Schmidtling *et al.*, 1999), *Shorea robusta* (Suoheimo *et al.*, 1999), *Rhus trichocarpa* (Chung *et al.*, 1999), *Nothofagus nervosa* (Marchelli and Gallo, 2000), *Pinus massoniana* (Huang and Zhang, 2000), *Pinus nigra* (Tolun *et al.*, 2000), *Camellia japonica* (Chung and Chung, 2000) and *Taxus cuspidata* (Lee *et al.*, 2000).

2.3.1.3. Isozyme studies in sandal

In *S. album*, the isozyme technique was first employed in 1977 at erstwhile Sandal Research Centre, Bangalore. A study of the isozyme pattern in respect of peroxidase, malate dehydrogenase and esterase in leaves of different types of sandal plants showing variation in their leaf shape (ovate, lanceolate, linear and elliptic) had indicated the existence of characteristic differences in the pattern of peroxidase and malate dehydrogenase at vegetative as well as flowering stages. The plants with normal-ovate-wavy and normal-ovate-non-wavy leaves were found to be very close genetically (Parthasarathi *et al.*, 1985). The activity of a specific peroxidase isozyme in the living bark tissue was found to bear a strong negative correlation with the oil content in the heartwood of mature sandal plants (Parthasarathi *et al.*, 1986). This technique could be used to predict the oil-bearing capacity of a sandal plant even at a young age. Kamala *et al.* (1986) reported changes in peroxidase isozyme patterns associated with deficiency of trace

elements. Angadi and Ananthapadmanabha (1988) observed variations in isozyme patterns in the sandal plants affected with spike disease.

The isozymes could be used as a powerful tool in analyzing the population/provenance structure. Egerton-Warburton (1990) utilized the efficiency of isozyme markers to describe the genetic structure of provenances in Western Australia. It has also been employed to estimate genetic distance in sandal plants between and within natural populations in Timor (Brand, 1994). Genetic distance between Indian and Indonesian *S. album* populations were analysed using eight major isozyme systems (Fox *et al.*, 1994) and detected a large genetic distance between West Timor and Indian *S. album* populations.

2.3.2. Randomly amplified polymorphic DNA

2.3.2.1. Historical aspects

In the 1970's, discovery and use of restriction enzymes and DNA hybridization allowed the analysis of restriction fragment length polymorphism (RFLP). RFLP technique involves the digestion of target DNA by restriction enzymes and the resulting fragments can be size fractionated by electrophoresis and visualised by hybridizing with labeled known base sequences called probes (Botstein *et al.*, 1980). The subsequent advent of modern techniques of molecular biology brought out several methods for the detection of DNA polymorphisms viz. random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), microsatellites (SSRs) (Weber and May, 1989; Morgante and Olivieri, 1993), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), etc. Microsatellite or SSR consists

of segments of DNA containing numerous tandem repeats of a short 'motif' usually of one to six bases (Beckman and Weber, 1992). AFLP technique is intermediate between RFLP and RAPD and combines the power of restriction enzyme digestion and the efficiency of PCR amplification (Vos *et al.*, 1995; Karp *et al.*, 1997). All these DNA markers allow investigation of both coding and non-coding regions of whole genome, independent of growth and developmental stages and are considered as the most powerful tool in the study of population genetics. Deciding which DNA marker to use depends on the financial resources, availability of equipment, skilled personnel and on the study objectives. Because of these unique advantages, molecular markers are gaining enormous popularity day by day (Ahuja, 2001).

The RAPD method, because of its technical simplicity, has attracted wide spread interest in detecting DNA polymorphisms in many species of forests trees (Williams *et al.*, 1990; Deragon and Landry, 1992; Hadrys *et al.*, 1992). RAPDs are generated by applying PCR to genomic DNA samples using randomly constructed oligonucleotides as primers. Each RAPD reaction cycle involves three steps; DNA denaturation (90°C-94°C), primer annealing (30°C-36°C) and primer extension (72°C). The extension step involves the addition of nucleotides using target sequences as template, resulting in the synthesis of a new copy of the target sequence in each cycle. The cycle is repeated generally for 20-45 times and the amplification process follows geometric progression (Welsh and Mc Clelland, 1990). The technique is relatively easy to apply to a wide array of plant and animal

taxa and the number of loci that can be examined is essentially unlimited (Lynch and Milligan, 1994). DNA variation down to the level of a single base substitution can be detected through electrophoretic gels followed by ethidium bromide staining. Because of the dominant nature, RAPD reveals low information content per locus and easily prone to artifacts by contamination of the sample DNA. However, the technique is widely accepted because it offers advantages in speed, technical simplicity, requires only nanogram amounts of DNA and reveals high levels of sequence polymorphisms. There are several applications for RAPD assay, each of these techniques exploits the efficiency of detection of DNA sequence based polymorphisms (Williams *et al.*, 1993).

2.3.2.2. Applications in tree species

2.3.2.2.1.Genetic diversity/variation

Determining nature and extent of genetic diversity in population of forest trees has been the focus of many studies over the past three decades (Wickneswary *et al.*, 1996; Karp *et al.*, 1997). RAPDs have been used as an efficient method to reveal the genetic structure and diversity pattern of trees in natural populations, reserve forests and plantations and the outcome could readily be used to select priority areas for conservation, to design and monitor management strategies in protected areas and to plan conservation strategies for genetic resources (Newbury and Ford-Lloyd, 1993; Young *et al.*, 2000; Uma Shaanker *et al.*, 2001). RAPDs have been successfully used for estimation of genetic diversity/variation and relatedness in *Gliricidia* (Chalmers *et al.*, 1992), *Coffea* (Lashermes *et al.*, 1993; Anthony *et al.*,

2001), *Malus* (Harada *et al.*, 1993), *Elaeis* (Shah *et al.*, 1994), mahoganies (Chalmers *et al.*, 1994), *Populus* (Yeh *et al.*, 1995; Rajagopal *et al.*, 2000), *Grevellia* (Rossetto *et al.*, 1995), *Eucalyptus* (Nesbitt *et al.*, 1995), *Prunus* (Warburton and Bliss, 1996), Brazil nut (Kanashiro *et al.*, 1997), pistachio (Hormaza *et al.*, 1998), *Quercus* (Kremer and Zenetto, 1997), plums (Shimada *et al.*, 1999), tea (Kaundun *et al.*, 2000; Wachira *et al.*, 2001), *Anacardium* (Mneney *et al.*, 2001), mango (Hemanthkumar *et al.*, 2001) and in *Pinus* (Fazekas and Yeh, 2001).

2.3.2.2.2. Linkage mapping

Genetic linkage maps of molecular markers offer a powerful tool to investigate the genetic architecture of quantitatively inherited traits and assist in breeding through identification and manipulation of the individual genetic factors controlling such traits. One of the practical uses of RAPD is in the creation of high-density genetic maps, since RAPD markers can be shown to segregate in Mendelian fashion. Genetic maps allow extensive coverage of genomes for detailed genetic analysis, the decomposition of complex traits into its discrete Mendelian components and the canalization of linkage information between markers and traits of interest into breeding strategies (Neale and Williams, 1991). Genetic maps also enable marker assisted breeding; since forest trees have particularly long breeding cycles, a tool that helps shorten the breeding cycle could be quite valuable. Many desirable attributes require several years to develop; if individuals can be selected for controlled crossing based on RAPD markers linked to the desired trait, then

breeding cycle could be shortened appreciably (Neale and Harry, 1994). RAPDs have been successfully used in the construction of linkage maps in *Populus* (Bradshaw and Stettler, 1994), *Eucalyptus* (Byrne *et al.*, 1995; Grattapaglia *et al.*, 1995; Vaillancourt *et al.*, 1995; Verhaegen and Plomion, 1996; Grattapaglia *et al.*, 1996; Byrne *et al.*, 1997b), *Pinus* (Plomion *et al.*, 1995; Yazdani *et al.*, 1995) and larch (Arcade *et al.*, 2000).

2.3.2.2.3. Genetic fingerprinting

RAPDs, in view of high levels of polymorphism are particularly useful for fingerprinting genotypes where there is a need to identify cultivars of a variety. The technique has been used in olive (Bogani *et al.*, 1994; Febbri *et al.*, 1995; Mekuria *et al.*, 1999), *Mangifera* (Schnell *et al.*, 1995; Adato *et al.*, 1995), plums (Ortiz *et al.*, 1997), almond (Bartolozzi *et al.*, 1998), chestnut (Galderisi *et al.*, 1998), *Prunus* (Gerlach and Stosser, 1997) and in peach (Cheng *et al.*, 2001). RAPDs have been used in identifying parental lines and inter and intra specific hybrid genotypes in apples (Nybom and Schaal, 1990), *Populus* (Heinz, 1998), olive (Claros *et al.*, 2000) and in *Santalum* (Mc Comb and Jones, 1998).

2.3.2.2.4. Clonal fidelity

The reproducibility of RAPD markers and their ability to differentiate between individual genotypes have been utilized in verification, identification and characterization of clones of forest trees. Such type of clonal identification was performed in *Eucalyptus* (Lange *et al.*, 1993; De Lala *et al.*, 2000), *Populus* (Lin *et*

al., 1994; Sigurdsson *et al.*, 1995); *Salix* (Auriol *et al.*, 1994), *Hevea* (Varghese *et al.*, 1997) and in *Pinus* (Scheepers *et al.*, 1997).

2.3.2.2.5. Resistance breeding

Breeding for resistance to both abiotic and biotic stresses requires the development of suitable and reliable screening techniques and identification of heritable resistance characters. Molecular markers are considered to be an important screening tool in disease resistance breeding programmes. Identification and cloning of resistance genes could find application in evolving transgenic plants (Licy *et al.*, 2000). An efficient and relatively simple way to generate molecular markers for disease and pest resistance is to search for randomly amplified polymorphic DNAs (RAPD) and this strategy has already proven valuable along with restriction fragment length polymorphism (RFLPs) (Lefebvre *et al.*, 1997). In association with the appropriate planting material such as bulks of F_2 , doubled-haploid individuals, near-isogenic lines etc., RAPD markers have been efficiently used to unveil the genomic regions near specific loci (Paran and Michelmore, 1993). RAPDs have been used to identify markers linked to nematode resistance gene in tomato (Klein-Lankhorst *et al.*, 1991), downy mildew resistance gene (Dm5/8) in lettuce (Michelmore *et al.*, 1991; Paran and Michelmore, 1993), mildew resistance gene in *Hevea* (Chen *et al.*, 1994; Chen *et al.*, 1999), tomato spotted wilt virus in *Capsicum* (Black *et al.*, 1996), club root-resistance gene in *Brassica* (Kuginuki *et al.*, 1997) and potato Y potyvirus (PVY) resistance gene (Ry_{adg}) in *Solanum* (Kasai *et al.*, 2000). Marker assisted selection with RAPD is not always

possible because RAPDs designed for one population are not always polymorphic for another. To overcome this problem, it is possible to convert a RAPD fragment to a RFLP probe or sequence characterized amplified region (SCAR) (Paran and Michelmore, 1993). To retrieve the monomorphic amplifications of SCAR, CAPS (cleaved amplified polymorphic sequence) has been developed from SCAR in *Arabidopsis* (Konieczny and Ausubel, 1993).

2.3.2.2.6. Genetic fidelity in tissue culture

RAPDs have been used to test the genetic fidelity of tissue culture raised plants. Various species of plants regenerated by callus formation may show genetic variability known as somaclonal variation (Brown, 1989; Karp, 1991; Godwin *et al.*, 1997; Hashmi *et al.*, 1997). Somaclonal variation, which occurs during the period of cell differentiation in the regeneration process, can be caused by changes in chromosome number or structure (deletions and rearrangements), DNA amplification, DNA modifications and activation of transposable elements. Some changes in the genome can not be observed at morphological or physiological levels because different gene structures may not alter the biological activity enough to modify the phenotype (Sabir *et al.*, 1992). Molecular markers can be used in the juvenile phase of the plant before mature characteristics appear, allowing for early selection of true-to-type plants. RAPD has been used in the rapid screening of somaclonal variants in rice (Muller *et al.*, 1990), *Triticum (*Morere-Le Paven *et al.*, 1992; Brown *et al.*, 1993), *Picea* (Isabel *et al.*, 1993), *Populus* (Rani *et al.*,

1995), *Piper* (Parani *et al.*, 1997), *Andrographis* (Padmesh *et al.*, 1999) and sugarbeet (Jazdzewska *et al.*, 2000).

Recently, much has been achieved in understanding the structure and organization of plant genomes by uncovering and exploiting molecular forms of polymorphism employing molecular markers (Grattapaglia *et al.*, 1992). A key future challenge is to devise appropriate technology for exploiting this variability in a wider range of tree crops and the molecular information acquired can be integrated into the different tree breeding programmes (Soller and Beckmann, 1983; Thormann and Osborn, 1992). Finally, linkage maps of molecular markers have been important tools to tackle many problems starting from conventional breeding practices to transgenic breeding. RAPD, together with the other available molecular markers can be used to answer a range of questions in the field of forest genetics (Mohapatre and Singhal, 2000).

2.3.3. Tissue culture

2.3.3.1. Historical aspects

Plant tissue culture plays an important role in crop improvement by which a plant breeder or grower is no longer constrained by the season for developing large number of plants with desirable characters within a short span of time and space (Gupta *et al.*, 1993). Plant tissue culture is a collection of techniques by which isolated cells or tissues from either root, stem, leaf or any other part of plant body, if provided with a suitable condition, would develop into a plantlet in a test tube (Bajaj, 1995).

The science of plant tissue culture began in 1902 with the attempt to culture plant cells by Haberlandt (1902). The possibility to cultivate plant tissue for unlimited periods of time was announced independently and simultaneously by White (1939), Nobecourt (1939) and Gautheret (1939). Skoog and Miller (1957) discovered the interaction between auxin and cytokinin and regeneration of entire plant was achieved by Steward (1958) from callus tissue of carrot. Vasil and Hildebrandt (1966) have demonstrated for the first time, cellular totipotency in the single isolated cell of a tobacco hybrid. In 1960, Cocking isolated protoplasts by enzymatic digestion of cell wall. Later, in 1971, Takebe *et al.* (1971) achieved division and regeneration of protoplasts. This was followed by first successful fusion of protoplast and recovery of hybrid plant (Carlson *et al.*, 1972).

Gautheret made the first reports of organogenesis in tree tissue cultures in 1940, who obtained formation of adventitious buds in cambial cultures of *Ulmus campestris*. Later Jacquiot (1951) made further observations on similar phenomena. These observations helped to lay the foundation for many of the experimental systems used to obtain organogenesis in woody angiosperms (Bonga and Durzan, 1982). Mathes (1964) was the first researcher to achieve growth of both shoots and roots in callus cultures of *Populus tremuloides*. A few years later Wolter (1968) showed that shoots could be initiated on callus of *Populus tremuloides*. Regeneration of a complete plantlet in a tree species for the first time was reported by Winton (1968) in *Populus*.

2.3.3.2. Applications in tree species

Forest destruction and industrialization have resulted in drastic increase in world demand for wood and consequently created an urgent need for superior planting materials. Hence, rapid propagation by various *in vitro* techniques is one of the possibilities that can be translated into reality to meet this challenge (Cheliak and Rogers, 1990). In view of the continued interest and urgency for biofuel energy, micropropagation has far reaching implications in mass production, propagation and tree improvement programmes (Jain *et al.*, 1995; Bajaj, 1995). The micropropagation involves three stages, establishment of cultures, regeneration of plants and transfer of plants from test tube to soil (Bajaj, 1986). The *in vitro* plant regeneration may be either directly from explants such as segments, buds, meristems etc. or indirect via initiation and differentiation of callus (Thorpe, 1995).

2.3.3.2.1. Direct regeneration

Direct regeneration of plantlets or the formation of somatic embryos from the explants such as buds, meristems, cuttings, etc. ensures the cloning of genetic stocks. For producing true-to-type plantlets, success of direct regeneration is reported in a number of tree species such as *Alnus* (Brown and Sommer, 1982), *Tectona* (Gupta *et al.*, 1980; Bonal and Monteuuis, 1997), *Castanea* (Vieitez *et al.*, 1983), *Santalum* (Rao and Bapat, 1978; Bapat and Rao, 1984; Lakshmi Sita, 1986; Rao *et al.*, 1984; Das *et al.*, 1998; Sanjaya *et al.*, 1998; Pradhan and Saiju, 1999), apple (Karhu, 1997), *Dendrocalamus* (Ravikumar *et al.*, 1998), Albizzia (Kumar *et*

al., 1998; Sinha *et al.*, 2000), chestnut (Gonialves *et al.*, 1998), almond (Ainsley *et al.*, 2000), *Aegle* (Islam *et al.*, 2000), *Dalbergia* (Pattnaik *et al.*, 2000), etc.

The explants from young and juvenile trees grow easier than those from mature and old trees. Nevertheless, examples of regeneration from 100-year-old trees of *Sequoia* (Boulay *et al.*, 1979) and *Tectona* (Gupta *et al.*, 1980) are known. Moreover, there are considerable differences in the regeneration and morphogenetic potential of various parts of the plant. Embryo culture has been employed as a tool for direct regeneration in trees where seeds are dormant, recalcitrant or if they abort at early stages of development (Islam *et al.*, 2000).

2.3.3.2.2. Indirect regeneration

Indirect regeneration is the regeneration of plants via callus. Callus cultures can be started from any type of explant material possessing parenchymatous cells capable of renewed cell division to form an unorganized mass of proliferating cells. From such callus mass, shoot or root regeneration can be induced by manipulating the culture environment (Bonga and Durzan, 1982).

In contrast to the direct method, which ensures clonal stability, the indirect regeneration of plants from callus may result in genetic variability. The callus cultures on periodic subculturing undergo genetic changes such as mutations, changes in ploidy, etc. The advantage of callus cultures is the large number of plants that can be produced from a single culture, as every cell is a potential plant (Gupta, 1991). Callus regeneration is efficiently exploited in many tree species such as *Citrus* (Chaturvedi and Mitra, 1974), *Prunus* (Druart, 1980), *Sapium*

(Venkateswaran and Gandhi, 1982), pine (Tang *et al.*, 1998), apple (Rugini and Mugano, 1998) and pear (Zhu and Welander, 2000). In view of the enormous number of propagules, this system of regeneration can be used in reforestation programmes where the quantity and not necessarily the quality of the biomass is of importance (Zhu and Welander, 2000).

2.3.3.2.3. Somatic embryogenesis

Somatic embryogenesis is the most important and efficient mode of micropropagation (Tulecke, 1987; Thorpe, 1995). Induction of somatic embryos is either direct from explant as in coconut (Pannetier and Morel, 1982; Verdeil *et al.*, 1994), *Populus* (Michler and Bauer, 1991), *Camellia* (Pedroso and Pais, 1993), *Quercus* (Cuenca *et al.*, 1999) or through callus cultures as in *Santalum* (Lakshmi Sita *et al.*, 1979; 1980; 1982; Lakshmi Sita, 1992; Bapat and Rao, 1979; Bapat *et al.*, 1985), Douglas fir (Durzan, 1982), *Mangifera* (Litz *et al.*, 1984), *Eucalyptus* (Muralidharan *et al.*, 1989), olive (Rugini and Caricato, 1995), oil palm (Karun and Sajini, 1996), date palm (Veramendi and Navarro, 1996), *Hevea* (Cailloux *et al.*, 1996), *Coffea* (Berthouly and Ferriere, 1996), chestnut (Carraway and Merkle, 1997), *Citrus* (Carimi *et al.*, 1998; Carimi *et al.*, 1999), neem (Murthy and Saxena, 1998), *Pinus* (Haggmann *et al.*, 1999), *Prunus* (Talleux *et al.*, 1999), oak (Wilhelm, 2000) and *Acacia* (Sane *et al.*, 2000; Xie and Hang, 2000).

Somatic embryogenesis, especially in cell suspensions in bioreactors would enable the production of somatic embryos in millions. As several millions of plantlets are needed for reforestation, bioreactor technology can be efficiently used

to meet the increased demand (Gupta *et al.*, 1991). This technique has been utilized for large-scale multiplication of trees as in black spruce (Tautorus, 1992), *Santalum* (Bapat *et al.*, 1990; Rao and Bapat, 1992; Rao and Bapat, 1995; Mujib *et al.*, 1998; Dey, 2001), etc. It has been suggested that such embryos could be encapsulated to form so-called artificial seeds, which can be stored until used (Bajaj, 1986). There is also the possibility of long term storage of embryos through cryopreservation techniques as suggested for *Santalum* (Bapat and Rao, 1988; Fernandez *et al.*, 1994), Scots Pine (Haggman *et al.*, 1999), chestnut (Jekkel *et al.*, 1998), Strawberry (Hirai *et al.*, 1998), *Citrus* (Olivares Fuster *et al.*, 2000), *Pinus* (Ford *et al.*, 2000), etc. Freeze preservation of cells, tissues and organs is regarded as a meaningful tool for the long-term conservation especially in the case of trees with recalcitrant seeds, establishment of gene banks and international exchange of germplasm (Bajaj, 1995).

In spite of all this excitements in the area of somatic embryogenesis, some basic problems needed further attention. Plants regenerated through somatic embryogenesis exhibit phenotypic variation, which may have a genetic, or non-genetic basis. Genetic variation has been termed somaclonal variation (Scowcroft and Larkin, 1982). For obtaining true-to-type propagules from a selected genotype, somaclonal variation is undesirable. On the other hand, somaclonal variation offers prospects for the recovery of beneficial variant and creates variability useful for plant improvement (Larkin and Scowcroft, 1981; Svobodova *et al.*, 1999). Difficulties for induction of somatic embryos in some species/genotypes have been

reported (Bonga and Durzan, 1982; Tang, 2001). Problems with maturation and conversion of somatic embryos and poor development of viable somatic seedlings are also reported in a number of forestry species (Faure *et al.*, 1996; Tang, 2001; Ilah *et al.*, 2002). The anatomical, biochemical and molecular basis for such abnormalities have to be elucidated for further normalization, improvement and proper utilization of the technique.

Current research and development concentrate on genetic engineering of plants for herbicide resistance, insect resistance, disease resistance, etc., and genetic transformation of plant cell using co-cultivation with *Agrobacterium* or biolistic projectile techniques. The DNA transformation technology can entirely replace the long term recurrent selection procedure exercised in a conventional breeding programme to introgress a gene. Useful transformation with *Agrobacterium* has been achieved in many forest trees such as *Populus* (Han *et al.*, 1997), *Paulownia* (Bergmann *et al.*, 1998), *Eucalyptus* (Moralejo *et al.*, 1998), peach (Hayama *et al.*, 2000), etc. Tissue culture techniques thus have opened up new possibilities in conservation and commercialization of large number of tree species and eventually for an increased production of biomass energy.

CHAPTER 3

ISOZYME BASED GENETIC VARIATION IN SANDAL POPULATIONS

3. ISOZYME BASED GENETIC VARIATION IN SANDAL POPULATIONS

3.1. INTRODUCTION

Any genetic improvement programme depends on the existence, nature and extent of genetic variation available for manipulation within the species. *S. album* is a facultatively selfing and preferentially outcrossing species (Shepherd, 1992). Breeding system can influence the amount of genetic variation within a species. But in *S. album,* the relative extent of outcrossing and selfing within population has not yet been studied. It is of paramount importance to evaluate and maintain genetic variability within and between populations because populations consisting of genetically uniform individuals may be vulnerable to major climatic changes and disease and pest outbreaks (Aradhya and Phillips, 1993).

Isozymes have proven to be the most efficient and inexpensive tool for the study of genetic variation in tree species as compared to other molecular markers or morphological characteristics (Soltis and Soltis, 1989). Being multiple molecular forms of enzyme proteins, isozymes are primary gene products. Variation in their structure gives reliable information about the variability in the genome (Hoelzel, 1991). Isozymes appear on electrophoretic gels as multiple bands and genetic interpretations can be made from the banding profile.

Studies using morphological markers and progeny trials indicated that significant amount of intra specific variation exists in South Indian *S. album* populations (Kulkarni and Srimathi, 1982; Bagchi and Veerendra, 1987).

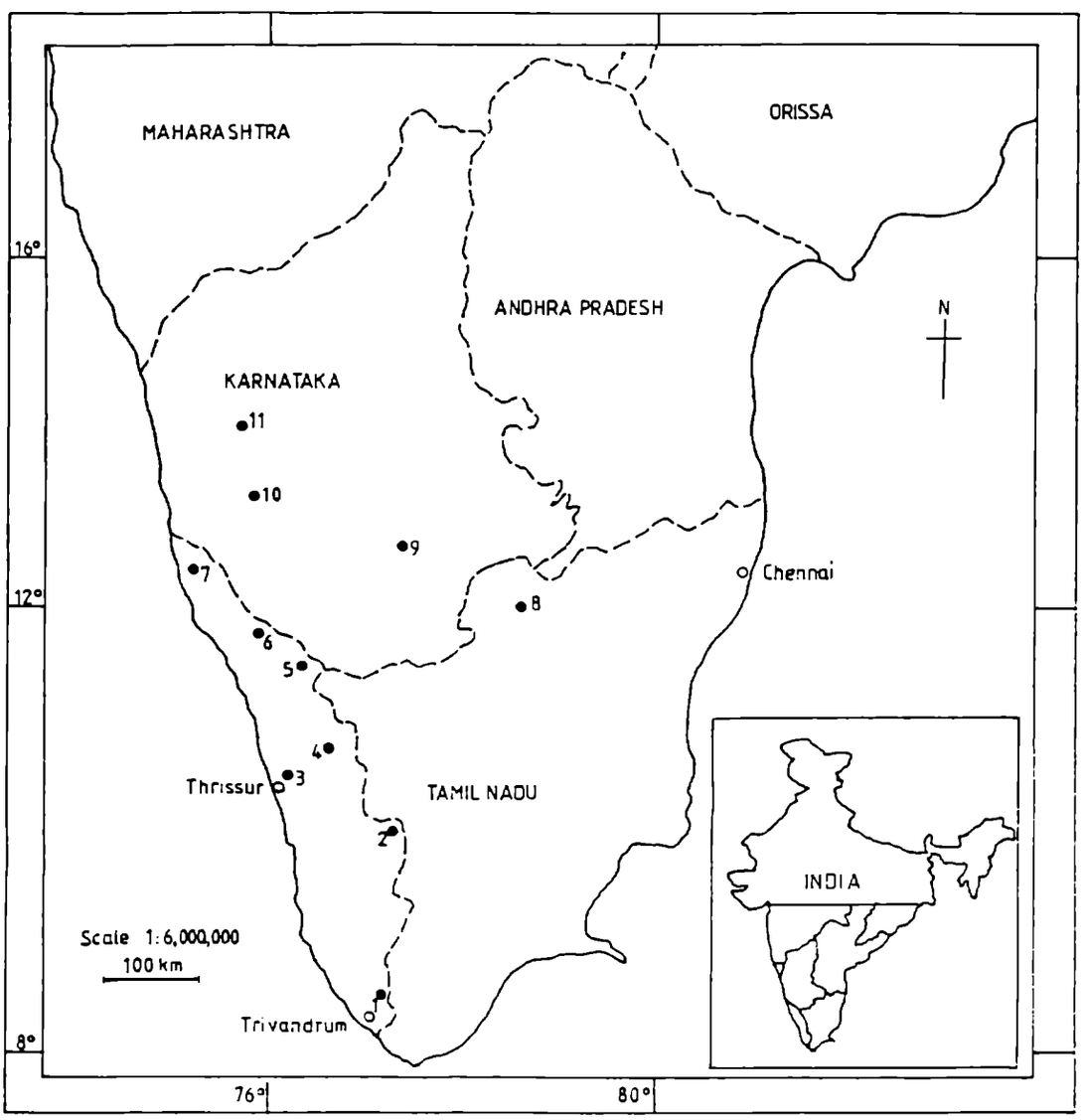
Variations occur in vegetative and reproductive parts such as leaf structure, leaf shape, bark structure and seed polymorphism. Amount of heartwood present varies from tree to tree in proportion to sapwood; oil content varies from 0.5 per cent to 5 per cent (Veerendra and Ananthapadmanabha, 1998). Preliminary studies on isozyme analysis in sandal were carried out by Parthasarathi *et al.* (1985) in order to correlate isozyme activity and leaf variations. Subsequently, peroxidase activity in bark tissue was reported as a marker for the oil-bearing capacity (Parthasarathi *et al.*, 1986). Isozyme techniques have been used in *S. album* to estimate genetic variation between sandal plants within the same and different populations in Timor (Brand, 1994) and to identify provenances in Western Australia (Egerton-Warburton, 1990). But the pattern of genetic variation within and between sandal population in South India is virtually unknown.

This chapter reports the level of genetic variation within and between 11 Southern Indian *S. album* populations using five major metabolic enzyme systems viz. peroxidase (PRX), esterase (EST), malate dehydrogenase (MDH), shikimate dehydrogenase (SKDH) and gluco phosphate isomerase (GPI) detected through Poly Acrylamide Gel Electrophoresis (PAGE).

3.2. MATERIALS AND METHODS

Eleven populations viz. Marayoor, Wayanad, Thenmalai, Wadakkanchery, Ottappalam, Kannavam and Kasargod of Kerala state, Bangalore, Shimoga and Chikkamagalur of Karnataka state, and Thirupattur of Tamil Nadu state in South India were selected for the study (Fig. 3.1.). These populations vary from

Figure 3.1. Map showing the location of different selected populations of *Santalum album* L. in South India. (1-Thenmalai, 2-Marayoor, 3-Wadakkanchery, 4-Ottappalam, 5-Wayanad, 6-Kannavam, 7-Kasargod, 8-Thirupattur, 9-Bangalore, 10-Chikkamagalur, 11-Shimoga).



fragmented discrete patches to large areas separated from each other. Authentic data on the area of each sandal population was not available. The distance between two nearest populations varied from approximately 25km (Wadakkanchery and Ottappalam) to more than 200km (Thenmalai and Marayoor). Seed samples were collected from more than 20 parent trees randomly, covering approximately the entire range of each population and the seeds were pooled together. From this bulk, samples were drawn randomly for raising seedlings. Juvenile leaves of seedlings at four-leaf-stage (3-week-old) were used for the present study. Twenty samples (1 gram each) from each of the eleven populations were ground using mortar and pestle with 1 ml of the modified extraction buffer containing 0.1M Tris HCl, pH 7.5, 1 mM EDTA, 0.01 M $MgCl_2$, 4 per cent (w/v) PVP-40 and 0.1 per cent (v/v) β-mercapto ethanol at ice cold temperature (Fox *et al.*, 1994). The resulting crude extract was centrifuged at 18,000 rpm for 10 minutes at 4°C and the supernatant stored at -20°C until used for electrophoresis.

3.2.1. Electrophoresis

Samples were electrophoretically analyzed using polyacrylamide gel electrophoresis procedure (Laemmli, 1970) on a minigel (Biorad, USA). 30 µl sample of supernatant was loaded into wells and electrophoresed at 4°C in a refrigerator for 140 minutes at 200 V with an initial current of 15 mA in stacking gel (4%) and 20 mA in separating gel (7.5%) using Tris HCl electrode buffer, pH 8.3.

3.2.2. Enzyme visualization

The five enzyme systems chosen for the study were peroxidase (PRX), esterase (EST), malate dehydrogenase (MDH), shikimate dehydrogenase (SKDH) and gluco phosphate isomerase (GPI). Staining procedures were adapted from Wendel and Weeden (1989) (Table 3.1.). The gels were immersed in the respective stain solutions and were kept in an incubator at 37°C till the bands were visible. The stained gels were visualised under bright light Trans-illuminator and photographed using Kodak DC120 gel electrophoresis documentation and analysis system (Kodak, USA). Deciphering the genetic basis of the observed banding patterns and interpretation of the banding profiles were carried out according to the basic principles and guidelines suggested by May (1994).

3.2.3. Statistical analysis

3.2.3.1. Genetic variation within populations

In order to determine the amount of genetic variation within a population, number of alleles (na), proportion of polymorphic loci (p.loci), expected heterozygosity (Exp. Het.) and observed heterozygosity (Obs. Het.) were estimated for the populations (Nei, 1973). In addition, depending on the locus resolved, the observed average heterozygosity of an individual in a population (H_I), the expected average heterozygosity of an individual in a population (H_S) and the expected heterozygosity of an individual, overall populations (H_T) were estimated in accordance with the Hardy-Weinberg expectations (Nei, 1987). The deviations from Hardy-Weinberg equilibrium in populations and genetic differentiation of

Table 3.1. Stain buffers, substrates and stains of the active enzyme systems.

Enzyme	Stain buffer	Substrate	Stain
Peroxidase (PRX)	0.1 M phosphate buffer (pH 7.0)	Hydrogen peroxide	Guaicol
Esterase (EST)	0.1 M phosphate buffer (pH 6.0)	1- Naphthyl acetate 2- Naphthyl acetate	Fast blue RR salt
Malate Dehydrogenase (MDH)	50 mM Tris HCl (pH 8.5)	Maleic acid	10 mg NAD 10 mg NBT 2 mg PMS
Shikimate Dehydrogenase (SDH)	50 mM Tris HCl (pH 8.5)	Shikimic acid	5 mg NADP 10 mg NBT 2 mg PMS
Gluco phosphate isomerase (GPI)	50 mM Tris HCl (pH 8.0)	Fructose 1-6 diphosphate Glucose 6-phosphate dehydrogenase	10 mg NAD 10 mg MTT 2 mg PMS

populations were calculated according to Nei (1987) using Popgene version 1.31 software (Yeh *et al.*, 1999). The Wright's F- Statistics (Wright, 1951) parameters viz. F_{IS} = inbreeding coefficient within population [$F_{IS}=(H_S - H_I)/ H_S$], F_{IT} = inbreeding coefficient over the total population [$F_{IT}=(H_T - H_I) / H_T$] and F_{ST} = Reduction in inbreeding coefficient due to differences among populations or genetic differentiation of populations [$F_{ST}=(H_T - H_S)/ H_T$] were calculated for this purpose.

3.2.3.2. Genetic diversity between populations

The estimates of Nei's standard genetic identity and genetic distance unbiased for sample size (Nei, 1978) for all pair-wise population comparisons were calculated to show the genetic relationships between the studied populations. All these estimates were carried out using Popgene 1.31 (Yeh *et al.*, 1999). The values were used to construct a UPGMA dendrogram (Nei, 1978). The populations with the highest similarity (least genetic distance) were merged, forming nested classes (clusters). This process was repeated until all the populations belonged to a single cluster.

3.3. RESULTS

The five enzyme systems representing 11 loci (1 locus for PRX; 2 loci for SKDH; 2 loci for GPI; 3 loci for MDH and 3 loci for EST) were resolved with sufficient consistency and clarity, which permitted to state whether one locus is mono or polymorphic. For the estimates of genetic variability measures, the genotypes of all the 11 loci in 20 individuals of each of the 11 populations were utilized. Isozyme

profiles of ten individuals for each of all the eleven populations are given in Figs. 3.2; 3.3; 3.4; 3.5 and 3.6.

