Ex-situ conservation, molecular diversity of *Pterocarpus santalinus* L.f and *Rauvolfia serpentina* (L.) Benth. ex Kurz using RAPD markers: Endangered medicinal plants

Thesis submitted to the University of Hyderabad
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By

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Declaration

I hereby declare that the work presented in the thesis titled "*Ex-situ* conservation, molecular diversity of *Pterocarpus santalinus* L.f and *Rauvolfia serpentina* (L.) Benth. ex Kurz using RAPD markers: Endangered medicinal plants" has been carried out by me under the supervision of Prof. M.N.V. Prasad, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, and that this work has not been submitted for any other degree or diploma to any University or Institute.

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Certificate

This is to Certify that the research work in the thesis titles "***Ex-situ* conservation, molecular diversity of *Pterocarpus santalinus* L.f and *Rauvolfia serpentina* (L.) Benth. ex Kurz using RAPD markers: Endangered medicinal plants"** has been carried out by Ms. Padmalatha K. under my supervision for the full period prescribed under the Ph.D ordinance of this University and that this work has not been submitted for any other degree or diploma to any University or Institute.

12.6.05

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Abbreviations

A	Absorbance
AC	Activated charcoal
A.P	Andhra Pradesh
BAP	6-Benzyl amino purine
BGCI	Botanical Garden Conservation International
B5	Gamborg' s medium.
$CHCl_3$	Chloroform
CTAB	Hexa decyl cetyl trimethyl ammonium bromide
dNTP	Deoxy nucleotide tri-phosphate
2,4 D	2,4- Dichlorophenoxyacetic acid
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium Bromide
Gm	gram
$g\ Kg^{-1}$	Gram/Kilogram
hr	Hour
IAA	Indole acetic acid
IBA	Indole butyric acid
IPRs	Intellectual Property Rights
IUCN	International Union of Conservation of Nature and Natural Resources
kg/ha	Kilogram/Hectare
KN	Kinetin
L	Litre
$LiCl_2$	Lithium Chloride
M	Molarity
MAPS	Medicinal and Aromatic Plants
mgL^{-1}	milligram/litre
mM	millimolar
µM	micromolar
µl	microlitre
µg	microgram
min	minimum
max	maximum

MS	Murashige and Skoog' s medium
mm	millimeter
MPCA	Medicinal Plant Conservation Assessment Center
MW	Molecular weight.
NAA	α-Naphthalene acetic acid
ng	nanogram
OD	Optical density
PB	Polymorphic bands
PCR	Polymerase chain reaction
PGR	Plant Genetic Resources
PMR	Plant microreserves
PVPP	Polyvinyl poly pyrrolidone
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
rpm	rotations/revolutions per minute
SDS	Sodium dodecysulphate
SE	Standard error
Sec	seconds
Taq	*Thermus aquaticus*
TE	Tris EDTA
TB	Total bands
Tris	Tris (Hydroxymethyl aminomethane)
TDZ	1-phenyl-3-(1,2,3-thiadiazol-5-yl) Urea (Thidiazuron)
UH	University of Hyderabad
UP	Uttar Pradesh
UPGMA	Unweighted Pairwise Group Matrix for Arithmetic Average.
UV	Ultra violet
VAM	Vesicular Arbuscular Mycorhiza
V/V	Volume/Volume
W/V	Weight /Volume
Z	Zeatin

1. Conservation of medicinal plants – general introduction

India is floristically rich and is recognized as one of the twelve mega biodiversity centers of the world, ranking 10th among the plant resources rich nations of the world and 4th among the countries of Asia. India is the 7th largest country in the world and Asia's 2nd largest nation with an area of 3,287,263 sq Km, and is an example of diverse ecosystems. (Swingland, 2001). It is endowed with a rich heritage of medicinal plant wealth. Based on the ethnomedicinal traditional knowledge, utilization and conservation of medicinal and aromatic plants has received considerable attention in recent times, especially in south India. Forests are the primary source of a variety of medicinal plants, while a number of the medicinal plants are also cultivated (FAO, 2003).

Conservation is the process of management of biosphere in order to obtain the greatest benefit for the present generation and maintaining the potential for future. Conservation of plant resources is of global concern because we don't know what we are losing and what we will need in future.

Conservation methods vary with many biological and environmental factors (Rajasekharan and Ganeshan, 2002). Small isolated populations, endemic and rare species in particular are subjected to genetic drift, inbreeding and their genetic variation is consequently expected to be low compared to that of larger populations which may lead to a decrease in species ability to survive environmental changes and demographic fluctuations, both in short and long term (Bilington, 1991; Gaston and Cunin, 1997a; Karron, 1997). Hence, the maintenance of genetic variation is

essential for long-term protection of a taxon (Hamrick and Godt, 1989; Simberloff, 1988).

1.1. Threats to medicinal plants

There are different primary and secondary factors that pose threat to many medicinal plants. The threats are degradation of habitat due to expanding human activity, forest decline, destructive collection of plant species, invasion of exotic species that compete with native species, increased spread of diseases, industrialization, over exploitation, human socioeconomic change and upheaval, changes in agricultural practices, excessive use of agrochemicals, natural and man-made calamities, genetic erosion etc., In South India, it is estimated that about 70-80 out of the estimated 300 medicinal plants are either endangered or threatened. Hence, there is a necessity to strike a balance between conservation and utilization of these medicinal plants (Rajasekharan and Ganeshan, 2002).

1.2. Need for conservation of medicinal plants

To meet the requirements of expanding regional and international markets healthcare products and needs of growing populations, large quantities of medicinal plants are harvested from forests (Desilva, 1997). In India large number of medicinal plants are extracted from the wild to meet the increasing demand for raw material needed for domestic consumption and for export. As a result, the natural sources are rapidly depleting. Medicinal plants contribute to health, income, agroforestry system, cultural identity and livelihood security. Hence there is a need for conservation, cultivation, maintenance and assessment of germplasm for future use.

Conservation of biological diversity involves protecting, restoration and enhancing the variety of life in an area so that the abundance and distribution of species and communities contributes to sustainable development. The ultimate goal of conservation biology is to maintain the evolutionary potential of species by maintaining natural levels of diversity which is essential for species and populations to respond to long and short term environmental changes in order to overcome stochastic factors failing which would result in extinction.

1.3. Conservation strategies for medicinal plants

The two main strategies are *ex situ* (protection of species outside their natural habitats) and *in situ* (in their natural surroundings) conservation. There is a need for coordinated conservation efforts based on these strategies (Figure 1). More information is required on medicinal plant production, utilization, trade, monitoring the stock of medicinal plants, development of sustainable harvesting practices, preservation of traditional knowledge and intellectual property rights.

World Conservation Union (formerly known as the International Union for Conservation of Nature and Natural Resources) categorized plants Red Data List Categories" (IUCN, 2001) based on the detailed knowledge of the population dynamics and genetics of the species "viz., extinct, extinct in wild, threatened (critically endangered, endangered and vulnerable) and low risk (conservation dependent, near threatened and least concern) and indeterminate where the data is

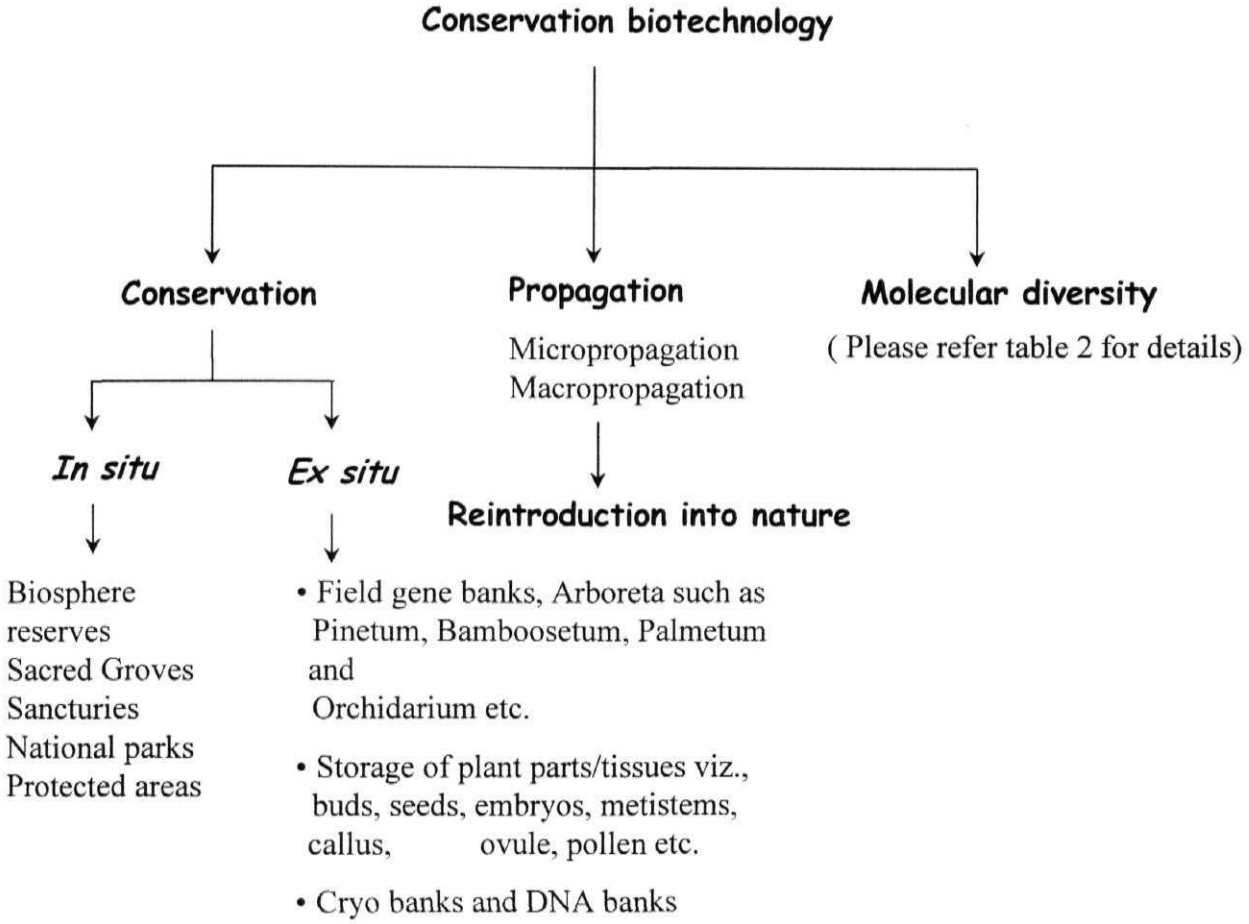

Figure 1: Plant genetic resources - Advancing conservation biotechnology

insufficient. Conservationists focus their attention exclusively on species extinction rather than genetic erosion within individual gene pools, and the latter may be of equal importance in terms of loss of biodiversity (Maxted, 2001).

Hence, it is imperative that viable strategies to conserve the populations and genetic resources of medicinally important species is a must to avoid further loss. On going efforts in India include both *in situ* and *ex situ* conservation measures *viz,* plant tissue culture, introduction of new crop genetic resources, research in habitat restoration, pollution abatement, seed storage and tissue banking etc. (Jackson and Sutherland, 2000).

1.3.1. *In situ* conservation

In situ or on site conservation involves maintaining genetic resources in their natural habitats i.e., within the ecosystem to which it is adapted, whether as wild or crop cultivar in farmer's field as components of the traditional agricultural systems (Damania, 1996) (Figure 1). The key operational steps for establishing *in situ* gene banks for conservation of prioritized medicinal plants include: Threat assessment, establishment of a network of medicinal plant forest reserves, involving local stakeholders, botanical, ecological, trade and ethno-medical surveys, assessing intra-specific variability of prioritized species, designing species recovery programmes, establishment of a medicinal plant seed center etc. Conclusively, no *in situ* conservation project can succeed without the complete cooperation and involvement of local people (Srinivasamurthy and Ghate, 2002).

1.3.2. *Ex situ* conservation

Ex situ conservation, involves conservation of biodiversity outside the native or natural habitat where the genetic variation is maintained away from its original location (Figure 1) The *ex situ* genetic conservation fulfills the requirement of present or future economic, social and environmental needs. Conservation also includes propagation and assessment of molecular diversity (Olorode, 2004)

Conservation of medicinal plants include a combination of methods, depending on factors such as geographic sites, biological characteristics of plants, available infrastructure, and network having an access to different geographical areas, human resources and number of accessions in a given collection (Rajasekharan and Ganeshan, 2002).

1.3.2.1. In vitro regeneration

In vitro regeneration include plant/explant growth, maintenance under disease free condition, retention of regenerative potential, genetic stability, and ensuring that there is no damage to the live material. It offers a number of advantages over the *in vivo* method:

a) great savings in storage space and time
b) possibility of maintaining species for which seed preservation is impossible or unsuitable and
c) disease-free transport and exchange of germplasm, since cultures are maintained under phytosanitary conditions (Natesh, 2000)

In vitro multiplication protocols for fast propagation of a number of red listed medicinal, aromatic and recalcitrant taxa that are difficult to propagate through conventional means would be very useful. Usually, shoot tips or axillary buds are cultured on a nutrient medium containing (i) high levels of cytokinins or (ii) low concentrations of auxin coupled with high-cytokinin content. Somatic embryos, or even axillary buds are encapsulated in hydrosoluble gels to form 'artificial seeds' and have used for rapid propagation of the species. Even more important is the reintroduction of *in vitro* raised material into their natural habitat and monitoring its performance over several years, to ensure fidelity with respect to active compounds or the marker chemical, vis-a-vis the parents (Natesh, 2000).

The cell culture process itself can result in genetic changes in the regenerated plants. These heritable genetic changes are termed as somaclonal variation. The presence of an undifferentiated callus phase in the regeneration protocol enhances the chances for somaclonal variation among the regenerated plants. These variations can result from simple DNA sequence differences. The cell environment appears to induce a very high frequency of such mutations. Other types of changes that frequently occur in regenerated plants could be due to chromosomal, structural and number changes due to rearrangements in multi-gene families, gene silencing due to changes in DNA methylation, action of jumping genes etc. Hence, it is necessary to avoid the use of auxin and auxin like substances in the meristem multiplication protocols. It is also mandatory to check the fidelity of the plants multiplied from the meristem cultures and plants multiplied from cryo preserved meristems by using RAPD markers.

1.3.2.2. Cryobanks for conservation

Cryopreservation of plant cells and meristems is an important tool for long-term storage of germplasm or experimental material without genetic alteration using a minimum space and maintenance. The development of methods to store apical meristems in liquid nitrogen successfully is needed to aid in the conservation of genetic resources. Cryobanks are basically meant for storage of germplasm. For long-term preservation, cryogenic storage at ultra low temperatures under liquid nitrogen (-150 to -196°C) is the method of choice. Relatively new to plants, cryopreservation has followed advances made in the mammalian systems is achieved either through slow cooling or vitrification. Encapsulation/dehydration is another new technique that offers practical advantages. It is based on the technology originally developed for production of synthetic seeds, i.e., somatic embryos encapsulated in a hydrosoluble gel. Several types of *in-vitro* raised materials such as meristems/shoot tips, cell suspensions, protoplasts, somatic embryos and pollen embryos of medicinal and aromatic species have been studied from the cryopreservation perspective (Natesh 2000).

1.3.2.3. Low temperature germplasm storage

Preservation by under-cooling has recently been applied to plant tissue cultures. The objective of this approach is to maintain tissues at low temperatures (-10 to -20 ^{0}C) but in the absence of ice crystallization. The plant tissues are immersed in immiscible oil and the emulsion thus formed can be under cooled to relatively low temperatures thereby circumventing ice formation, one of the most injurious consequences of low temperature storage. Although good recovery has been reported in certain species, this

has only been achieved using a temperature of -10^0 C and for relatively short storage periods (6-48 hours).

Recently, vitrification, simplified freezing, and encapsulation-dehydration methods have been used for storage of valuable germplasm. These new procedures may replace freeze-induced cell dehydration by removal of all or of a major part of freezable water from cells at room temperature or at 0^0 C. In the encapsulation-dehydration technique, extraction of water results in progressive osmotic dehydration, additional loss of water is obtained by evaporation and the subsequent increase of sucrose concentration in the beads. In the technique, preculturing encapsulated meristems in medium enriched with sucrose before dehydration induces resistance to dehydration and deep-freezing. The vitrification procedure for cryopreserving meristems involves preculture and/or loading and osmotic dehydration by short exposure of meristems to highly concentrated mixture of cryoprotectants. The encapsulation-dehydration technique is easy to handle and alleviates dehydration process.

1.3.2.4. Seed storage modules

Usually seeds, being natural perennating structures of plants, represent a condition of suspended animation of embryos, and are best suited for storage. By suitably altering their moisture content (5-8%), they can be maintained for relatively long periods at low temperatures (-18 ^{o}C or lower). However, in several species, rhizome/bulb or some other vegetative part may be the site of storage of active ingredients, and often, such species do not set seed. If seeds set, they may be sterile or recalcitrant i.e., intolerant of reduction in moisture or temperature, or, otherwise

unsuitable for storage. It is now possible to store materials other than seed, such as pollen or clones obtained from elite genotypes/cell lines with special attributes, *in-vitro* raised tissues/organs, or, genetically transformed material (Natesh 2004).

1.4. Constraints for conservation

The IUCN Red Data book lists 34,000 plants with endangered status. The Botanical Garden Conservation International (BGCI) 2000 database indicates that there are about 1846 botanic gardens. In-order to put efforts for *ex-situ* conservation; these botanical gardens have to cultivate several hundreds of endangered, rare and vulnerable plant species, which requires elaborate facilities and extraordinary efforts. Therefore, biologists feel that the *ex situ* conservation should be considered as a complimentary measure of i*n situ* conservation for holistic strengthening of conservation.

2. Need for molecular and morphological diversity analysis

The term "diversity" refers to the range of variation, variety or differences among set of parameters of different populations or individuals. Biodiversity specifically refers to the variety and variations within assemblages including the genetic differences among them whether naturally occurring or induced articifcially. Biodiversity has also been defined as the variety of life and its processes that encompasses various living organisms, the communities and ecosystem in which they occur (Keystone Center, 1991).

The basic building blocks are the genes contained in plants and animals, which by their diversity can enable the whole organisms to adapt to the changing environment. Plant genetic diversity is a useful parameter that can be transmitted genetically from parents to offspring, the source of tremendous variations in plants support all other forms of life on earth and covers a wide range at the evolutionary and ecological level. The diversity in plants is the basis for food and human needs for millennia and it continues to be so for the development of plant characters useful to human needs (Tanto and Demissie, 1996). It is important for broadening the genetic base and may be exploited via heterosis (Melchinger, 1999).