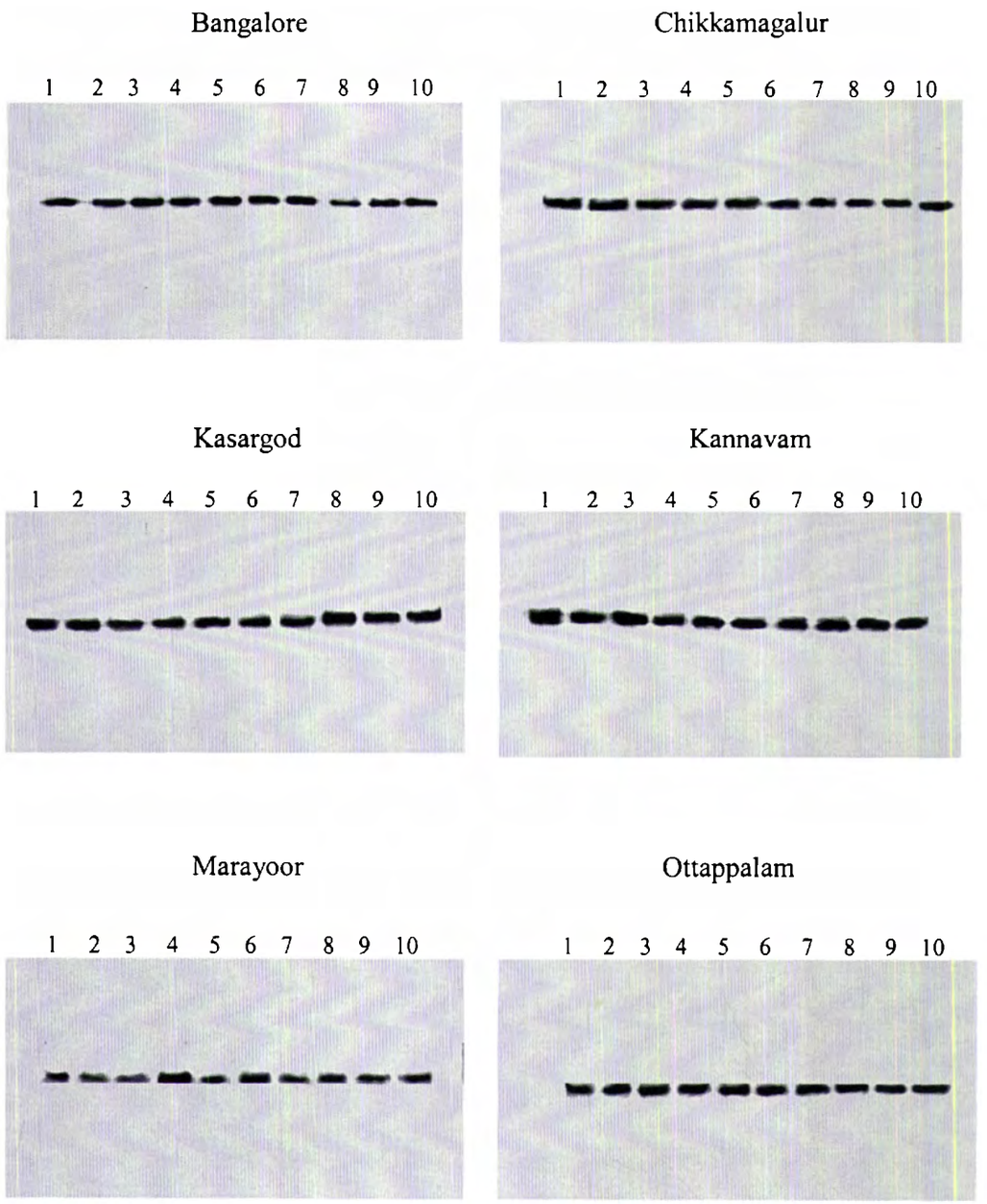
3.3.1. Genetic variation within populations

Population specific allele frequencies obtained are tabulated in table 3.2. The overall allelic frequencies at the level of loci (Table 3.3.) range from 0.0023 (B allele - EST-3) to 1.000 (A allele - MDH-3). The mean number of alleles per locus (na) (Table 3.4.) range from 1.000 to 3.000 (viz. MDH-3 has only one allele, EST-3 has 3 alleles and all the other loci viz. PRX-1, SKDH-1, SKDH-2, GPI-1, GPI-2, MDH-1, MDH-2, EST-1 and EST-2 have 2 alleles each). Of the 11 loci that were consistently scorable in all the populations analysed, except MDH-3 locus, all the ten loci (90.9%) were found to be polymorphic.

Depending on locus, the values for observed heterozygosity (H_I), expected average heterozygosity (H_S), total heterozygosity (gene diversity) (H_T) and F-statistics (F_{IS}, F_{IT} and F_{ST}) are listed in table 3.4. The observed heterozygosity (H_I) ranged from zero in PRX-1, GPI-1, MDH-1 and MDH-3 to 0.5000 in EST-1. The average expected heterozygosity (H_S) values varied from zero (PRX-1, GPI-1 and MDH-3) to 0.2670 (EST-1) and were lower than the observed heterozygosity values in most of the loci. The total heterozygosity (gene diversity) (H_T) values ranged from zero (MDH-3) to 0.4959 (PRX-1).

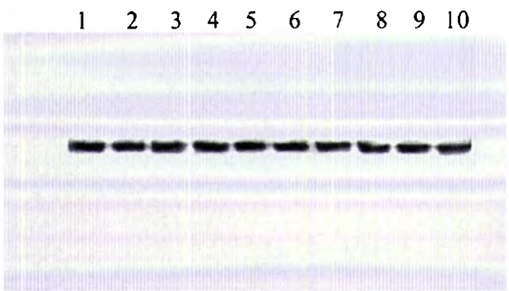
F-statistics (Table 3.4.) provided the measure of deviations in gene frequencies from Hardy-Weinberg expectations. If all the populations are in HWE, F_{IS} will be zero and F_{IT} will be equal to F_{ST}. In the present study, except for MDH-1

Figure 3.2. Peroxidase isozyme profiles of eleven sandal populations: lane 1-10 represents individual samples.

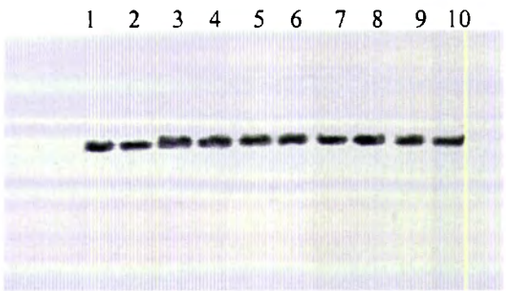


continued..

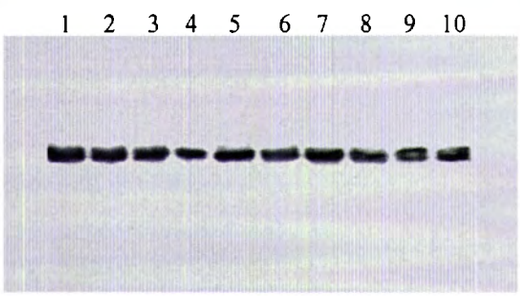
Shimoga



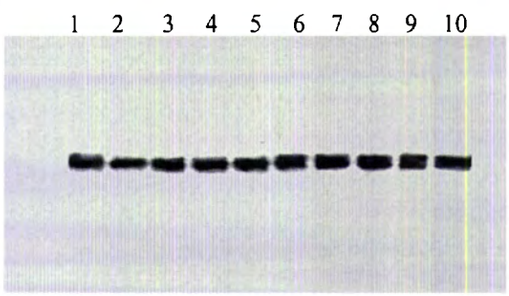
Thenmalai



Thirupattur



Wadakkanchery



Wayanad

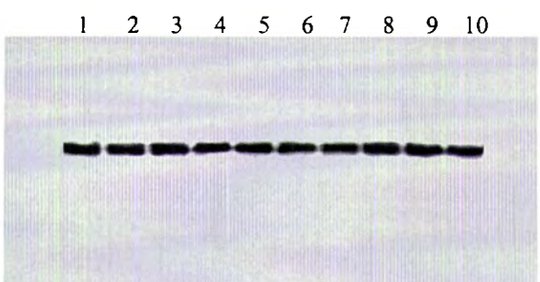
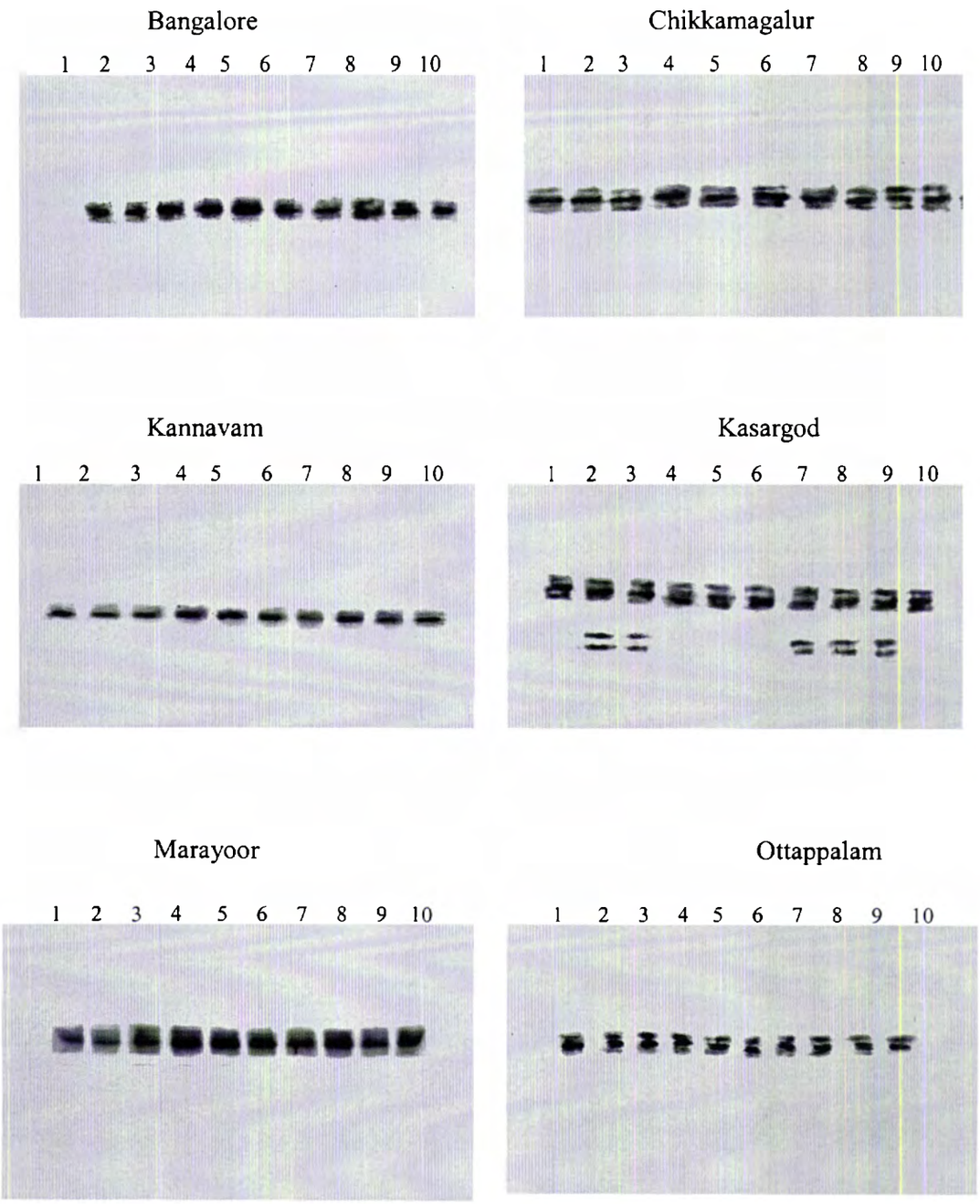
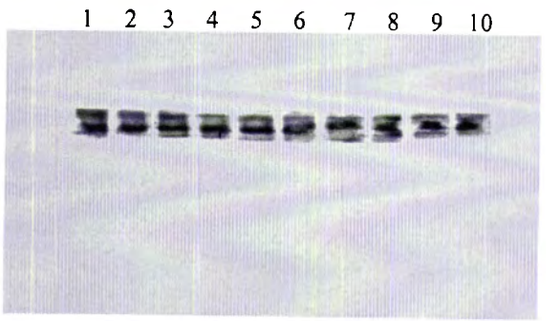


Figure 3.3. Esterase isozyme profiles of eleven sandal populations: lane 1-10 represents individual samples.

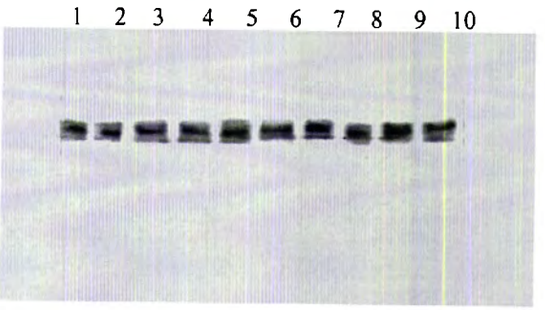


continued...

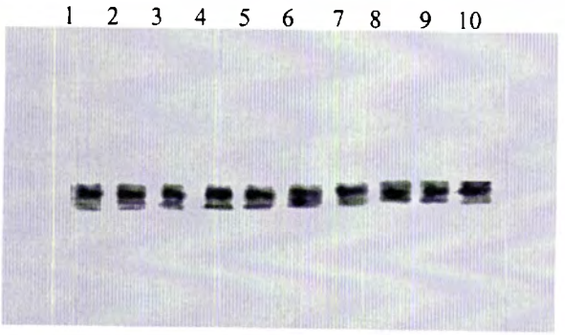
Shimoga



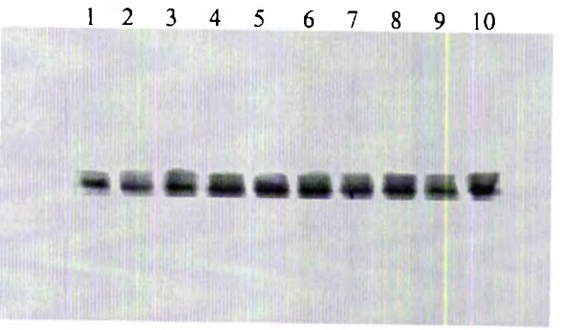
Thenmalai



Thirupattur



Wadakkanchery



Wayanad

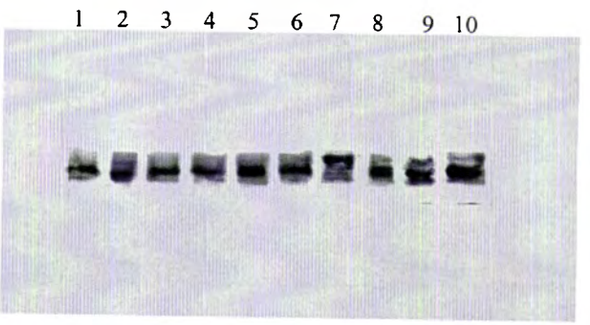
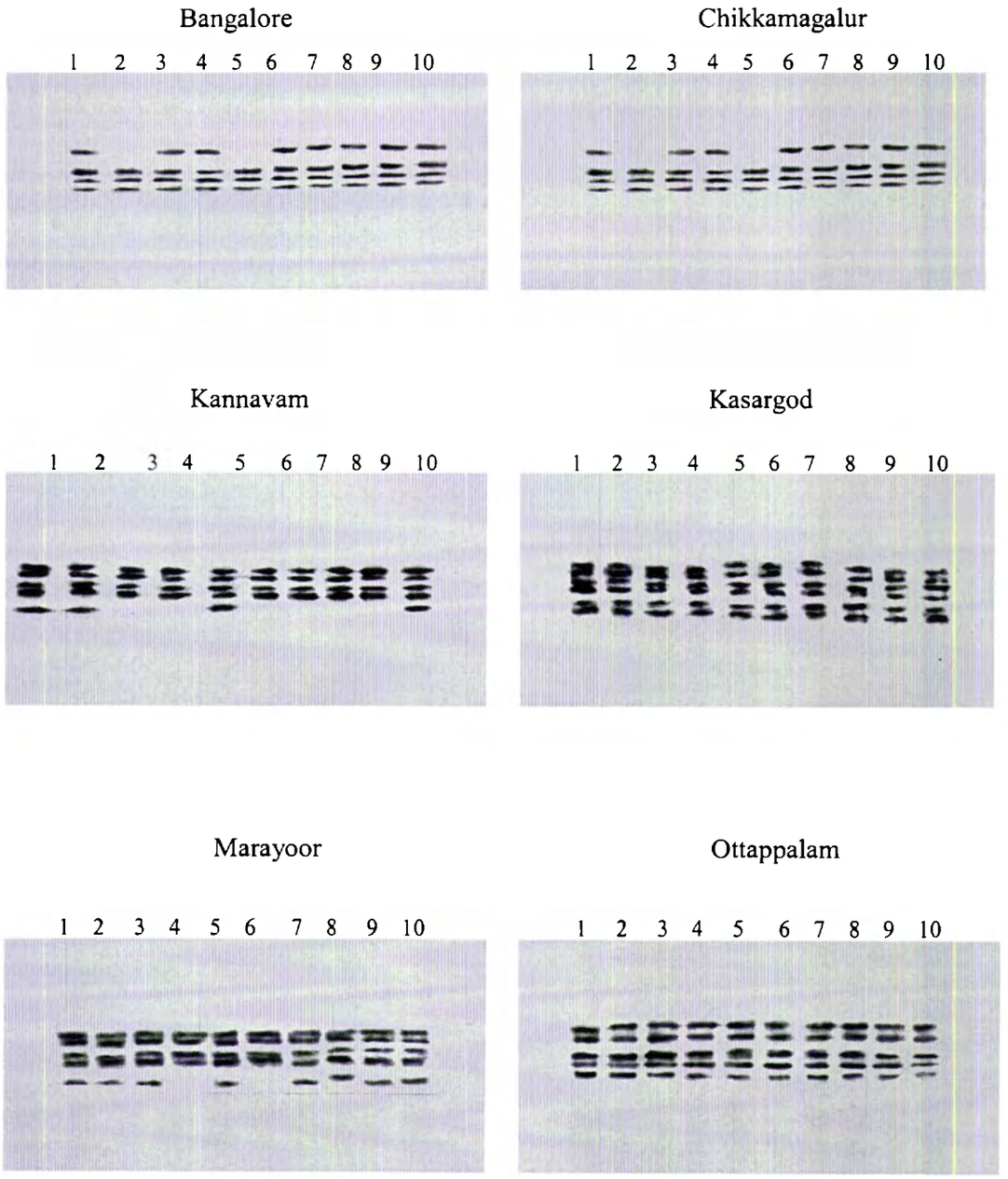
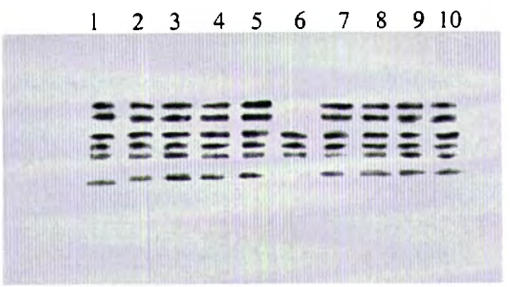


Figure 3.4. Malate dehydrogenase isozyme profiles of eleven sandal populations: lane 1-10 represents individual samples.

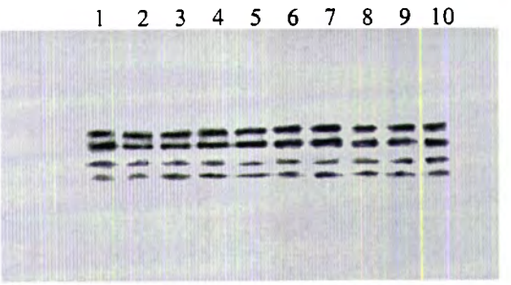


continued..

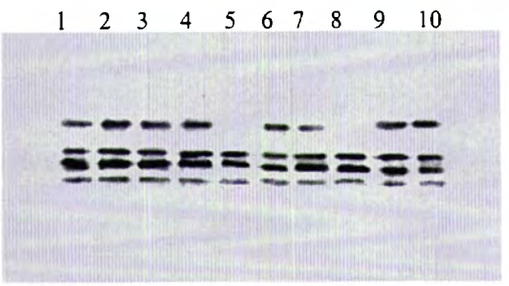
Shimoga



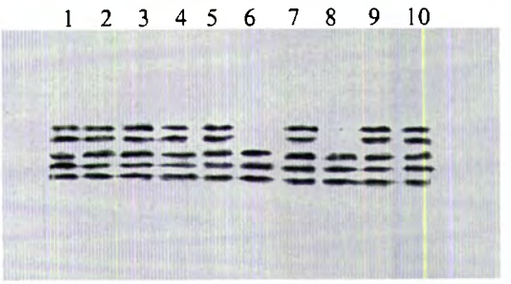
Thenmalai



Thirupattur



Wadakkanchery



Wayanad

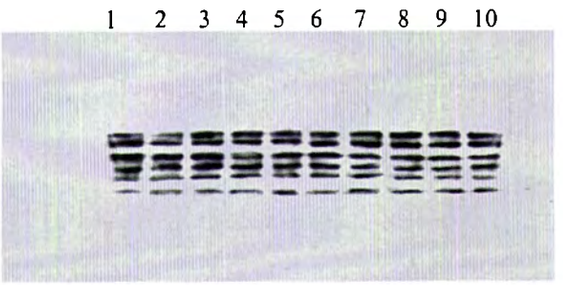
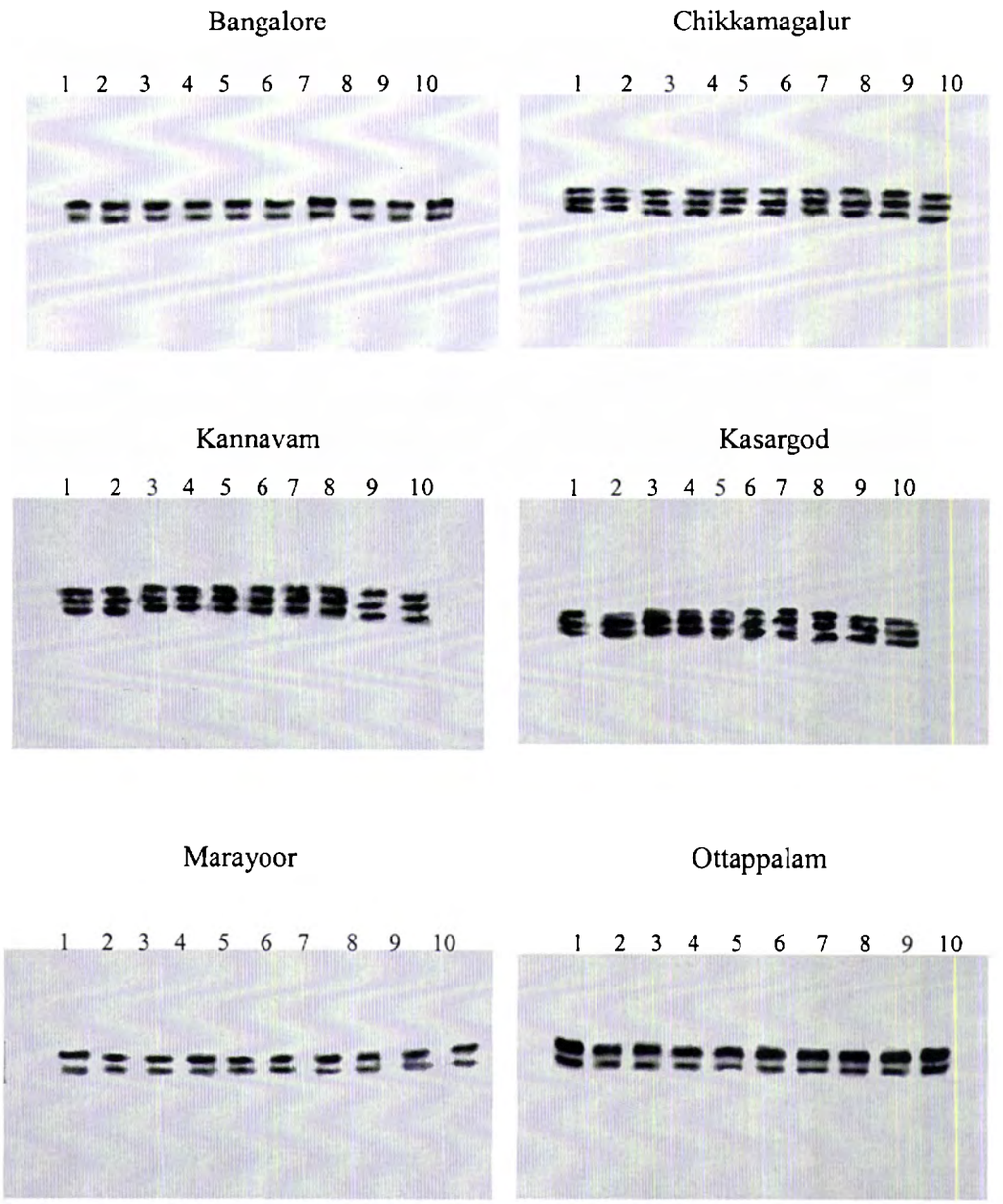
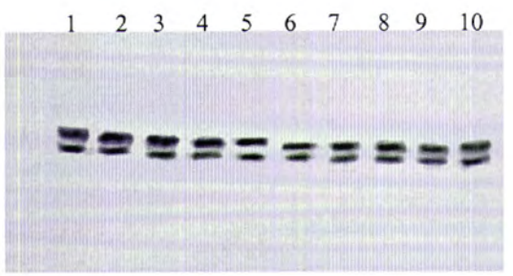


Figure 3.5. Shikimate dehydrogenase isozyme profiles of eleven sandal populations: lane 1-10 represents ten individual samples.

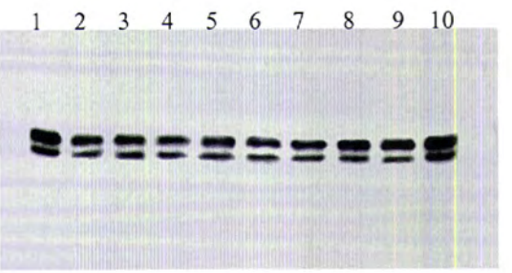


continued..

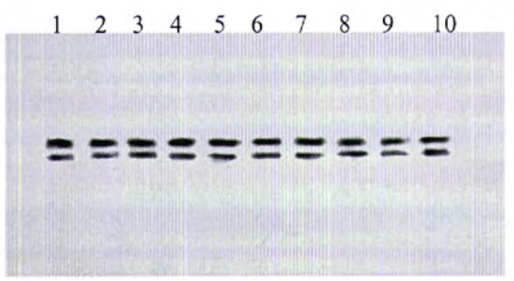
Shimoga



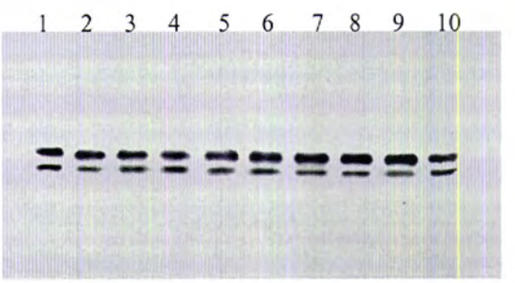
Thenmalai



Thirupattur



Wadakkanchery



Wayanad

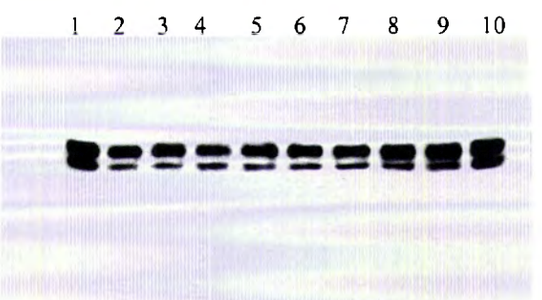
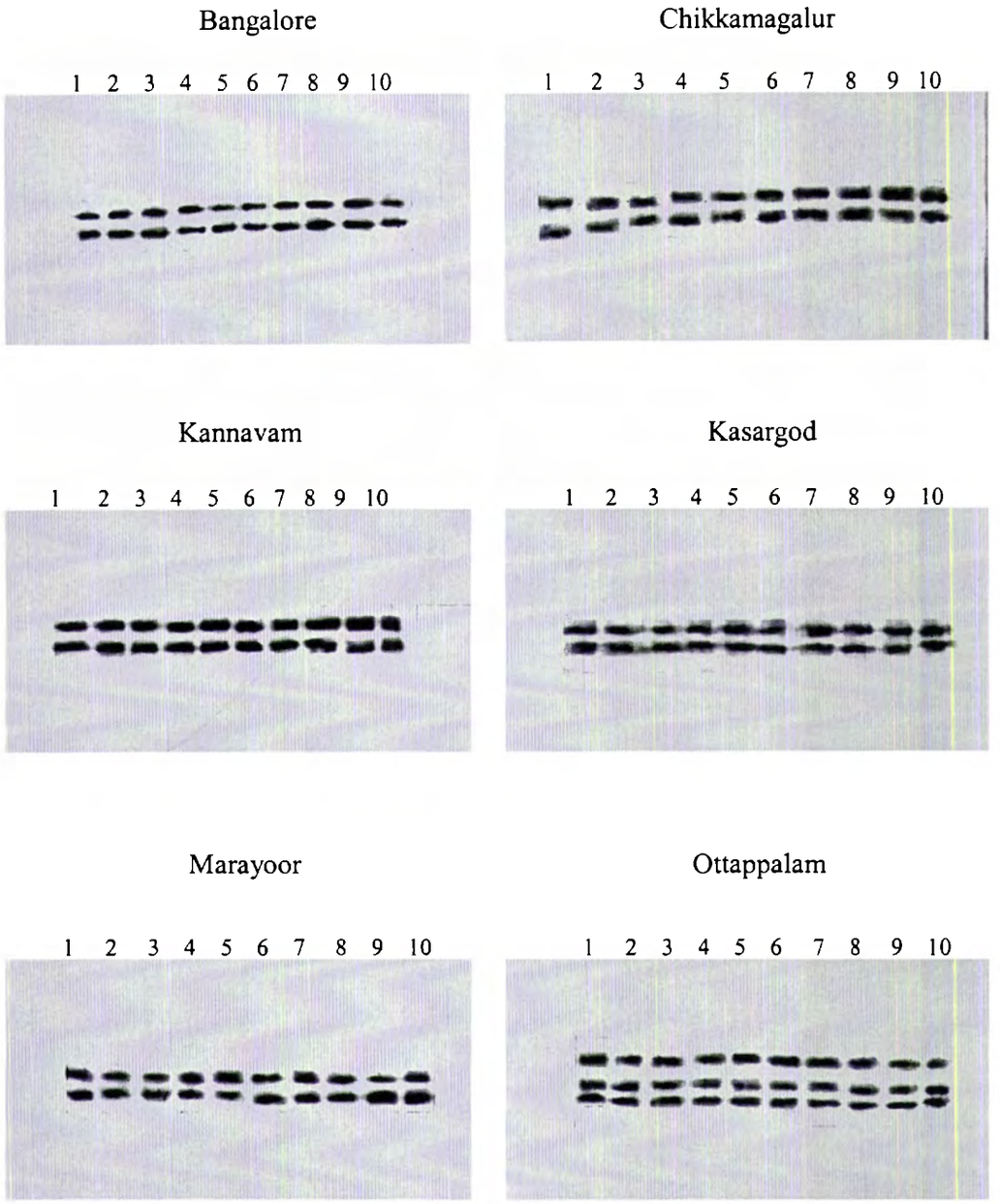
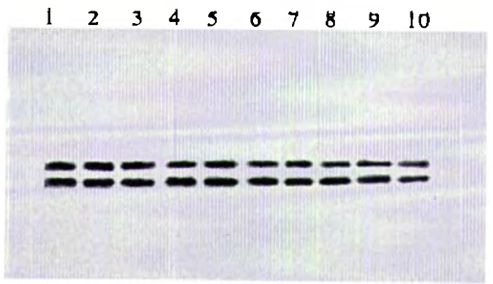


Figure 3.6. Gluco phosphate isomerase isozyme profile of eleven sandal populations: lane 1-10 represents individual samples.

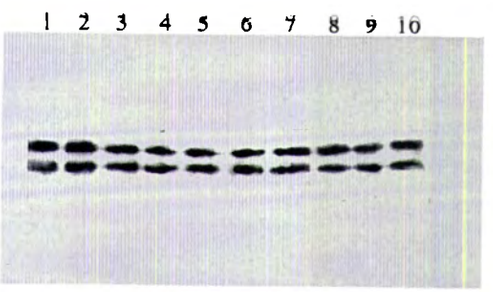


continued...

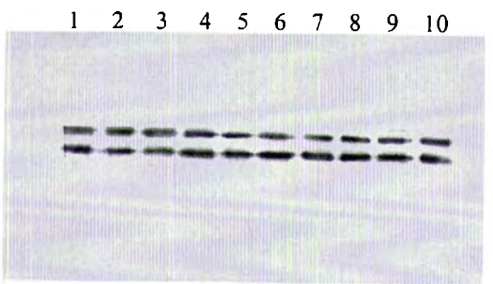
Shimoga



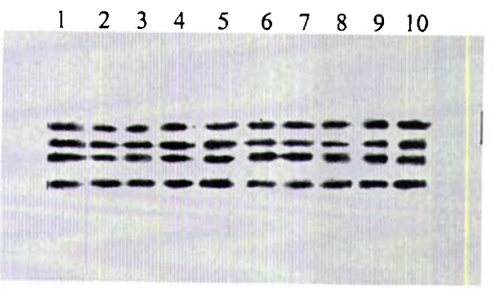
Thenmalai



Thirupattur



Wadakkanchery



Wayanad

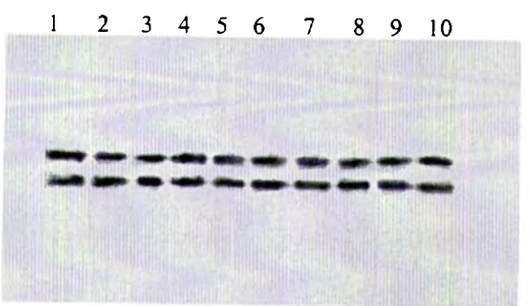


Table 3.2. Allele frequencies for 11 loci in eleven sandal populations.
(Pop 1-Marayoor, Pop 2-Wayanad, Pop 3-Thenmalai, Pop 4-Wadakkanchery, Pop 5-Ottappalam, Pop 6-Kannavam, Pop 7-Kasargod, Pop 8-Bangalore, Pop 9-Shimoga, Pop 10- Thirupattur, Pop 11-Chikkamagalur).

Locus	allele	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	Pop11
PRX-1	A	1.0000	0.0000	1.0000	0.0000	1.0000	1.0000	1.0000	0.0000	0.0000	0.0000	1.0000
	B	0.0000	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	1.0000	1.0000	1.0000	0.0000
SKDH-1	A	1.0000	0.0000	0.0000	0.0000	0.0000	0.5000	0.5000	0.0000	0.0000	0.0000	1.0000
	B	0.0000	1.0000	1.0000	1.0000	1.0000	0.5000	0.5000	1.0000	1.0000	1.0000	0.0000
SKDH-2	A	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5000
	B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000
GPI-1	B	0.0000	1.0000	0.0000	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0000	1.0000
	C	1.0000	0.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
GPI-2	A	0.0000	1.0000	0.0000	0.5000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0000	1.0000
	B	1.0000	0.0000	1.0000	0.5000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
MDH-1	C	0.6111	0.0000	1.0000	0.6000	1.0000	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000
	D	0.3889	1.0000	0.0000	0.4000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000
MDH-2	B	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5000	1.0000	1.0000
	C	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000	0.0000	0.0000
MDH-3	A	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
EST-1	C	0.5000	0.5250	0.0000	0.5000	0.6750	0.5000	0.5000	0.0000	0.0000	0.0000	0.0000
	D	0.5000	0.4750	1.0000	0.5000	0.3250	0.5000	0.5000	1.0000	1.0000	1.0000	1.0000
EST-2	B	1.0000	0.9500	0.8000	1.0000	1.0000	1.0000	1.0000	0.5000	0.5000	0.5000	0.5000
	C	0.0000	0.0500	0.2000	0.0000	0.0000	0.0000	0.0000	0.5000	0.5000	0.5000	0.5000
EST-3	A	0.9750	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0000
	B	0.0250	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	D	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000

Table 3.3. Overall allele frequencies of 11 loci.

Allele	PRX-1	SKDH-1	SKDH-2	GPI-1	GPI-2	MDH-1	MDH-2	MDH-3	EST-1	EST-2	EST-3
A	0.5455	0.2727	0.9545		0.6818			1.0000			0.9068
B	0.4545	0.7273	0.0455	0.6364	0.3182		0.9545			0.7955	0.0023
C				0.3636		0.7441	0.0455		0.2909	0.2045	
D						0.2559			0.7091		0.0909

Table 3.4. Genetic diversity measures depending on the locus; Observed number of alleles (Na), Observed heterozygosity (H_I), Expected average heterozygosity (H_S), Total heterozygosity (H_T), Wright's F-inbreeding coefficients [F_{IS} (within population), F_{IT} (over the total population) and F_{ST} (genetic differentiation)] and Nm (rate of gene flow) per locus.