2.1. Monitoring genetic integrity and germplasm health

2.1.1. Need for maintenance of genetic diversity

Conservation of plant genetic diversity has recently generated a lot of interest in the tropics as a result of many years of mismanagement, adverse environment as well as socio-economic changes. Population genetic theory predicts that the decrease

in the genetic diversity limits a species ability to keep pace with the changing selection pressure (Young and Merriam, 1992). Plant species especially the perennials such as trees, rely on the available genetic diversity for stability and survival under the ever changing environments (National Research Council, 1991). Understanding species population genetic structure is essential for their conservation, planning and sustainable management (Sun *et al*., 1998). Hence a common goal of conservation is to maintain genetic diversity in "red listed" species, which is crucial for long-term survival and evolutionary response to the changing environment (Hueneke, 1991).

In addition, genetic erosion would reduce the potential of the species improvement through selection, also required for effective incorporation into breeding strategies for the selection of diverse parents to obtain heterotic hybrids as well as for the conservation and characterization of germplasm and management of plant genetic resources (Pujar *et al*, 1999).

Research on genetic diversity need to be strengthened to improve understanding of the origin; evolution and variation patterns of crop gene pools (Engles, 1989). It is the basis for the ability of the organisms to adapt to changes in their environment through natural selection. Hence, gene conservation management aims to save adaptive genetic diversity based on the knowledge of the genetic basis of adaptation (www.fao.org). However, despite its cardinal role in evolutionary theory and application, the maintenance of genetic diversity is a challenge not withstanding the dramatic discoveries of molecular biology, which revealed abundant genetic diversity in nature. The levels of genetic diversity vary non randomly among

populations, species and higher taxa and also among ecological parameters (zone, geographical range, habitat type, range and climatic region), demographic parameters (species size and population structure, gene flow, and sociality), and life history characteristics (longevity, generation length, fecundity, origin and parameters related to the mating system and mode of reproduction). Ultimately the loss of genetic diversity can affect the viability of populations and can be an important consideration in evaluating extinction risks of the species as a whole (Flather *et al.*, 2003a).

Molecular biology permitted the characterization of genetic diversity among individuals, populations and species, which are the corner stones of evolution. It did so firstly by unraveling relationships between genes and proteins (Lewontin, 1974) and secondly, by elucidating the genetic basis of evolutionary change and the nature at the extra nuclear, nuclear coding and non coding DNA regions, the structure, expression, function, mechanism and evolution of genes, intergenic spacers, and multigene families by employing recombinant DNA methodologies (Avise, 1994), expression, meaning, transfer and regulation of information in biological systems, thus unraveling the blueprint and evolutionary forces driving life. However despite the ease of measuring and deciphering genetic diversity and the dramatic advances in comparative genomics, the evolutionary forces that generate and maintain segregating genetic diversity, preventing allele fixation or random elimination in nature, remain elusive.

Out of the many techniques available, PCR is a powerful tool for DNA fingerprinting and for effective measurement of genetic diversity within and between populations and thus has revolutionized population genetics by providing an

unprecedented amount of genetic diversity for critical analysis and hypothesis testing. These dramatic developments can be achieved by using molecular techniques like DNA markers based sequence polymorphism (extranuclear, nuclear, mitochondrial and chloroplast).

With due consideration to conserve maximum possible intra and inter specific genetic diversity for conservation of red listed medicinal plants, molecular marker technique i.e., RAPD marker system is routinely used for studying genetic polymorphism and clarifying phylogenetic relationship between taxa. The persistence of a species in the long term depends on the maintenance of enough genetic variation within and among populations to adapt to changing environmental conditions (Beardmore, 1983; Hueneke, 1991). In the short term, genetic isolation by distance may indirectly affect individual fitness and population variability. Distribution of genetic diversity within and among populations (intra- and inter-) of redlisted medicinal plant species is important to evolve conservation strategies (Epperson, 1992). Spatial autocorrelation analysis has advantages in that it includes all pair comparisons in samples and it requires no presumptions about the spatial scale of the structure within a plant population (Epperson, 1989; Heywood, 1991).

One important implication of this approach, from the viewpoint of conservation genetics, is that it could help us set sampling intervals of areas within populations to optimize the genetic diversity in collections from local populations of rare, endangered, or endemic plant species (Kang and Chung 1997; Maki and Yahara 1997; Chung and Park 1998; Chung *et al.*, 1998). For the purpose of conservation of plant species, most of the studies deal only with determination of genetic diversity in

individual populations (Chase *et al.*, 1996; Stewart and Excoffier 1996, Godt and Hamrick 1996; Fisher and Matthies, 1998; Gemmil *et al.*, 1998; Chung and Epperson 1999; Martinez-Palacious *et al.*, 1999). Few studies have analyzed both spatial structure within populations and the pattern of genetic variation among population (Chung *et al.*, 1998).

The distribution pattern of the genetic diversity and human activities at or near the site will inturn determine optimal conditions in suitable locations for *in situ* conservation of the target species (Damania, 1996) The reasons for loss of genetic diversity include deforestation, developmental activities such as hydroelectric projects, road laying, urbanization and changes in agricultural practices, overgrazing and globalization. Traditionally, provenance and progeny tests coupled with biometrical analysis of phenotypic traits have been the standard methods for describing and quantifying the genetic variation in forest tree species (National Research Council, 1991). However due to the high cost involvement and slow technique, strong environmental conditions and occurrence of lots of developmental stages, recent development of molecular markers has complemented in generating information required in making conservation and management decisions.

2.1.2. Assessment of phenotypic/morphological diversity

A phenotype commonly refers to the physical appearance of an organism but it can also describe non-visual properties of the organism, such as physiology or behavior. Plant populations may show morphological variations as adaptation to different selection pressures (Morrison and Weston, 1985; Nevo *et al*, 1986; Hageman and Fahselt, 1990), which may result from phenotypic plasticity or genetic

differentiation due to natural selection and evolutionary forces. Traditional morphological observation alone cannot determine the roles of phenotypic plasticity and genetic differentiation on population variation and adaptation. Phenotypic differences between organisms may be due to environmental and genetic differences, and the latter are the result of genetic divergence or polymorphism. Classical phenotypic methods of identification are not always sufficient to solve these problems because of the instability of the morphological characters i.e., clonal and environmental variability as well as an inability to use such information for identification at juvenile stages or of isolated plant parts.

Genetic diversity is partly correlated and predictable by three or four variable combinations of ecological, demographic, and life history variables. Although no quantitative data is available, co-evolution, or at least adaptation to interspecific interactions, has unquestionably enhanced the phenotypic diversity among species. The cooperation and conflict inherent in mutualism likewise had immense effects on phenotypic diversity. New methods were developed to study the genetic basis of adaptation and about the relationship between genetic and phenotypic differences among the organisms. Natural selection can only operate where phenotypic variation exists. Evolution results from changes but these are the consequences rather than the cause of phenotypic differences. A phenotypic response to environmental conditions may allow a genetically non-adapted population to survive long enough to accumulate variants and then adapt genetically (Baldwin, 1896, Osborn, 1897).

2.1.3. Need for molecular diversity assessment in conservation studies

Morphological diversity being evaluated on phenotypic and biochemical performance is largely influenced by the environmental and developmental changes hence molecular markers have been adopted for diversity analysis in plant species. Molecular markers have been used to identify groups from which core collection accessions can be selected or to monitor the effectiveness of one of the other strategy in capturing genetic diversity found in the whole collection. PCR based markers have been used extensively for assessing genetic variation within the species to measure the genetic diversity (Virk *et al.*, 1995). DNA based molecular markers have acted as versatile tool and found their own position in various fields like taxonomy, physiology, embryology and genetic engineering, They offer several advantages over traditional phenotypic markers, as they provide data that can be analysed objectively (Joshi *et al.*, 1999). Current focus is on chemotype driven molecular techniques so that an optimal characterization of botanical materials is possible (Joshi *et al.*, 2004).

Molecular methods are playing an increasingly important role in conservation and use of plant genetic resources. Recently, Hodgkin *et al.*, in 2001 reviewed the techniques available and the ways in which they are used for analysis of diversity to support Plant Growth Response (PGR) activities. The concept of germplasm conservation demands that collection methods initially capture variation and subsequently, conservation and regeneration techniques minimize losses through time. To this effect, PGR conservation activities comprise collection, conservation and management, identification of potentially valuable material by characterization, and evaluation for subsequent use. The analysis can be performed at any growth stage

using any plant part and requires only small amounts of material. In particular genetic diversity data provides information on gaps in terms of coverage in gene pools as well as redundancies i.e., material with similar characteristics demanding high resources and low productivity (cost prohibitive management).

2.1.4. RAPD markers for assessment of molecular diversity

RAPD markers which are used for identification of duplicates, superior seeds and also improves the efficiency of seed health tests, besides sensitivity or specificity, assessment of genetic diversity, genetic similarity, phylogenetic studies etc. in crop plants (Gates and Boulter, 1979, Aguirre *et al* 1999, Fuentes *et al.*, 1999).

RAPD is a very convenient tool for assessment of closely related accessions, particularly in plant species it is the most suitable method for estimating diversity, monitoring genetic erosion, and removing duplicates from germplasm collections (Virk *et al.*, 1995; Ghany and Zaki, 2003). Therefore, RAPD is a powerful technique for genetic analysis (Williams *et al.*, 1990;Chapco *et al.*, 1992; Kiss *et al.*, 1993; Landry *et al.*, 1993; Wight *et al.*, 1993).

2.1.4.1. Principle of RAPD technology

RAPD is a PCR based DNA marker technique (Williams *et al.*, 1990), which allows the amplification of DNA sequences at random within a genome. The standard RAPD technology utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of anonymous stretches of total genomic DNA under low annealing temperatures by PCR. Amplification products are generally separated on agarose gels and stained with ethidium bromide. Decamer primers are commercially available from various sources. At an appropriate annealing

temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products, if these priming sites are within an amplifiable distance of each other.

The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNA will result in the presence or absence of bands because of changes in the priming sites. One cause of RAPD polymorphisms is chromosomal rearrangements such as insertions or deletions. Therefore amplification product from the same alleles in a heterozygote differs in length and will be detected as presence or absence of bands in the RAPD profile. The DNA segment is amplified from a locus that is heterozygous or homozygous. RAPD markers are therefore considered as dominant. The key point about this technique is that the identity of the amplification products is extremely useful as markers in genetic diversity studies.

There are both advantages and disadvantages of RAPD markers: The advantages are: 1) It is a fast, simple, easy and relatively cost effective method for detecting polymorphisms, 2) It is not a technically demanding method and moreover need for hybridization with radioactive probes can be avoided 3) Very less amount of DNA (ng) is required and 4) prior knowledge of sequence information of the genome is not required in order to design primers. The limitations are 1) they are dominant markers i.e., homozygotes and heterzygotes cannot be distinguished, 2) It has got the problems of reproducibility and is sensitive to alterations in PCR conditions and 3)

Problems with interpreting banding patterns eg., problems of co-migration is high. The principle of RAPD is mentioned schematically (Figure 2).

The RAPD reaction is more sensitive than conventional PCR because of the length of a single arbitrary primer used to amplify anonymous regions of a genome. The reproducibility problem is usually in the case for bands with lower intensity. The reasons for bands with high or lower intensity are still not known. Perhaps some primers do not perfectly match the priming sequence, amplifications in some cycles might not occur, inadequate preparation of the template DNA the thermocycler used, laboratory practice, and handling by different persons applying template DNA the thermocycler used, laboratory practice, different persons applying the method, source of primer, *Taq* polymerase, and the PCR program used (Pennar *et al.*, 1993; Chen *et al.*, 1997; Mizukami *et al.*, 1998; Ellinghaus *et al.*, 1999;) differentiation and the physiological state of a tissue (Bitonti *et al.*, 1996; Bogani *et al.*, 1996; Chen *et al.*, 1997;Schaffer and Arnholdt-Schimitt, 2001) may play an important role. The chance of these bands being sensitive to reaction conditions, of course would be higher than those with higher intensity amplified with primers perfectly matching the priming sites.

2.1.4.2. Applications of RAPDs

RAPD markers have found a wide range of applications as stated below mainly due to the speed, cost and efficiency to generate large numbers of markers in a short period compared with previous methods. Despite some limitations, the RAPD method probably will be important as long as other DNA based techniques remain

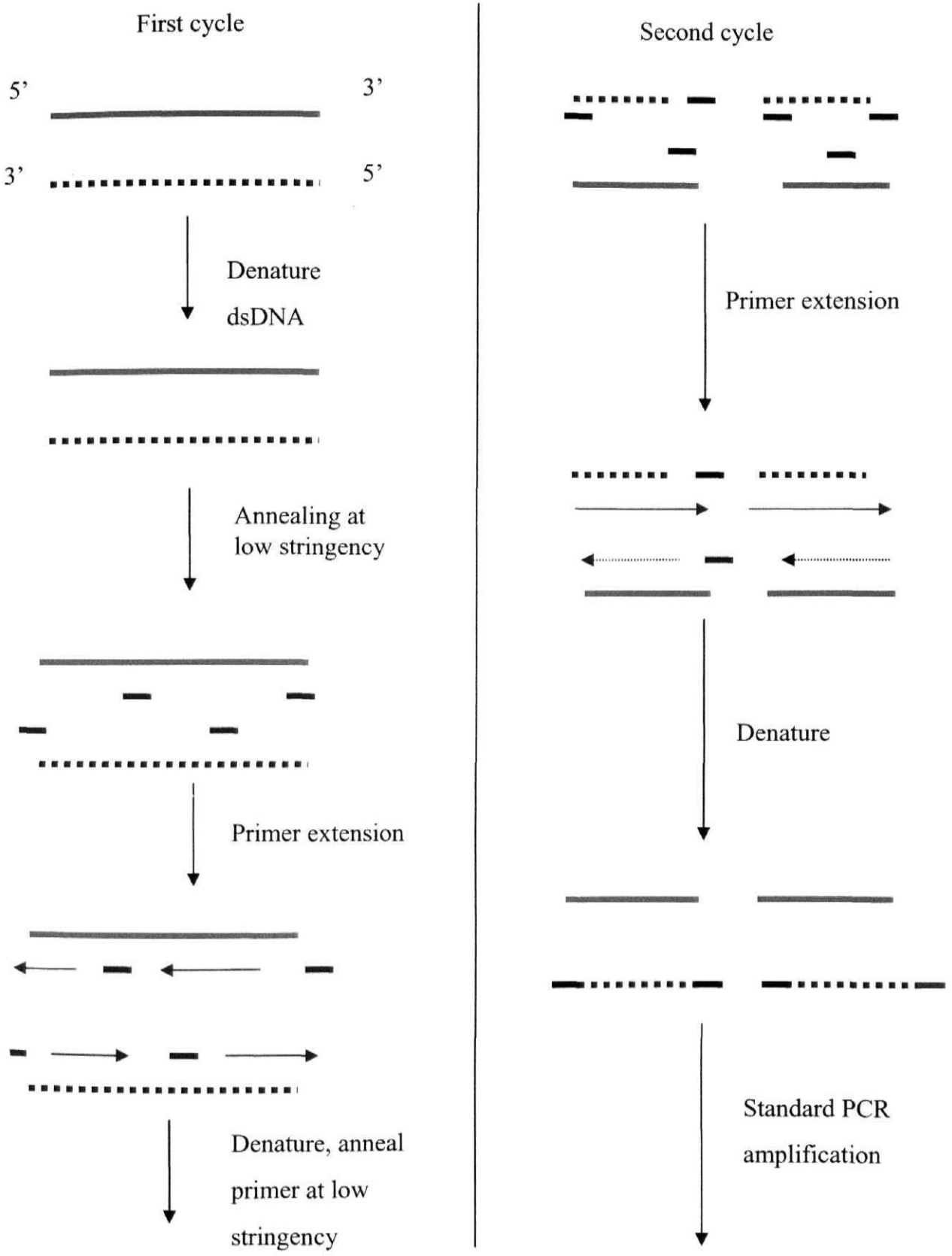

Figure 2: Schematic diagram of RAPD reaction (Welsh and Mc Clelland, 1991)

unavailable in terms of cost, time and labour. Various applications of RAPDs are as follows:

1) F1 hybrid seed purity
2) Gene mapping
3) Population genetics
4) Molecular evolutionary genetics
5) Plant breeding
6) Taxonomic (identity / levels)
7) Assess kinship relationship
8) Detect interspecific gene flow
9) Analyze hybrid speciation
10) Create specific probes for further analysis
11) Identification of asexually reproduced plant varieties
12) Forensic or agricultural
13) Ecological importance
14) Degree of mismatching between synthetic and actual offspring
15) Detection of quantitative trait loci

2.1.4.3. Future scope of RAPDs

Dominant RAPD markers can be converted into codominant markers (Paran and Michelmore, 1993). This approach involves cloning and sequencing of a single RAPD amplification product. The nucleotide sequence is used to design pairs of primers of 24 nucleotides that result in the amplification of a single locus in the polymerase chain reaction. The genomic DNA fragment amplified is referred to as

sequence characterized amplified region (SCAR). The use of long primers often allows both the alleles of a locus to be amplified. Variation within the amplification products can be identified by restriction enzyme digestions, denaturing gradient gel electrophoresis (DGGE) or single-strand conformational polymorphism assay (SSCP). The cloning of RAPD amplification products and the design of primers for the generation of SCARS involves the following steps:

a) Purification of the RAPD amplification product of interest.

b) Reamplification of the purified RAPD amplification product.

c) Ligation of the purified RAPD amplification product, transformation of *E. coli* with recombinant DNA molecule.

d) Rapid PCR screening of recombinant clones i.e., bacterial colonies.

e) Sequence analysis of the cloned RAPD amplification product.

f) Design of primers and PCR analysis.

The references for medicinal plants and their importance are mentioned in Table 1. There are many advantages and disadvantages of RAPD technology over other molecular techniques (table 2). To chart the molecular diversity of many MAPs RAPD technique was used, of which few are mentioned in table 1.