Locus	Na	(H_I)	(H_S)	(H_T)	F_{IS}	F_{IT}	F_{ST}	Nm
PRX-1	2	0.0000	0.0000	0.4959	****	1.0000	1.0000	0.0000
SKDH-1	2	0.1818	0.0909	0.3967	-1.0000	0.5417	0.7708	0.0743
SKDH-2	2	0.0909	0.0455	0.0868	-1.0000	-0.0476	0.4762	0.2750
GPI-1	2	0.0000	0.0000	0.4628	****	1.0000	1.0000	0.0000
GPI-2	2	0.0909	0.0455	0.4339	-1.0000	0.7905	0.8952	0.0293
MDH-1	2	0.0000	0.0868	0.3785	1.0000	1.0000	0.7706	0.0744
MDH-2	2	0.0909	0.0455	0.0868	-1.0000	-0.0476	0.4762	0.2750
MDH-3	1	0.0000	0.0000	0.0000	****	****	0.0000	****
EST-1	2	0.5000	0.2670	0.4126	-0.8723	-0.2119	0.3527	0.4588
EST-2	2	0.4000	0.2195	0.3254	-0.8219	-0.2292	0.3253	0.5184
EST-3	3	0.0045	0.0044	0.1694	-0.0256	0.9732	0.9738	0.0067
Mean	2	0.1236	0.0732	0.2962	-0.6869	0.5820	0.7522	0.0824

(F_{IS}=1.0000) which showed a positive value, all the loci got negative values for F_{IS} (the inbreeding coefficient within population). These results indicated that all the loci showed deviations from Hardy-Weinberg expectations. Similarly, F_{IT}, the inbreeding coefficient over the total population varied between -0.2292 (EST-2) and 1.000 (PRX-1) and was not equal to F_{ST} ($F_{IT} \neq F_{ST}$). The genetic differentiation of populations (F_{ST}) was at par with Nei's G_{ST} (coefficient of genetic differentiation). The mean F_{ST} was 0.7522 meaning that 75.22 per cent of the total variation was between populations and only 24.78 per cent was observed within populations. The average rate of gene flow was found to be 0.0824.

For the populations, the estimated parameters that were used to describe the genetic variation are given in table 3.5. The per cent of polymorphic loci varied from 9.09 in Thenmalai, Ottappalam, Bangalore and Thirupattur to 27.27 in Wayanad and Wadakkanchery populations. The mean number of alleles per locus had the lowest value in Thenmalai, Ottappalam, Bangalore and Thirupattur (1.0909) whereas, it was the highest in Wayanad and Wadakkanchery (1.2727) populations. Among the populations, the heterozygosities calculated directly from the genotypes (Obs. Het.) varied from 0.0364 in Thenmalai to 0.1818 in Wadakkanchery, Kannavam, Kasargod, Shimoga and Chikkamagalur. Whereas, expected heterozygosity estimated (Exp. Het.) varied from 0.0291 in Thenmalai to 0.1345 in Wadakkanchery populations of sandal.

Table 3.5. Population genetic diversity parameters. Na: Observed number of alleles; Ne: Effective number of alleles; Obs. het.: Observed heterozygosity; Exp. Het.: Expected heterozygosity (Nei, 1973); No.P.loci: Number of polymorphic loci; % P.loci: Per cent of polymorphic loci.

Populations	Na	Ne	Obs. Het.	Exp. Het.	No.P.loci	% P.loci
Marayoor (Pop1)	1.1818	1.1733	0.0909	0.0887	2	18.18
Wayanad (Pop2)	1.2727	1.1047	0.0818	0.0584	3	27.27
Thenmalai (Pop3)	1.0909	1.0428	0.0364	0.0291	1	09.09
Wadakkanchery (Pop4)	1.2727	1.2657	0.1818	0.1345	3	27.27
Ottappalam (Pop5)	1.0909	1.0711	0.0591	0.0399	1	09.09
Kannavam (Pop6)	1.1818	1.1818	0.1818	0.0909	2	18.18
Kasargod (Pop7)	1.1818	1.1818	0.1818	0.0909	2	18.18
Bangalore (Pop8)	1.0909	1.0909	0.0909	0.0455	1	09.09
Shimoga (Pop9)	1.1818	1.1818	0.1818	0.0909	2	18.18
Thirupattur (Pop10)	1.0909	1.0909	0.0909	0.0455	1	09.09
Chikkamagalur (Pop11)	1.1818	1.1818	0.1818	0.0909	2	18.18

3.3.2. Genetic diversity between populations

Genetic identity/distance coefficients are provided in table 3.6. For all pair wise populations, genetic similarity coefficients showed a higher value than distance coefficients. Of all the populations analysed Kannavam (pop6) and Kasargod (pop7) showed the highest value for genetic identity (1.0013) between them, while Wadakkanchery (pop4) and Chikkamagalur (pop11) showed the highest value for genetic distance (0.7503).

Nei's (1978) unbiased measures of genetic identity/distance were used to construct UPGMA dendrogram (Fig. 3.7.) showing the clustering of all the 11 populations. It was possible to distinguish 2 main clusters within the *S. album* populations. The first main cluster consisted of 10 populations, formed by the merging of two sub clusters, one contained four populations Marayoor, Thenmalai, Wadakkanchery and Thirupattur and the other contained six populations Wayanad, Shimoga, Bangalore, Ottappalam, Kannavam and Kasargod. This major cluster in turn linked with Chikkamagalur to form the final cluster. The branches in the dendrogram reflect the genetic differentiation among populations.

3.4. DISCUSSION

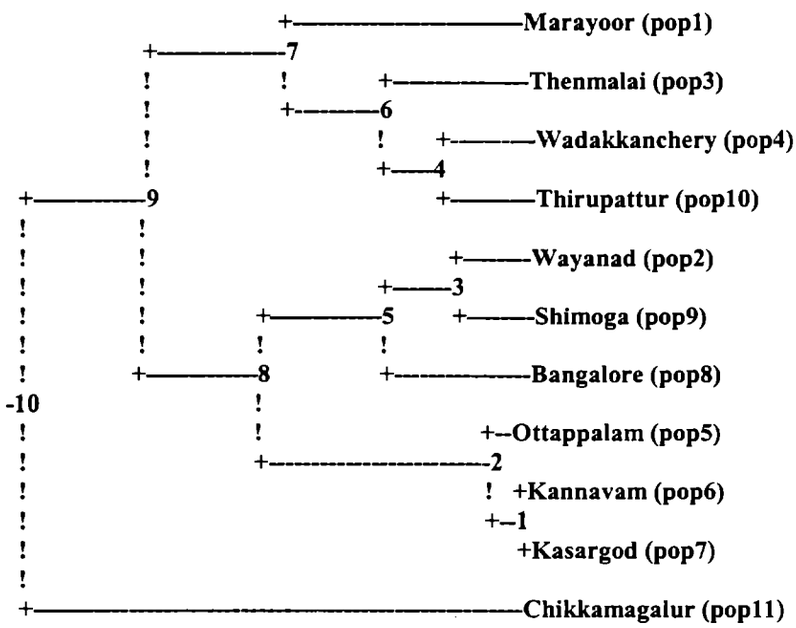
Trees from different geographic areas within the range of a species may vary in growth rate, form, and adaptation to environmental conditions and resistance to insects and disease incidence (Hamrick and Nason, 2000).

Table 3.6. Nei's (1978) unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal)coefficients.

(Pop 1-Marayoor, Pop 2-Wayanad, Pop 3-Thenmalai, Pop 4-Wadakkanchery, Pop 5-Ottappalam, Pop 6-Kannavam, Pop 7-Kasargod, Pop 8-Bangalore, Pop 9-Shimoga, Pop 10- Thirupattur, Pop 11-Chikkamagalur).

Populations	1	2	3	4	5	6	7	8	9	10	11
1	****	0.5712	0.8619	0.7713	0.6917	0.7611	0.7611	0.5474	0.4889	0.7426	0.6111
2	0.5601	****	0.5917	0.8395	0.8069	0.7795	0.7795	0.8588	0.9295	0.6669	0.5385
3	0.1486	0.5247	****	0.8340	0.7654	0.7554	0.7554	0.7086	0.5811	0.8975	0.5811
4	0.2597	0.1749	0.1815	****	0.7589	0.7289	0.7289	0.8112	0.7597	0.9114	0.4722
5	0.3685	0.2146	0.2674	0.2759	****	0.9739	0.9739	0.8385	0.7134	0.6485	0.7134
6	0.2729	0.2491	0.2805	0.3163	0.0264	****	1.0013	0.8303	0.7009	0.6349	0.8010
7	0.2729	0.2491	0.2805	0.3163	0.0264	-0.0013	****	0.8303	0.7009	0.6349	0.8010
8	0.6025	0.1522	0.3445	0.2092	0.1761	0.1860	0.1860	****	0.8791	0.8100	0.6838
9	0.7156	0.0731	0.5429	0.2749	0.3377	0.3554	0.3554	0.1288	****	0.6838	0.5507
10	0.2977	0.4051	0.1081	0.0928	0.4331	0.4542	0.4542	0.2107	0.3801	****	0.4884
11	0.4924	0.6189	0.5429	0.7503	0.3377	0.2219	0.2219	0.3801	0.5966	0.7166	****

Figure 3.7. Dendrogram based on Nei's (1978) genetic distance coefficients.



Between	And	Length
10	9	5.77555
9	7	6.85525
7	pop1	11.76587
7	6	4.52666
6	pop3	7.23922
6	4	2.59800
4	pop4	4.64121
4	pop10	4.64121
9	8	5.79136
8	5	5.80436
5	3	3.37004
3	pop2	3.65536
3	pop9	3.65536
5	pop8	7.02540
8	2	11.50942
2	pop5	1.32035
2	1	1.32035
1	pop6	0.00000
1	pop7	0.00000
1	pop11	24.39667

Conservation as well as utilization of this variation is extremely important for the genetic improvement of any tree species. Sandal trees in Indian forests have been depleted considerably due to illicit felling of trees, absence of regeneration caused by repeated fires and excessive grazing, and also greatly due to mortality caused by spike disease (Nageswara Rao *et al.*, 2001). Information on genetic variation within and between geographically isolated populations of *S. album* was unknown previously.

The present study provided considerable amount of information on the magnitude and pattern of genetic variation existing within eleven sandal populations. The genetic "richness" as measured by the number of alleles indicated that EST-3 is the most genetically rich locus amongst the eleven loci analysed and MDH- 3, the least rich. Wayanad and Wadakkanchery were found to be the genetically rich populations and Thenmalai, Ottappalam, Bangalore and Thirupattur were the least genetically rich among the analysed populations. Similarly, the per cent of polymorphism also varied significantly in the eleven analysed populations and Wayanad got the highest amount of polymorphism. Generally, in forest trees, the allelic richness and per cent of polymorphism were reported to be influenced by local environmental conditions and by gene flow (Prus-Glowacki and Stephan, 1994). Prober and Brown (1994) found a significant positive relationship between population size and allelic richness in population of *Eucalyptus alben* BENTH. Young *et al.* (1999) observed lower number of

allozyme alleles with reduced population size in *Rutidosis leptorrhynchoides*. The variation in the per cent of polymorphism and number of alleles in the different populations in *S. album* can be attributed to the difference in population size, environmental conditions and also to the difference in rate of gene flow.

Expected heterozygosity is the expected proportion of heterozygotes, assuming Hardy-Weinberg equilibrium (Hamrick, 1987). Within each of the analysed populations (averaged overall locus) and at the locus level (averaged overall populations), the observed amount of heterozygosity was found to have a higher value than what is expected under random mating (Table 3.4.). Genetic heterozygosity is often influenced by founder effects, genetic drift, population size, natural and man-made selection, and local environmental variations (Van Boren and Harper, 1996)). Fragmented distribution and poor rate of gene flow favour genetic drift (Brown and Moran, 1981). Consequently, populations evolve independently as single discrete units, resulting in a high amount of observed heterozygosity. Such increased levels of observed heterozygosity was also reported in *Arctomecon humilis* (Alphin *et al.*, 1998) The sandal populations under study are separated by considerable distance and hence chance for gene flow are minimum. It is reported that the possibilities of gene flow between natural populations of most species diminish rapidly with distance (Mayr, 1963; Varela and Eriksson, 1995). Gene flow seemed to be an important factor in preventing the differentiation of populations (Hamrick and Nason, 2000). Thus the population might have evolved separately as discrete independent units. Young and Brown (1999) found

significant changes in mating patterns with reduced size and increased isolation of remnant population of the daisy *Rutidosis leptorrhynchoides*. Prober and Brown (1994) found that the changes due to fragmentation immediately affect mating system parameters such as outcrossing rates in *Eucalyptus albens*.

Genetic differentiation among populations was high with F_{ST} = 0.7522, implying that 75.22 per cent of total variation was between populations and 24.78 per cent of total genetic variation was within populations. Therefore, *S. album* exhibited a pattern of genetic diversity characterized by a moderate degree of intrapopulation variation and a rather high inter-population genetic diversity. These results also pointed out towards the more discrete nature of sandal population with least amount of gene flow.

In order to estimate the deviations from Hardy-Weinberg expectations, Wright's (1951) F-statistics (Yeh, 2000) were used. Depending on the locus, the average inbreeding coefficient within population (F_{IS}) was -0.6869 (Table 3.4.), meaning that within population heterozygotes were 68.69 per cent higher than expected due to the lower levels of inbreeding within the population. While, the inbreeding coefficient over the total populations (F_{IT}) was 0.5820, indicating that 58 per cent of the heterozygotes were lower than expected under Hardy-Weinberg equilibrium. This might be due to the mating or gene flow between similar genotypes due to some natural evolutionary selection processes. The low rate of gene flow might be the reason for the high level of genetic differentiation among the populations. Generally, in forest tree species inter population gene flow are

usually high. However, due to fragmentation, the remaining populations become geographically isolated and the original rate of gene flow cannot be maintained (Hamrick and Loveless, 1985). The small, localized and fragmented nature of *S. album* populations and their geographic separation might have diminished the rate of gene flow and intrapopulation variation is also reported to be small in those cases (Tennakoon *et al.*, 2000). Gene flow is mediated by seed and pollen migration and this in turn depends on the geographical separation of the populations (Gontcharenko *et al.*, 1993).

The eleven populations covered a broad geographic range, differing in rainfall (700-4000mm) and altitude (60-1000m) and thus enzymatic differentiation is expected to exist among them. The genetic relatedness of the eleven populations analysed, as given in the dendrogram, showed clustering of some of the neighbouring populations but not fully in agreement with geographic separation. Similar pattern of genetic relatedness has also been reported for populations of *Picea abies* (Lundkvist and Rudin, 1977). According to Endler (1977), various regimes of selection either directly via linkage, migration and genetic drift could produce these patterns. Jourde (1980) reported that geographic distance, which restricts the gene flow, is the major cause of population difference. In the case of *S. album* populations, Wadakkanchery and Chikkamagalur are the most genetically distant populations, and also geographically separated considerably, about 400 km. Similarly Kannavam and Kasargod are the most genetically identical populations, which are also closely located geographically, only about 70 km.

Forest tree populations are often considered large and continuous, but many tree species as in *Acacia mangium* (Moran *et al.*, 1989) and *Eucalyptus* (Moran, 1992), occur naturally as small isolated populations. Large populations maintain ample genetic variability, which assures a readiness to respond to current selection pressures. Many formerly large populations are experiencing reductions in population size owing to human disturbances. Small populations may lose variability and their ability to respond to stress conditions (Karkkainen *et al.*, 1996). As the population size increases, recurrent mutation can restore the genetic variability, but it takes place very slowly. The low amount of genetic variation within *S. album* populations and high amount of genetic variation between populations as evidenced from F_{ST} values, might be due to the fragmentation of a previously large population and differentiation of populations due to random drift and low amount of gene flow.

CHAPTER 4

RAPD BASED GENETIC VARIATION IN SANDAL POPULATIONS

4. RAPD BASED GENETIC VARIATION IN SANDAL POPULATIONS

4.1. INTRODUCTION

Genetic variation in natural populations is a resource for the survival and future evolution of a species as well as for improving its productivity (Frankel *et al.*, 1995). In the past, forest genetic research involved classical approaches for the assessment of genetic variation by using either morphological or physiological traits (Michelmore *et al.*, 1991). Forest tree species are generally characterized by outbred mating systems and long generation cycles. Many of the traits of commercial importance are expressed late in the breeding cycle and subjected to varying degrees of environmental modifications and interactions. These characteristics have limited the development of classical morphological markers, which in turn hampered the progress in tree breeding programs as compared to the other crop species (Byrne *et al.*, 1995).

The advent of isozyme techniques provided new genetic markers that could be used to address questions concerning genetic resources, mating systems and population structure (Adams, 1983; Hamrick *et al.*, 1992; Conkle, 1992). However, the numbers of available loci have not been sufficient for application in breeding strategies (Ayala, 1983). Recently, DNA based markers such as RFLPs, RAPDs, AFLPs and microsatellites have been applied to the detection of genetic polymorphism in plants (Botstein *et al.*, 1980; Litt and Luty, 1989; Hedrick, 1992; Lisitsyn *et al.*, 1993).

RAPDs constitute a class of genetic markers which produce arbitrary fragment length polymorphisms and utilize single, arbitrary decamer DNA

oligonucleotide primers to amplify regions of the genome using the polymerase chain reaction (Williams *et al.*, 1990; Hedrys *et al.*, 1992; Newbury and Ford-Lloyd, 1993). Priming sites are thought to be randomly distributed throughout the genome and polymorphism in these regions results in differing amplification products (Williams *et al.*, 1993). RAPD analysis with agarose gel electrophoresis has been used successfully to identify and discriminate species, varieties, hybrids and cultivars (Dawson *et al.*, 1996; Ortiz *et al.*, 1997; Bartolozzi *et al.*, 1998; Mc Comb and Jones, 1998), for QTL mapping (Yazdani *et al.*, 1995; Byrne *et al.*, 1997a; Bundock *et al.*, 2000) and to analyze population genetic structure (Chalmers *et al.*, 1994; Burner *et al.*, 1997; Hormaza *et al.*, 1998). RAPD markers are dominant and hence heterozygotes cannot be distinguished; but, compared to other markers, they provide a fast and cheap means of generating large numbers of loci randomly spread throughout the genome (Rafalski and Tingey, 1993).

Genetic conservation of *S. album* is a subject of great concern recently, owing to the loss of significant amount of genetic diversity by spike disease and illegal felling. Assessment of genetic diversity is therefore of crucial importance for estimating more accurately the conservation status of particular populations, quantifying the affects of illegal cutting on the gene pool and for developing integrated, scientifically based genetic improvement and conservation strategies (Jain *et al.*, 1998). Hence, the main objective of this study was to determine genetic diversity and pattern of variation in South Indian *S. album* populations using RAPD markers.

4.2. MATERIALS AND METHODS

4.2.1. Sample collection

RAPD analysis was carried out in eleven populations viz. Marayoor, Wayanad, Thenmalai, Wadakkanchery, Ottappalam, Kannavam and Kasargod of Kerala State, Bangalore, Shimoga and Chikkamagalur of Karnataka State and Thirupattur of Tamil Nadu State in South India (Fig. 3.1.). Seed samples were collected from more than 20 parent trees randomly, covering almost the entire range of population and the seeds were pooled together. From this bulk, 12 samples were drawn randomly for raising seedlings. Seedlings at four-leaf-stage (3-week-old) were used as the source material.

4.2.2. DNA extraction

Genomic DNA was extracted from seedling tissue following the modified method of Doyle and Doyle (1990). Samples were homogenated in hot (65° C) CTAB buffer containing 2 per cent CTAB, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl and the sample homogenates were incubated at 60° C on a water bath for half an hour. The samples were extracted with chloroform/isoamyl alcohol (24:1) followed by low speed centrifugation (1600 g) for 5 minutes and the aqueous phase eluted out. This step was repeated again and to the aqueous layer, double the volume of cold (-20° C) absolute alcohol was added. After incubation at -20° C for 12 hours, the DNA precipitate was centrifuged for 10 minutes at low speed (1600 g). Ethanol (95%) was added to the DNA pellet and recentrifuged at the same speed. The supernatant was discarded and the air-dried pellet was dissolved in 100 µl double distilled water.

Total DNA was estimated at A_{260} (1 OD = 50 μg ml-1) (Gallagher, 1996) using UV-spectrophotometer.

4.2.3. Polymerase Chain Reaction

Twenty decamer oligonucleotides from kit A (Operon Technologies, Almeda, Calif.) were screened for genetic polymorphism by PCR amplification on agarose gel. Comparison of resolved bands on agarose gel was performed with three selected primers from kit A (OPA 07, OPA 09 and OPA 16). DNA was amplified in 25 μl reaction mixtures containing 100-125 ng of template DNA, 100 μM each of dATP, dTTP, dCTP and dGTP, 0.5 units of Taq polymerase, 1μl (250 ng) of each primer and 5 μl Taq buffer with 1.5 mM $MgCl_2$ (Genei, Bangalore). The incubation mixture was overlaid with one or two drops of mineral oil (Genei, Bangalore) and subjected to 45 cycles of amplification in PTC-150 Minicycler (MJ Research Inc., USA), each of 60 S denaturation (94° C), 60 S annealing (36° C) and 120 S extension (72° C). The last cycle was followed by incubation for 10 minutes at 72° C.

4.2.4. Separation and visualization of the amplification products

The PCR amplification products were electrophoresed on mini-sub gel GT (Biorad, USA) in 1.5 per cent horizontal agarose gel (Biorad, USA) in TBE buffer (40 mM Tris-borate, 1 mM EDTA, pH 8.0). The gel, after the completion of electrophoresis was stained with ethidium bromide and bands were compared with a 100-bp DNA ladder (Genei, Bangalore). The gels were documented using Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Kodak, USA).

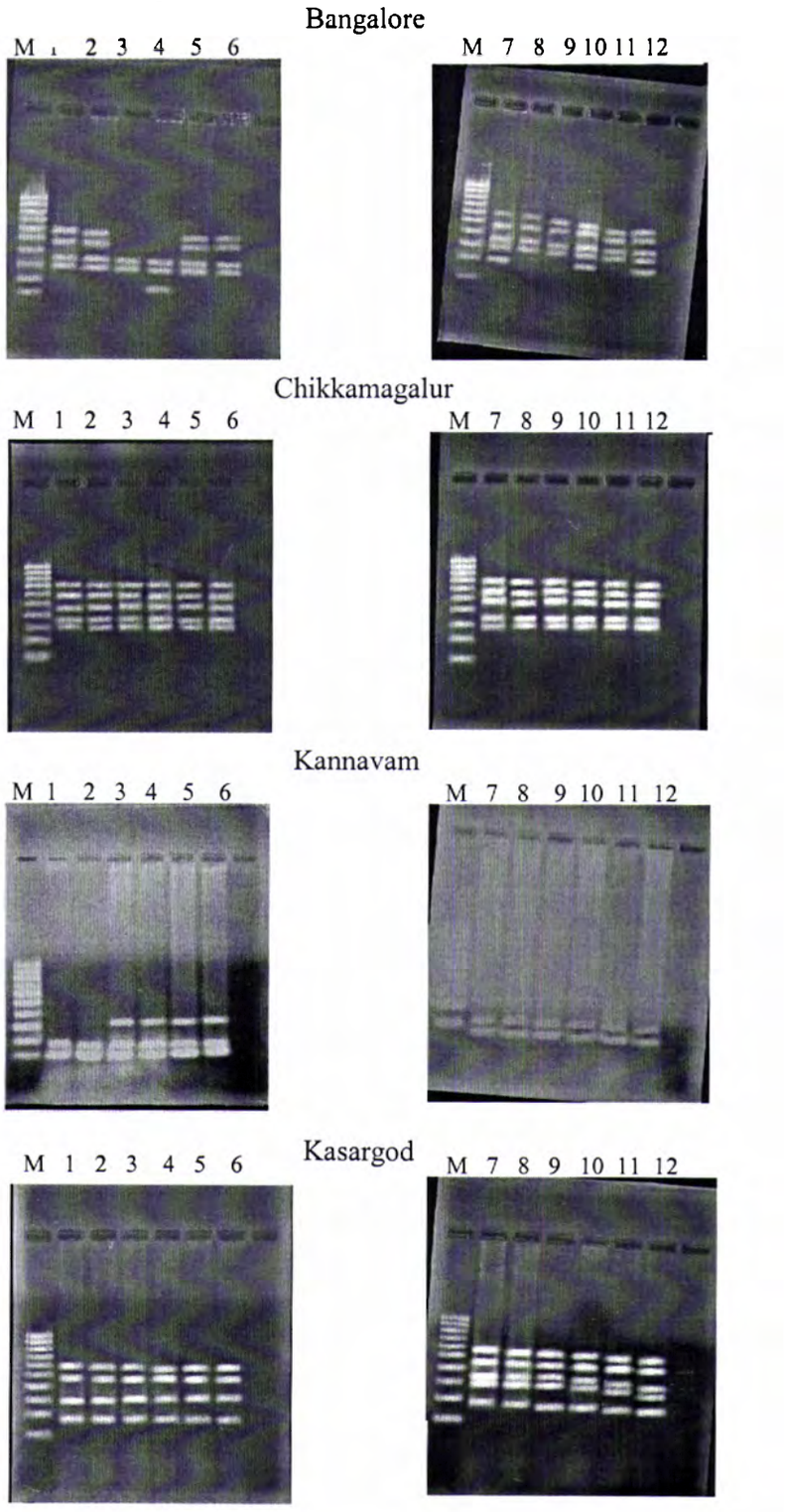
4.2.5. Data Analysis

The DNA fragment sizes were estimated comparing DNA size markers run on the same gel and the data scored for RAPD analysis. The bands were scored '1' for their presence and '0' for absence in each DNA sample to create binary data matrices. The data matrices were entered into the Popgene version 1.31 computer package and pair-wise comparison of populations were made (Yeh *et al.*, 1999). The observed number of alleles (na), expected number of alleles (ne), per cent of polymorphic loci (polymorphic bands per total bands) and Nei's G statistics (H_T, H_S and G_{ST}) were determined (Nei, 1987). Nei's genetic distance (D) was calculated and used to construct UPGMA dendrogram.

4.3. RESULTS

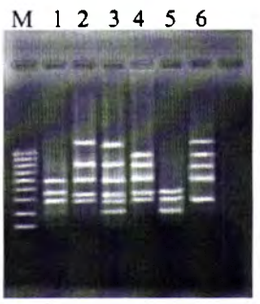
Three primers from OPA series (OPA 07, OPA 09 and OPA 16) were chosen, out of the 20 OPA series primers tested, on the basis of number of resolved bands and frequency of polymorphism among the samples. Twelve individuals from each of the eleven populations were screened for variation using the three selected primers and 36 RAPD products from each population were scored for presence or absence. The molecular weight of the products generally ranged from 100-1000 base pairs. Assuming that each RAPD product represented a single locus, all the loci [viz. 11 loci for OPA 07 (Fig. 4.1.), 12 for OPA 09 (Fig. 4.2.) and 13 for OPA 16 (Fig. 4.3.)] were found to be polymorphic at least in one of the individuals analysed.

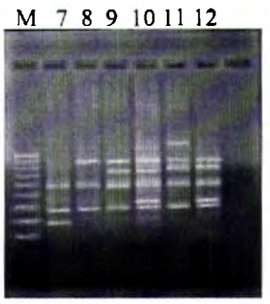
Figure 4.1. RAPD profiles of eleven sandal populations amplified by OPA 07 primer: lane M: DNA marker (100bp ladder), lane 1-6: profiles of 1-6 samples, lane 7-12: profiles of 7-12 samples.



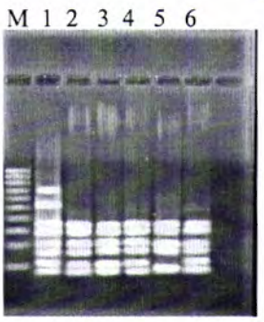
continued..

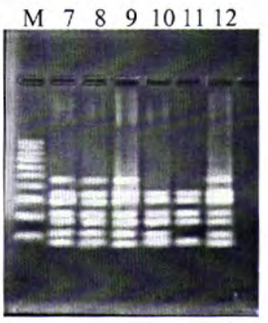
Marayoor



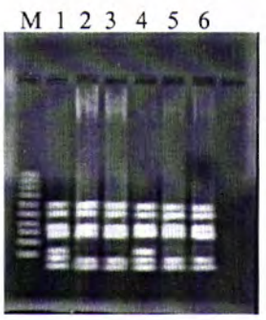


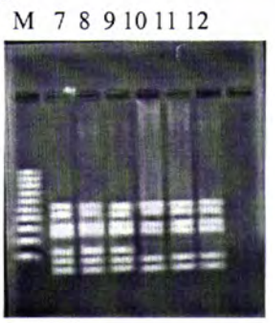
Ottappalam



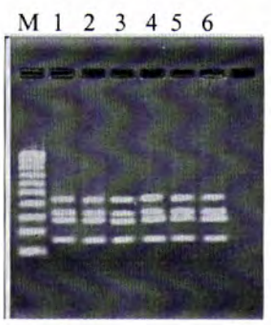


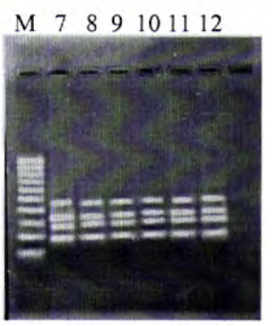
Shimoga





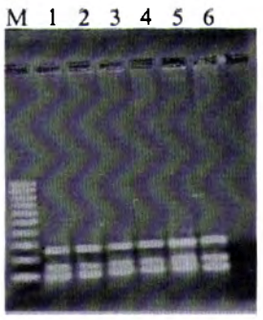
Thenmalai

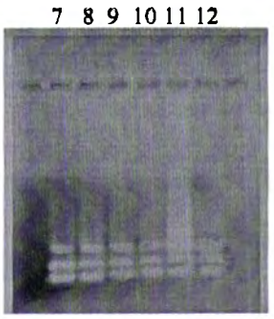




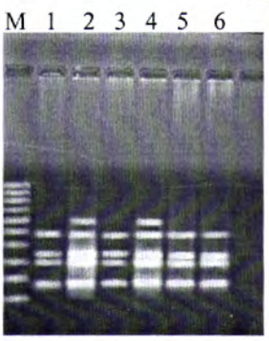
continued..

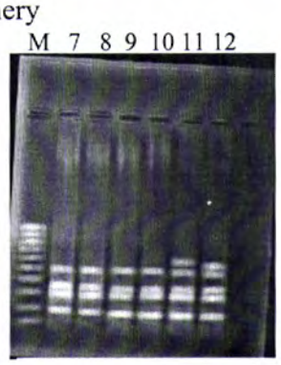
Thirupattur



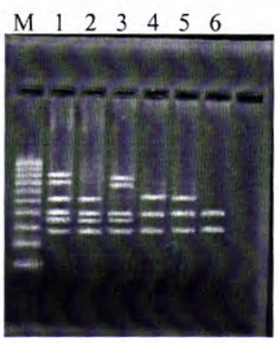


Wadakkanchery





Wayanad



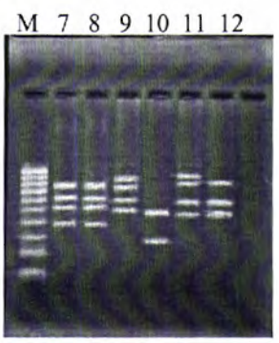
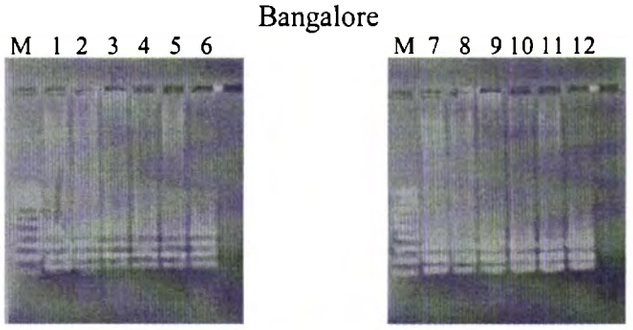
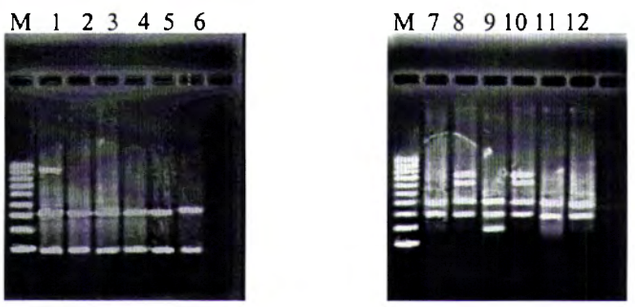


Figure 4.2. RAPD profiles of eleven sandal populations amplified by OPA 09 primer; lane M: DNA marker (100bp ladder), lane 1-6: 1-6 individual samples, lane 7-12: 7-12 individual samples.

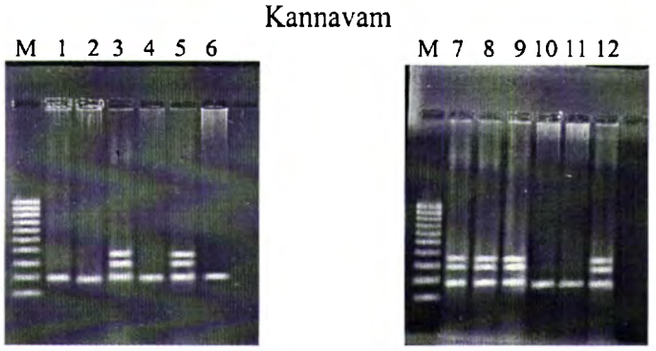
Bangalore



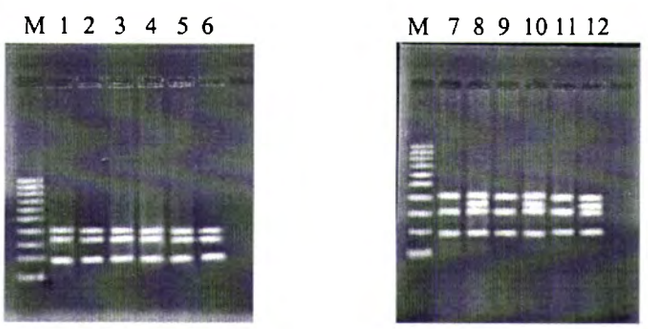
Chikkamagalur



Kannavam

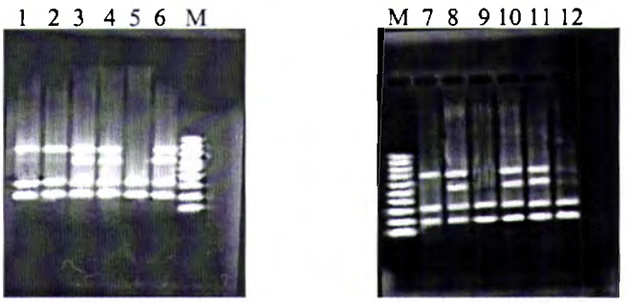


Kasargod

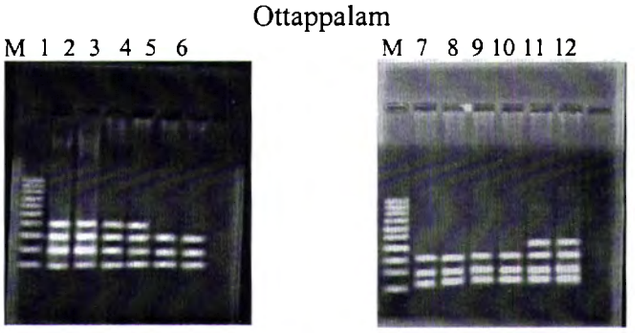


continued..

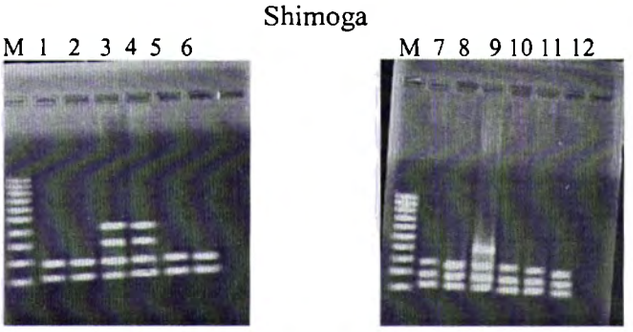
Marayoor



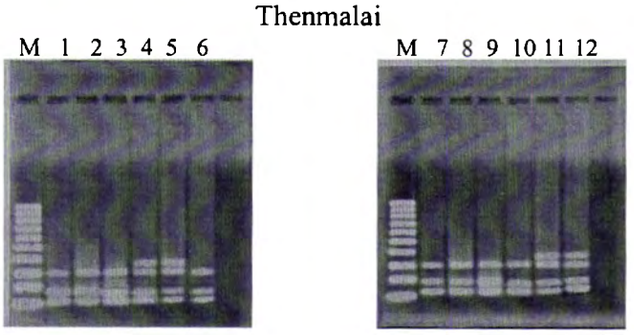
Ottappalam



Shimoga

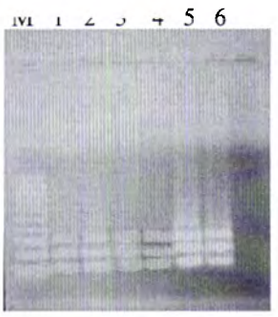


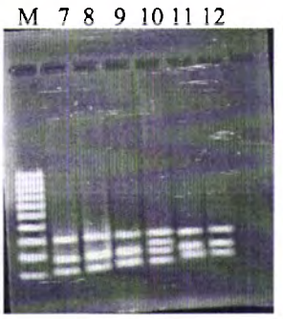
Thenmalai



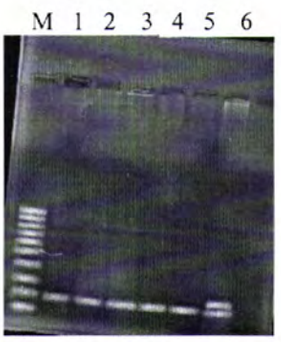
continued..