Table 1: Importance of RAPD analysis for understanding the molecular diversity of selected MAPS

Taxon	MedicinalImportance	References
Phyllanthus amarus (Fabaceae)	Viral hepatitis, Oedema, Anorexia	Jain *et al.*, 2003
Zingiber officinalis (Zingiberaceae)	Asthama, Leprosy, Cepalgia, Cholera, Skin diseases, Pharygopathy	Rout *et al.*, 1998
Podophyllum hexandrum (Podophyllaceae)	Purgative, lung cancer, Testicular cancer, Kaposis sarcoma, Gymphoma, Leukamia, Brain tumour, Vaginal warts	Singh *et al.*, 2000
Aloe vera (Liliaceae)	Leprosy, Piles, Mental disorders, Skin diseases, Liver ailments, Jaundice, Menstrual problems, Dysentry, Joint pains	Darokar *et al.*, 2003
Medicago sativa (Fabaceae)	Tumours, Anemia, Fatique, Peptic ulcers, pituitary problems, Narcotics, building general health, Fatique.	Gherardi *et al.*, 1998
Hypericum perforatum (Clusiaceae)	Bronchitis, internal bleeding, healing wounds, septic wounds, to ease depression, headaches, hysteria, neuralgia, shingles, in swellings, abscesses, and bad insect stings, AIDS.	Haluskova *et al.*, 2003
Leucadendron elimense (Proteaceae)	-	Tansley *et al.*, 2000
Vicia pisiformis (Fabaceae)	-	Samuelsson *et al.*, 1997.
Digitalis obscura (Scrophulariaceae)	Arrhythmia, Arterial premature heart, Auricular fibrillation, cardiotonic, congressive heart failure.	Nebauer *et al.*, 1999
Andrographis paniculata (Acanthaceae)	Antipyretic, Antiperiodic, Anti inflammatory, Ulcers, Chronic fevers, Bronchitis, Skin diseases, Leprosy, Intestinal worms, Haemorrhoids, jaundice, Stomach ulcers.	Padmesh *et al.*, 1999
Scutellaria sp. (Lamiaceae)	Chronic and acute diseases that affect the nerves, regulates sexual desireslessen the affects of epilepsy, aids in easing insomnia and restlessness, is a remedy for	Hosokawa *et al.*, 2000

Table 2: Different molecular marker techniques, their advantages and limitations in diversity analysis of plant species.

Advantages	Limitations
1. Random Amplified Polymorphic DNA (RAPD) (Williams *et al*, 1990)	
Easy, less amount of DNA, no sequence information required, inexpensive, moderate equipment required. Anonymous origin	Dominant, do not distinguish between homo and heterozygotes, no potential for candidate gene mapping, low to medium reproducibility, simplicity
2. Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al.*, 1980)	
Codominant, genome and QTL and comparative mapping potential is good, Moderate equipment potensial required, anonymous and genetic origin, very high reproducibility	Difficult, limited by the restriction site, requires very high amount of DNA, limited candidate gene mapping potential
3. Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995)	
Moderate ease of development, very good genome and QTL mapping potential, moderate to expensive equipment required, Anonymous origin, medium to high reproducibility	Moderate to difficult, dominant, limited by the restriction site, medium to High DNA concentration required, very limited comparative mapping potential, useless for candidate gene mapping potential
4. Arbitrary Primed PCR (AP-PCR) (Welsch and Mac Clelland, 1990)	
Similar to RAPD except the size of the primer variation (5-8 oligonucleotides) Easy, less amount of DNA, no sequence information required, inexpensive, moderate equipment required. Anonymous origin	Dominant, do not distinguish homo and heterozygotes, no potential for candidate gene mapping, low to medium reproducibility
5. Simple Sequence Repeats (SSR) (Hearne *et al.*, 1992)	
Codominant, easy to moderate, genome and QTL mapping potential is good, easy, medium to high reproducibility, anonymous origin	Limited by the size of the genome and number of simple sequence repeats, medium to high amount of DNA required, limited comparative mapping potential, useless for candidate gene mapping, moderate to expensive equipment required, development is difficult
6. Sequence Characterised Amplified Regions (SCARs) (Williams *et al.*, 1991)	
Specific locus representing a single RAPD fragment, Codominant, highly reproducible	Prior sequence information is required, specific primers are required.
7. Single Primer Amplification Reaction (SPARs) (Williams *et al.*, 1991)	
Core motifs of microsatellite DNA more or less similar to RAPDs	Similar to RAPDs

8. Single Stranded Conformational Polymorphism (SSCP) (Orita *et al.*, 1989)	
Similar sized DNA fragments can be distinguished, based on the mobility of the single stranded DNA, cost effective.	Polymorphism levels will be restricted.
9. Sequence Tagged Microsatellites (STMS) (Beckmann and Soller, 1990)	
Single locus, multiallelic, co-dominant, highly reproducible clanking regions of the micro satellites are used for designing primers	Prior sequence information is required, Robustness is involved
10.Sequence Tagged Site / Expression Sequence Tagged Site. STS/EST (Fukova *et al.*, 1994)	
Codominant, high reproducibility, moderate ease of development, good genome and QTL mapping potential and excellent candidate gene mapping potential	Limited by the number of enzyme genes and histochemical enzyme assays, less DNA required, easy, development is expensive, moderate to expensive equipment is required
11. Thermal Gel Gradient Electrophoresis (TGGE)	
Separation of fragments according to their mobilities, better resolution can be achieved	Denaturation conditions are high which may generate lot of heat.
12.Variable nucleotide tandem repeats (VNTR)	
Variable number of tandem repeats generated, mostly on microsatellites	Samples of identical or closely related species only can be analyzed.
13.Cleaved amplified polymorphic sequences (CAPS) (Lyamichev *et al.*, 1993)	
Specific for a particular locus and codominant,	Limited by the restriction site, prior sequence information ifs required
14. Sequence Amplified Polymorphic Loci (SAMPL)	
Large scale analysis of genome, yields the best fingerprints when prior information on SSRs is not available, hypervariable loci are targeted, more discriminative	Variation in between very closely related genotypes can be determined
15. Microsatellite primed polymerase chain reaction (MP-PCR) (Meyer *et al.*, 1993)	
Primer length is varied (10-20 oligonucleotides) similar to SSRs	Limitations similar to SSR markers.
16. Arbitrary Primed polymerase chain reaction (AMP- PCR) (Meyer *et al.*, 1993)	
Specificity of the primer annealing is enhanced	Large number of microsatellite primers have to be screened and optimal ones have to be selected
17. Inter Simple Sequence Repeats (ISSR) (Hearne *et al.*, 1992)	
MP-PCR with 3' anchored primers are referred as ISSR	Polymorphism levels may be low due to absence of hypervariable regions
18. Amplicon Length Polymorphism (ALP) (Ghareyazie *et al.*, 1995)	
More amount of variation can be detected	No specificity is observed

19. Allele Specific PCR (AS-PCR) (Sarkar *et al.*, 1990)	
Specificity is more and is applicable in applied aspects	Wide variations cannot be detected and it is very specific
20. DNA amplified fingerprints (DAF) (Caetano-Anolles *et al.*, 1991)	
Short primers (5 bp), large number of variations can be detected	No specificity is observed and binds to the DNA at random
21. Randomly Amplified Microsatellites (RAMS) (Ender *et al.*, 1996)	
Amplification is at random, large variations which may be useful are detected	No specificity observed, binds at random to the entire genome
22. Retrotrasposon Microsatellite Amplified Polymorphism (REMAP) (Kalender *et al.*, 1999)	
Useful in detecting variations in the transposable elements can be further useful in expression studies	The variation in the transposon sometimes may not be useful which may be futile
23. Specific – Amplicon Polymorphism (SAP) (Williams *et al.*, 1991)	
Very specific and helpful in detecting the variations at specific level	Variations at random cannot be detected
24. Single Nucleotide Polymorphism (SNP) (Nikiforov *et al.*, 1994)	
Very specific and can detect polymorphism at the nucleotide level which may be helpful in applied aspects	Not applicable for diversity analysis
25. Microsatellite Simple Sequence Length Polymorphism (SSLP) (Rongwen *et al.*, 1995)	
Detects variations at random with short sequences of primers	No specificity is observed , variations detected are at random
26. Minisatellite Simple Sequence Length Polymorphism (SSLP) (Jarwan and Wells, 1989)	
There is less specificity as the primer length is more comparatively. Variations can be useful at applied level	No specificity is observed, the variations are detected at random

3. Medicinal importance of the selected plants for this study

3.1. *Pterocarpus santalinus* L.f

Pterocarpus santalinus L.f (Fabaceae), commonly named in trade as Red sanders, known for its medicinal and commercial value, is considered as the "Pride of India" and "State tree" of Andhra Pradesh. It is reported to be a native of Africa, but its entry into a restricted part of India remains a mystery. It is an "endangered tree ", and occurs gregariously in patches in some regions of Southern Eastern Ghats (Ahmed and Nayar, 1984, Jadhav *et al.*, 2001). In India four species of *Pterocarpus* are distribted in different parts. *P. santalinus, P. dalbergiodes and P. indicus* are valued for a red pigment viz. santalin and *P.marsupium* for firewood (Purnachandrarao and Solomanraju, 2002).

Two lignans from the heartwood of *P. santalinus* were isolated by activity guided fractionation and investigated for their biological properties and their molecular mechanism of action. One of the lignan called 'Savinin' was found to inhibit tumour necrosis factor –production and T cell proliferation and the other compound was 'Calocedrin' (Cho *et al.*, 2001). The chloroform extract of the heartwood of *P. santalinus* yielded a mixture of the red pigments which could be separated by a polyamide column chromatography into two major compounds, santalin-A and santalin-B (Ravindranath and Seshadri, 2001). A new isoflavone together with liquiterigenin and isoliquitiriginin was also isolated from the heartwood of *P. santalinus* based on the spectral methods and the structure has been elucidated (Krishnaveni and Srinivasarao, 2000).

It has a very restricted natural range of 15,540 sq. Km. in the Southeast portion of the Indian Peninsula (Sarma, 1993), is a tropical forest species confined to $13^{0}30'$-$15^{o}N$ latitude and $78^{o}45'$-$79^{0}30'E$ longitude (Kesavaraju and Jagdishwararao, 1991) and is endemic to Rayalaseema region i.e., Kadapa (Cuddapah), Chittoor, Nellore and Prakasam Districts of A.P where 30% of the total trees in these areas is Red sanders, whereas in the "House of Red sanders'' *i.e.* Kadapa (Cuddapah), 80% of the forests comprise of these trees. Apart from the above-mentioned districts in A.P it is also sparsely distributed in forests of North Arcot and Chingleput districts of Tamil Nadu (Krishnamurthi, 1988).

Earlier reports claim that it is not found naturally in any other part of the world. Although the species is well adapted in nature, it is categorized as endangered, principally a result of many primary factors like habitat loss, introduction of new species, overexploitaion, indiscrete harvesting, unscientific methods of extraction and adulteration of active principle and commercially useful plant parts, environmental catastrophies, geographic effects, genetic loss of variations, stimulation of deleterious mutations (genetic erosion), urbanization, deforestration, over testing etc., which lead to rapid demographic and geographical decline (Martin and Bermejo, 2000). Red sanders is at the verge of depletion mainly due to indiscriminate felling for a long time, because of its quality timber and multi-utilitarian characters.

It flowers during dry season i.e., from April to May and seeds are formed by February- March (Sarma, 1993). Pollination occurs with the aid of a few species of bees. (*Apis dorsata, A.cerana* var. *indica* and *A. florae). Apis dorsata* is the main pollinator, shows facultative xenogamy, but mostly from selfpollinated flowers,

degenerate fruits are seen. The natural fruit set rate was very low (6%) may be attributed to different factors like self incompatibility, poor pod set etc., (Purnachandrarao and Solomanraju, 2002).

Red sanders tolerate a maximum shade temperature of 45°C to 47.5°C or possibly more and a minimum temperature of 7.3°C to 12.3°C (Ramakrishna, 1962), with a normal rainfall ranging between 89-106 cm (Troup, 1921). It is usually associated at the lower elevations with species like *Anogeissus latifolia*, *Chloroxylon sweitinia, Hardwickia binata* and at higher elevations with plants like *Terminalia coriacea*, *T. chebula, T. alata, Syzygium alternifolium, Buchanania lanzan, Tectona grandis* etc. The presence of *Cymbopogan coloratus* (Boda grass) is the characteristic feature of the Red Sanders habitat.

Overexploitation, without commensurate replanting poses a severe threat to the very existence of this precious timber tree (Ahmed and Nayar, 1984). Being a dry deciduous forest tree, it thrives on poor, dry and shallow soils though exhibits better form of growth on deep and fertile soils. Naturally, the tree grows typically on dry hilly, often rocky ground at elevations of 100-1000 m above the sea-level, mainly on stony or gravelly soil on formations of gneiss, quartzite, shale or laterite soils, particularly grows well on lateritic loam soil, does not tolerate stiff water logged soil, but has been planted with success on rich alluvial grounds (Troup, 1921). It is a strong light demander and does not tolerate overhead shade. The medicinal properties of *P. santalinus* plant parts are shown in Table 3.

Table 3: Medicinal properties of *P. santalinus*

Part used/Alkaloid	Medicinal uses
Wood powder	Astringent, tonic, antipyretic, antihelminthic, antiperiodic, diaphoretic, alexeritic, spider poisoning, freckles, defects of vision, bone fractures, leprosy, scorpion sting, hiccough, ulcers, general debility, mental aberrations, bleeding piles. vomiting, eye diseases, headache, heamophilic disorders, inflammation, ulcers, blood purifier, skin diseases, fever, inflammation, toothache, hemicrania
Wood + Bark brew	Chronic dysentery, worms, blood vomiting, weak vision, hallucination
Wood + Fruit extracts	Astringent, diaphoretics, inflammations, headache, skin diseases, bilious infections, chronic dysentery.
Wood powder + Dust	Fish preservative
Wooden chips + Water	Diabetes
Stem bark powder + Soft porridge	Diarrhoea
Bark powder	Astringent, blood purifier, antihelmimthic, antipyretic, antidiabetic, curing arecanuts .
Condensed bark powder	0.38-0.45% of mild smelling essential oil
Condensed bark powder + alcoholic HCl	1.98-2.25% of mild smelling essential oil
Distil of wood	Medicine for heart diseases, blood purifier
Fruit decoction	Astringent, tonic, chronic dysentery
Pods decoction	Astringent, tonic-chronic dysentery, psoriasis
Santalin	Colouring pharmaceutical preparations, food stuffs, high class alcoholic liquors, paper pulp etc.,
Roots and stumps	Dyeing cotton and leather, staining the woods
Roots and stumps	Dyeing cotton and leather, staining the woods

3.2 Rauvolfia serpentina (L.) Benth. ex Kurz

Rauvolfia (Apocynaceae) is also called as "snakeweed" includes about 50 species. now attained world wide popularity, finds mention in ancient, is a source of reserpine which is medicinally very important. It is commonly known as "Sarpagandha", one of the important medicinal plants, under Endangered category (Jadhav *et al.*, CAMP 2001). In India it is listed as an endangered species in the North Western Himalayas (Gupta, 1986). It is a Red listed medicinal plant occurring in MPCAs of A.P i.e., in Marudumilli of East Godavari District (Jadhav *et al.*, 2001). It is included in the negative list of export and import policy of foreign trade (1997-2002). Export of the plants, its parts, their derivatives and extracts are prohibited (Reddy *et al.*, 2001).

It is distributed in India, Malaysia, Sub-Himalaya, Pakistan, Bangladesh, Sri Lanka, Myanmar, Thailand and Java. In India it is present in moist deciduous forests in Karnataka, Kerala and Tamil Nadu. It prefers tropical and sub tropical areas with mean annual rainfall between 1500 to 4000 mm and mean annual temperature ranging from 20^0C-30^0C. It can grow upto an altitude of 1300 m (Singh *et al.*, 2002) The major soil types for its natural growth are sandy alluvial loam, red laterite loam and in some places stiff dark loam. Good growth is always associated with large percentage of humus rich in nitrogenous and organic matter, ensuring uniform moisture level. Alkaline soils are reported to be not suitable for commercial cultivation. Over exploitation for commercial use is the major cause of threat, which has brought the species to the brink of extinction in the wild, although the species is

cultivated in many of the medicinal plant gardens, and thus the plant has become rare in most of the areas of its natural occurrence (Jain and Sastry, 1980). It is included under the list of wild species relaxed for export by CITES regulations from 1997-2002 (Singh *et al.*, 2002).

Roots are sold under the trade name Sarpagandha at a price of @ 110-150 NR kg^{-1} (Ravikumar *et al.*, 2000) and seed rate is 10 Kg / average, yield is 2700 to 3300 Kg dried roots / ha and 8-10 Kg of seeds. Approximately 600 tonnes of dry roots are produced yearly, mainly from forest areas (Singh *et al.*, 2002). According to the estimates 400-500 tons of roots of are being collected annually mostly from the forests in India, Bangladesh, Sri Lanka and Thailand (Hossain *et al.*, 1993).

Under irrigated conditions the dry root yield varies from 15-25 q/ha. The average total alkaloid content is 2.4% in the root bark and 0.4% in the root wood. Stem and leaves also contain alkaloids ranging between 0.45%-0.55%. Sometimes, roots of *R. tetraphylla*, *R. densiflora* and *R. micrantha* are found mixed in the market samples. Roots contain an active principle reserpine, an alkaloid that is medicinally important (Table 4). The other notable alkaloids apart from reserpine are rescinamine and deserpidine, which have been extensively researched in India, Europe and North America, which are also important medicinally. The Ayurvedic preparations of *R. serpentina* are "Sarpagandha ghanavati", "Sarpagandha yoga", "Sarpagandha churna" and "Maheshvari vati" etc,. The other alkaloids are Ajmaline and Ajmalicine which have been worked out phytochemically and used for medicinal purposes (Table 4).

Table 4: Represents the parts and alkaloid used and its medicinal properties in *R. serpentina.*

Part used/Alkaloid	Medicinal uses
Roots (Ayurveda and Unani)	Hypnotic, sedative, reducing blood pressure, Central nervous disorders (Psychic / Motor, Anxiety, Psychosis, Schizophrenia, Epilepsy, stress, Depression), Insomnia, Giddiness, dyspepsia and vitiated conditions of Kapha and Vata, Malaria, Fever
Root powder	Antidote to snake venom, insect stings, rat bites, poisons
Root extract	Abdominal, Intestinal disorders like diarhoea, dysentery, antihelminthic, laxative, diuretic, and thermogenic.
Root extract + Other plant extracts	Cholera, colic and fever, wounds, strangury
Root decoction	Helps in child birth (Stimulates uterine contractions)
Leaf extract	Opacity of the cornea
Dried fruits + Black pepper + Ginger	Regulates menstruation
Rescinnamine	Hypertension, psychiatry disorders
Ajmaline	Cardiac arrhythmic (Heart diseases)
Ajmacline	Circulatory diseases

3.3. *Rauvolfia tetraphylla* L.f

R. tetraphylla (Apocynaceae) is also one of the medicinally important plant. It is native to West Indies and introduced to India where it is cultivated in gardens in Uttar Pradesh, West Bengal, Tamil Nadu and Kerala. It has become naturalized in many localities and is distributed on moist habitats. The roots are medicinally important although they reportedly contain low concentrations of the alkaloid reserpine and are mostly reported to have hypotensive property and contains lower quantities of reserpine also can cure many diseases (Parrotta, 2001).

R..tetraphylla was one of 17 plant aqueous extracts traditionally used in Guatemala for the treatment of 'blood disorders' and parasitic infections. The aggregation of washed human platelets induced by thrombin (0.075 U/mL) was inhibited by extracts of *R. tetraphylla* (Villar *et al.*, 1998). The plant extract and castor oil is used in curing chronic and refractory ailments and its bark decoction is used for chronic refractory skin ailments and in deworming.