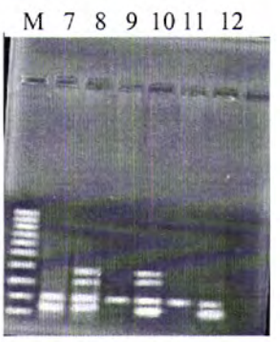
Thirupattur



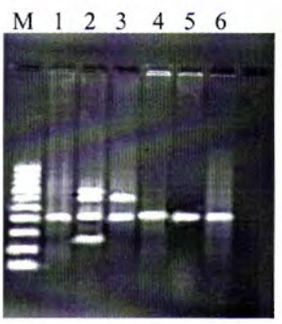


Wadakkanchery





Wayanad



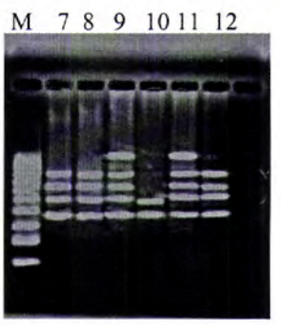
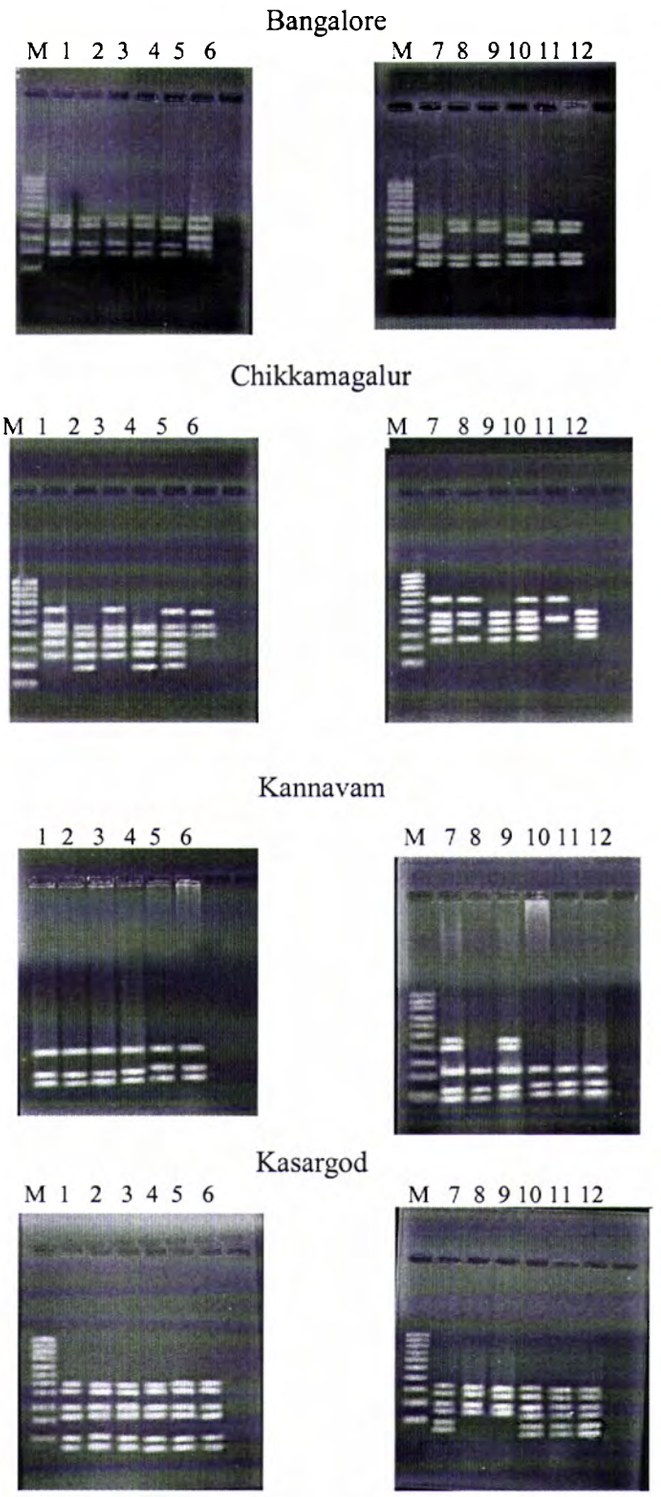
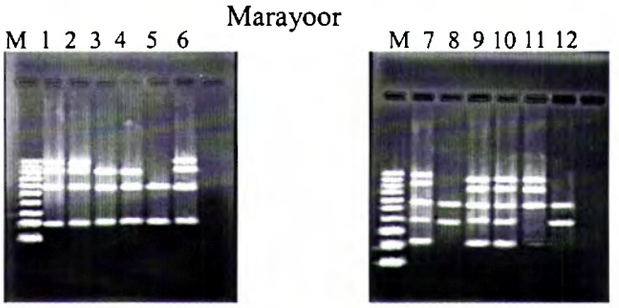


Figure 4.3. RAPD profiles of eleven sandal populations amplified by OPA 16; lane M: DNA marker (100 bp ladder), lane 1-6: 1-6 individual samples, lane 7-12: 7-12 individual samples.

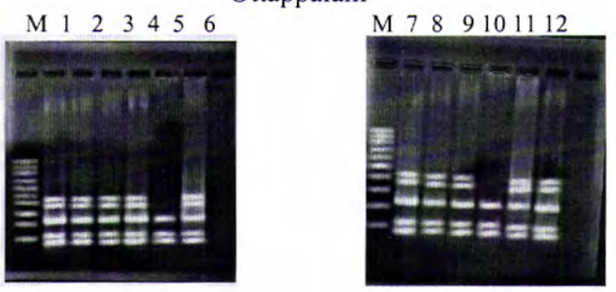


continued...

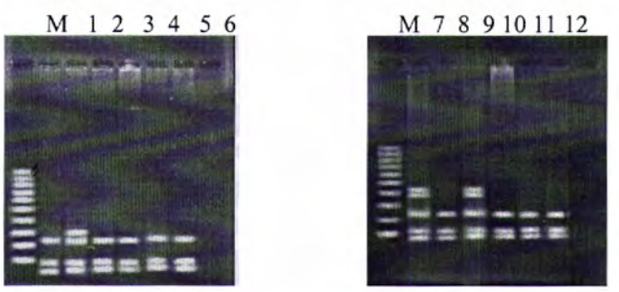
Marayoor



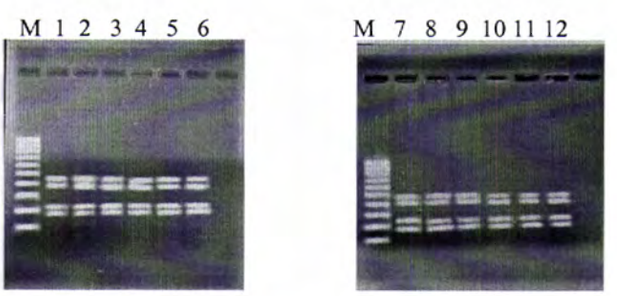
Ottappalam



Shimoga

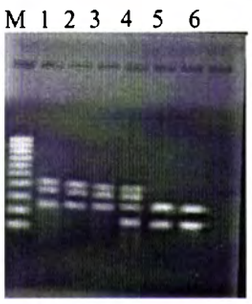


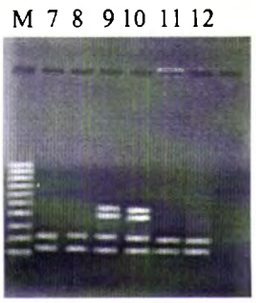
Thenmalai



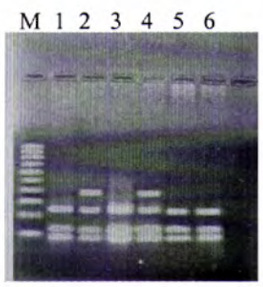
continued..

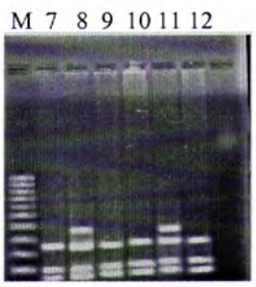
Thirupattur



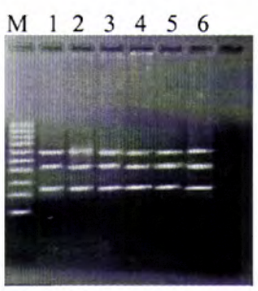


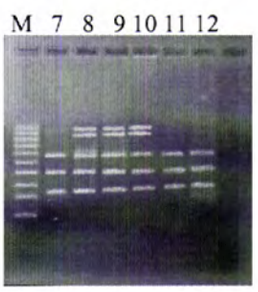
Wadakkanchery





Wayanad





At the population level, the per cent of polymorphic loci (ppl) varied from a minimum of 8.33 per cent (Kasargod and Thenmalai) to a maximum of 38.89 per cent (Marayoor and Wayanad) (Table 4.1.). The average observed number of alleles (na) range from 1.0833 (Kasargod and Thenmalai) to 1.3889 (Marayoor and Wayanad). The effective number of alleles (ne) range from 1.0433 (Wadakkanchery) to 1.2362 (Marayoor). Similarly Nei's (1973) gene diversity index (h) varied from 0.0316 (Wadakkanchery) to 0.1384 (Marayoor). Depending on locus, the observed number of alleles (na) and effective number of alleles (ne) are given in table 4.2. The average observed number of alleles per locus was 2. The average effective number of alleles per locus was 1.4531 and the mean gene diversity (h) was 0.2610.

The mean expected heterozygosity (H_S), the mean total genetic diversity (H_T) and genetic differentiation (G_{ST}) are given in table 4.3. The mean expected heterozygosity (H_S) was 0.0715, the mean total genetic diversity (H_T) was 0.2610 and the relative magnitude of genetic differentiation among population (G_{ST}) was 0.7262 indicating that 72.62 per cent of the total diversity was among populations while the rest (27.38%) of the total variations occurred within populations. The gene flow estimated from G_{ST} value, was found to be very low, 0.1855.

The standard genetic distance (D) unbiased for sample size (Nei, 1978) for all pair wise population comparisons were estimated (Table 4.4.). The genetic distance varied from 0.0776 (between Kasargod and Kannavam) to 0.5924 (between Wayanad and Ottappalam). An Unweighted Pair Group

Table 4.1. Comparison of populations for various genetic diversity measures.

Populations	Na	ne	h	npl	ppl
Marayoor	1.3889	1.2362	0.1384	14	38.89
Wayanad	1.3889	1.1748	0.1116	14	38.89
Thenmalai	1.0833	1.0643	0.0353	3	8.33
Wadakkanchery	1.1389	1.0433	0.0316	5	13.89
Ottappalam	1.1667	1.1253	0.0672	6	16.67
Kasargod	1.0833	1.0638	0.0352	3	8.33
Kannavam	1.1389	1.0582	0.0383	5	13.89
Bangalore	1.2778	1.1821	0.1023	10	27.78
Shimoga	1.1667	1.0473	0.0325	6	16.67
Chikkamagalur	1.3333	1.1915	0.1114	12	33.33
Thirupattur	1.1389	1.0620	0.0400	5	13.89

na = Observed number of alleles

ne = Effective number of alleles

h = Nei's (1973) gene diversity

npl = Number of polymorphic loci

ppl = Percentage of polymorphic loci

Table 4.2. Summary of genic variation statistics for all loci.

Locus	Sample Size	na	ne	h
OPA07-1	132	2	1.6575	0.3967
OPA07-2	132	2	1.9169	0.4783
OPA07-3	132	2	1.9993	0.4998
OPA07-4	132	2	1.4358	0.3035
0PAO7-5	132	2	1.9836	0.4959
OPA07-6	132	2	1.7473	0.4277
OPA07-7	132	2	1.9988	0.4997
OPA07-8	132	2	1.5657	0.3613
OPA07-9	132	2	1.2767	0.2167
OPA07-10	132	2	1.1816	0.1537
OPA07-11	132	2	1.0491	0.0468
OPA09-1	132	2	1.9995	0.4999
OPA09-2	132	2	1.1980	0.1653
OPA09-3	132	2	1.0078	0.0077
OPA09-4	132	2	1.8592	0.4621
OPA09-5	132	2	1.1271	0.1128
OPA09-6	132	2	1.7582	0.4312
OPA09-7	132	2	1.0715	0.0667
OPA09-8	132	2	1.0970	0.0884
OPA09-9	132	2	1.0339	0.0328
OPA09-10	132	2	1.0339	0.0328
OPA09-11	132	2	1.0160	0.0157
OPA09-12	132	2	1.0835	0.0771
OPA16-1	132	2	1.9997	0.4999
OPA16-2	132	2	2.0000	0.5000
OPA16-3	132	2	1.1508	0.1310
OPA16-4	132	2	1.8378	0.4559
OPA16-5	132	2	1.4967	0.3319
OPA16-6	132	2	1.1054	0.0954
OPA16-7	132	2	1.9912	0.4978
OPA16-8	132	2	1.8953	0.4724
OPA16-9	132	2	1.5332	0.3478
OPA16-10	132	2	1.0326	0.0316
OPA16-11	132	2	1.0696	0.0650
OPA16-12	132	2	1.0665	0.0624
OPA16-13	132	2	1.0339	0.0328
Mean	132	2	1.4531	0.2610
St. Dev		0.0000	0.3957	0.1937

sample size = Total number of populations x number of samples
na = Observed number of alleles
ne = Effective number of alleles
h = Nei's (1973) gene diversity

Table 4.3. Expected heterozygosity (H_S), total genetic diversity (H_T), genetic differentiation (G_{ST}) and rate of gene flow (Nm) per locus.

Locus	Sample Size	H_T	Hs	G_{ST}	Nm
OPA07-1	132	0.3967	0.0000	1.0000	0.0000
OPA07-2	132	0.4783	0.0416	0.9130	0.0476
OPA07-3	132	0.4998	0.0490	0.9019	0.0544
OPA07-4	132	0.3035	0.0701	0.7689	0.1503
0PAO7-5	132	0.4959	0.0000	1.0000	0.0000
OPA07-6	132	0.4277	0.0439	0.8973	0.0572
OPA07-7	132	0.4997	0.1272	0.7455	0.1707
OPA07-8	132	0.3613	0.1479	0.5907	0.3465
OPA07-9	132	0.2167	0.0557	0.7427	0.1732
OPA07-10	132	0.1537	0.0898	0.4155	0.7032
OPA07-11	132	0.0468	0.0430	0.0817	5.6180
OPA09-1	132	0.4999	0.0617	0.8765	0.0704
OPA09-2	132	0.1653	0.0000	1.0000	0.0000
OPA09-3	132	0.0077	0.0074	0.0389	12.3690
OPA09-4	132	0.4621	0.1339	0.7102	0.2040
OPA09-5	132	0.1128	0.0921	0.1830	2.2316
OPA09-6	132	0.4312	0.1587	0.6319	0.2913
OPA09-7	132	0.0667	0.0521	0.2187	1.7860
OPA09-8	132	0.0884	0.0588	0.3345	0.9949
OPA09-9	132	0.0328	0.0272	0.1697	2.4472
OPA09-10	132	0.0328	0.0272	0.1697	2.4472
OPA09-11	132	0.0157	0.0145	0.0798	5.7625
OPA09-12	132	0.0771	0.0561	0.2725	1.3345
OPA16-1	132	0.4999	0.1024	0.7953	0.1287
OPA16-2	132	0.5000	0.1085	0.7830	0.1386
OPA16-3	132	0.1310	0.0712	0.4568	0.5945
OPA16-4	132	0.4559	0.0817	0.8208	0.1092
OPA16-5	132	0.3319	0.0496	0.8505	0.0879
OPA16-6	132	0.0954	0.0662	0.3056	1.1363
OPA16-7	132	0.4978	0.2517	0.4943	0.5114
OPA16-8	132	0.4724	0.2805	0.4062	0.7309
OPA16-9	132	0.3478	0.0518	0.8511	0.0875
OPA16-10	132	0.0316	0.0285	0.0974	4.6309
OPA16-11	132	0.0650	0.0539	0.1713	2.4182
OPA16-12	132	0.0624	0.0416	0.3330	1.0015
OPA16-13	132	0.0328	0.0272	0.1697	2.4472
Mean	132	0.2610	0.0715	0.7262	0.1885
St. Dev		0.0375	0.0039		

Sample size = number of populations x number of individuals

Table 4.4. Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal).
(Pop1-Marayoor, Pop2-Wayanad, Pop3-Thenmalai, Pop4-Wadakkanchery, Pop5-Ottappalam, Pop6-Kannavam, Pop7-Kasargod, Pop8-Bangalore, Pop9-Shimoga, Pop10- Thirupattur, Pop11-Chikkamagalur).

pop ID	1	2	3	4	5	6	7	8	9	10	11
1	****	0.8907	0.7411	0.7316	0.6487	0.7068	0.7703	0.7932	0.7045	0.8436	0.7465
2	0.1158	****	0.6297	0.6266	0.5530	0.7221	0.6803	0.6779	0.6010	0.7549	0.6209
3	0.2996	0.4625	****	0.8589	0.8990	0.7757	0.8451	0.8873	0.8358	0.7774	0.7832
4	0.3126	0.4675	0.1521		0.9007	0.7725	0.8694	0.8073	0.8688	0.7328	0.7706
5	0.4328	0.5924	0.1064	0.1046	****	0.8009	0.8471	0.8502	0.8988	0.7055	0.8745
6	0.3471	0.3256	0.2540	0.2581	0.2220	****	0.9253	0.7611	0.7677	0.7152	0.7585
7	0.2610	0.3852	0.1683	0.1399	0.1659	0.0776	****	0.7982	0.8585	0.7196	0.8304
8	0.2317	0.3888	0.1195	0.2141	0.1623	0.2730	0.2254	****	0.7902	0.8867	0.8657
9	0.3503	0.5092	0.1793	0.1407	0.1067	0.2643	0.1526	0.2355		0.7052	0.8590
10	0.1701	0.2812	0.2518	0.3109	0.3488	0.3352	0.3291	0.1202	0.3493	****	0.7286
11	0.2924	0.4766	0.2444	0.2605	0.1341	0.2764	0.1859	0.1442	0.1520	0.3167	****

Method with Arithmetic means (UPGMA) dendrogram is constructed to estimate the differentiation of the eleven populations using Nei's genetic distance (1978). Two main clusters with clear separation were observable in the dendrogram (Fig. 4.4.). The first main cluster consisted of 3 populations, of which Marayoor and Wayanad formed one cluster, which in turn was linked to Chikkamagalur. The second main cluster was a combination of three sub clusters each of 2 populations viz. Kasargod and Kannavam, Wadakkanchery and Ottappalam, Thenmalai and Bangalore, together with two linked single populations.

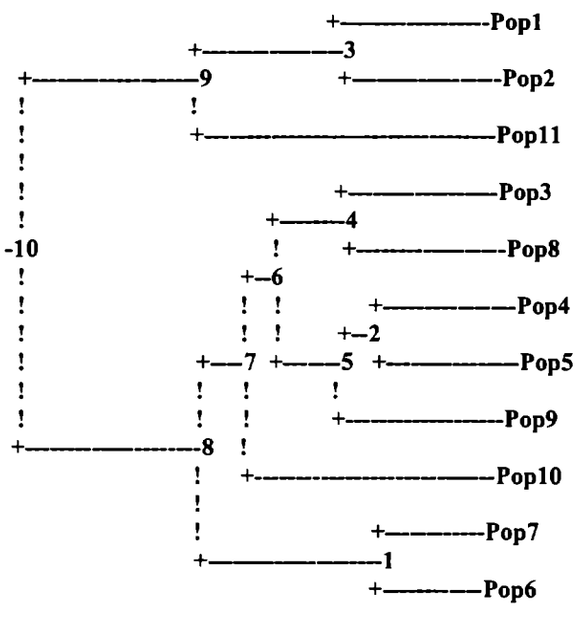
4.4. DISCUSSION

The rate of depletion of sandalwood resources in India is very rapid and the gap between the supply and demand is widening over time (Ananthapadmanabha, 2000). The understanding of the level of genetic variation and distribution of that variation in *Santalum album* populations is therefore crucial. The use of RAPD assay to identify genetic variation is preferred over the conventional, morphological and biochemical markers, since RAPD markers are unaffected by environmental changes and the growth stage of the experimental material; thus making them highly reliable (Newbury and Ford-Lloyd, 1993). The RAPD results reported here provide new information regarding the organization of genetic variation in natural populations of *S.album* in South India.

The various genetic diversity measures within populations viz. per cent of genetic polymorphism, number of alleles per locus and amount of

Figure 4.4. Dendrogram based on Nei's (1978) genetic distance.

(Pop1-Marayoor, Pop2-Wayanad, Pop3-Thenmalai, Pop4-Wadakkanchery, Pop5-Ottappalam, Pop6-Kannavam, Pop7-Kasargod, Pop8-Bangalore, Pop9-Shimoga, Pop10- Thirupattur, Pop11-Chikkamagalur).



Between	And	Length
10	9	6.42166
9	3	5.49265
3	pop1	5.78843
3	pop2	5.78843
9	pop10	11.28108
10	8	6.92874
8	7	1.42174
7	6	0.60404
6	4	2.77138
4	pop3	5.97685
4	pop8	5.97685
6	5	2.56291
5	2	0.95414
2	pop4	5.23118
2	pop5	5.23118
5	pop9	6.18532
7	pop11	9.35227
8	1	6.89472
1	pop6	3.87929
1	pop7	3.87929

heterozygosity were determined. The mean number of alleles was 2 which was higher than the average of 1.84 in outcrossing wind pollinated woody plants (Ibrahim, 1996). The effective number of allele (1.4531) was lower than actual number of alleles. This means that there were some genes in which the frequencies were low and not contributing much to the population genetic variation as reported by Huang and Zhang (2000) in Masson pine. Similarly the mean expected heterozygosity (0.0715) was lower than that of the outcrossing wind-pollinated woody plants (0.154) reported by Moran (1992), House and Bell (1994) and Martins-Corder and Lopes (1997).

In the present study, the mean total genetic diversity of the analysed populations was 0.2610 and the partitioning of genetic diversity among populations, had a high value (G_{ST}=0.7262). High G_{ST} values were also reported in some pine species (Schiller, 1986; Hamrick and Godt, 1989). High G_{ST} values were reported to happen when large single population was fragmented into small individual breeding units as a result of human disturbances, random genetic drift and differential natural selection (Gibson and Hamrick, 1991). The result of the present study is in contrast to earlier findings of other tree species in which most of the genetic diversity resides within rather than between populations such as in *E. urophylla* (House and Bell, 1994), *Azadirachta indica* (Kundu, 1999) and *Eucalyptus grandis* (Grattapaglia *et al.*, 1997).

The estimated amount of gene flow (0.1855) was found to be very low. Hamrick and Nason (2000) suggested 4 migrants per generation (Nm = 3.54) as the minimum to prevent differentiation due to drift. In the isolated populations where such gene exchange cannot exist, the population must have a size

size sufficient to avoid the risk of genetic erosion. Most of the analysed populations of *S. album* occur as fragmented widely scattered populations with varying densities. The restricted amount of gene flow between the isolated populations might have resulted in increased inbreeding within the populations. Consequently, the amount of genetic variation within population might be reduced and ultimately resulted in the genetic differentiation of the isolated populations. The comparatively higher genetic diversity measures in Marayoor and Wayanad might be due to the high density population as compared to others and thus evolutionary forces can operate on a large number of genotypes resulting in an increased amount of gene diversity within the population.

It is generally known that genetic diversity within population is influenced by the geographic distribution of the species, mating system and the method of seed dispersal (Hamrick *et al.*, 1992). The eleven populations under study covered a broad geographic range differing in rainfall (700-4000mm) and altitude (60-1000m) (Fig. 3.1.). Of the analysed eleven populations, the most genetically similar populations, Kasargod and Kannavam are geographically separated only by a distance of about 70 km while the genetically distant populations, Wayanad and Wadakkanchery are separated by about 225 km.

The UPGMA dendrogram revealed the genetic relatedness of the eleven populations. The genetic separation of the populations was partly in agreement with geographic isolation. The Kasargod and Kannavam, which are the closely located populations geographically, were the most genetically similar populations by RAPD analysis and formed a single cluster. Even though Shimoga and Thenmalai are separated far geographically, Wayanad and

Wadakkanchery populations had the highest genetic distance coefficient. The lack of correlation between geographic and genetic distance in some cases might be due to the geographic isolation of the area and the evolutionary forces like mutation, migration and natural selection operating in the area (Huang and Zhang, 2000). This partial disagreement in correlation between geographic and genetic distance has been observed in many tree species, such as *Eucalyptus globulus* (Nesbitt *et al*, 1995), Brazil nut (Kanashiro *et al*, 1997) and Himalayan poplar (Rajagopal *et al*., 2000).

Marayoor and Wayanad are the only populations with above 30 per cent genetic polymorphism. However, given the fact that genetic variation in *S. album* is distributed more or less randomly among populations, sampling or preservation of any one population may not insure that all the variation in *S. album* is captured. Some populations such as Marayoor included in the study have been severely disturbed recently, by the spike disease and indiscriminate felling of superior trees. In these cases, outplanting of seedlings raised from seeds, back to the disturbed sites would help maintain an effective population size and the evolutionary forces naturally operating can restore the genetic diversity of the natural populations.

CHAPTER 5

PROTEIN, ISOZYME AND RAPD PROFILES OF SPIKE DISEASE EVADED AND INFECTED SANDAL TREES

5. PROTEIN, ISOZYME AND RAPD PROFILES OF SPIKE DISEASE EVADED AND INFECTED SANDAL TREES

5.1. INTRODUCTION

Spike disease, the most serious disease of sandal is characterized by extreme reduction in size of leaves accompanied by stiffening and reduction in length of internodes; in advanced stage the whole shoot looks like a spike inflorescence (Fig. 5.1).

Figure 5.1. Healthy and Spiked sandal trees

Diseased trees die within 1-3 years after the appearance of the symptoms (Rangaswamy and Griffith, 1941; Ghosh *et al.*, 1985; Ghosh *et al.*, 1992). The causative agent of the disease is a non-culturable phytoplasma found in the phloem of the infected trees (Dijkstra and Le, 1969; Hull *et al.*, 1969; Verma *et al.*, 1969). The disease was reported from Coorg in 1899 (Barber, 1903). Now the disease is

prevalent in almost all the major sandal growing Southern states of India. The spread of the disease is supposed to be through insect vectors. Ghosh *et al.* (1985) reported *Redarator bimaculatus* as the insect vector in Marayoor forest range of Kerala state. Within the heavily infected patches of Marayoor forest reserve, a few trees devoid of any disease symptoms for the past 20 years were observed. These had either chance-evaded the disease or were capable of resisting the disease (Balasundaran, 1998).

For a long-term solution to the problem of spike disease and for sustainable supply of superior sandalwood, development of spike disease resistant trees through breeding could be the best method (Venkatesh and Kedarnath, 1963; Venkatesh, 1978; Srimathi *et al.*, 1980). The disease evaded/resistant trees found in the spike disease affected area of Marayoor could be used as an ideal starting material for this purpose. But before using the disease evaded trees for further tree improvement programmes, it has to be confirmed whether the disease evasion is only a chance occurrence or whether it has got any stable genetic basis.

Molecular markers provide a new alternative to conventional disease resistance screening. These markers allow us to identify resistance alleles without the use of pathogens and without the influence of environmental factors (Kuginuki *et al.*, 1997). As the quantitative nature of disease resistance markers are less amenable to classical genetic analysis, more powerful methods of analysis, such as marker-assisted analysis are required (Jorge *et al.*, 2000).

In the present investigation, protein, isozyme and RAPD analysis were carried out in disease evaded and infected trees found in spike disease affected forests of Marayoor to investigate whether the disease evasion has got any genetic basis.

5.2. MATERIALS AND METHODS

5.2.1. Sample collection

Leaf samples were collected from 12 mature trees, >20-year-old, selected randomly from each group of disease evaded and infected trees identified in the diseased tract of sandal reserve 51 of Marayoor range in Kerala. Samples were brought to laboratory either in liquid N_2 or by dipping the excised branches vertically in water and covered with wet polyethylene bags. The samples were subjected to protein, isozyme and RAPD analysis.

5.2.2. Total protein extraction

Total proteins were extracted according to modified Lowry's (Lowry *et al.*, 1951) procedure. The samples were homogenized by grinding in a pre-chilled mortar in 0.1M phosphate buffer, pH 7, centrifuged at 5000 rpm for 10 minutes and the total proteins precipitated by adding 100 per cent trichloro acetic acid to the supernatant and keeping overnight at ice cold temperature. The precipitated protein, after acetone wash, resuspended in Laemmli's (1970) sample extraction buffer containing 0.5 M Tris HCl, 10 per cent SDS, 1 per cent glycerol and 2 per cent 2-mercaptoethanol and heated on a water bath at 95°C for complete denaturation.

5.2.2.1. SDS-PAGE electrophoresis

Total proteins were subjected to one-dimensional SDS-PAGE, according to the method of Laemmli (1970) on a minigel (Biorad, USA) system. After electrophoresis, gels were stained for 45 minutes in coomassie brilliant blue R-250 and then destained with several changes of 40 per cent methanol to remove background staining. The protein molecular weight marker (Genei, Bangalore) was used as standard in the first lane of the gel to facilitate protein molecular weight determination.

5.2.3. Isozyme analysis

Isozyme analysis was carried out in 12 samples of both infected and disease evaded trees for the four isozyme systems viz. peroxidase, esterase, malate dehydrogenase and shikimate dehydrogenase. The methods and protocols followed for sample preparation, electrophoresis and gel staining were same as described in paragraph 3.2., 3.2.1. and 3.2.2.

5.2.4. RAPD analysis

RAPD analysis was carried out in 12 samples of both infected and disease evaded trees using the following three primers viz. OPA 07 (GAAACGGGTG), OPA 09 (GGGTAACGCC) and OPA 16 (AGCCAGCGAA) from twenty primers of OPA series and one primer, OPB 03 from OPB series (CATCCCCCTG) selected based on the number and reproducibility of amplification products. The methods and protocols for DNA extraction, PCR amplification, gel electrophoresis and staining were described in paragraph 4.2.2., 4.2.3. and 4.2.4.

5.3. RESULTS

5.3.1. Total protein analysis

SDS-PAGE of total proteins of 12 genotypes belonging to each group of the disease evaded and infected trees showed a maximum number of 17 polypeptide bands of homogenous molecular weights, protein profiles of 3 samples from each group is provided in figure 5.2. The molecular weights ranged from 14.3 kD to 97.4 kD and R_f values ranged from 0.17 to 0.84 with uniform intensity, except for one band corresponding to $R_f = 0.53$, which showed an increase in intensity in diseased trees.

5.3.2. Isozyme analysis

All the four analysed isozymes viz. peroxidase, esterase, malate dehydrogenase and shikimate dehydrogenase showed the same banding profiles in both infected and disease evaded trees. Isozyme profiles of only 5 samples from each group is provided in figure 5.3. Peroxidase and malate dehydrogenase isozymes showed a single band of activity at $R_f = 0.37$ and 0.42 respectively, esterase and shikimate dehydrogenase isozymes showed two bands at $R_f = 0.54$ and 0.56 and $R_f = 0.67$ and 0.72 respectively. No significant difference was noticed in infected and disease evaded samples.

5.3.3. RAPD analysis

A total of 17 discrete PCR amplified products ranging in size form 100bp to 600bp were obtained i.e. six bands for OPA 07, three bands for OPA 09, three bands for OPA 16 and five bands for OPB 03. For the purpose of comparison, six

Figure 5.2. Protein profiles of spike diseased and disease evaded sandal trees; lane M: protein molecular weight marker in kilo Daltons, lane 1-3: protein profiles of infected trees, lane 4-6: protein profiles of disease evaded trees.

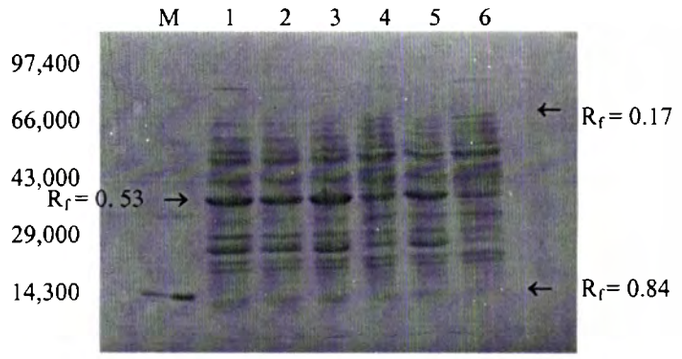
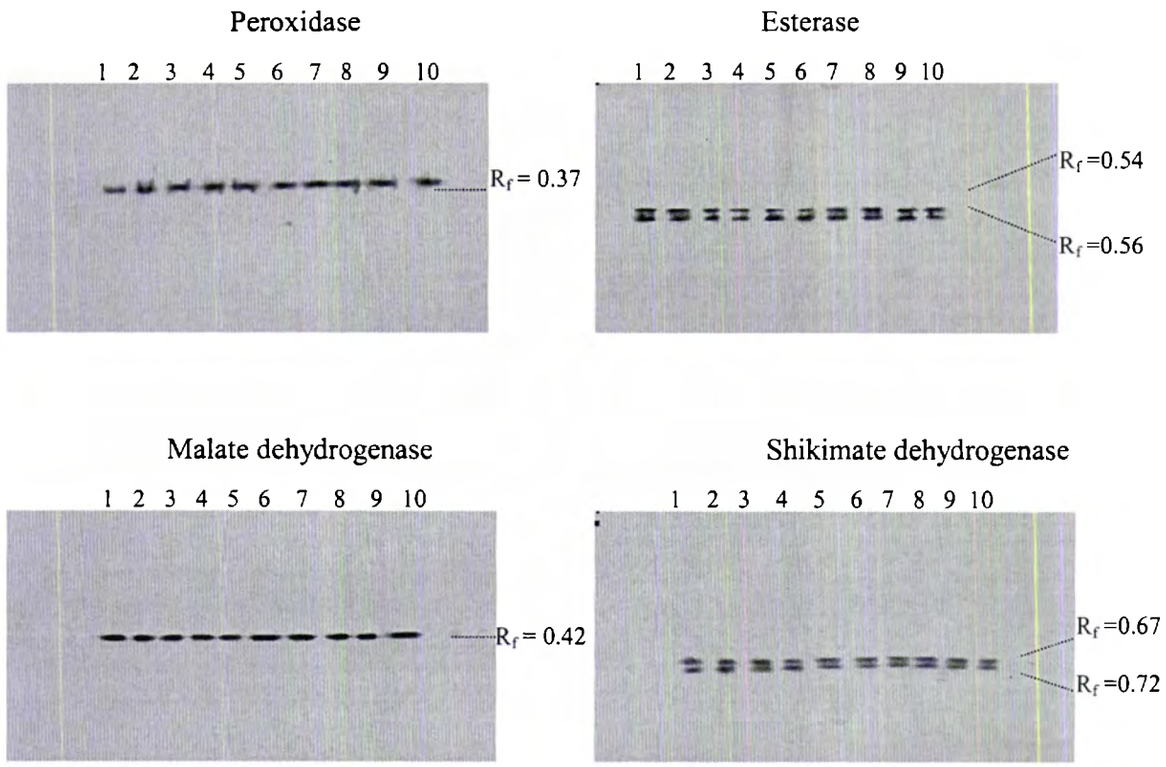


Figure 5.3. Isozyme profiles of spike diseased and disease evaded sandal trees; lane 1-5: isozyme profiles of diseased trees, lane 6-10: isozyme profiles of disease evaded trees.