4. Problems, prospects and objectives

Reddy and Srivasuki (1990) reported various limitations involved in propagation of Red sanders by natural and conventional means. Natural propagation has some constraints such as prolonged dormancy and poor seed germination (Kalimuthu and Lakshmanan, 1995). Owing to the hardness of the pods, germination is difficult and the percentage of success is comparatively low. Poor pod set was noticed under natural self pollination and pod maturity takes about eleven months after flower opening (Dayanand, 1988). Past experiments on air layering yielded less promising results but the improved techniques with modifications has given 100% success (Reddy and Srinivasuki, 1990).

Earlier studies using conventional clonal propagation methods like grafting and rooted cuttings were not very successful (Dayanand and Lohidas, 1988). Though the tree coppices well, producing root suckers freely, the regeneration and growth is very slow (Ahmed and Nayar, 1984). Vegetative propagation using IBA has been standardized for *P. santalinus* with a view to establish clonal seed orchards, which was not effective (Reddy, 1991). Studies on the development and maturity of the pods in Red sanders revealed that the pods harvested and sown in March gave better results with respect to germination and establishment of seedlings (Dayanand, 1988).

Preliminary investigations on micropropagation of Red sanders was done using nodes and shoot tips derived from *in vitro* regenerated seedlings as explants and shoots differentiated from cotyledonary callus on ¼ MS medium with BAP ($3x10^{-6}$ M) and adenine ($4x10^{-4}$ M), which was better than other media combinations.

Adventitious shoot formation from the cotyledonary callus occurred on MS medium with BAP ($3x10^{-6}$ M) at 28±2°C (Patri *et al.*, 1988). Studies on micropropagation of *P. santalinus* revealed best response on MS medium with shoot multiplication and leaf differentiation (Mithila and Srivasuki, 1992). Rapid multiplication of "Red sanders" was achieved by culturing mesocotyl explants on B5 medium fortified with 3.0 mgL^{-1} BAP and 1.0 mgL^{-1} NAA within a six-week culture period. Shoot tip necrosis expressed in regenerated shoots was controlled. Shoots treated with IAA, NAA and IBA ($1mgL^{-1}$) each, prior to transferring them to the rooting medium, exhibited better rooting than those with no prior treatment (Anuradha and Pullaiah, 1999).

Successful development of plantlets of "Red sanders" by induction of multiple shoots from shoot tips and transfer of micropropagated plants to soil has been reported on MS medium with 0.2 mgL^{-1} BAP and 0.1 mgL^{-1} KN (Lakshmisita *et al.*, 1992). *In vitro* regeneration in *P. santalinus* was also achieved when detached cotyledons from *in vitro* germinated seedlings were cultured on MS medium containing NAA ($0.1mgL^{-1}$), KN ($1mgL^{-1}$) and BAP ($1mgL^{-1}$). The regenerated shoots rooted on ½ strength MS medium with IAA ($1mgL^{-1}$) and the fully developed plantlets were successfully established in the soil (Arockiasamy *et al,* 2000). Studies on *ex situ* conservation of *P. santalinus* revealed that tissue culture can be used as one of the tools for the conservation of this endangered tree species (Murughesh *et al.*, 1999).

In *P. santalinus* another problem to be investigated is regarding seed dormancy. The causes for seed dormancy may be due to the exo, endo or combined

factors has still not been reported. In previous reports a dormancy period of one year was stated, hence in order to enhance the germination capacity, seed germination studies were planned to carry out both in *in vivo* and *in vitro* conditions.

Germplasm in the form of seed storage was also considered as one of the strategy for conservation for all the selected species i.e, *P. santalinus, R. serpentina* and R. *tetraphylla.*

Conservation of biodiversity on planent earth is a priority, and so is molecular diversity. Polymorphism, a vital component of genetic diversity is considred as important factor for the survival of a species. It is important not only in the evolutionary point of view, but also for the need of applied biological research. . In fact polymorphism patterns of many species with large effective population sizes have been shaped during millions of years by the action of very small selection intensities. Mutations will be lost if such populations dwindle. Therefore, conservation of endangered plant species is a topic of priority area of research.

Analysis and characterization of genetic variation is fundamental to any conservation strategy, whether *in situ* or *ex situ.* In the past the genetic diversity analyses of morphological variants was detected by biometrical approaches or protein isoenzyme profiles. The complimentary DNA sequencing and whole array of molecular marker techniques are relevant and helpful for investigating molecular diversity. Hence, the accessions of *P.santalinus* genetic diversity was detected using morphological and molecular markers. In case of *R. serpentina* and *R. tetraphylla,* investigations on genetic diversity and similarity are relevant. *R. serpentina* is a conservation dependent species in various locations. In contrast *R. tetraphylla* is a

weed. Therefore, information on genetic identity between R. *serpentina* and *R. tetraphylla* RAPD analysis would be helpful, possibly for genetic improvement of *R. tetraphylla.* Molecular markers are now routinely used in the management of genetic resources. The objectives of the present investigation are:

a) Extensive field survey and collection of germplasm of *P. santalinus, R. serpentina* and *R. tetraphylla* from various locations in different districts of Andhra Pradesh.
b) *Ex situ* conservation of collected germplasm in the experimental site, field gene bank and seed bank.
c) To examine the viability of the seeds of *P. santalinus* collected from various locations.
d) To study the morphological and molecular variations among the collected accessions of *P. santalinus.*
e) To analyse the molecular variations in interpopulations from 6 locations from A.P (India) and intrapopulations (Dulapally, Medak District) of *R. serpentina* collected from various locations.
f) To study the molecular variations in interpopulations of *R. tetraphylla* collected from various locations.
g) To understand the genetic similarity between *R. serpentina* and *R. tetraphylla* using RAPD markers.

5. Materials and methods

5.1. Germplasm collections of the chosen species

Most of the germplasm was collected from Deccan ecoregion (Figure 3). In the present investigation, an extensive field survey has been carried out. The plants were identified based on regional, local and the flora of A.P State (Pullaiah and Chenniah, 1998). Germplasm of *Pterocarpus santalinus,* seeds and seedlings were collected from 27 different locations of Andhra Pradesh, India (Figure 4). Two accessions were collected from nurseries in Hyderabad (Andhra Pradesh.. , India). These plants were supposed to have been collected from forests of Kerala and Karnataka Sates of India which were used for comparison with accessions from A.P. Seeds and seedlings of *Rauvolfia serpentina* (6 accessions) and *R. tetraphylla* (7 accesions) were collected from different locations of A.P (Figure 5).

5.2. Conservation of germplasm

Germplasm in the form of seedlings and plants was grown in plastic covers, pots and on the ground with proper irrigation, sunlight, shade, humidity and temperature and is maintained in the field experimental site in net house, glass house and open shade and in the field gene bank, which were further used for experimental studies. Seeds were stored in seed bank at $4^{o}C$.

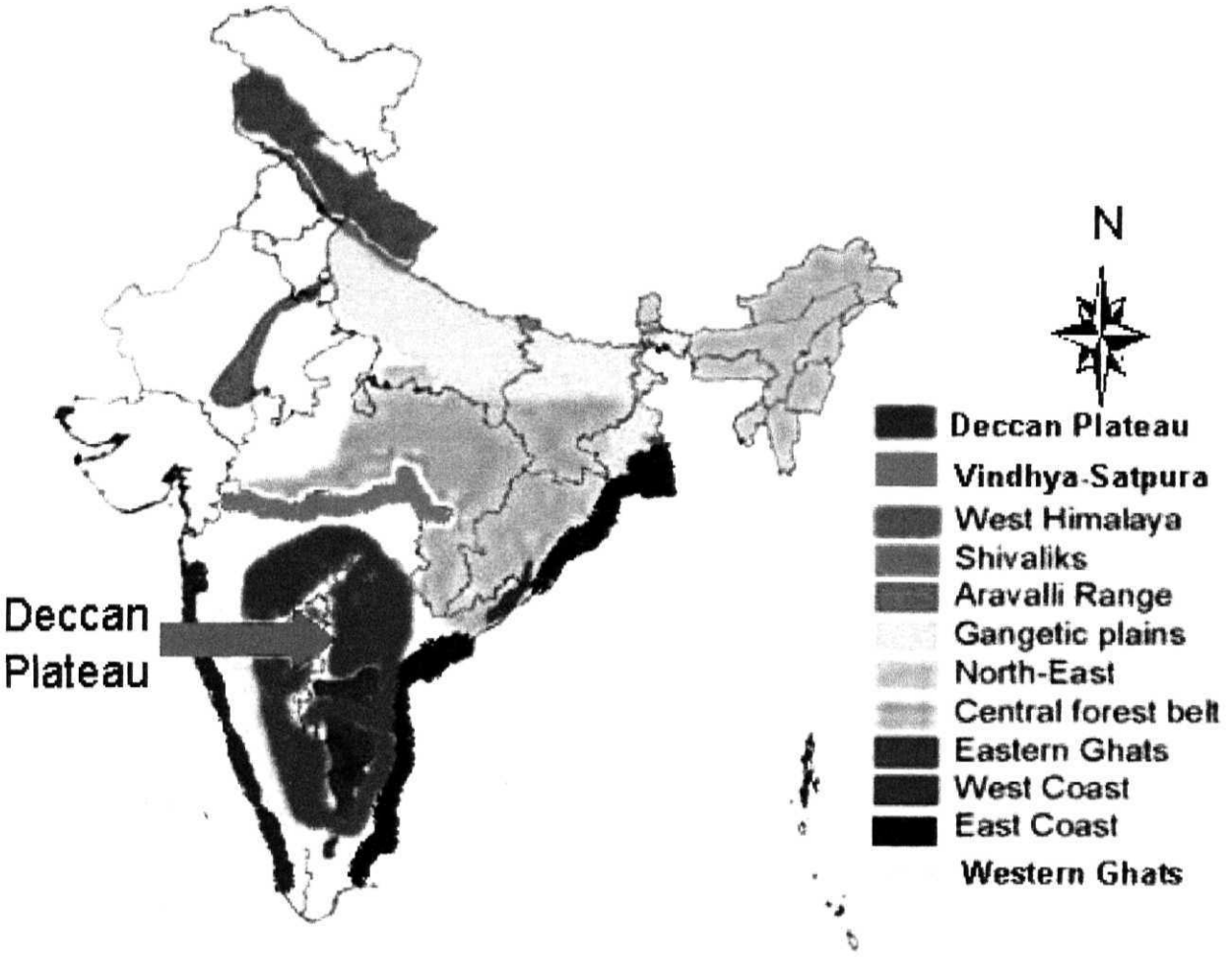

Figure 3: Ecoregions of India. National Biodiversity Strategy and Action Plan (NBSAP) of the Ministry of Environmnet and Forests (1999) recognized recognized the above ecoregions