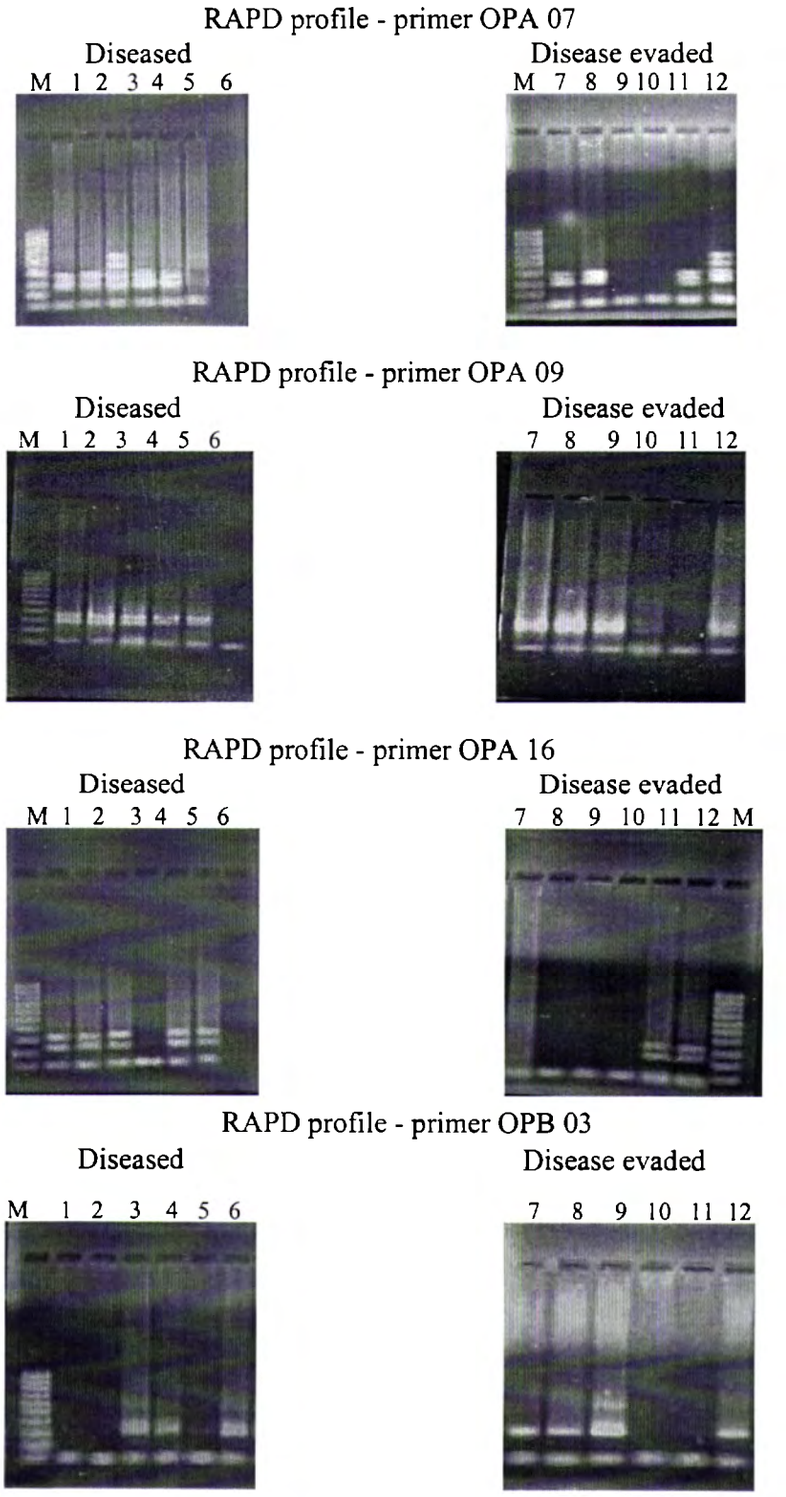
samples each of the diseased and disease evaded trees are provided in figure 5.4. Of these OPA 09 and OPB O3 gave two types of banding patterns due to genotypic polymorphism. The majority of these RAPD products were identical both in the disease evaded and infected trees. The genotypic differences in banding patterns amplified by OPA 09 and OPB 03 primers were present in both diseased and evaded trees.

5.4. DISCUSSION

Breeding for disease resistance and restocking the forest with resistant varieties is an effective approach to minimize the depletion of forest stock due to illicit felling and disease problems. Utilizing spike disease evaded trees located in highly diseased tract for resistance breeding has been suggested by Venkatesh and Kedarnath (1963) and Venkatesh (1978). Such an effort was made with the premise that the disease free trees might have escaped the disease either due to genetic resistance against the pathogen/vector or due to a chance evasion of infection. Eventhough, the outcome will not be conclusive, the present study is an attempt to investigate whether the disease evaded trees show any peculiarity with respect to total proteins, isozymes and RAPD profiles when compared with those of infected trees.

Invasion of host tissue by the pathogen results in drastic changes in cellular metabolism and the cell to cell interaction, and cell wall metabolism are most

Figure 5.4. RAPD profiles of spike diseased and disease evaded sandal trees; lane M: DNA marker, lane 1-6: diseased samples, lane 7-12: disease evaded samples.



probably changed after pathogen infestation (Van Loon, 1985). This results in the presence of new polypeptides and enzymes (isozymes) which could be part of many reactions in the intra cellular fluids of cells after pathogen infestation (Parent and Asselin, 1983). The pathogen related protein might play a role in the localization of the pathogen as well as in the acquired systemic resistance. It was suggested that these pathogen related proteins could act as genetic markers and they show polymorphism, differ in electrophoretic mobility in PAGE and in most cases has got a monogenic inheritance (Redolfi *et al.*, 1982). Four new molecular proteins appeared in relatively higher amounts in total soluble protein profiles of TMV infected *Nicotiana* (Ahl *et al.*, 1982). Five polymorphic protein bands (24 - 58 kD) were reported in green leaf hopper resistant rice genotypes (Padmavathi *et al.*, 1999). However, the absence of specific polypeptide bands in spike disease evaded trees in the present investigation might be due to a probable non-genetic basis of the spike disease evasion.

Isozymes may function in disease resistance indirectly by affecting biochemical process which in turn influence disease resistance (Ye *et al.*, 1990). The isozymes play an integral part in the biosynthesis of plant cell wall components including lignin, suberin and cross linked extensions as well as in a variety of cellular processes. Peroxidase plays an important role in lignification and wall thickening which are well known defense responses to pathogens. Isozyme activity linked to resistance has been reported in many plant species; a systemic increase in peroxidase activity is associated with induced systemic resistance in *Cucumber*

(Hammerschmidt *et al.*, 1982); changes in polyphenol oxidase in *Fusarium* infected *Cucumis* is reported by Rueveni (1983). Other reports include changes in maize peroxidase associated with variation in susceptibility to *Bipolaris maydis* (Akthar and Garraway, 1987), peroxidase isozyme markers linked to TMV resistance reported in *Nicotiana* (Ye *et al.*, 1990), use of peroxidase activity as a biochemical marker for resistance to *Pseudoperonospora* in *Cucumis* (Rueveni *et al.*, 1992) and peroxidase activity during susceptible and resistant interaction between cassava and *Xanthomonas cassavae* reported by Periera *et al.*, 2000. Like the protein profiles, the four isozyme profiles (viz. peroxidase, malate dehydrogenase, esterase and shikimate dehydrogenase) of major metabolic pathways were also found to be identical in disease evaded and susceptible trees of sandal in the present study.

In order to use RAPD markers for resistant breeding, at least a single locus linked to the resistance must be identified unequivocally. Several genes conferring resistance to pathogen have been characterized at the molecular level and most of these genes are probably involved in signal transduction mechanisms (Barker, 1997). RAPD markers linked to QTLs concerned with disease resistance have been detected for *Pseudomonas* resistance in tomato (Martin *et al.*, 1991), to downy mildew resistant gene in lettuce (Paran and Michelmore, 1993), blast disease resistance in rice (Naqvi *et al.*, 1995), *Plasmodifora* resistance in *Brassica* (Grandclement and Thomas, 1996), tomato spotted virus resistance in *Capsicum* (Moury *et al.*, 1997), nematode resistance in *Pinus* (Susumu Goto, 1998) and *Xanthomonas* infection in rice (Li *et al.*, 1999).

In the present study, no specific locus linked to disease resistance could be detected in the RAPD products of disease evaded/infected trees analysed with the four primers. The minor differences observed in RAPD profiles were present in both disease evaded and infected trees and hence cannot be attributed to resistance. The polymorphism might be due to genotypic differences unrelated to spike disease. It had been reported earlier that there was no natural resistance to spike disease (Iyengar, 1955; Nayar, 1986). However, with the present study, it was not possible to prove the absence of genetic resistance against spike disease conclusively. Adequate number of mature trees were not available to have a detailed study. Further conclusive evidence will be possible only through a wider study of disease evaded parents and their progenies showing co-segregating markers linked to disease resistance.

CHAPTER 6

PROTEIN, ISOZYME AND RAPD PROFILES DURING ONTOGENY OF SOMATIC EMBRYOS

6. PROTEIN, ISOZYME AND RAPD PROFILES DURING ONTOGENY OF SOMATIC EMBRYOS

6.1. INTRODUCTION

Spike disease, the most serious phytoplasmal disease of sandal is prevalent in Karnataka, Tamil Nadu and Kerala, the most important sandal growing states in India (Ghosh *et al.*, 1985). Among the heavily infected patches of Marayoor in Kerala, there are a few disease evaded trees which are presumed to be either disease resistant or escapes. These trees were considered as ideal starting material for reforestation of the disease affected areas (Balasundaran, 1998) eventhough the disease evaded trees are yet to be proved as genetically resistant to spike disease through protein, isozyme and RAPD studies (Chapter 5). In order to produce large number of true-to-type planting stock, the method of micropropagation of the disease evaded trees were attempted.

In India, work on tissue culture of sandal was carried out mainly in two laboratories, Indian Institute of Science, Bangalore (Lakshmi Sita, 1986, 1992; Lakshmi Sita *et al.*, 1994) and Bhabha Atomic Research Centre, Mumbai (Bapat and Rao, 1979, 1984, 1998; Rao and Raghavaram, 1983). Although, shoot morphogenesis and multiple shoot formation were reported (Rao *et al.*, 1984; Sanjaya *et al.*, 1998; Pradhan and Saiju, 1999), regeneration of plantlets through somatic embryogenesis was suggested to be the most efficient method of mass propagation in *Santalum album* (Bapat and Rao, 1984). Sandal has got a very high frequency of somatic embryogenesis but the successful utilization of the technique

is hampered due to the high per cent of abnormal embryogenic plantlets and their poor rate of survival (Rao and Bapat, 1995). Attempts to multiply sandal through somatic embryogenesis, carried out in our laboratory also did not yield encouraging results (Balasundaran and Muralidharan, 2001). The embryos were highly abnormal and the plantlets formed did not establish. A better knowledge of the biochemical and molecular events leading to the morphogenetic differentiation of somatic cell to a whole plant is expected to enable us to understand whether the abnormal development has a genetic/molecular basis.

Karyological analysis at different developmental stages of plant tissue culture can often reveal significant chromosomal abnormalities such as alterations in ploidy levels and structural rearrangements of segments of chromosomes (Edallo *et al.*, 1981; Karp and Bright, 1985; Karp, 1991). However, study of chromosomal aberrations cannot reveal alterations in individual genes and gene products (Brown *et al.*, 1993). Studies of total protein (Sung and Okimoto, 1981; Komamine *et al.*, 1992) and isozyme analysis (Chawla, 1988; Fransz *et al.*, 1989; Feirer and Simon, 1991; Bapat *et al.*, 1992; Martinelli *et al.*, 1993; Samantaray *et al.*, 1999) provide a relatively convenient tool for examining biochemical changes. Since isozymes are codominantly expressed and developmentally regulated, their study and analysis are important steps towards establishing the biochemical events, which trigger the induction and subsequent development of somatic embryos (Bapat *et al.*, 1992).

A precise determination of changes in a particular gene sequence resulting from tissue culture can be obtained by RFLP analysis (Muller *et al.*, 1990; Brown

et al., 1991; Morere-Le Paven *et al.*, 1992; Jazdzewska *et al.*, 2000). However, the method is time consuming and laborious, particularly due to the difficulty in the sequence identification and construction of probes (Brown *et al.*, 1993). The efficiency of PCR based RAPD can be easily and effectively applied to determine the level of variability in plant material at all stages of culture and growth (Brown *et al.*, 1991; Jazdzewska *et al.*, 2000) and to assess genetic integrity of tissue culture raised plants (Isabel *et al.*, 1993; Rani *et al.*, 1995; Parani *et al.*, 1997; Padmesh *et al.*, 1999).

In this chapter, we provide evidence for the stage-specific changes associated with somatic embryogenesis in *S. album*, at both the DNA and protein levels, through biochemical (protein/isozyme analysis) and molecular (RAPD) techniques.

6.2. MATERIALS AND METHODS

6.2.1. Somatic embryogenesis

The internodes collected from > 20-year-old, spike disease evaded trees (12 trees) located at Marayoor were used as materials for the present study. The explants were washed in tap water with extran (Merck), kept for 15 minutes in running tap water, sterilized with 0.1 per cent $HgCl_2$ for 8 minutes and washed in sterile water four times before inoculation onto culture medium.

Murashige and Skoog's (1962) basal medium was used at all stages of culture. The pH of the medium was adjusted to 5.7 prior to autoclaving at 120°C at 15-lbs/in^2 pressure for 20 minutes. The auxins (2,4-D and IAA) and cytokinins

(BAP and Kinetin) either singly or in combinations along with growth adjuvants, abscisic acid (ABA), casein hydrolysate (CH) and activated charcoal were used at different stages of embryogenesis. All cultures on semi-solid media were routinely incubated at 25±1°C under diffused fluorescent light with 15 hour photoperiod. Maintenance transfers to fresh medium were on a monthly schedule.

6.2.2. Biochemical and molecular analysis

6.2.2.1. Total protein analysis

The tissues at four sequential stages viz. compact callus stage, embryo induction stage, embryo maturation stage and fully developed embryos were analysed for total proteins. The protocols followed for sample preparation and electrophoresis were the same as described in paragraph 5.2.2. and 5.2.2.1.

6.2.2.2. Isozyme analysis

Samples at five different developmental stages of somatic embryogenesis (viz. compact callus, friable callus, embryo induction stage, embryo maturation stage and mature embryos) were used for analysis of four isozymes viz. peroxidase, esterase, malate dehydrogenase and shikimate dehydrogenase. The protocols followed for sample preparation, electrophoresis and gel staining were the same as described in paragraph 3.2., 3.2.1. and 3.2.2.

6.2.2.3. RAPD analysis

RAPD analysis was carried out at the five different developmental stages of somatic embryogenesis (viz. compact callus, friable callus, embryo induction stage, embryo maturation stage and mature embryos) using three primers viz OPA 07

(GAAACGGGTG), OPA 09 (GGGTAACGCC) and OPA 16 (AGCCAGCGAA) from twenty primers of OPA series, selected based on the number and reproducibility of amplification products. The methods and protocols for DNA extraction, PCR amplification, gel electrophoresis and staining were same as described in paragraph 4.2.2., 4.2.3. and 4.2.4.

6.3. RESULTS

6.3.1. Callus initiation

Compact proliferating callus developed from internode explants on MS medium fortified with 2,4-D (0.1mg/l) and Kinetin (1mg/l) in 90 per cent of the cultures (Fig. 6.1.). The compact callus was routinely subcultured onto the same medium at 30 days interval. After 5-6 subcultures, white friable soft calli appeared from the sides and surface of the brown compact callus (Fig. 6.2.). This friable callus could be maintained for a long period without loosing its embryogenic capacity.

6.3.2. Embryo induction

The friable soft callus, when subcultured onto MS basal medium supplemented with IAA/BAP either singly or in combinations gave rise to white friable granular embryogenic callus (Fig. 6.3.). Of the different combinations tried, IAA (0.5 mg/l) and BAP (0.5 mg/l) were found to be suitable for the induction of embryogenic callus. After 2 weeks, the embryogenic callus showed somatic embryo induction in the same medium (Fig. 6.4.)

Figure 6.1. Compact callus

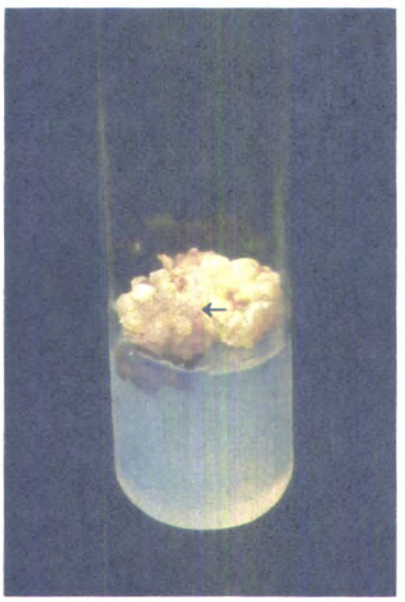


Figure 6.2. Friable callus

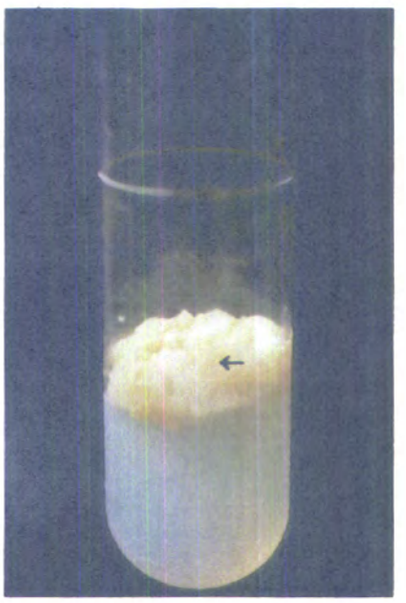


Figure 6.3. Embryogenic callus

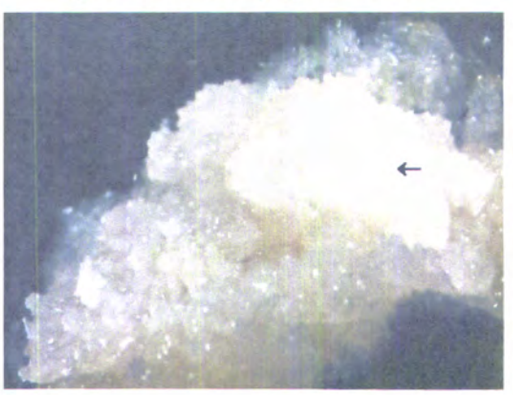
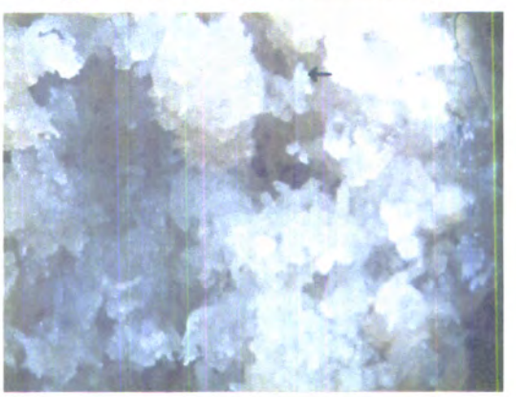


Figure 6.4. Embryo induction stage



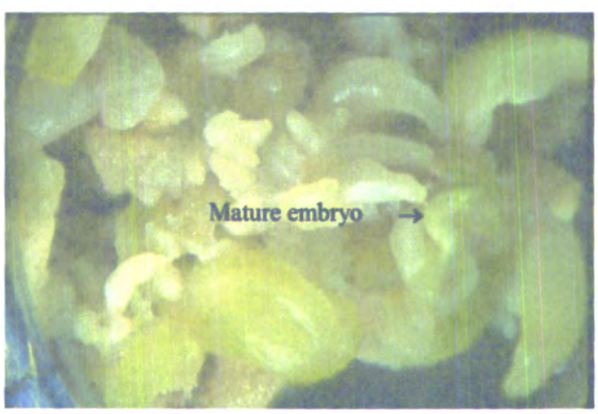
Somatic embryos followed the same stages of development as that of zygotic embryos (globular, heart or torpedo). Somatic embryos of different stages of development were found in the same culture vessel and adventitious embryo

formation in which embryos formed adventitiously on somatic embryos were also observed.

6.3.3. Embryo maturation

Many of the somatic embryos thus produced, developed asynchronously and germinated precociously, some with atypical morphology. This type of precocious germination (without passing through characteristic stages of development) in somatic embryos of sandal was found to be controlled to some extent by the addition of abscisic acid (ABA) to the induction medium at concentrations ranging from 0.1μM - 0.5μM. Addition of ABA at 3μM concentration gave rise to embryos which were mostly normal in appearance (Fig. 6.5.).

Figure 6.5. Embryo maturation stage

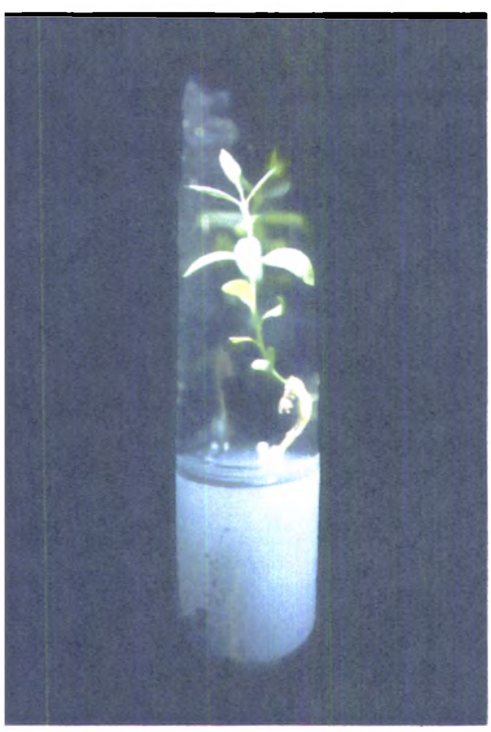


6.3.4. Embryo conversion to plantlets

Eventhough, somatic embryos were produced in higher frequency, only 20 per cent of the normal embryos were converted to plantlets in the final stage. The development of plantlets from somatic embryos was attempted by transferring

single embryos at the dicotyledonous stage to a medium with lower concentration of sucrose and growth regulators. This separation of embryos was found to have a stimulating effect on their germination. Among the combinations of hormones tried, quarter strength MS medium containing IAA (0.5 mg/l), IBA (0.5 mg/l) and GA_3 (1mg/l) was found to be ideal for embryo conversion to plantlets. Along with normal plantlets with well-developed taproot and shoot (Figs. 6.6. and 6.7.), abnormal plantlets either with prominent shoot and no root or with well developed taproot and no shoot were also observed.

Figure 6.6. Mature somatic plantlet. **Figure 6.7.** Plantlet growing in vermiculite



6.3.5. Total protein analysis

Analysis of protein patterns revealed approximately 26 polypeptides (Fig. 6.8.). The protein profile at all the developmental stages showed an array of proteins with molecular weight ranging from 14.3 kD to 97.4 kD. However, at the friable stage where the induction of embryo development took place, there was a relative abundance in the expression of low molecular weight proteins (<43 kD). As development proceeded, the proteins were expressed in a much less intensity.

6.3.6. Isozyme analysis

The isozyme analysis at five sequential stages of embryogenesis revealed specific differences in the banding profile. The zymograms of the four isozymes viz. peroxidase, esterase, malate dehydrogenase and shikimate dehydrogenase are given in Fig. 6.9.

6.3.6.1.Peroxidase

Peroxidase isozyme showed a single zone of activity (R_f = 0.37) at all stages of somatic embryo development while friable and mature embryo stages were characterised by 4 isoforms at a single locus (R_f= 0.37, 0.41, 0.43 and 0.45).

6.3.6.2. Esterase

The esterase isozyme showed a single zone of activity uniformly (R_f = 0.54) at all stages while, from the embryo induction stage onwards showed an additional zone of activity (R_f = 0.74) and the mature embryo showed a further additional band (R_f = 0.80).

6.3.6.3. Malate dehydrogenase

Malate dehydrogenase activity differed in different developmental stages: one zone of activity with single band (R_f = 0.42) in compact callus stage, one zone of activity with two bands (R_f = 0.42 and 0.52) in soft friable callus stage, two zones of activity with two bands (R_f = 0.37 and 0.42) in the first zone and one band (R_f = 0.58) in the second zone in friable granular stage, and only one zone of activity with two bands (R_f = 0.56 and 0.58) in the embryoid stage. The activity of the enzyme was located to 3 zones, with 2 bands in upper zone (R_f = 0.096 and 0.13), 4 bands in middle zone (R_f = 0.29, 0.37, 0.42 and 0.52) and with 2 bands in lower zone (R_f = 0.56 and 0.58) in mature embryos.

6.3.6.4. Shikimate dehydrogenase

The enzyme showed only a single zone of activity with one band (R_f = 0.67) in compact and friable callus stages. In the next stages of development in embryo induction and maturation stages, the activity of the enzyme was meagre. The mature embryos, like the initial stages of development showed a single zone of activity with two bands (R_f = 0.65 and 0.67).

6.3.7. RAPD analysis

RAPD analysis demonstrated a remarkable degree of heterogeneity between the five different culture stages (Fig. 6.10.). The RAPD profile with OPA 07 primer showed three DNA fragments between 600bp to >1000bp in the undifferentiated stages (compact and soft friable callus stages) and embryo

Figure 6.8. Protein profiles at sequential stages of somatic embryogenesis; lane M: protein molecular weight marker (14.3 kD - 97.4 kD), lane 1-4 represents protein profiles of compact callus stage, friable embryo induction stage, embryo maturation stage and mature embryo stage respectively.

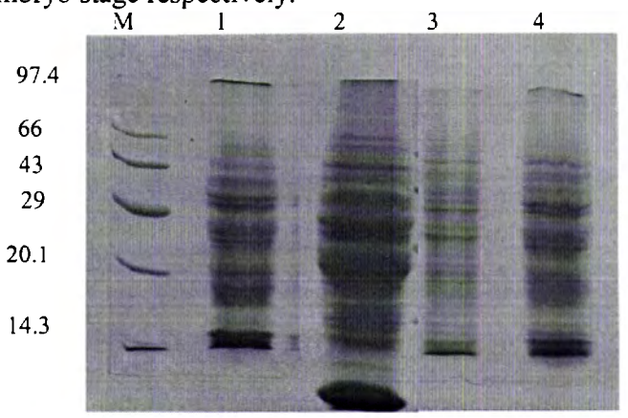


Figure 6.9. Isozyme profiles at sequential stages of somatic embryogenesis; lane 1-5 represents isozyme profiles of compact callus, friable callus, embryo induction, embryo maturation and mature embryo stages respectively.

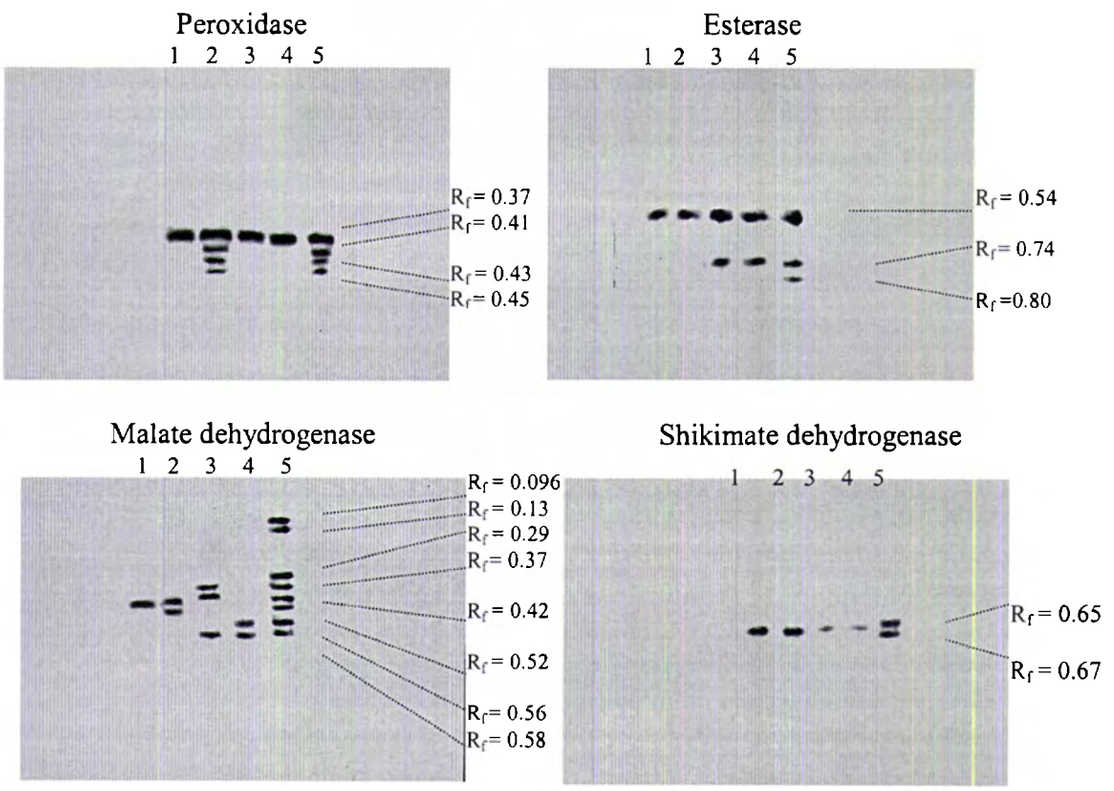
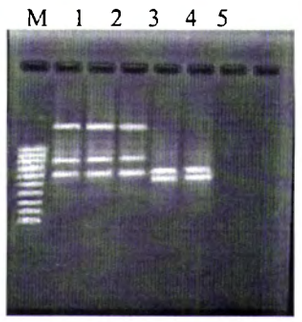
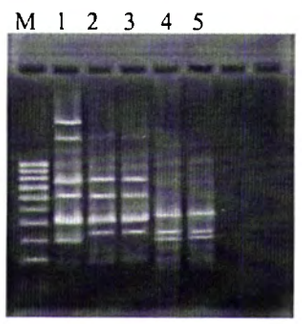


Figure 6.10. RAPD profiles at sequential stages of somatic embryogenesis; lane M: DNA marker (100bp ladder), lane 1-5: RAPD profiles of compact callus, friable callus, embryo induction, embryo maturation and mature embryo stages.

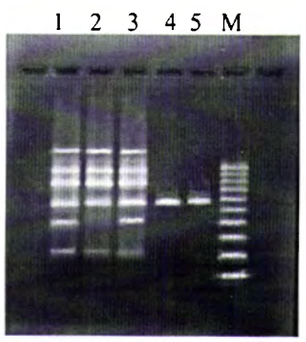
RAPD profile: primer - OPA 07



RAPD profile: primer - OPA 09



RAPD profile: primer - OPA 16



induction stage while only two bands were present in the embryo maturation and mature embryo stages. With OPA 09, DNA of the compact callus gave 8 bands between 200bp to >1000bp while 6 bands were present in the soft friable stage and in the embryo induction stage. Five bands characterized the immature and mature embryo stages. OPA 16 primer showed six bands between (200bp - >1000bp) in compact callus and embryo induction stage while only five bands were present in the friable callus stage. Only a single band characterized embryo maturation and mature embryo stages. Of the 20 resolved loci (4 for OPA 07, 10 for OPA 09 and 6 for OPA 16), 17 loci were found to be polymorphic at least in one of the stages with 85 per cent of polymorphism.

6.4. DISCUSSION

The *in vitro* multiplication of forest trees either through direct organogenesis or somatic embryogenesis has acquired great importance and substantial progress has been made in many species. (Ahuja, 1993; Thorpe, 1995). The induction of somatic embryogenesis in *S. album* is one of the earliest reports on somatic embryogenesis in forest trees (Lakshmi Sita *et al.,* 1979; Bapat and Rao 1979, 1984). Disagreement has existed in the literature concerning the frequency of aberrant/abnormal embryo phenotype and subsequent low conversion rate of somatic embryos into normal plantlets (Bapat and Rao, 1984; Bapat *et al.*, 1992; Rao and Bapat, 1995). Rao and Bapat (1995) suggested that the metabolic process leading to efficient germination of somatic embryos has to be elucidated to improve

the conversion rate of somatic embryos and survival rate of the embryogenic plantlets. They also observed that though culture conditions required for somatic embryogenesis are well defined in *S. album*, the molecular and biochemical events underlying the phenomenon are quite unknown. Thus, with the objective to provide evidence for the occurrence of stage specific variation at DNA and protein levels, biochemical and molecular analysis were carried out at the sequential stages of embryogenesis.

The morphogenetic differentiation of a somatic cell to whole plant is a complex developmental process that involve morphogenetic, biochemical, physiological and molecular changes (Thorpe, 1995). In an effort to examine changes in gene expression, proteins were monitored at different developmental stages of somatic embryos. The relative abundance of low molecular weight proteins i.e. < 43 kD (Fig. 6.8.) observed in friable callus stage may be characteristic of embryogenic potential. This type of low molecular weight embryogenic proteins were reported in somatic embryos of *Hordeum* (Ramgopal, 1989) and *Daucas carota* (Cordewiner *et al.*, 1991).

Though, the above results pointed out the presence of embryogenic marker proteins which showed quantitative increase in expression, there is no conclusive evidence to suggest that whether they constitute only one protein type or more. Further studies in 2-dimensional electrophoresis are needed to confirm quantitative separation of proteins.

The enzyme pattern of an organism changes during development and differentiation. Changes of enzyme pattern in samples of a particular organ or tissue during development are seen by the appearance and disappearance of individual isozymes. Such changes in isozymes suggest that the genes involved in the synthesis of these enzymes are differentially activated during development as suggested by Chawla (1988).

Peroxidase activity was found to be modulated during developmental phases. In friable callus state, just before the induction of embryos, there was an increase in the activity of peroxidase. Peroxidase represents the enzymatic complex involved in the metabolism of auxin and the synthesis of lignin (Thorpe and Gaspar, 1978). In the developing embryos, peroxidase activity was found to be low. This type of characterization, which is essential for the elucidation of differential gene expression, was also reported in wheat (Gaspar *et al.*, 1977) and in pumpkin (Rasol *et al.*, 1982). In most systems, the induction of somatic embryos is achieved on a culture medium different to callus maintenance medium containing 2,4-D. It is well known that 2,4-D influences the activity and pattern of peroxidase isozymes (Paul and Ohrman, 1980; Blanco *et al.*, 1997). So in the present study, the difference in peroxidase banding profile could be attributed to presence and absence 2,4-D in the medium as well as due to the different stages of organization and development.

Esterase isozyme activity was also found to be stimulated in the later stages of development as indicated by the appearance of two additional bands. Esterase,

appeared to be less active during early phase, was reported to be triggered in the later stages of development through genetic responses induced by growth regulators (Everett *et al.,* 1985). Similarly, because of the influence of developmental regulators, the malate and shikimate dehydrogenase also might be showing a differential activity in different developmental stages.

RAPD analysis was carried out to determine specifically the molecular changes taking place in different stages of development. The results of this work demonstrated that the DNA fingerprinting using RAPD is specific enough to demonstrate that with a suitable primer/cycling regime, polymorphisms can be detected at different stages of somatic embryo development. Polymorphism of the 17 loci out of 20 resolved loci (85%) could be considered as an indication for the occurrence of genic variation in the different developmental stages of somatic embryos. All the loci except three (OPA 07-3, OPA 09-5 and OPA 16-4) had undergone some modifications in the genetic material during the onset of embryogenesis as evidenced from the banding profiles. The RAPD profiles at different sequential stages of embryogenesis may be indicative of the hypervariable gene sequences due to tissue culture induced stress. Accepted mechanism for the molecular changes induced by tissue culture ranged from base pair changes like insertion, deletion, duplication, translocation and inversion to activation of transposons (Brown *et al.*, 1991).

Hake and Walbot (1980) reported the presence of unique sequences in cultured maize induced by genetic stress. Zheng *et al.* (1987) observed quantitative

changes in gene expression in suspension cultured cells of *Oryza sativa*. The variation in DNA fragments from the undifferentiated callus stage to mature regenerants is also reported in maize. Generally the callus culture was reported to be genetic mosaic of cells owing to rapid changes of DNA during cell divisions. From this, selection for genetic fitness takes place and consequently, the regenerants attain a normal genotype almost true to the parent (Edallo *et al.*, 1981). Morere-Le Paven *et al.* (1992) reported *in vitro* induced reorganization of genome in the initial undifferentiated stages of wheat cultures. Many of such cryptic structural changes of DNA were reported to be heterozygous and recessive and consequently do not appear until the plants have been selfed and the progeny examined (Gobel *et al.*, 1985).

Although, in sandal, more work is needed to correlate the RAPD variation to the developmental anomalies, the presence of unique DNA fragments provides evidence for the occurrence of culture induced genetical changes during development and differentiation of somatic embryos.

CHAPTER 7

CONCLUSIONS AND FUTURE IMPLICATIONS

7. CONCLUSIONS AND FUTURE IMPLICATIONS

The Indian sandalwood, *Santalum album* L. is acclaimed worldwide because of its unique fragrant wood and oil. India is the chief exporter of the best heartwood oil, accounting for more than 90 per cent of the world trade (Nageswara Rao *et al.*, 2001). In India, sandal genetic resources are now under severe threat because of the illegal logging by smugglers, inadequate regeneration due to over grazing, repeated fires and encroachments, and the devastating spike disease. Because of the resource decline, the annual production of sandalwood has plummeted from 4000 tonnes during 1965-1970 to merely 1000 tonnes during 1999-2000. Correspondingly, the oil production has also reduced from a constant rate of 60 tonnes during 1981-1994 to 40 tonnes during 1999-2000 (Ananthapadmanabha, 2000). This alarming situation has necessitated an urgent need for mass conservation of existing genetic resources and for this purpose a comprehensive conservation programme has to be planned.

Forest trees have long generation intervals and hence a prior information on genetic variation/diversity and improved characterization of genotype can hasten the progress of both conservation and tree improvement programmes (Erikson *et al.*, 1993). One of the fundamental requirements for any genetic improvement programme is to understand the biological dynamics of genetic variation and the extent and pattern of genetic diversity within and between species (Changtragoon and Szmidt, 1993). Isozymes being codominant genetic markers, provide information about the allelic evenness of the locus and can be used to evaluate the

genetic variation and diversity of forest trees in a relatively shorter time. DNA based molecular markers such as RAPD, RFLP, AFLP and microsatellites are also efficient markers as they provide an ample and precise information about the genome (Glaubitz and Moran, 2000).

The isozyme and RAPD analysis of eleven natural populations of *S. album* in South India provided vital information regarding the population genetic structure and pattern of genetic diversity. The results of the analysis can answer major conservation issues, as to what to conserve and how to conserve, regarding conservation of sandal genetic resources.

7.1. CONSERVATION STRATEGIES FOR SANDAL

The results of the present study reveal the affect of illegal felling and devastating spike disease on the genetic diversity status of sandal populations studied. Population genetic structure describes the partitioning of genetic variance into two components namely variance among populations and within populations (Rajora *et al.*, 2000). In the sandal populations of South India, both the isozyme and RAPD marker studies revealed that more than 70 per cent of the genetic variation is distributed between the populations and only the rest of the variation is located within populations. Genetic diversity measures based on allelic richness (no. of alleles/locus), gene diversity index, per cent of polymorphism and the gene flow revealed the levels of diversity in each of the 11 analysed populations. These results can be utilized for planning more efficient and comprehensive conservation strategies.

7.1.1. *In situ* conservation

Forest genetic resources can be conserved either *in situ* or *ex situ* (Ledig, 1988; Finkeldey and Gregarious, 1994). *In situ* conservation should be focussed on populations, which are hotspots of genetic variability as measured in terms of per cent of polymorphism, allelic richness, gene diversity index and gene flow. It is found that Marayoor and Wayanad are the most genetically diverse populations followed by Chikkamagalur, Bangalore, Shimoga and Thirupattur. Kasargod population has got the least amount of diversity. *In situ* conservation represent a more evolutionarily dynamic approach compared with more static *ex situ* conservation method (Finkeldey and Gregarious, 1994).

7.1.2. *Ex situ* conservation

For *ex situ* conservation purpose, seed samples should be selected from populations with high genetic diversity so that long-term fitness of the progenies can be achieved. Therefore, care needs to be exercised in elimination of seed stocks of highly inbred population for *ex situ* gene conservation purposes. An understanding of the population genetic differentiation is important in developing sampling strategies for gene conservation and for capturing a representative sample. Since genetic variation in *S. album* is distributed more or less randomly across the populations, sampling or preservation of any one population may not insure that all the variation in *S. album* is captured. As far as forest tree populations are concerned *ex situ* conservation in most cases is not sustainable because of small population size and consequent intermating (Nason and Hamrick, 1997).

One alternate strategy for both *ex situ* and *in situ* conservation is the establishment of gene banks. Conceptually, the forest gene bank serves as an *in situ* bank in which genes from as many diverse populations of the species are conserved and dynamically maintained. Operationally, in these gene banks, an *in situ* site serves as the 'sink' population into which genes from distinct 'source' populations are infused. Apart from maintaining full allelic set of the species *in situ*, the forest gene banks facilitate a continuous turnover of the genetic material within and among populations (Uma Shaanker *et al.*, 2001).

7.2. SPIKE DISEASE EVASION/RESISTANCE

Gap filling of the degraded forests with seedlings of elite sandal trees with desirable characteristics including disease resistance is regarded a promising method to improve the productivity of the species (Rajora and Mosseler, 2001). Marayoor provenance is considered as the best sandal provenance in India. But, the damage due to spike disease is severe in a few reserve forests (Ghosh and Balasundaran, 1995; Srimathi *et al.*, 1995). Among the heavily infected sandal reserves of Marayoor, a few trees are devoid of disease symptoms for the past 25 years. These trees were presumed to be capable of evading/resisting spike disease and were considered to be an ideal starting material for the production of disease resistant stock (Balasundaran, 1998).

Comparison of spike disease affected and evaded trees using protein, isozyme and RAPD markers could not locate any specific marker exclusively in the disease evaded plants of Marayoor. However, this finding cannot be considered as

conclusive, since the studies were made only in 12 trees using four primers. Hence, a more detailed study using a wider range of enzymes and random primers needs to be carried out in sufficiently large number of disease evaded parent trees and their progenies to detect possible inheritance of markers linked to disease resistance. Probably studies using SSR, RFLP or AFLP markers will be able to provide a more precise information related to the genetic basis of spike disease resistance.

7.3. GENETIC BASIS OF ABNORMAL/IRREGULAR SOMATIC EMBRYOGENESIS

To reverse the decline of forest resources, afforestation of degraded forests using genetically superior and ecologically adapted planting stock is considered as an effective method (Adams and Burczyk, 2000). In sandal, clonal propagation through rooting of juvenile root suckers is an important technique for producing true-to-type plants of superior characters. However, the limited number of juvenile shoots available as root suckers and the low per cent of rooting hampers the efficiency of the technique in sandal (Balasundaran, 1998). *In vitro* regeneration has the advantage of surmounting the above problem and somatic embryogenesis using juvenile and mature explants is identified as an important tool with several potential advantages for mass production of superior genotypes (Bajaj, 1986). However, the successful utilization of somatic embryogenesis in sandal is hampered by the high frequency of abnormal embryos, poor conversion rate of somatic embryos and low survival per cent of embryonic plantlets (Bapat *et al.*, 1990; Das *et al.*, 1999; Ilah *et al.*, 2002). Genetic characterization of sequential

stages of somatic embryogenesis using protein, isozyme and RAPD markers revealed differential activity of proteins and isozymes, and polymorphism in RAPD banding patterns. The abnormalities associated with different stages of somatic embryogenesis might be due to culture induced variations at the DNA level.

Even after three decades of research in somatic embryogenesis of sandal, still the proper utilization of the technique is not fully exploited. Further exchange of ideas and concerted efforts are needed for achieving rapid synchronized production of normal plantlets. Also, there is relatively little information available regarding the fidelity and performance of somatic embryo-derived plants. These aspects are essential to further improve the efficacy of woody plant somatic embryogenesis in general (Wann, 1988; Eastman *et al.*, 1991; Gupta *et al.*, 1991).

CHAPTER 8

SUMMARY

8. SUMMARY

Santalum album L., the root hemi-parasitic tree belonging to the family Santalaceae, has been rated as one of the most valuable tree in Indian forestry for its highly scented heartwood and precious oil. Regeneration problems, spike disease caused by phytoplasma and illegal harvesting by smugglers have resulted in the depletion of sandal forests in India. The annual production of sandalwood has dwindled from 4000 tonnes in 1965-1970 to merely 1000 tonnes in 2000-2001 and the oil production has also reduced to 40-50 tonnes per year during 1999-2000 from a constant rate of 60 tonnes per year during 1981-1994. Mass conservation of remaining sandal genetic resources has got considerable significance in these circumstances. An understanding of the genetic diversity pattern of the existing population is of profound importance before initiating any genetic improvement programme. The present study was undertaken with a view to investigate the genetic diversity pattern in eleven sandal populations in South India through isozyme and RAPD markers, to identify a possible genetic basis of spike disease evasion/resistance and to ascertain whether the developmental anomalies (abnormal plantlets and poor survival rate of embryogenic plantlets) associated with somatic embryogenesis in *S. album* has got any genetic basis.

8.1. ISOZYME BASED GENETIC VARIATION IN SANDAL POPULATIONS

Peroxidase, esterase, malate dehydrogenase, shikimate dehydrogenase and phosphogluco isomerase were used as isozyme markers to study variation/diversity

from random samples of eleven South Indian sandal populations viz. Marayoor, Wayanad, Thenmalai, Wadakkanchery, Ottappalam, Kasargod and Kannavam from Kerala, Bangalore, Shimoga and Chikkamagalur from Karnataka, and Thirupattur from Tamil Nadu. Five isozymes were resolved into 11 loci, of which 10 (90.9%) were found to be polymorphic. High genetic diversity existed within sandal populations of Marayoor, Wadakkanchery and Wayanad as evidenced from the per cent of polymorphism, number of alleles and amount of heterozygosity. Higher per cent (75.22%) of the genetic variation was found to be distributed between populations than within population (24.78%). Genetic relatedness revealed by the cluster analysis grouped the 11 populations broadly into two groups. Of the 11 populations, Wadakkanchery and Chikkamagalur were the most genetically distant and Kasargod and Kannavam were the most identical populations.

8.2. RAPD BASED GENETIC VARIATION IN SANDAL POPULATIONS

Randomly amplified polymorphic DNA (RAPD) analysis was carried out in the same populations mentioned above. Out of the 20 primers of OPA series screened initially for genetic polymorphism, analysis was carried out using three selected primers of OPA series (viz. OPA 07, OPA 09 and OPA 16), based on the number and reproducibility of the amplified products. Of the eleven analysed populations, Marayoor and Wayanad had the highest genetic diversity as measured by per cent of polymorphic loci and gene diversity index. High proportion of genetic diversity was observed between the populations (72.62%) while variation within population was low (27.38%). The dendrogram depicting the genetic relationship showed the

clustering of 11 populations into two main clusters. Kasargod and Kannavam were the most genetically similar and Ottappalam and Wayanad were the genetically distant ones as revealed by genetic similarity/distance coefficients.

8.3. COMPARISON OF SPIKE DISEASE EVADED/SUSCEPTIBLE TREES AT PROTEIN, ISOZYME AND RAPD LEVELS

Samples were collected from spike disease evaded and susceptible (diseased) trees in an infected tract for total protein, isozyme and RAPD analysis. Except for an increased intensity in one of the polypeptide, the rest of the total protein profiles and isozyme profiles were identical in disease evaded and susceptible trees. The RAPD analysis was carried out using three selected primers of OPA series (OPA 07, OPA 09 and OPA 16) and one selected primer of OPB series (OPB 03), based on the number and reproducibility of bands. RAPD profiles of disease evaded and susceptible trees showed no remarkable difference in banding patterns. The minor differences observed with some of the analysed primers were present in both disease evaded and diseased trees and might be an indication of the genotypic differences unrelated to spike disease.

8.4. PROTEIN, ISOZYME, RAPD PROFILES DURING THE ONTOGENY OF SOMATIC EMBRYOS IN *S. ALBUM* L.

The internodes collected from disease evaded trees of Marayoor were used as explants for induction of somatic embryos. Compact proliferating callus was developed from internode explants on MS medium fortified with 2,4-D (0.1mg/l) and Kinetin (1mg/l). After 5-6 subcultures, white friable soft calli appeared from sides and surface of brown compact callus and gave rise to granular embryonic

callus on MS media supplemented with various concentrations of IAA/BAP. Somatic embryos developed asynchronously and germinated precociously. Only twenty per cent of the embryos were converted to plantlets.

Total protein, isozyme and RAPD analysis were carried out at sequential stages of embryogenesis viz. compact callus, friable callus, embryo induction stage, embryo maturation stage and fully developed embryos. Differential activity of proteins and isozymes were observed in the different sequential stages of somatic embryogenesis. Remarkable degree of heterogeneity observed in RAPD profiles at the different stages of somatic embryogenesis provided evidence for the culture induced variations at the DNA level.

8.5. CONCLUSIONS AND FUTURE IMPLICATIONS

The RAPD and isozyme analysis revealed the affect of indiscriminate felling, lack of regeneration and devastating spike disease on the genetic diversity of existing sandal populations. The pattern and distribution of genetic diversity provided a few implications for practical considerations. *In situ* conservation efforts could be focussed on populations with highest genetic diversity and for *ex situ* conservation purposes, samples could be drawn according to the partitioning of genetic diversity.

Comparison of spike disease evaded and disease susceptible trees using molecular markers could not provide any conclusive evidence for a possible genetic basis for spike disease evasion. No unique isozyme or DNA profile was obtained for disease evaded trees. Hence, these trees have to be subjected to isozyme and RAPD analysis with a wider range of enzymes and random primers respectively, to

bring out the uniqueness of the genome if there is any. Studies using SSR, RFLP or AFLP markers also will be able to provide the required information.

The occurrence of culture induced genetical changes at different stages of somatic embryogenesis in *S. album,* as unveiled by this study, have hampered the successful utilization of the technique for mass production of plantlets. High per cent of abnormal embryos, low conversion rate of somatic embryos and poor rate of survival of embryonic plantlets might be due to these changes at the DNA level. Further efforts are needed to achieve rapid, uniform maturation and conversion of the somatic embryos into plantlets in order to use them in future planting programme.

REFERENCES

Adams, W.T. 1983. Application of isozymes in tree breeding. In: Isozymes in Plant Genetics and Breeding (Tanksley, S.D. and Orton, T.J., Eds.), pp. 381-400. Elsevier Science Publishers, Amsterdam.

Adams, W.T. 1992. Gene dispersal within forest tree populations. *New Forests* **6**: 217-240.

Adams, W.T. and Burczyk, J. 2000. Magnitude and implications of gene flow in gene conservation reserves. In: Forest Conservation and Genetics Principles and Practice (Young, A., Boshier, T. and Boyle, T., Eds.), pp. 215-224. CSIRO Publishing, CABI Publishing, Australia.

Adams, W.T., Strauss, S.H., Copes, D.L. and Griffin, A.R. 1992. Population Genetics of Forest Trees. Kluwer Academic Publishers, Dordrecht.

Adams, W.U. and Joly, R.J. 1980. Genetics of allozyme variants in loblolly pine. *J. Hered.* **71**: 33-40.

Adato, A., Sharon, D. and Lavi, U. 1995. Application of DNA fingerprints for identification and genetic analysis of mango (*Mangifera indica*) genotypes. *J. Amer. Soc. Hort. Sci.* **120**: 259-264.

Ahl, P., Cornu, A. and Gianinazzi, S. 1982. Soluble proteins as genetic markers in studies of resistance and phylogeny in *Nicotiana*. *Phytopathol.* **72**: 80-85.

*Ahuja, M.R. 1993. Micropropagation a la Carte. In: Micropropagation of Woody Plants (Ahuja, M.R., Ed.), pp. 3-9. Kluwer Academic Publishers, Dordrecht, Germany.

Ahuja, M.R. 2001. Recent advances in molecular genetics of forest trees. *Euphytica* **121**: 173-195.

Ainsley, P.J., Collins, C.G. and Sedgley, M. 2000. Adventitious shoot regeneration from leaf explants of almond (*Prunus dulcis* Mill.). *In Vitro Cell Dev. Biol.* **36**: 470-475.

Akthar, M. and Garraway, M. O. 1987. Changes in maize peroxidase associated with variation in susceptibility to *Bipolaris maydis* race T. (Abstr.). *Phytopathol.* **77**: 1739.

Alphin, L., Windham, M.D. and Harper, K.T. 1998. Genetic diversity and gene flow in the endangered dwarf bear poppy, *Arctomecon humilis* (Papaveraceae). *Am. J. Bot.* **85**: 1251-1261.

Amaral, W. 2001. Characterization, evaluation and conservation of forest genetic resources: The potential and limitation of new biotechnology tools. In: Forest Genetic Resources-Status, Threats and Conservation Strategies (Uma Shaanker, R., Ganeshaiah, K.N. and Kamaljit S. Bawa, Eds.), pp. 115-125. Oxford and IBH Publishing Co., New Delhi.

Ananthapadmanabha, H.S. 2000. Sandalwood and its marketing trend. *My Forest* **36**: 147-151.

Angadi, V.G. and Ananthapadmanabha, H.S. 1988. Variations in isoenzyme pattern associated with spike disease of sandal. *Ind. J. For.* **11**: 37-38.

Annamalai, R. 1999. Participatory learning and action and microplanning for joint forest management. Sriram Computer Prints and Offset, Salem, p. 333.

Anthony, F., Bertrand, B., Quiros, O., Wilches, A., Lashermes, P., Berthaud, J. and Charrier, A. 2001. Genetic diversity of wild coffee (*Coffea arabica* L.) using molecular markers. *Euphytica* **118**: 53-65.

Aradhya, K.M. and Phillips, V.D. 1993. Genetic variability in fourteen provenances of *Eucalyptus* species in Hawaii. *Silvae Genet.* **42**: 9-15.

Arcade, A., Anselin, F., Rampant, P.F., Lesage, M.C., Paques, L.E. and Prat, D. 2000. Application of AFLP, RAPD and SSR markers to genetic mapping of European and Japanese larch. *Theor. Appl. Genet.* **100**: 299-307.

Auriol, J.C., Metivier, E. and Lebacq, P. 1994. Method for genetic certification of poplar, *Comptes-Rendus- de- l, Academie-d, Agriculturede-france.* **80**: 27-31.

Ayala, F.J. 1983. Enzymes as taxonomic characters. In: Protein Polymorphism: Adaptive and Taxonomic Significance (Oxford, G.S. and Rollonson, D., Eds.), (The Systematic Association Special Volume No. 24), pp. 3-26. Academic Press, New York.

Bagchi, J.K. and Veerendra, H.C.S. 1987. Study on intra-tree and inter-tree variations in leaves of *Santalum album*. *My Forest* **21**: 33-39.

Bajaj, Y.P.S. 1986. Biotechnology in Agriculture and Forestry Vol.1, Trees 1, Springer- Verlag, Berlin.

Bajaj, Y.P.S. 1995. Biotechnology in Agriculture and Forestry Vol.30, Somatic embryogenesis and synthetic seed 1, Springer- Verlag, Berlin.

Balasundaran, M. 1998. A method for clonal propagation of sandal. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 126-129. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Balasundaran, M. and Muralidharan, E.M. 2001. Development of spike disease resistant sandal seedlings through Biotechnology involving ELISA technique and tissue culture. DBT project completion report, Kerala Forest Research Institute, Peechi, Trichur, Kerala, p. 64.

Bapat, S.A., Rawal, S.K. and Mascarenhas, A.F. 1992. Isozyme profiles during ontogeny of somatic embryos in wheat (*Triticum aestivum* L). *Curr. Sci.* **82**: 235-242.

Bapat, V.A., Fulzele, D.P., Rao, P.S. and Heble, M.R. 1990. Production of somatic embryos of sandalwood (*Santalum album* L.) in bioreactors. *Curr. Sci.* **59**: 746-748.

Bapat, V.A, Gill, R. and Rao, P.S. 1985. Regeneration of somatic embryos and plantlets from stem callus protoplasts of sandalwood tree (*Santalum album* L.). *Curr. Sci.* **54**: 978-982.

Bapat, V.A. and Rao, P.S. 1979. Somatic embryogenesis and plantlet formation in tissue cultures of sandalwood (*Santalum album* L.). *Ann. Bot.* **44**: 629-630.

Bapat, V.A. and Rao, P.S. 1984. Regulatory factors for *in vitro* multiplication of sandalwood tree (*Santalum album* L.) 1. Shoot bud regeneration and somatic embryogenesis in hypocotyl cultures. *Proc. Ind. Acad. Sci.* **93**: 19-27.

Bapat, V.A. and Rao, P.S. 1988. Sandalwood plantlets from 'synthetic seeds'. *Plant Cell Rep.* **7**: 434-436.

Bapat, V.A. and Rao, P.S. 1998. Biotechnological approaches for propagation of sandal (*Santalum album* L). In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 42-45. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Barber, C.A. 1903. Report on spike disease in sandalwood tree in Coorg. *Ind. For.* **29**:1.

Barker, H., 1997. Extreme resistance to potato virus V in clones of *Solanum tuberosum* that are also resistant to potato virus Y and A: Evidence for a locus conferring broad-spectrum poly-virus resistance. *Theor. Appl. Genet.* **95**: 1258-1262.

Bartolozzi, F., Warburton, M.C., Arulsekar, S. and Gradziel, T.M. 1998. Genetic characterization and relatedness among California almond cultivars and breeding lines detected by randomly amplified polymorphic DNA analysis. *J. Amer. Soc. Hort. Sci.* **123**: 381-387.

*Bateson, W. and Punnett, R.C. 1905. Experimental studies in the physiology of heredity. *Reports to Evol. Comm. Royal Soc.* **2**: 1-131.

Beckman, J.S. and Weber, J.L. 1992. Survey of human and rat microsatellites. *Genomics* **12**: 627-631.

Benettka, V., Mottl, J., Vackova, K., Pospiskova, M. and Dubsky, M. 1999. Estimation of the introgression level in *Populus nigra* L. population by means of isozyme gene markers. *Silvae Genet.* **48**: 218-223.

Bergmann, B.A., Lin, X.H. and Whetten, R. 1998. Susceptibility of *Paulownia elongata* to *Agrobacterium* and production of transgenic calli and hairy roots by *in vitro* inoculation - research note. *Plant Cell Tiss. Org. Cult.* **55**: 45-53.

Bergmann, F. 1987. Characterization of multiclonal aspen cultivars using isozyme electrophoresis. *For. Ecol. Manag.* **22**: 167-172.

Bergmann, F. and Hattemer, H. 1998. Isozymes in forest genetic research. In: Forest Genetics and Tree Breeding (Mandal, A.K. and Gibson, G.L., Eds.), pp. 227-238. CBS Publishers, New Delhi.

Berthouly, M. and Ferriere, N.M.M. 1996. High frequency somatic embryogenesis in *Coffea canephora*. *Plant Cell Tiss. Org. Cult.* **44**: 169-176.

Black, L.L., Hobbs, H.A. and Kammerlohr, D.S. 1996. Resistance of *Capsicum* Chinese lines to tomato spotted wilt virus isolates from Louisiana, U.S.A., and inheritance of resistance. *Acta Hort.* **431**: 393-401.

Blanco, M.D.A., Nieves, N., Sanchez, M., Carlos, G., Castillo, R., Justo, L., Escalona, M., Baez, E.L. and Hemandez, Z. 1997. Protein changes associated with plant regeneration in embryogenic calli of sugar cane (*Saccharam* sp.). *Plant cell Tiss. Org. Cult.* **51**: 153-158.

Bogani, P., Cavalieri, D., Petrucelli, R., Polsenelli, L. and Roselli, G. 1994. Identification of olive tree cultivars by using random amplified polymorphic DNA. *Acta Hort.* **356**: 98-101.

Bonal, D. and Monteuuis, O. 1997. *Ex vitro* survival, rooting and initial development of *in vitro* rooted vs unrooted microshoots from juvenile and mature *Tectona grandis* genotypes. *Silvae Genet.* **46**: 301-307.

Bonga, J.M and Durzan, D.J. 1982. Tissue Culture in Forestry. Nijhoff Publishers, Dodrecht.

Boshier, D.H. and Billingham, M.R. 2000. Genetic variation and adaptation in tree populations. In: Ecological Consequences of Habitat Heterogeneity (Hutchings, M.J., Ed.), pp. 51-65. Black Well Science, UK.

Boshier, D.H. and Young, A.G. 2000. Forest conservation genetics: limitations and future directions. In: Forest Conservation Genetics. Principles and Practice. (Young, A., Boshier, D. and Boyle, T., Eds.), pp. 289-297. CSIRO Publishing, Australia.

Boskovic, R., Tobutt, K.R. and Nicol, F.J. 1997. Inheritance of isoenzymes and their linkage relationships in two interspecific cherry progenies. *Euphytica* **93**: 129-143.

Botstein, D., White, R.L., Skolnick, M. and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* **32**: 314-331.

Boulay, M., Chaperon, H., David, A., Faye, M., Isemukali, K., Durand, R., Boudet, A.J., Franclet, A. and Rancillac, M. 1979. Micropropagation of forest trees. AFOCEL Report, Nangis, France.

Bournival, B.L. and Korban, S.S. 1987. Electrophoretic analysis of genetic variability in the apple. *Sci. Hort.* **31**: 233-243.

Boyle, S.B. and Morgenstein, E.K. 1985. Inheritance and linkage relationships of some isozymes of black spruce in New Brunswick. *Can. J. For. Res.* **15**: 992-996.

Boyle, T.J. 2001. Conserving forest genetic resources: from theory to practice. In: Forest Genetic Resources-Status, Threats and Conservation Strategies (Uma Shaanker, R., Ganeshaiah, K.N. and Kamaljit S. Bawa, Eds.), pp. 253-261. Oxford and IBH Publishing Co., New Delhi.

Bradshaw, H.T. and Stettler, R.F. 1994. Molecular genetics for growth and development in *Populus*. II. Segregation distortion due to genetic load. *Theor. Appl. Genet.* **89**: 551-558.

Brand, J.E. 1994. Genotypic variation in *Santalum album* L. *Sandalwood Research Newsletter* **2**: 2-4.

Brennan, B. and Merlin, M. 1993. Biogeography and traditional use of *Santalum* in the pacific region. In: Sandalwood in the pacific region. Proceedings of a symposium held on 2nd June at the XVII pacific science congress (McKinnell, F.H., Ed.), Honolulu, Hawaii, pp. 31-40. CPN Publications, Australia.

Brown, A.H.D. 1978. Isozymes, plant population genetic structure and genetic conservation. *Theor. Appl. Genet.* **52**: 145-157.

Brown, A.H.D. and Allard, R.W. 1969. Inheritance of isozyme differences among inbred parents of a reciprocal recurrent selection population of maize. *Crop Sci.* **9**: 72-75.

Brown, A.H.D. and Moran, G.F. 1981. Isozymes and genetic resource of forest trees. In: Isozymes of North American Forest Trees and Forest Insects. (Conkle, M.T., Ed.), pp. 1-10. Burkley CA, USA.

Brown, C.L. and Sommer, H.E. 1982. Vegetative propagation of dicotyledonous trees. In: Tissue Culture in Forestry (Bonga, J.M. and Durzan, D.J., Eds.), pp. 109-149. Nijhoff, The Hague.

Brown, P.T.H. 1989. DNA methylation in plants and its role in tissue culture. *Genome* **31**: 717-729.

Brown, P.T.H., Gobel, S. and Lorz, H. 1991. RFLP analysis of *Zea mays* callus cultures and their regenerated plants. *Theor. Appl. Genet.* **81**: 227-232.

Brown, P.T.H., Lange, F.D., Kranz, E. and Lorz, H. 1993. Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. *Mol. Gen. Genet.* **237**: 311-317.

Bundock, P.C., Hayden, M. and Vaillancourt, R.E. 2000. Linkage maps of *Eucalyptus globulus* using RAPD and microsatellite markers. *Silvae Genet.* **49**: 223-232.

Burner, D.M., Bao, P.Y., Webster, R.D. and Pan, V.B. 1997. Genetic diversity of North America and old world *Saccharam* assessed by RAPD analysis *Genetic Res. Crop. Evol.* **44**: 230-240.

Byrne, M., Moran, G.F., Stukely, M., Emebiri, L.C. and Williams, E.R. 1997a. Identification of QTL for resistance to *Phytophthora cinnamomi* in *Eucalyptus marginata*, pp. 111. Proceedings of the Australian Plant Pathology Society, 11TH Biennial Conference, Perth, Australia.

Byrne, M., Murnell, J.C., Allen, B. and Moran, G.F. 1995. An integrated genetic linkage map for eucalypts using RFLP, RAPD and isozyme markers. *Theor. Appl. Genet.* **91**: 869-879.

Byrne, M., Murrell, J.C., Owen, J.V., Kriedeman, P., Williams, E.R. and Moran, G.F. 1997b. Identification and mode of action of quantitative trait loci affecting seedling height and leaf area in *Eucalyptus nitens*. *Theor. Appl. Genet.* **94**: 674-681.

Cabrita, L.F., Aksoy, A., Hepaksoy, S. and Leitao, J.M. 2001. Suitability of isozyme, RAPD and AFLP markers to assess genetic differences and relatedness among fig (*Ficus carica* L.) clones. *Sci. Hort.* **87**: 261-275.

Cailloux, F., Guerrier, J.J., Linossier, L. and Coudret, A. 1996. Long-term somatic embryogenesis and maturation of somatic embryos in *Hevea Brasiliensis*. *Plant Sci.* **120**: 185-196.

Carimi, F., Pasquale, F.D. and Crescimanno, F.G. 1999. Somatic embryogenesis and plant regeneration from pistil thin cell layers of *Citrus*. *Plant Cell Rep.* **18**: 935-940.

Carimi, F., Tortorici, M.C., Pasquale, F.D., Crescimanno, F.G. 1998. Somatic embryogenesis and plant regeneration from undeveloped ovules and stigma/style explants of sweet orange navel group (*Citrus sinensis* L.) Osb. *Plant Cell Tiss. Org. Cult.* **54**: 183-191.

Carlson, P.S., Smith, H.H. and Dearing, R.D. 1972. Parasexual interspecific plant hybridisation. *Proc. Natl. Acad. Sci.* **69**: 2292-2294.

Carraway, D.T. and Merkle, S.A. 1997. Plantlet regeneration from somatic embryos of American chestnut. *Can. J. For. Res.* **27**: 1805-1812.

Chalmers, K.J., Newton, A.C., Waugh, R., Wilson, J. and Powell, W. 1994. Evaluation of extent of genetic variation in mahoganies (Meliaceae) using RAPD markers. *Theor. Appl. Genet.* **89**: 504-508.

Chalmers, K.J., Waugh, R., Sprent, J.I., Simons, A.J. and Powell, W. 1992. Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculata* using RAPD markers. *Heredity*: **69**: 465-472.

Changtragoon, S. and Szmidt, A.E. 1993. An integrated population genetic approach to conserve forest tree diversity in Thailand. In: ASEAN Seminar on Management and Conservation of Biodiversity. Session 111, pp. 217-224. ASEAN Institute of Forest Management, Kuala Lumpur, Malaysia.

Chaturvedi, H.C. and Mitra, G.C. 1974. Clonal propagation of *Citrus* from somatic callus cultures. *Hort. Sci.* **9**: 118-120.

Chawla, H.S. 1988. Isozyme modifications during morphogenesis of callus from barley and wheat. *Plant cell Tiss. Org. Cult.* **12**: 299-304.

Cheliak, W.M. and Pitel, J.A. 1984. Electrophoretic identification of clones in trembling aspen. *Can. J. For. Res.* **14**: 740-743.

Cheliak, W.M. and Rogers, D.L. 1990. Integrating biotechnology into tree improvement programmes. *Can. J. For. Res.* **20**: 452- 463.

Cheliak, W.M., Skroppa, T. and Pitel, J.A. 1987. Genetics of polycross 1. Experimental results from Norway spruce. *Theor. Appl. Genet.* **73**: 321-329.

Chen, S., Shao, H., Hu, D., Lin, S. and Zheng, X. 1994. Identification of mildew resistant gene from *Hevea* tree by RAPD technique. *Chinese Journal of Tropical Crops.* **15**: 26.

Chen, S., Shao, H., Hu, D., Zheng, X. and Fu, S. 1999. Identifying, cloning and sequencing of a RAPD marker linked to mildew resistant gene of *Hevea Brasiliensis*. IRRDB Rubber Conference, China.

Cheng, H.Y., Yang, W.C. and Hsiao, J.Y. 2001. Genetic diversity and relationship among peach cultivars based on random amplified microsatellite polymorphism (RAMP). *Bot. Bull. Acad. Sin.* **42**: 201-206.

Chevreau, G., Leuliette, S. and Gallel, M. 1997. Inheritance and linkage of isozyme loci in pear (*Pyrus communis* L.). *Theor. Appl. Genet.* **94**: 498-506.

Chrampach, A. and Rodbard, D. 1971. Poly acrylamide gel electrophoresis. *Science* **172**: 440-451.

Chung, M.G. and Chung, M.V. 2000. Levels and partitioning of genetic diversity of *Camellia japonica* (Theaceae) in Korea and Japan. *Silvae Genet.* **49**:119-124.

Chung, M.G., Chung, J.M. and Epperson, B.K. 1999. Spatial genetic structure of allozyme polymorphisms within populations of *Rhus trichocarpa* (Anacardiaceae). *Silvae Genet.* **48**: 223-227.

Claros, M.G., Crespillo, R., Auilar, M.L. and Canovas, F.M. 2000. DNA fingerprinting and classification of geographically related genotypes of olive tree (*Olea europaea* L.). *Euphytica* **116**: 131-142.

Cocking, E. 1960. A method for the isolation of plant protoplasts and vacuoles. *Nature* **187**: 962-963.

Conkle, M.T. 1992. Genetic diversity - seeing the forest through the trees. *New Forests* **6**: 5-22.

Conkle, M.T., Hodgskiss, P.D., Nunnally, L.B. and Hunter, S.C. 1982. Starch gel electrophoresis of conifer seeds: a laboratory manual. USDA For. Serv. Gen. Tech. Rep., PSW- 64, p. 18.

Coolidge, T.B. 1939. A simple cataphoresis apparatus. *J. Biol. Chem.* **127**: 551-553.

Cordewiner, J., Booij, H., Vander Zandt, H., Van Kammen, A. and De verges, S. 1991. Camicamycin inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isozymes. *Planta* **184**: 478-486.

Cousineau, J.C., Anderson, A.K. and Daubeny, H.A. 1993. Characterization of red raspberry cultivars and selections using isozyme analysis. *Hort. Sci.* **28**: 1185-1186.

Cuenca, B., Sanjose, M., Martinez, M.T., Ballester, A. and Vieitez, A.M. 1999. Somatic embryo from stem and leaf explants of *Quercus robur* L. *Plant Cell Rep.* **18**: 538-544.

Das, S., Das, S., Mujib, A., Pal, S. and Dey, S. 1998. Influence of carbon source and pH on rapid mass propagation of *Santalum album* L. by somatic embryogenesis: the application of Biotechnology in agro-forestry. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 66-68. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Das, S., Das, S., Pal, S., Mujib, A., Sahoo, S., Dey, S., Ponde, N.R. and Dasgupta, S. 1999. A novel process for rapid mass propagation of *Santalum album* L. in liquid media and bioreactors. *Acta Hort.* **50**: 281-288.

Dawson, I.K., Simons, A.D., Waugh, R. and Powell, D. 1996. *Gliricidia sepium* x *G. maculata* in Meso-America revealed by PCR based assays. *Mol. Ecol.* **5**: 89-98.

De Lala, M.L., Gomes, E.A., Esbrisse, E.J. and De Araujo, E.F. 2000. Random amplified polymorphic DNA (RAPD) analysis of genotypic identities in *Eucalyptus* clones. *Silvae Genet.* **49**: 239-243.

Deragon, J.M. and Landry, B.S. 1992. RAPD and other PCR based analyses of plant genomes using DNA extracted from small leaf discs. *PCR Methods and Applications.* **1**: 175-180.

Dey, S. 2001. Mass cloning of *Santalum album* L. through somatic embryogenesis: scale up in bioreactor. *Sandalwood Research Letter* **13**: 1-3.

Dijkstra, J. and Le, T.S. 1969. Presence of mycoplasma like bodies in the phloem of sandal affected with spike disease. *Netherlands J. Plant Pathol.* **75**: 374-378.

Douglas, J.S., Hart, R.A.J. and Ranganathan, G.S. 1982. Forest farming-Prosperity for India. Natraj Publishers, Dehra Dun, India, p. 227.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of DNA from fresh plant tissue. *Focus* **12**: 13-15.

Druart, P.H. 1980. Plantlet regeneration from root callus of different *Prunus* sp. *Sci. Hort.* **12**: 339-342.

Durrum, E.L. 1950. A microelectrophoretic and microionophoretic technique. *J. Am. Chem. Soc.* **72**: 2943-2948.

Durzan, D.J. 1982. Somatic embryogenesis and sphaeroblasts in conifer cell suspensions. In: Plant Tissue Culture (Fujiwara, A., Ed.), pp. 113-114. 5th International Congress on Plant Tissue and Cell Culture, Maruzen, Tokyo.

Eastman, P.A.K., Webster, F.B., Pitel, J.A. and Roberts, D.R. 1991. Evaluation of somaclonal variation during somatic embryogenesis of interior spruce (*Picea glauca*) using culture morphology and isozyme analysis. *Plant Cell Rep.* **10**: 425-430.

Echt, C.S. and Nelson, C.D. 1997. Linkage mapping and genome length in eastern white pine (*Pinus strobus* L.). *Theor. Appl. Genet.* **94**: 1031-1037.

Edallo, S., Zucchini, Y.C., Penenzin, M. and Salamini, F. 1981. Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* culture and plant regeneration in maize. *Maydica* **26**: 39-56.

Egerton-Warburton, L.M. 1990. Techniques for assessing isoenzyme activity in *Santalum album* L. and preliminary results. *Mulga Research Centre Journal* **10**: 56-60.

Elena-Rossello, J.A. and Cabrera, G. 1996. Isozyme variation in natural population of cork oak (*Quercus suber* L.). Population structure, diversity, differentiation and gene flow. *Silvae Genet.* **45**: 229-235.

El-Kassaby, Y.A., Barnes, S., Cook, C. and Mac Leod, D.A. 1993. Supplemental mass pollination success rate in a mature Douglas-fir seed orchard. *Can. J. For. Res.* **23**: 1096-1099.

Endler, J.A. 1977. Geographic variation, species and clines. Princeton Univ. Press, Princeton, New Jersey.

Erikson, G., Namkoong, G. and Roberds, J.H. 1993. Dynamic gene conservation for uncertain future. *For. Ecol. Manag.* **62**: 15-37.

Everett, N.P., Wach, M.J. and Ashworth, D.J. 1985. Biochemical markers of embryogenesis in tissue cultures of the maize inbred 373. *Plant Sci.* **41**: 133-140.

FAO, 1999. State of world's forests. Food and Agricultural Organization of the United States. Rome, Italy, p. 154.

Faure, O., Aarrouf, J. and Nougarede, A. 1996. Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L.): proembryogenesis. *Ann. Bot.* **78**: 23-28.

Fazekas, A.J. and Yeh, F.C. 2001. Random amplified polymorphic DNA diversity of marginal and central populations in *Pinus contorta* subsp. *latifolia*. *Genome* **44**: 13-22.

Febbri, A., Hormoza, J.I. and Polito, V.S. 1995. Random amplified polymorphic DNA analysis of olive (*Olea europea* L.) cultivars. *J. Amer. Soc. Hort. Sci.* **120**: 538-542.

Feirer, R.P. and Simon, P.W. 1991. Biochemical difference between carrot inbreds differing in plant regeneration potential. *Plant Cell Rep.* **10**: 152-155.

Feret, P.P. and Bergmann, F. 1976. Gel electrophoresis of proteins and enzymes. In: Modern Methods in Forest Genetics (Miksche, J., Ed.), pp. 49-77. Berlin-Heidelberg, Springer Verlag.

Fernandez, P.C., Bapat, V.A. and Rao, P.S. 1994. Effect of crushed seed homogenate on germination of synthetic seeds of *Santalum album* L. *Ind. J. Exp. Bot.* **32**: 840-841.

Finkeldey, R. and Gregarious, H.F. 1994. Genetic resources: selection criteria and design. In: Conservation and Manipulation of Genetic Resources in Forestry (Kim, Z.S. and Hattemer, H.S., Eds.), pp. 322-347. Kwang Moon Kag, Seoul.

Ford, C. S., Jones, N.B. and Vanstaden, J. 2000. Optimization of a working cryopreservation protocol for *Pinus patula* embryonic tissue. *In Vitro Cell Dev. Biol.* **36**: 366-376.

Fox, J.E.D., Brand, J.E., Barrett, D.R. and Effendi, M. 1994. Genetic variation in *Santalum album* in Timor, Indonesia, pp. 79-103. Sandalwood Workshop, New Caledonia, ACIAR contribution No.4, CIRAD/FAO, Rome.

Frankel, O.H. 1983. The place of management in conservation. In: Genetics and Conservation A Reference for Managing Wild Animals and Plant Populations (Schonewald-Cox, C.M., Chambers, S.M., Mac Bryde, B. and Thomas, L., Eds.), pp. 1-14. Benjamin/Cummings, Menlo Park, CA, USA.

Frankel, O.H., Brown, A.H. and Burds, J.J. 1995. The conservation of plant biodiversity, Cambridge University Press, Cambridge

Fransz, P.F., De Ruijter, N.C.A. and Schel, J.H.M. 1989. Isozymes as biochemical and cytochemical markers in embryogenic callus cultures of maize (*Zea mays* L.). *Plant Cell Rep.* **8**: 67-70.

Galderisi, U., Cipollaro, M., Dibernado, C., Demasi, L., Galano, G. and Cascino, A. 1998. Molecular typing of Italian sweet chestnut cultivars by random amplified polymorphic DNA analysis. *J. Hort. Sci. Biotechnol.* **73**: 259-263.

Gallagher, S.R. 1996. Quantification of DNA and RNA with absorption and fluorescence spectroscopy. In: Current Protocols in Molecular Biology, Vol. III. (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., Eds.), pp. A.3D.1 - A.3D.8 John Wiley and Sons, USA.

Gaspar, T., Wyndaele, R., Bouchet, M. and Ceulemans, E. 1977. Peroxidase and α-amylase activities in relation to germination of dormant and non-dormant wheat. *Physiol. Plant.* **40**: 11-14.

*Gautheret, R.J. 1939. Sur la possibelitiede realiser la culture indefenie des tissue de tubercules decarotte. *Cr. Acad. Sci.* **8**: 118-120.

*Gautheret, R.J. 1940. Nouvelles recherches sur le bouregeonnement du tissu cambial d" *Ulmus campestris* cultive *in vitro*. *Cr. Acad. Sci.* **210**: 744-746.

Gerlach, H.K. and Stosser, R. 1997. Patterns of random amplified polymorphic DNAs for sweet cherry (*Prunus avium* L.) cultivar identification. *J. Appl. Bot.* **71**: 212-218.

Ghosh, S.K. and Balasundaran, M. 1995. Occurrence of spike disease of sandal in Kerala. In: Recent Advances in Research and Management of *Santalum album* L. in India (Srimathi, R.A., Kulkarni, H.D. and Venkatesan, K.R., Eds.), pp. 119-129. Associated Publishing Company, New Delhi.

Ghosh, S.K., Balasundaran, M. and Ali, M.I.M. 1985. Studies on the spike disease of sandal. KFRI Research Report 37, Kerala Forest Research Institute, Thrissur.

Ghosh, S.K., Balasundaran, M. and Ali, M.I.M. 1992. Sandal spike disease. In: Plant Diseases of International Importance - Diseases of Sugar, Forest and Plantation crops, Vol. IV (Mukhopadhyay, A.N., Kumar, J., Chaube, H.S. and Singh, U.S., Eds.), pp. 296-310. Prentice Hall, New Jersey.

Gibson, J.P. and Hamrick, J.L. 1991. Genetic diversity and structure in *Pinus pungens* population. *Can. J. For. Res.* **21**: 635 - 642.

Glaubitz, J.C. and Moran, G.F. 2000. Genetic tools: the use of biochemical and molecular markers. In: Forest Conservation Genetics. Principles and Practice. (Young, A., Boshier, D. and Boyle, T., Eds.), pp. 39-59. CSIRO Publishing, Australia.

Gobel, E., Brown, P.T.H. and Lorz, H. 1985. *In vitro* cultures of *Zea mays* and analysis of regenerated plants. In: Nuclear Techniques and *In Vitro* Culture for Plant Improvement, pp. 21-27. IAEA/FAO, Vienna.

Godwin, I.D., Sangduen, N., Kunanuvatchaidach, R., Piperidis, G. and Adkins, S.W. 1997. RAPD polymorphism among variant and phenotypically normal *rice (Oryza sativa* var. *indica*) somaclonal progenies. *Plant Cell Rep.* **16**: 322-324.

Gonialves, J.C., Diogo, G. and Amancio, S. 1998. *In vitro* propagation of chestnut (*Castanea satie* x *C. cnenato*): Effects of treatments on plant survival, peroxidase activity and anatomical changes during adventitious root formation. *Sci. Hort.* **72**: 265-277.

Gontcharenko, G.G., Padutov, V.E. and Silin, A.E. 1993. Allozyme variation in natural populations of Eurasian pines.1. Population genetic structure, genetic variation and differentiation in *Pinus pumila* (Pall.). Regel from Chukotsk and Sakhalin. *Silvae Genet.* **42**: 237-246.

Gottileb, L.D. 1977. Electrophoretic evidence and plant systematics. *Ann. Missouri bot. Gard.* **64**: 161-180.

Grandclement, C. and Thomas, G. 1996. Detection and analysis of QTLs based on RAPD markers for polygenic resistance to *Plasmodifora brassicae* Woron in *Brassica oleraceae* L. *Theor. Appl. Genet.* **93**: 86-90.

Grattapaglia, D., Bertolucci, F.L. and Sederoff, R. 1995. Genetic mapping QTLs controlling vegetative propagation in *Eucalyptus grandis* and *E. urophylla* using a pseudo testcross strategy and RAPD markers. *Theor. Appl. Genet.* **90**: 933-947.

Grattapaglia, D., Bertolucci, F.L., Penchel, R. and Sederoff, R. 1996. Genetic mapping of quantitative trait loci controlling growth and wood quality traits in *Eucalyptus grandis* using a maternal half-sib family and RAPD markers. *Genetics* **144**: 1205-1214.

Grattapaglia, D., Clampi, A.Y., Collevatti, R., Reis, A.M.M., Walter, B.M., Gandara, F.B. and Brondani, R.P.V. 1997. Development and application of genomic technologies for genetic conservation of forest trees. Proceedings of IFS workshop on 'Recent advances in biotechnology for tree conservation and management", 15 to 19 September, Florianopolis, Brazil.

Grattapaglia, D., O'Mally, D.M. and Dvorak, W. 1992. Multiple applications of RAPD markers to genetic analysis of *Eucalyptus* sp. In: Proceedings of the IUFRO International Conference on Breeding Tropical Trees, section 2.02.08, pp. 436-450. Columbia.

Gupta, P.K. 1991. Development of an embryonic system for automated propagation of forest trees. In: Scale-up and Automation in Plant Propagation (Vasil, I.K., Ed.), pp. 76-90. Academic press, New York.

Gupta, P.K., Mascarenhas, A.F. and Jagannathan, V. 1980. Tissue culture of forest trees: clonal multiplication of *Tectona grandis* L. (teak) by tissue culture. *Plant Sci. Lett.* **17**: 259-268.

Gupta, P.K., Pullmann, G., Timmis, R., Kreitinger, M., Carlson, W.C., Grob, J. and Welty, E. 1993. Forestry in the 21st century: the biotechnology of somatic embryogenesis. *Biotechnology* **11**: 454-459.

Gupta, P.K., Timmis, R., Pullman, G., Yancey, M., Kreitinger, M., Carlson, W. and Carpenter, C. 1991. Development of an embryonic system for automated propagation of forest trees. In: Cell Culture and Somatic

Embryogenesis of Plants Vol. 8 (Vasil, I.K., Ed.), pp. 75-88, Academic Press, San Diego.

*Haberlandt, G. 1902. Kuterversuche mit iso lierten pflanzen zellen Sitzungsber Acad. Wiss Wien Math. Naturwiss. KI; Ab. 1. 111: 69-92.

Hadrys, H., Balick, M. and Schierwater, B. 1992. Applications of random amplified polymorphic DNA (RAPD). *Mol. Ecol.* **1**: 55-64.

Haggmann, H., Jokele, A., Krajnakova, J., Kauppi, A., Niemi, K. and Aronen, T. 1999. Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction. *J. Exp. Bot.* **50**:1769-1778.

Hake, S. and Walbot, V. 1980. The genome of *Zea mays*, its composition and homology to related grasses. *Chromosoma* **7**: 70.

Hammerschmidt, R., Nuckles, E. M. and Kuc, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium. Physiol. Plant Pathol.* **20**: 73-82.

Hamrick, J.L. 1987. Gene flow and distribution of genetic variation in plant populations. In: Differentiation Patterns in Higher Plants (Urbanska, K., Ed.), pp. 53-76. Academic Press, New York.

Hamrick, J.L. and Godt, M.J.W. 1989. Allozyme diversity in plant species. In: Plant Population genetics, Breeding and Gene Resources (Brown, A.H.D., Clegg, M.T., Kahler, A.L. and Wier, B.S., Eds.), pp. 43-63. Sinauer Associates Inc., Sunderland, Massachusetts.

Hamrick, J.L., Godt, M.J.W. and Sherman-Broyles, S.L. 1992. Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6**: 95-124.

Hamrick, J.L. and Loveless, M.D. 1985. Genetic structure of tropical populations: associations with reproductive biology. In: Evolutionary Ecology of Plants. (Brock, J.H. and Linhart, Y.B., Eds.), pp. 34-46. Westview Press, Boulder, CO, USA.

Hamrick, J.L. and Nason, J.D. 2000. Gene flow in forest trees. In: Forest Conservation Genetics. (Young, A., Boshier, D. and Boyle, T., Eds.), pp. 81-89. CSIRO Publishing, Wallingford, UK.

Han, K.H., Gordon, M.P. and Strauss, S.H. 1997. High frequency transformation of cotton wood (genus *Populus*) by *Agrobacterium rhizogenes*. *Can. J. For. Res.* **27**: 464-471.

Harada, T., Matsukawa, K., Sato, T., Ishikawa, R., Niizeki, M. and Saito, K. 1993. DNA- RAPDs detect genetic variation and paternity in *Malus*. *Euphytica* **65**: 87-91.

Hardy, G.H. 1908. Mendelian proportions in a mixed population. *Science* **28**: 49-50.

Harris, H. 1966. Enzyme polymorphisms in man. *Proc. Roy. Soc.* **164**: 298-310.

Hashmi, G., Huettel, R., Meyer, R., Krusberg, L. and Hammerschlag, F. 1997. RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. *Plant Cell Rep.* **16**: 624-627.

Hayama, H., Shimada, T., Haji, T., Ito, A., Kashimura, Y. and Yoshikawa, H. 2000. Molecular cloning of a ripening related expansin c DNA in peach: evidence for no relationship between expansin accumulation and change in fruit firmness during storage. *J. Plant Physiol.* **157**: 567-575.

Hedrick, P. 1992. Shooting the RAPDs. *Nature* **355**: 679-680.

Hedrys, H., Balick, M. and Schierwater, B. 1992. Applications of randomly amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* **1**: 55-63.

Heinz, B. 1998. PCR based chloroplast assays for the identification of native *Populus nigra* and introduced poplar hybrids in Europe. *J. For. Gen.* **5**: 31-38.

Hemanthkumar, N.V., Narayanaswamy, P., Theertha Prasad, D. Mukunda, G.K. and Sondur, S.L. 2001. Estimation of genetic diversity of commercial mango (*Mangifera indica* L.) cultivars using RAPD markers. *J. Hort. Sci. Biotechnol.* **76**: 529- 533.

Hewson, H. and George, A. 1984. Santalaceae. In: Flora of Australia (George, A., Ed.), pp. 29-66. Australian Government Publishing Service, Canberra.

Hirai, D., Shiraj, K., Shirai, S. and Sakai, A. 1998. Cryopreservation of *in vitro* grown meristems of strawberry (*Fragaria xananassa* Duch.) by encapsulation-vitrification. *Euphytica* **101**: 109-117.

Hoelzel, A.R. 1991. Molecular genetic analysis of populations - A practical approach. Oxford University Press, Oxford, New York, p. 315.

Hormaza, J.I., Pinney, K and Polito, V.S. 1998. Genetic diversity of pistachio (*Pistacia vera* L.) germplasm based on randomly amplified polymorphic DNA (RAPD) markers. *Econ. Bot.* **52**: 78-87.

Hosoya, T. 1960. Turnip peroxidase. Purification and physiochemical properties of multiple components in turnip peroxidase. *J. Biochem.* **47**: 369-381.

House, A.P.N. and Bell, J.C. 1994. Isozyme variation and mating system in *Eucalyptus urophylla* S. T. Blake. *Silvae Genet.* **43**: 167-176.

Huang, H., Dane, F. and Norton, J.D. 1994. Genetic analysis of 11 polymorphic isozyme loci in chestnut species and characterization of chestnut cultivars by multi-locus allozyme genotypes. *J. Am. Soc. Hort. Sci.* **119**: 840-849.

Huang, H. and Layne, A.R. 1997. Using isozyme polymorphisms for identifying and assessing genetic variation in cultivated pawpaw (*Asimina triloba* (L.) Dunal.). *J. Am. Soc. Hort. Sci.* **124**: 504-511.

Huang, Q.Q. and Zhang, Y.K. 2000. Study on the genetic structures in *Pinus massoniana* (Masson Pine) populations. *Silvae Genet.* **49**: 190-195.

Hubby, S.L. and Lewontin, R.C. 1966. A molecular approach to the study of heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura. Genetics* **54**: 577-594.

Hull, R., Horne, R.W. and Nayar, R.M. 1969. Mycoplasma like bodies associated with sandal spike. *Nature* **224**: 1121-1122.

Hunter, R.L. and Markert, C.L. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science* 125: 1294-1295.

Ibrahim, A.M. 1996. Genetic variation in *Faidherbia albida*: implications for conservation of genetic resources and tree improvement. Univ. Helsinki *Tropic. Forest. Rep.* **11**: 86.

ICFRE, 2000. Forest statistics India, Directorate of Statistics, M/s Valley Offset Printers, Dehra Dun, p. 308.

Ilah, A., Abdin, M.Z. and Mujib, A. 2002. Somatic embryo irregularities in *in vitro* cloning of sandal (*Santalum album* L.). *Sandalwood Research Newsletter* **15**: 2-3.

Isabel, N., Tremblay, L. Michaud, M., Tremblay, F.M. and Bousquet, J. 1993. RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill.) B.S.P. *Theor. Appl. Genet.* **86**: 81-87.

Islam, R., Hossain, M., Khalekuzzaman, M. and Joarder, O.I. 2000. *In vitro* plant regeneration from zygotic embryos of *Aegle marmelos* Carr. *Ban. J. For.* **29**: 177-181.

Iyengar, A.V V. 1955. The physiology of the spike disease of sandal. *Rev. App. Mycol.* **34**: 254.

*Jaccquiot, C. 1951. Action du mesoinositol et de 1' adenine sur la formation de bourgeons par le tissu cambial d' *Ulmus campestris* cultive *in vitro*. *Cr. Acad. Sci.* Paris **240**: 557-558.

Jain, S., Gupta, P. and Newton, R. 1995. Somatic embryogenesis in woody plants, Vol. 1, Kluwer Academic Publications, Dordrecht.

Jain, S.H., Angadi, V.G., Rajeevalochan, A.N., Sankaranarayana, K.H., Theagarajan, K.S. and Rangaswamy, C.S. 1998. Identification of provenances of sandal in India for genetic conservation. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 117-120. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Jazdzewska, E., Sadoch, Z., Niklas, A. and Majewska-Sawka, A. 2000. Plant regeneration from sugar beet leaf protoplast: analysis of shoots by DNA fingerprinting and restriction fragment length polymorphism. *Can. J. Bot.* **78**:10-18.

Jeeva, V., Saravanan, S., Devaraj, P. and Lakshmidevi, R. 1998. Malady and remedy of sandal cultivation in farmlands and private lands an overview. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 16-19. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Jekkel, Z., Gyulai, G., Kiss, J., Kiss, E. and Heszky, L.E. 1998. Cryopreservation of horse chestnut (*Aesculus hippocastanum* L.) somatic embryos using three different freezing methods. *Plant Cell Tiss. Org. Cult.* **52**: 193-197.

Jimenez, P., Alia, R. and Gil, L. 1999. Genetic variation in central and marginal populations of *Quercus suber* L. *Silvae Genet.* **48**: 276-284.

Jorge, V., Fregene, M.A., Duque, M.C., Bonierbale, M.W., Tohme, J. and Verdier, V. 2000. Genetic mapping of resistance to bacterial blight disease in cassava (*Manihot esculenta* Crantz.). *Theor. Appl. Genet.* **101**: 865-872.

Jourde, L.B. 1980. The genetic structure of subdivided human populations. A review. In: Current Developments in Anthropological Genetics. Vol. 1. (Mielke, J.H. and Crawford, M.H., Eds.), pp. 135-208. Plenum Press, New York.

Kamala, B.S., Angadi, V.G., Parthasarathi, K. and Rai, S.N. 1986. Symptoms and changes in the pattern of multi molecular forms of peroxidase associated with trace element deficiency in sandal (*Santalum album* Linn.) seedlings. *Van Vigyan* **24**: 49-55.

Kanashiro, M., Harris, S.A. and Simons, A. 1997. RAPD diversity in Brazil nut (*Bertholletia excelsa* HUMB. and BONPL., Lecythidaceae). *Silvae Genet.* **46**: 219-223.

Karhu, S.T. 1997. Sugar use in relation to shoot induction by sorbitol and cytokinin in apple. *J. Am. Soc. Hort. Sci.* **122**: 476- 480.

Karkkainen, K., Koshi, V. and Savolainen, O. 1996. Geographical variation in the inbreeding depression of Scots pine. *Evolution* **50**: 111-119.

Karp, A. 1991. On the current understanding of somaclonal variation. *Plant Mol. Cell Biol.* **77**: 1-58.

Karp, A. and Bright, S.W.J. 1985. On the causes and origins of somaclonal variation. *Plant Mol. Cell Biol.* **2**: 199-234.

Karp, A., Kresovich, S., Bhat, K.V., Ayad, W.G. and Hodkin, W.G. 1997. Molecular tools in plant genetic resource conservation: A guide to the technologies, IBPGRI Technical Bulletin No.2, IBPGRI, Rome.

Karun, A. and Sajini, K.K. 1996. Plantlet regeneration from leaf explants of oil palm seedlings. *Curr. Sci.* **71**: 922-955.

Kasai, K., Morikawa, Y., Sorri, V.A., Valkonen, J.P.T., Gebhardt, C. and Watanabe, K.N. 2000. Development of SCAR markers to the PVY resistance gene *RY adg* based on a common feature of plant disease resistance genes. *Genome* **43**: 1-8.

Kaundun, S.S., Zhyvoloup, A. and Park, Y.G. 2000. Evaluation of the genetic diversity among elite tea (*Camellia sinenesis* var. *sinenesis*) accessions using RAPD markers. *Euphytica* **115**: 7-16.

Kertadikara, A.W.S. and Prat, D. 1995. Genetic structure and mating system in teak (*Tectona grandis* L.F.) provenance. *Silvae Genet.* **44**: 104-110.

Kjaer, E.D., Seigismund, H.R. and Suangatho, V.A. 1996. Multivariate studies on genetic variation in teak (*Tectona grandis* L.). *Silvae Genet.* **45**: 361-368.

Klein-Lankhorst, R.M., Vermunt, A., Weide, R., Liharska, T. and Zabel, P. 1991. Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD). *Theor. Appl. Genet.* 83: 108-114.

Komamine, A., Kawahara, R., Masumoto, M., Sunabori, S., Toya, T., Fujiwara, M., Tuskahara, M., Smith, J., Ito, M., Fukuda, K., Momura, K. and

Fujimura, T. 1992. Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry and molecular biology. *In Vitro Cell. Dev. Biol.* **28**: 11-14.

Konieczny, A. and Ausubel, F.M. 1993. A procedure for mapping *Arabidopsis* mutations using codominant ecotype-specific PCR-based markers. *Plant Journal* **4**: 403-410.

Kremer, A. and Zenetto, A. 1997. Geographical structure of gene diversity in *Quercus petraea* (Matt.) Liebl. 11. Multilocus pattern of diversity. *Heredity* **78**: 476-489.

Kuginuki, Y., Ajisaka, H., Yui, M., Yoshikawa, H., Hida, K. and Hirai, M. 1997. RAPD markers linked to a club root resistance locus in *Brassica rapa* L. *Euphytica* **98**:149 - 154.

Kulkarni, H.D. 1995. Studies on variations and tree improvement aspects of sandal (*Santalum album* L.), Ph. D thesis submitted to the University of Mysore, p. 226.

Kulkarni, H.D. and Srimathi, R.A. 1982. Variation in foliar characteristics in sandal In: Biometric Analysis in Improvement of Forest Biomass (Khosla, P.K., Ed.), pp. 63-69. International Book Distributors, Dehra Dun.

Kulkarni, R.S., Fakrudin, B. and Shashidhar, K.S. 1998. Tree improvement efforts in sandal: the need to employ novel techniques. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 151-153. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Kumar, S., Sarkar, A.K. and Kunhikannan, C. 1998. Regeneration of plants from tissue culture raised safed siris (*Albizzia procera*). *Plant Cell Tiss. Org. Cult.* **54**: 137-145.

Kundu, S.K. 1999. Comparative analysis of seed morphometric and allozyme data among four populations of neem (*Azadirachta indica*). *Genet. Res. Crop Evol.* **92**: 192-197.

Kushalappa, K.A. 1983. Sandal as a species for social forestry. *My Forest* **19**: 185-187.

Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lakshmi Sita, G. 1986. Sandalwood. In: Biotechnology in Agriculture and Forestry Trees 1 (Bajaj, Y.P.S. Ed.), pp. 363-374. Springer Verlag, Berlin.

Lakshmi Sita, G. 1992. Performance of tissue cultured plants of sandalwood. *Curr. Sci.* **66**: 194.

Lakshmi Sita, G. and Bhattacharya, A. 1998. cDNA cloning and characterization of a proline (or hydroxyproline) rich protein from *Santalum album* L. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K. Eds.), pp. 29-35. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Lakshmi Sita, G., Mridula, S. and Gopinathan, K.P. 1994. Molecular analysis of somatic embryogenesis in sandalwood (*S. album*) In: Biotechnology in Germplasm Conservation, pp. 231-243. Proc. of the IV th Asia Pacific Symposium.

Lakshmi Sita, G., Raghavaram, N.V. and Vaidyanathan, C.S. 1979. Differentiation of embryoids and plantlets from shoot callus of sandalwood. *Plant Sci. Lett.* **15**: 265-270.

Lakshmi Sita, G., Shobha, J. and Vaidyanathan, C.S. 1980. Regeneration of whole plants by embryogenesis from cell suspension cultures of sandalwood. *Curr. Sci.* **49**: 196-198.

Lakshmi Sita, G., Vaidyanathan, C.S. and Ramakrishnan, T. 1982. Applied aspects of tissue culture with special reference to tree improvement. *Curr. Sci.* **51**: 89-92.

Lange, W.J., Wingfield, B.D., Viljoen, C.D. and Wingfield, M.J. 1993. RAPD fingerprinting to identify *Eucalyptus grandis* clones. *South Afric. For. J.* **167**: 47-50.

Larkin, P.J. and Scowcroft, W.R. 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* **60**: 197-214.

Lashermes, P.J., Cros, P.M. and Charrier, A. 1993. Use of random amplified DNA markers to analyze genetic variability and relationship of coffee species. *Genet. Resour. Crop. Evol.* **40**: 91-99.

Ledig, F.T. 1988. The conservation of diversity in trees. *Bioscience* **38**: 471-479.

Lee, S.W., Choi, W.Y., Kim, W.W. and Kim, Z.S. 2000. Genetic variation of *Taxus cuspidata* SIEB et ZUCC. in Korea. *Silvae Genet.* **49**: 124-130.

Lefebvre, V., Caranta, C., Pflieger, S., Moury, B., Daubeze, A.M., Blattes, A., Ferriere, C., Phaly, T., Nemouchi, G., Ruffinato, A. and Palloix. 1997. Updated intra specific maps of pepper. *Capsicum and Eggplant Newsletter* **16**: 35-41.

Lewandowski, A. and Burczyk, J. 2000. Mating system and Genetic diversity in natural populations of European Larch (*Larix decidua*) and stone pine (*Pinus cembra*) located at higher elevations. *Silvae Genet.* **49**: 158-161.

Lewontin, R.C. and Hubby, J. 1966. A molecular approach to the study of genetic heterozygosity in natural populations. II. Amounts of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics* **54**: 595-609.

Li, Z.K., Luo, L.J., Mei, H.W., Paterson, A.H., Zhao, X.H., Zhong, D.B., Wang, Y.P., Yu, X.Q., Zhu, L., Tabien, R., Stansel, J.W. and Ying, C.S. 1999. A "defeated" rice resistance gene acts as a QTL against a virulent strain of *Xanthomonas oryzae* pv. *oryzae*. *Mol. Gen. Genet.* **261**: 58-63.

Libby, W.J. and Critchfield, W.B. 1988. Patterns of genetic architecture. *Ann. For.* **13**: 77-92.

Licy, J., Dickinson, M.J., Bligh, F.J., Power, J.B., Saraswathyamma, C.K. and Davey, M.R. 2000. An approach to identify disease resistance gene analogues in *Hevea*. *Indian Journal Natural Rubber Research* **13**: 79-85.

Lin, D., Hubbes, M. and Zsuffa, L. 1994. Differentiation of poplar and willow clones using RAPD fingerprints. *Tree Physiol.* **14**: 1097-1105.

Lisitsyn, N., Lisitsyn, N. and Wigler, M. 1993. Cloning the differences between two complex genomes. *Science* **259**: 946-956.

Litt, M. and Luty, J.A. 1989. A hyper variable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* **44**: 398-401.

Litz, R.E., Knight, R.J. and Gazit, S. 1984. *In vitro* somatic embryogenesis from *Mangifera indica* L. callus. *Sci. Hort.* **22**: 233-240.

Longauer, R., Zheler, P., Paule, L. and Gomory, D. 1992. The mating system outcrossing rate and genetic differentiation in natural scots pine (*Pinus sylvestris* L.) populations from Bulgaria. *Biologia Bratislava* **47970**: 539-547.

Lowry, O.H., Rosebrough, N.J., Farr, A.l. and Randall, R.J. 1951. Protein measurement with the Folin phenol Reagent. *J. Biol. Chem.* **193**: 265-275.

Lundkvist, K. and Rudin, D. 1977. Genetic variation in 11 populations of *Picea abies* as determined by isozyme analysis. *Hereditas* **85**: 67-74.

Lynch, M. and Milligan, B.G. 1994. Analysis of population genetic structure with RAPD markers. *Mol. Ecol.* **3**: 91-99.

Mandal, A.K. and Gibson, G.L. 1998. Forest Genetics and Tree Breeding. CBS Publishers and Distributors, New Delhi, p. 268.

Mandal, A.K., Ennos, R.A. and Fugg, C.W. 1994. Mating system analysis in a natural population of *Acacia nilotica* subspecies *leiocarpa*. *Theor. Appl. Genet.* **89**: 931-935.

Marchelli, P. and Gallo, L.A. 2000. Genetic analysis of isozyme variants in open pollinated families of Southern Beech *Nothofagus nervosa* (PHIL.) DIM. *et.* MIL. *Silvae Genet.* **49**: 90-98.

Markert, C.L. and Moller, F. 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci.* **45**: 753-763.

Marriette, S., Lefranc, M., Legrand. P., Taney hill. D., Frescaria-Lacoste, N. and Macnon, N. 1997. Genetic variability in wild cherry populations in France. Effects of colonizing processes. *Theor. Appl. Genet.* **94**: 904-908.

Marshall, D.R. and Allard, R.W. 1969. The genetics of electrophoretic variants in *Avena*. I. The esterase E4, E9, E10, phosphatase P5 and anodal peroxidase APX 5 loci in *Avena barbata*. *J. Hered.* **60**: 17-19.

Martin, G.B., Williams, J.G.K. and Tanksley, S.D. 1991. Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near isogenic lines. *Proc. Natl. Acad. Sci.* **88**: 2336-2340.

Martinelli, L., Scienza, A., Villa, P., Ponti, P.D. and Gianazza, E. 1993. Enzyme markers for somatic embryogenesis in *Vitis*. *Pl. Physiol.* **141**: 476-481.

Martins-Corder, M.P. and Lopes, C.R. 1997. Isozyme characterization of *Eucalyptus urophylla* (S.T.Blake) and *Eucalyptus grandis* (HILL ex MAIDEN) populations in Brazil. *Silvae Genet.* **46**: 192 - 197.

Mathes, M.C. 1964. The *in vitro* formation of plantlets from aspen cultures. *Phyton* **21**: 137-141.

Maurer, W.D. and Tabel, V. 1995. A methodical study to improve the isozyme analysis for identification of clones of *Tilia* (Linden Syn. Lime Tree). *Silvae Genet.* **44** : 351-361.

May, B. 1994. Starch gel electrophoresis of allozymes. In: Molecular Genetic Analysis of Populations - a Practical Approach (Hoelzel, A.R., Ed.), pp. 1-27. Oxford University Press Inc, New York.

Mayr, E. 1963. Animal Species and Evolution. Harvard Univ. Press, Cambridge, p. 521.

Mc Comb, J.A. and Jones, M.G.K. 1998. Interspecific hybridization between *Santalum album* and *S. spicatum*. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and

Satyanarayana Rao, K., Eds.), ACIAR Proceedings No. 84, pp. 36-41. ACIAR, Canberra, Australia.

Mc Cune, D.C. 1961. Multiple peroxidases in corn. *Ann. New York Acad. Sci.* **94**: 723-730.

Mc Mullan, E.E. and Ebell, L.F. 1970. Disc electrophoresis of soluble proteins of conifer foliage. *Phytochem.* **9**: 2281-2285.

McKinnell, F.H. 1990. Status of management and silviculture research on sandalwood in Western Australia and Indonesia. In: Proc. of the Symposium on Sandalwood in the Pacific (Hamilton, L. and Conrad, C.E., Eds.), pp. 19-25. US Department of Agriculture, General Technical Report PSW-122, Hawaii.

Mekuria, G.T., Collins, G.G. and Sedgley, M. 1999. Genetic variability between different accessions of some common commercial olive cultivars. *J. Hort. Sci. Biotechnol.* **74**: 309-314.

Michelmore, R.W., Paran, I. and Kessel, R.V. 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis. A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci.* **88**: 9828-9832.

Michler, C.H. and Bauer, E.O. 1991. High frequency embryogenesis from leaf tissues of *Populus* sp. *Plant Sci.* **77**: 111-118.

Millar, C.I. and Marshall, K.A. 1991. Allozyme variation of pot-orford cedar (*Chamaecyparis lawsoniana*): implications for genetic conservation. *For. Sci.* **37**: 1060-1077.

Mitton, J.B. 1998. Allozymes in tree breeding research. In: Forest Genetics and Tree Breeding (Mandal, A.K. and Gibson, G.L., Eds.), pp. 239-251. CBS Publishers and Distributors, New Delhi.

Mneney, E.E., Mantell, S.H. and Bennett, M. 2001. Use of random amplified polymorphic DNA (RAPD) markers to reveal genetic diversity within and between populations of cashew (*Anacardium occidentale* L.). *J. Hort. Sci. Biotechnol.* **76**: 375-383.

Mohapatre, K.P. and Singhal, V. 2000. Role of molecular markers in biodiversity conservation. *Ann. For.* **8**: 1-7.

Moralejo, M., Rochange, F., Boudet, A.M. and Teulieres, C. 1998. Generation of transgenic *E. globulus* plantlets through *Agrobacterium tumefaciens* mediated transformation. *Aus. J. Pl. Physiol.* **25**: 207-213.

Moran, G.F. 1992. Patterns of genetic diversity in Australian tree species. *New Forests* **6**: 49-66.

Moran, G.F. and Griffin, A.R. 1985. Non-random contribution of pollen in polycrosses of *Pinus radiata* D. Don. *Silvae Genet.* **34**: 117-121.

Moran, G.F., Muona, O. and Bell, J.C. 1989. *Acacia mangium*: a tropical forest tree of the coastal low lands with low genetic diversity. *Evolution* **43**: 231-235.

Morere-Le Paven, M.C., De Buyser, J., Henry, Y., Corre, F., Hartmann, C. and Rode, A. 1992. Multiple patterns of mitochondria DNA reorganization in plants regenerated from different *in vitro* cultured explants of a single wheat variety. *Theor. Appl. Genet.* **85**: 9-14.

Morgante, M. and Olivieri, A.M. 1993. PCR amplified microsatellites as markers in plant genetics. *Plant Journal* **3**: 175-182.

Moss, D.W. 1982. Isoenzymes. Capman and Hall, London and New York.

Mosseler, A., Egger, K.N. and Hughes, G.A. 1992. Low level genetic diversity in red pine confirmed by randomly amplified polymorphic DNA markers. *Can. J. For. Res.* **22**: 1332-1337.

Moury, B., Palloix, A., Gebre Selassie, K. and Marchoux, G. 1997. Hypersensitive resistance to tomato spotted wilt virus in three *Capsicum chinense* accessions is controlled by a single gene and is overcome by virulent strains. *Euphytica* **94**: 45-52.

Mujib, A., Pal, S. and Dey, S.1998. Biotechnological roots of micropropagation of *Santalum album* L. In: Role of Biotechnology in Medicinal and Aromatic Plants (Khan, I.A. and Khan, A., Eds.), pp. 83-94. Ukaaz Publication, Hyderabad.

Mukerji, K.G. and Bhasin, J. 1986. Plant Diseases of India - a Source Book. Tata McGraw Hill Publishing Company, New Delhi.

Muller, E., Brown, P.T.H., Harhke, S. and Lorz, H. 1990. DNA variation in tissue culture derived rice plants. *Theor. Appl. Genet.* **80**: 673-679.

Mullis, K. and Faloona, F. 1987. Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. *Methods Enzymol.* **55**: 335-350.

Muralidharan, E.M., Gupta, P.K. and Mascarenhas, A.F. 1989. Plantlet production through high frequency somatic embryogenesis in long-term cultures of *Eucalyptus citriodora*. *Plant Cell Rep.* **8**: 41.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 478-497.

Murthy, B.N.S. and Saxena, P.K. 1998. Somatic embryogenesis and plant regeneration of neem (*Azadirachta indica* A. Juss.). *Plant Cell Rep.* **17**: 469-476.

Muthana, M.A. 1995. Spike disease of sandal (*S. album* L.) *Ind. For.* **81**: 500-508.

Nageswara Rao, M., Ganeshaiah, K.N. and Uma Shaanker, R. 2001. Mapping genetic diversity of sandal (*Santalum album* L.) in South India: Lessons for *in situ* conservation of sandal genetic resources. In: Forest Genetic Resources - Status, Threats and Conservation Strategies (Uma Shaanker, R., Ganeshaiah, K.N. and Kamaljit S. Bawa, Eds.), pp. 49-67. Oxford and IBH Publishing Co., New Delhi.

Nageswara Rao, M., Padmini, S., Ganeshaiah, K.N. and Uma Shaanker, R. 1999. Sandal genetic resources of South India: threats and conservation approaches. In: National Symposium on Role of Plant Tissue Culture in Biodiversity Conservation and Economic Development. Kosi-Katarmal, Almora, UP, p. 63.

Namkoong, G., Boyle, T., Gregorius, H.R., Joly, H.Y., Savolainen, O., Ratnam, R. and Young, A. 1996. Testing criteria and indicators for assessing the sustainability of forest management: genetic criteria and indicators. CIFOR, Bogor, Indonesia, p. 12.

Naqvi, N.I., Bonman, J.M., Mackill, D.J., Nelson, R.J. and Chattoo, B.B. 1995. Identification of RAPD markers linked to major blast resistant gene in rice. *Molecular Breeding* **1**: 341-348.

Nason, J.D. and Hamrick, J.L. 1997. Reproductive and genetic consequences of forest fragmentation: two case studies of neotropical canopy trees. *J. Hered.* **88**: 264-276.

Nayar, R. 1986. Natural resistance of *Santalum album* to spike disease. *J. Ind. Acad. Wood Sci.* **17**: 111-114.

Neale, D.B. and Harry, D.E. 1994. Genetic mapping in forest trees: RFLPs, RAPDs and beyond. *A Biotech. News and Information.* **6**: 107-114.

Neale, D.B. and Wlliams, C.G. 1991. Restriction fragment length polymorphism mapping in conifers and applications to forest genetics and tree improvement. *Can. J. For. Res.* **21**: 545-554.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci.* **70**: 3321-3323.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**: 583- 590.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Nesbitt, K.A., Potts, B.M., Vaillencourt, R.E., West, A.K. and Reid, J.B. 1995. Partitioning and distribution of RAPD variation in a forest tree species, *Eucalyptus globulus* (Myrtaceae). *Heredity* **74**: 628-637.

Newbury, H.J. and Ford-Lloyd, B.V. 1993. The use of RAPD for assessing variation in plants. *Plant Growth Reg.* **12**: 43-51.

*Nobecourt, P. 1939. Sur la perennite I' augmentation de volume des culture tissues vegetause. C.R. Seances. *Soc. Biol. Ses.* F.I. **130**: 1270.

Nybom, H. and Schaal, B.A. 1990. DNA fingerprints applied to paternity analysis in apples (*Malus* x *domestica*). *Theor. Appl. Genet.* **79**: 763-768.

Olivares Fuster, O., Asins, M.J., Duranvila, D. and Navarro, L. 2000. Cryopreserved callus, a source of protoplasts for *Citrus* improvement. *J. Hort. Sci. and Biotechnol.* **75**: 635-641.

Ornstein, B.J. 1964. Disc electrophoresis. I. Background and theory. *Ann. New York Acad. Sci.* **121**: 321-349.

Ortiz, A., Renaud, R., Calzada, I. and Ritter, E. 1997. Analysis of plum cultivars with RAPD markers. *J. Hort. Sci.* **72**: 1-9.

Padmavathi, G., Kole, C. and Siddiq, E.A. 1999. Detection of protein markers for identification of rice genotypes resistant to green leafhopper. *Indian J. Genet.* **59**: 417-421.

Padmesh, P., Sabu, K.K., Seeni, N. and Pushpangathan, P. 1999. The use of RAPD in assessing genetic variability in *Andrographis paniculata* Nees, a hepatoprotective drug. *Curr. Sci.* **76**: 833-835.

Paiva JR-de, Kageyama, P.V Vencovsky, R. and De-Paiva, J.R. 1994. Genetics of rubber tree (*H. brasiliensis* willd ex Ad. de Jusc.). Mull Arg 2. Mating system. *Silvae Genet.* **43**: 373-376.

Pannetier, C. and Morel, B.J. 1982. Production of somatic embryos from leaf tissues of coconut, *Cocos nucifera* L. In: Plant Tissue Culture (Fujiwara, A., Ed.), pp. 755-756. Maruzen, Tokyo.

Paran, I. and Michelmore, R.W. 1993. Development of reliable PCR based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.* **85**: 985-993.

Parani, M., Anand, A. and Parida, A. 1997. Application of RAPD finger printing in selection of micropropagated plants of *Piper longum* for conservation. *Curr. Sci.* **73**: 81-83.

Parent, J. G., and Asselin, A. 1983. Detection of pathogenesis-related proteins (PR or *b*) and other proteins in the intracellular fluid of hypersensitive plants infected with tobacco mosaic virus. *Can. J. Bot.* **62**: 1984.

Parthasarathi, K., Angadi, V.G., Sankaranarayana, K.H. and Rajeevalochan, A.N. 1986. Peroxidase isozyme activity in living bark tissue as a marker for oil bearing capacity in sandal. *Curr. Sci.* **55**: 831-834.

Parthasarathi, K., Rangaswamy, C.R. and Angadi, V.G. 1985. Leaf peroxidase, malate dehydrogenase and esterase isoenzyme pattern in ten sandal (*Santalum album* L.) types showing variation in leaf pattern. *Ind. For.* **111**: 441-449.

Pattnaik, S., Pradhan, C., Naik, S.K. and Chand, P.K. 2000. Shoot organogenesis and plantlet regeneration from hypocotyl derived cell suspensions of a tree legume, *Dalbergia sissoo* Roxb. *In Vitro Cell Dev. Biol.* **36**: 407-412.

Paul, K.G. and Ohrman, B. 1980. Inertness of horseradish peroxidase to 2,4-D. *Physiol. Plant.* **49**: 185-187.

Pedroso, M.C. and Pais, M.S. 1993. Direct embryo formation in leaves of *Camellia japonica* L. *Plant Cell Rep.* **12**: 639-643.

Periera, L.F., Goodwin, P.H. and Erickson, L. 2000. Peroxidase activity during susceptible and resistant interactions between cassava (*Manihot esculenta*) and *Xanthomonas axonopodis* pv. *manihotis* and *Xanthomonas cassavae*. J. *Phytopathol.* **148**: 575-577.

Plomion, C., O' Malley, D.M. and Durel, C.E. 1995. Genomic analysis in maritime pine (*Pinus pinaster*) - comparison of two RAPD maps using selfed and open pollinated seeds of the same individuals. *Theor. Appl. Genet.* **90**: 1028- 1034.

Pradhan, N. and Saiju, H.K. 1999. Propagation of *Santalum album* L. through tissue culture. Proc. Inter. Workshop BIO-REFOR, pp. 96-97. Nepal.

Prober, S.M. and Brown, A.H.D. 1994. Conservation of the grassy white boxwood lands - population genetics and fragmentation of *Eucalyptus albens*. *Conserv. Biol.* **8**: 1003-1113.

Prus-Glowacki, W. and Stephan, B.R. 1994. Genetic variation of *Pinus Sylvestris* from Spain in relation to other European populations. *Silvae Genet.* **43**:7-14.

Qiu, Y.L. and Parks, C.R. 1994. Disparity of allozyme variation level in three *Magnolia* (Magnoliaceae) species from the Southeastern United States. *Am. J. Bot.* **81**: 1300-1308.

Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K. 1998. Sandal and Its Products. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia, p. 264.

Rafalski, T.A. and Tingey, S.T. 1993. Genetic diagnostics in plant breeding-RAPDs, microsatellites and machines. *Trends in Genet.* **9**: 275-280.

Rai, S.N. 1990. Status and cultivation of sandalwood in India. In: Proceedings of a Symposium on Sandalwood in the Pacific (Hamilton, L. and Conrd, C.E., Eds.), pp. 66-71. Honolulu, Hawaii.

Rai, S.N. and Kulkarni, H.D. 1986. Sandalwood plantations. In: Plantations Crops Vol 1. Opportunities and Constraints (Srivastava, H.C., Bharatendu Vatsya and Menon, K.K.G., Eds.), pp. 295-300. Oxford and IBH Publishing Co., New Delhi.

Rajagopal, J., Bashyam, L., Bhatia, S., Khurana, D.K., Srivastava, P.S. and Lakshmikumaran, M. 2000. Evaluation of genetic diversity in the Himalayan poplar using RAPD markers. *Silvae Genet.* **49**: 60-71.

Rajora, O.P and Mosseler, A. 2001. Challenges and opportunities for conservation of forest genetic resources. *Euphytica* **118**: 197-212.

Rajora, O.P., Mosseler, A. and Major, J.E. 2000. Indicators for population viability in red spruce.11. Genetic diversity, population structure and mating behavior. *Can. J. Bot.* **78**: 941-956.

Ramgopal, S. 1989. Barley proteins associated with tissue differentiation *J. Plant Physiol.* **134**: 395-405

Rangaswamy, K.T. 1995. Studies on sandal spike disease. Ph. D thesis. Department of Plant Pathology, TNAU, Coimbatore, p. 226.

Rangaswamy, S. and Griffith, A.L. 1941. Demonstration of *Jassus indicus* (Walk.) as a vector of the spike disease of sandal (*Santalum album* L.). *Ind. For.* **67**: 387-394.

Rani, V., Parida, A. and Raina, S.N. 1995. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. *Plant Cell Rep.* **14**: 459-462.

Rao, C.S. 1995. Determination of oil content in small sandalwood samples - A new technique. In: Recent Advances in Research and Management of *Santalum album* L. in India (Srimathi, R.A., Kulkarni, H.D. and Venkatesan, K.R., Eds.), pp. 279-281. Associated Publishing Company, New Delhi.

Rao, P.S. and Bapat, V.A. 1978. Vegetative propagation of sandalwood plants through tissue culture. *Can. J. Bot.* **56**: 1153-1156.

Rao, P.S. and Bapat, V.A. 1992. Micropropagation of sandalwood (*Santalum album* L.). In: Biotechnology in Agriculture and Forestry (Bajaj Y.P.S., Ed.), pp. 193-210. Springer-Verlag, Berlin.

Rao, P.S. and Bapat, V.A. 1995. Somatic embryogenesis in sandalwood (*Santalum album* L) In: Somatic Embryogenesis in Woody Plants (Mohanjain, S., Gupta, P.K. and Newton, R.J., Eds.), pp. 153-170. Kluwer Academic Publishers, Netherlands.

Rao, P.S., Bapat, V.A. and Mhatre, M. 1984. Regulatory factors for *in vitro* multiplication of sandalwood tree (*S.album.*). II. Plant regeneration in nodal and internodal stem explants and occurrence of somaclonal variations in tissue culture raised plants. *Proc. Natl. Acad. Sci.* **50**: 196-202.

Rao, P.S. and Raghavaram, N.V. 1983. Propagation of sandalwood (*Santalum album* L.) using tissue and organ culture technique. In: Plant Cell Culture in Crop Improvement (Sen, K.S. and Giles, K.l., Eds.), pp. 119-124. Plenum Press, New York and London.

Rao, P.S. and Srimathi, R.A. 1976. Vegetative propagation of sandal (*S. album* L.). *Curr. Sci.* **46**: 276-277.

Rao, R. and Koskela, J. 2001. Action plans and research needs to conserve forest genetic resources in Asia. In: Forest Genetic Resources - Status, Threats and Conservation Strategies (Uma Shaanker, R., Ganeshaiah, K.N. and Kamaljit S. Bawa, Eds.), pp. 283-301. Oxford and IBH Publishing Co., New Delhi.

Rasol, M.K., Jelaska, S. and Serman, D. 1982. Isoperoxidases - early indicators of somatic embryoid differentiation in pumpkin tissue. *Acta Bot. Croat.* **41**: 33-39.

Ravikumar, R., Ananthakrishnan, G., Kathiravan, K. and Ganapathi, A. 1998. *In vitro* shoot propagation of *Dendrocalamus strictus*. *Plant Cell Tiss. Org. Cult.* **52**: 189-193.

Redolfi, P., Vecchiati, M. and Gianinazzi, S. 1982. Changes in soluble protein constitution of *Gomphrena globosa* during the hypersensitive reaction to different viruses. *Phytopathol.* **103**: 48-54.

Remadevi, O.K., Sivaramakrishnan, V.R. and Sarma, C.R. 1998. Control of arboreal termites on *S. album* L. in plantations. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 192-195. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Rink, G., Zhang, G., Jinghea, Z., Kung, F.H. and Carroll, E.R. 1994. Mating parameters in *Juglans nigra* L. seed orchard similar to natural population estimates. *Silvae Genet.* **43**: 261-263.

Rogers, D.L. and Boyle, T.J.B. 1991. Unequal paternal contributions in black spruce polycross seed lots. *Heredity* **67**: 373-379.

Rossetto, M., Weaver, P.K. and Dixon, K.W. 1995. Use of RAPD analysis in devising conservation strategies for the rare and endangered *Grevillea scapigera* (Proteaceae). *Mol. Ecol.* **4**: 321-329.

Rueveni, R. 1983. Resistance reaction of *Cucumis melo* to inoculation with *Pseudoperonospora cubensis*. *Ann. Appl. Biol.* **102**: 533-537.

Rueveni, R., Shimoni, M., Karchi, Z. and Kuc, J. 1992. Peroxidase activity as a biochemical marker for resistance of muskmelon (*Cucumis melo*) to *Pseudoperonospora cubensis*. *Phytopathol.* **82**: 749-753.

Rugini, E. and Caricato, G. 1995. Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea purpurea* L.) "Canino" and "Moraiolo". *Plant Cell Rep.* **14**: 257-260.

Rugini, E. and Mugano, M. 1998. A novel strategy for the induction and maintenance of shoot regeneration from callus derived from established shoots of apple (*Malus* x *domestica* Borkh.). *Plant Cell Rep.* **17**: 581-586.

Sabir, A., Newbury, H.J., Todd, G., Catty, J. and Ford-Lloyd, B.V. 1992. Determination of genetic stability using isozymes and RFLPs in beet plants regenerated *in vitro*. *Theor. Appl. Genet.* **84**: 113-117.

Samantaray, S., Rout, G.R. and Das, P. 1999. Changes in isozyme pattern during *in vitro* regeneration from cotyledon explants of *Brassica* species. *Biol. Plant.* **42**: 169-176.

Sane, D., Borgel, A., Verdeil, J.L. and Gassamadia, Y.K. 2000. Plantlet regeneration via somatic embryogenesis in immature zygotic embryo calli from a tree species adapted to arid land, *Acacia tortillis* subsp. *raddiana* (Savi.) Brenan. *Acta Bot. Gall.* **147**: 257-267.

Sanjaya, Ananthapadmanabha, H.S. and Ravishankar Rai, V 1998. *In vitro* shoot multiplication from the mature tree of *Santalum album* L. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 60-65. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Sawyer, B. and Jones, P. 2000. Western Australian sandalwood resource statement, Department of conservation and land management, Western Australia.

Sax, K. 1923. The association of size differences with seed coat protein and pigmentation in *Phaseolus vulgaris*. *Genetics* **8**: 552 - 560.

Scheepers, D., Eloy, M.C., and Briquet, M. 1997. Use of RAPD patterns for verification and in studying provenance relationship in Norway spruce (*Picea abies*). *Theor. Appl. Genet.* **94**: 480- 485.

Schiller, G. 1986. Isozyme variation among natural stand and plantations of alleppo pine in Israel. *Isr. J. Bot.* **35**: 161-174.

Schmidtling, R.C., Carroll, G. and La Farge. 1999. Allozyme diversity of selected and natural loblolly pine population. *Silvae Genet.* **48**: 35-45.

Schnell, R.J., Ronning, C.M. and Knight Jr., R.J. 1995. Identification of cultivars and validation of genetic relationships in *Mangifera indica* L. using RAPD markers. *Theor. Appl. Genet.* **90**: 269-274.

Schwartz, D. 1960. Electrophoretic and immunochemical studies with endosperm proteins of maize mutants. *Genetics* **45**: 1419-1427.

Schwartz, D., Fuchsmund, L., and Mc Grath, K.H. 1965. Allelic isozymes of the pH 7.5 esterase in maize. *Genetics* **52**: 1265-1268.

Scowcroft, W.R. and Larkin, P.J. 1982. Somaclonal variation: a new option for plant improvement. In: Plant improvement and somatic cell genetics (Vasil, I.K., Scowcroft, W.R. and Frey, K.J., Eds.), pp. 159-178. Academic Press, London, New York.

Shah, F.H., Rashid, O., Simons, A.J. and Dunsdon, A. 1994. The utility of RAPD markers for the determination of genetic variation in oil palm (*Elaeis guineensis*). *Theor. Appl. Genet.* **89**: 713-718.

Shankaranarayana, K.H., Kulkarni, H.D., Srimathi, R.A. and Venkatesan, K.R. 1995. Sandal oil estimation in small quantity of material through extract oil correlationships. In: Recent Advances in Research and Management of *Santalum album* L. in India (Srimathi, R.A., Kulkarni, H.D. and Venkatesan, K.R., Eds.), pp. 275-279. Associated Publishing Company, New Delhi.

Shankaranarayana, K.H., Ravikumar, G. and Rangaswamy, C.R. 1998. Content and composition of oil from the central and transition zones of sandalwood disc. In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 86-88. ACIAR Proceedings No. 84, ACIAR, Canberra, Australia.

Shepherd, D.P. 1992. The breeding systems of the Western Australian sandalwood, *Santalum spicatum* and the Indian sandalwood, *Santalum album*. Biology project, Curtin University of Technology, Perth, WA 6001, Australia.

Shimada, T., Hayama, H., Haji, T., Yamaguchi, M. and Yoshida, M. 1999. Genetic diversity of plums characterised by random amplified polymorphic DNA (RAPD) analysis. *Euphytica* **109**: 143-147.

Sigurdsson, V., Anamthawat, J.K. and Sigurgeirsson, A. 1995. DNA fingerprinting of *Populus trichocarpa* clones using RAPD markers. *New Forests* **10**: 197-206.

Sinha, B.K., Majumdar, K. and Sinha, S. 2000. *In vitro* differentiation and plant regeneration of *Albizzia chinensis* (Osb.) Merr. *In Vitro Cell Dev. Biol.* **36**: 370-374.

Skoog, F. and Miller, C.O. 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. In: The biological action of growth substances (Porter, H.K., Ed.), pp. 118-131. Academic Press, New York.

Smithies, O. 1955. Zone electrophoresis in starch gels. Group variation in the serum proteins of normal human adults. *Biochem. J.* **61**: 629-641.

Soller, M. and Beckmann, J.S. 1983. Genetic polymorphism in varietal identification and genetic improvement. *Theor. Appl. Genet.* **67**: 25-33.

Soltis, D.C. and Soltis, P.S. 1989. Isozymes in Plant Biology. Advances in Plant Sciences Series, Vol. 4. (Dudley, T. R., Ed). Discorides Press, Port land, Oregon, p. 268.

Srimathi, R.A., Kulkarni, H.D. and Venkatesan, K.R. 1977. Variation pattern in *Santalum album* L. In: Mycoplasma Diseases of Trees (Raychaudhuri, S.P., Ed.), pp. 27-31. Associated Publishing Company, New Delhi.

Srimathi, R.A., Kulkarni, H.D. and Venkatesan, K.R. 1980. Selection of sandal (*Santalum album* L.) for spike disease resistance and other qualities. *Ind. J. For.* **3**: 303-305.

Srimathi, R.A., Kulkarni, H.D. and Venkatesan, K.R. 1995. Recent advances in research and management of sandal (*Santalum album L.*) in India. Associated Publishing Co., New Delhi, p. 416.

Srinivasan, V.V Sivaramakrishna, V.R., Rangaswamy, C.R., Ananthapadmanabha, H.S. and Shankaranarayana, K.H. 1992. Sandal

(*Santalum album* L.), Indian Council of Forestry Research and Education, Dehradun, p. 233.

Steward, F.C. 1958. Interpretation of the growth from free cells to carrot plants. *Am. J. Bot.* **45**: 709-713.

Strauss, S.H., Lande, R. and Namkoong, G. 1992. Limitations of molecular marker aided selection in forest tree breeding. *Can. J. For. Res.* **22**: 1050-1061.

Sturtevant, A.H. 1965. A history of genetics. Harper and Row Publishers, New York.

Sung, Z.R. and Okimoto, R. 1981. Embryonic proteins in somatic embryos of carrot. *Proc. Natl. Acad. Sci.* **78**: 3683-3687.

Suoheimo, J., Li, C.H. and Luukkanen, O. 1999. Isozyme variation of natural populations of sal (*Shorea robusta*) in the Terai region, Nepal. *Silvae Genet.* **48**: 199-203.

Susumu Goto. 1998. Genetic fingerprinting of nematode resistant clones of Japanese black pine (*Pinus thunbergii* Parl.) using RAPD markers. *J. For. Res.* **3**: 1277-130.

Svobodova, H., Albrechtova, J., Kumstyrova, H., Vagner, M. and Vondrakova, Z. 1999. Somatic embryogenesis in Norway spruce; anatomical study of embryo development and influence of polyethylene glycol on maturation process. *Plant Physiol. Biochem.* **37**: 209-221.

Takebe, T., Labic, C. and Melchers, G. 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. *Nat. Wissenschaften* **58**: 318-320.

Talleux, L.R., Diemer, F., Sourdioux, M., Chapelain, K. and De March, G.G. 1999. Improvement of somatic embryogenesis in wild cherry (*Prunus avium* L.): Effect of maltose and ABA supplements. *Plant Cell Tiss. Org. Cult.* **55**: 199-209.

Tandon, J.N. 1995. Sandalwood oil industry. In: Recent Advances in Research and Management of *Santalum album* L. in India (Srimathi, R.A.,

Kulkarni, H.D. and Venkatesan, K.R., Eds.), pp. 186-193. Associated publishing company, New Delhi.

Tang, W. 2001. *In vitro* regeneration of loblolly pine and RAPD analysis of regenerated plantlets. *Plant Cell Rep.* **20**: 163-169.

Tang, W., Ouyanang, F. and Guo, Z.C. 1998. Plant regeneration through organogenesis from callus induced from zygotic embryos of loblolly pine. *Plant Cell Rep.* **17**: 557-561.

Tanksley, S. and Orton, T. 1982. Isozymes in Plant Genetics and Breeding. Elsevier Scientific publications, Amsterdam.

Tautorus, T.E. 1992. Bioreactor culture of black spruce and interior spruce somatic embryos. Abstract in Proceedings of the 3rd Canadian Workshop on Plant Tissue Culture and Genetic Engineering , pp. 36. University of Guelph, Canada.

Tennakoon, K.U., Ekanayake, S.P. and Etampawala, E.R.L.B. 2000. An overview of *Santalum album* research in Srilanka. *Sandalwood Research Newsletter* **11**: 1-4.

Thomas, S. 2000. Detection of sandal spike phytoplasma using immunological and molecular techniques. Ph. D thesis, Faculty of Environmental Studies, Cochin University of Science and Technology, Cochin, p. 187.

Thormann, C.E. and Osborn, T.C. 1992. Use of RAPD and RFLP markers for germplasm evaluation. Proceedings of the symposium on Applications of RAPD Technology to Plant Breeding, pp. 9-11. CSSA/ASHS/AGA.

Thorpe, T.A. 1995. Woody plants somatic embryogenesis. In: *In Vitro* Embryogenesis in Plants (Thorpe, T.A., Ed.), pp. 473-535. Kluwer Academic Publishers, Dordrecht, Germany.

Thorpe, T.A. and Gaspar, T. 1978. Changes in isoperoxidases during shoot formation in tobacco callus. *In Vitro* **14**: 522-526.

Tolun, A.A., Vetioglu, E., Nazlier Cengel, B. and Kaya, Z. 2000. Genetic structure of Black Pine (*Pinus nigra* ARNOLD subspecies *pallasiana*) populations sampled from the Bolkai mountains. *Silvae Genet.* **49**: 113-119.

Toumi, L. and Lumaret, R. 1998. Allozyme variations of cork oak (*Quercus suber* L.). The role of phylogeography and genetic introgression by other mediterranean oak species and human activities. *Theor. Appl. Genet.* **97**: 647-656.

Tulecke, W. 1987. Somatic embryogenesis in woody perennials. In: Cell and Tissue Culture in Forestry, Vol. 2. (Bonga, J.M. and Durzan, J.L., Eds.), pp. 61-81. Nijhoff, Dodrecht.

Uma Shaanker, R., Ganeshaiah, K.N. and Kamaljit, S.B. 2001. Forest Genetic Resources Status, Threats and Conservation Strategies, Oxford and IBH Publishing Co., New Delhi, p. 317.

Uma Shaanker, R., Ganeshaiah, K.N. and Nageswara Rao, M. 2000. Conservation of sandal genetic resources in India; problems and prospects. In: International Conference of Science and Technology for Managing Plant Genetic Diversity in the 21st Century, pp. 73. Kuala Lumpur, Malaysia.

Uniyal, D.F., Thapaliyal, R.C. and Rawat, M.S. 1985. Vegetative propagation of sandal by root cuttings. *Ind. For.* **111**: 145.

Vaillancourt, R.E., Potts, B.M., Manson, A., Eldridge, T. and Reid, J.B. 1995. Using RAPDs to detect QTLs in an interspecific F_2 hybrid of *Eucalyptus*. In: Eucalypt Plantations: Improving Fiber Yield and Quality (Potts, B.M., Ed.), pp. 432-433. Proc. CRC-IUFRO conf., Hobart, Australia.

Van Boren, R. and Harper, K.T. 1996. Genetic variation among populations of *Arctomecon*. Southwestern rare and endangered plants. In: Proceedings of the second conference. USDA Forest Service General Technical Report RM-GTR-**283**: 77-85.

Van Lear, B.H. and Smith, W.H. 1970. Soluble protein and enzyme patterns in shoots of slash pine under different nutritional regimes. *Phytochem.* **9**: 1929-1932.

Van Loon, L.C. 1985. Pathogenesis-related proteins. *Plant Mol. Biol.* **4**: 111-116.

Varela, M.C. and Eriksson, G. 1995. Multipurpose gene conservation in *Quercus suber* - a Portuguese example. *Silvae Genet.* **44**: 28-37.

Varghese, Y.A., Knaak, C., Sethuraj, M.R. and Ecke, W. 1997. Evaluation of random amplified polymorphic DNA (RAPD) markers in *Hevea brasiliensis*. *Plant Breeding* **116**: 47-52.

Vasil, I.K. and Hildebrandt, A.C. 1966. Growth and chlorophyll production in plant callus tissue growth *in vitro*. *Planta* **68**: 69-82.

Veerendra, H.C.S. and Bagchi, S.K. 1986. Analysis of inter tree variability in anatomical characters of *S. album* L. *Ind. For.* **112**: 80-84.

Veerendra, H.C.S. and Ananthapadmanabha, H.S. 1998. A further tree improvement strategy in sandal (*Santalum album* L.). In: Sandal and Its Products (Radomiljac, A.M., Ananthapadmanabha, H.S., Welbourn, R.M. and Satyanarayana Rao, K., Eds.), pp. 161-165. ACIAR proceedings No. 84, Canberra, Australia.

Venkatesan, K.R. 1980. A fresh look at the management of sandal. Proc. Of Second Forestry Conference, F.R.I. & Colleges, Dehra Dun.

Venkatesan, K.R. 1981. Note on artificial regeneration of sandal. Sandal Research Centre, Bangalore.

Venkatesan, K.R. 1995. Sandal and social forestry. In: Recent Advances in Research and Management of *Santalum album* L. in India (Srimathi, R.A., Kulkarni, H.D. and Venkatesan, K.R., Eds.), pp. 199-206. Associated Publishing Company, New Delhi.

Venkatesan, K.R., Srimathi, R.A. and Kulkarni, H.D. 1995. Survey of sandal populations. In: Recent Advances in Research and Management of *Santalum album* L. in India (Srimathi, R.A., Kulkarni, H.D. and Venkatesan, K.R., Eds.), pp. 3-53. Associated Publishing Company, New Delhi.

Venkatesh, C.S. 1978. Resistant breeding against sandal mycoplasma. *Ind. J. For.* **1**: 35-38.

Venkatesh, C.S. and Kedarnath, S. 1963. Breeding sandal for resistance to spike disease. *For. Genet.* **5**: 2-9.

Venkateswaran, S. and Gandhi, V. 1982. Mass propagation and genetic improvement of forest trees for biomass production by tissue culture. *Biomass* **2**: 5-15.

Veramendi, J. and Navarro, L. 1996. Influence of physical conditions of nutrient medium and source on somatic embryogenesis of date palm. *Plant Cell Tiss. Org. Cult.* **45**: 159-164.

Verdeil, J.L., Huet, C., Grosdemange, F. and Morel, J.B. 1994. Plant regeneration from cultured immature inflorescences of coconut (*Cocos nucifera* L.): evidence for somatic embryogenesis. *Plant Cell Rep.* **13**: 2118-2212.

Verhaegen, D. and Plomion, C., 1996. Genetic mapping in *Eucalyptus urophylla* and *Eucalyptus grandis* using RAPD markers. *Genome* **39**: 1051-1061.

Verma, A., Chenulu, V.V. Raychaudhuri, S.P. Prakash, N. and Rao, P.S. 1969. Mycoplasma-like bodies in tissues affected with sandal spike and brinjal little leaf. *Indian Phytopathol.* **22**: 289-291.

Vieitez, A.M., Ballester, A., Vieitez, M.L. and Vieitez, E. 1983. *In vitro* plantlet regeneration of mature chestnut. *J. Hort. Sci.* **58**: 457-463.

Vos, P., Hogers, R., Bleeker, M., Reijens, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**: 4407-4414.

Wachira, F., Tanaka, J. and Takeda, Y. 2001. Genetic variation and differentiation in tea (*Camellia sinensis*) germplasm revealed by RAPD and AFLP variation. *J. Hort. Sci. Biotechnol.* **76**: 557-563.

Wann, S.R. 1988. Somatic embryogenesis in woody plants. 1988. *Hort. Rev.* **10**: 153.

Warburton, M.L and Bliss, F.A. 1996. Genetic diversity in peach (*Prunus persica* L. Batch) revealed by Randomly amplified polymorphic DNA (RAPD) markers and compared to inbred coefficients. *J. Amer. Soc. Hort. Sci.* **121**: 1012-1019.

Weber, J.L and May, P.E. 1989. Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* **44**: 388-396.

*Weinberg, W. 1908. Uber den Nachweis der Vererbung beim Menscehen. Jahresh. Verein f. vaterl. Naturk. *Wiirttemberg* **64**: 368-382.

Welsh, J. and Mc Clelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**: 7213 - 7218.

Wendel, J.F. and Weeden, N.F. 1989. Visualization and interpretation of plant isozymes. In: Isozymes in Plant Biology. (Soltis, D.E. and Soltis, P.G., Eds.), pp. 5-45. Dioscorides Press, Oregon.

White, P.R. 1939. Potentially unlimited growth of excised callus in an artificial nutrient medium. *Am. J. Bot.* **26**: 59-64.

Wickneswary, R. 2001. Use of molecular markers to quantify genetic diversity parameters of forest trees. Forest Genetic Resources Status, Threats and Conservation Strategies (Uma Shaanker, R., Ganeshaiah, K.N. and Kamaljit S. Bawa, Eds.), pp. 127-139. Oxford and IBH Publishing Co., New Delhi.

Wickneswary, R., Norwati, M., Lee, S.L., Lee, C.T., Yeang, H.Y., Lokmal, N. and Rasip, A.G.A. 1996. Practical uses of molecular markers in tropical tree improvement. In: Tree Improvement for Sustainable Forestry (Dieters, M.J., Matheson, A.C., Nikles, D.G., Harwood, C.E. and Walker, S.M., Eds.), pp. 506-511. Proc. QFRI-IUFRO Conference, 27 October - 1 November, Calundra, Australia.

Wilhelm, E. 2000. Somatic embryogenesis in oak. *In Vitro Cell. Dev. Biol.* **36**: 349-358.

Williams, J.G.K, Kubelik, A.R., Livak, K.J., Rafalski, L.A. and Tingey, S.V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531-6535.

Williams, J.G.K., Hanafey, M.K., Rafalski, L.A. and Tingey, S.V. 1993. Genetic analysis using random amplified polymorphic DNA markers. *Meth. Enzymol.* **218**: 704-740.

Winton, L.L. 1968. Plantlets from aspen tissue cultures. *Science* **160**: 1234-12335.

Wiselogel, A.E. and Van Buijtenen, J.P. 1988. Probability of equal mating in polymix pollinations of loblolly pine (*Pinus taeda* L.). *Silvae Genet.* **37**: 184-187.

Wolter, K.E. 1968. Root and shoot initiation in aspen callus cultures. *Nature* **219**: 509-510.

Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics* **15**: 323-354.

Xie, D.Y. and Hang, Y. 2000. Regeneration of *Acacia mangium* through somatic embryogenesis. *Plant Cell Rep.* **20**: 34-41.

Yazdani, R., Yeh, F.C. and Rimsha, J. 1995. Genomic mapping in *Pinus sylvestris* L. using random amplified polymorphic DNA markers. *Forest Genetics* **2**: 109-116.

Ye, X.S., Pan, S.Q. and Kuc. J. 1990. Activity, isozyme pattern and cellular localization of peroxidase as related to systemic resistance of Tobacco to blue mold (*Peronospora tabacina*) and to Tobacco Mosaic Virus. *Physiology and Biochemistry* **80**: 1295-1299.

Yeh, F.C. 2000. Population genetics. In: Forest Conservation Genetics. Principles and Practice. (Young, A., Boshier, D. and Boyle, T., Eds.), pp. 21-37. CSIRO Publishing, Australia.

Yeh, F.C., Chong, D.K.X. and Yang, R.C. 1995. RAPD variation within and among natural populations of trembling aspen (*Populus tremuloides* Michx.) from Alberta. *J. Hered.* **86**: 454-460.

Yeh, F.C., Yang, R. and Boyle, T. 1999. POPGENE Version 1.31: Microsoft Window-based Freeware for Population Genetic Analysis. A Quick User's Guide. A joint project developed by Centre for International Forestry Research and University of Alberta, Canada, p. 25.

Yeh, F.C.H. 1989. Isozyme analysis for revealing population genetic structure for use in breeding strategies. In: Breeding Tropical Trees- Population Structure and Genetic Improvement Strategy in Clonal and Seedling

Forestry. Proceedings of IUFRO Conference, Pattaya, Thailand. (Gibson, G.L., Griffin, A.R. and Matheson, A.C., Eds.), pp. 119-131. Oxford Forestry Institute, Oxford, UK and Winrock International, Arlington, VA, USA.

Young, A., Boshier, D. and Boyle, T. 2000. Forest Conservation Genetics-Principles and Practices. CSIRO Publishing, Australia, p. 352.

Young, A.G. and Brown, A.H.D. 1999. Paternal bottlenecks in fragmented populations of the endangered grassland daisy *Rutidosis Leptorrhynchoides*. *Genet. Res.* **73**: 111-117.

Young, A.G., Brown, A.H.D. and Zich, F.C. 1999. Genetic structure of fragmented populations of the endangered grassland daisy *Rutidosis leptorrhynchoides*. *Conserv. Biol.* **13**: 256-265.

Zheng, K.L., Castiglione, S., Brasini, M.G., Morandi, C. and Sala, F. 1987. Nuclear DNA amplification in cultured cells of *Oryza sativa* L. *Theor. Appl. Genet.* **74**: 65-70.

Zhu, L.H. and Welander, M. 2000. Adventitious shoot regeneration of two dwarfing pea root stocks and the development of a transformation protocol. *J. Hort. Sci. Biotechnol.* **75**: 745-750.

*Originals not referred.