Boundaries for Deccan Plateau: North= Narmada, South = Cauvery, East = Coromandel coast (excluding eastern ghats), West = Western ghats

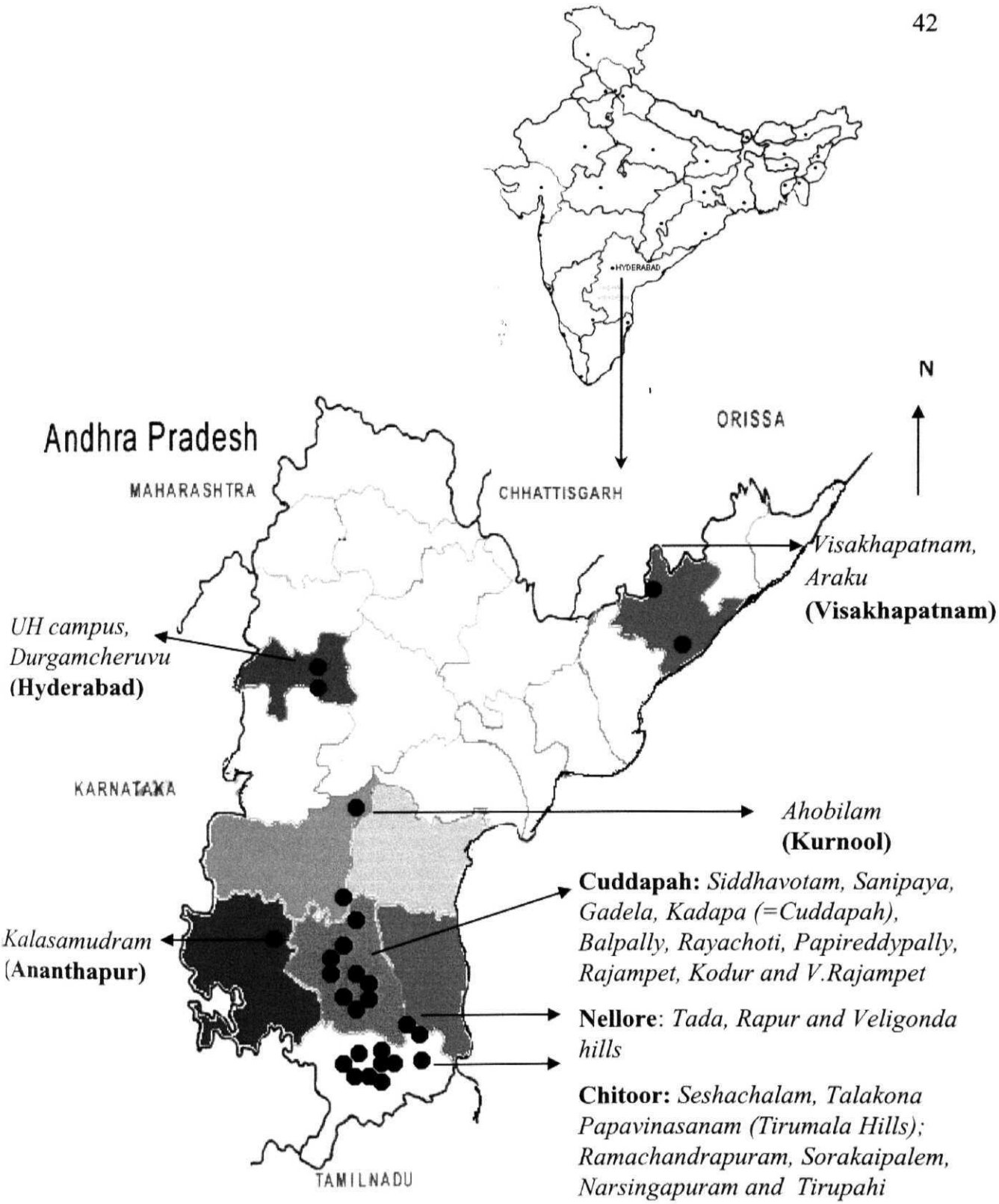

Figure 4: Locations in Andhra Pradesh (*in italics*) from where the germplasm of *Pterocrapus santalinus* was collected. The district name in A.P was mentioned in **bold face** in parenthesis.

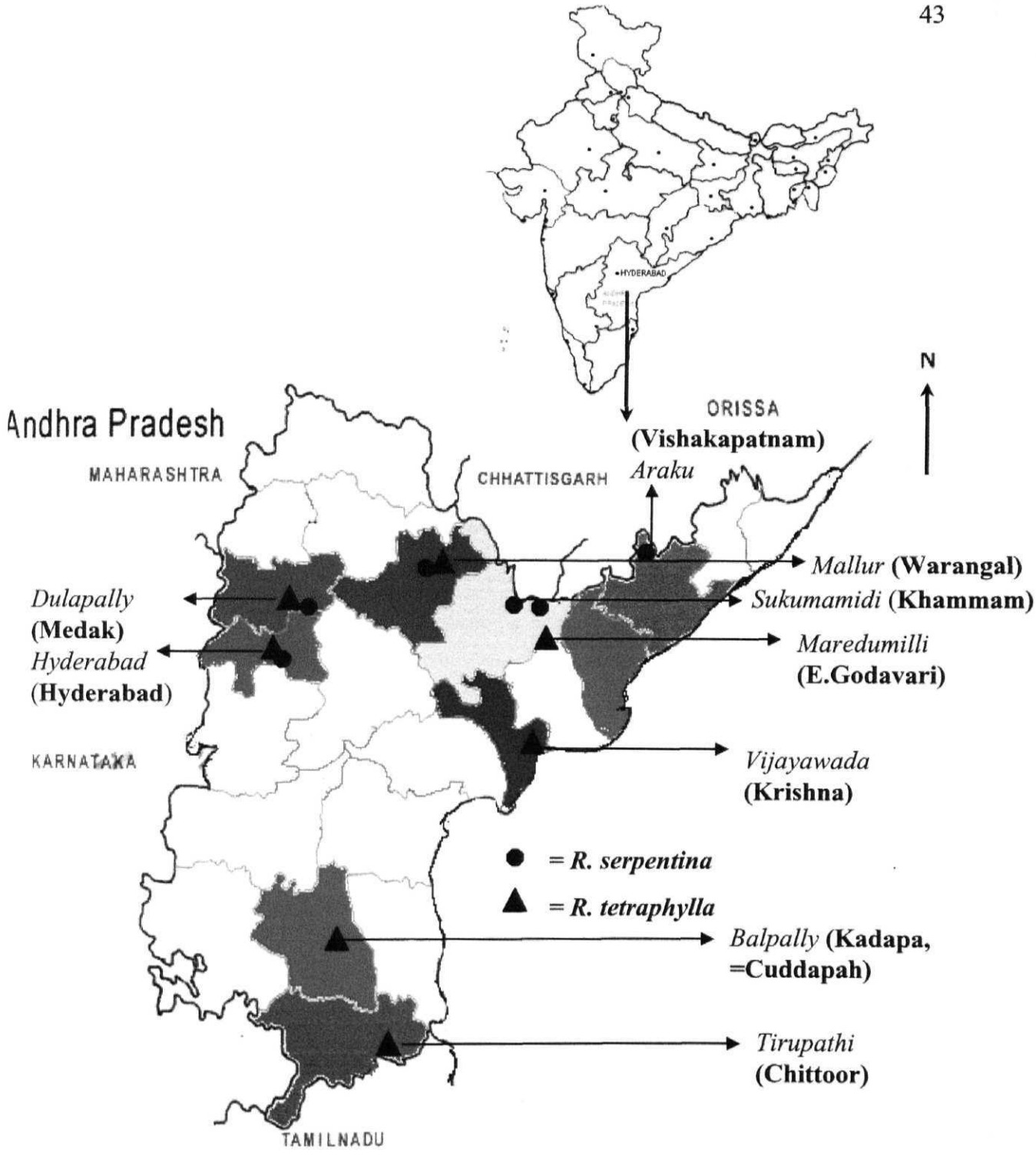

Figure 5: Locations in Andhra Pradesh (*in italics*) from where the germplasm of *Rauvolfia serpentina and R.tetraphylla* was collected. The district name in A.P was mentioned in **bold face** in parenthesis.

5.3. Seed germination

5.3.1. Studies on *in vivo* seed germination

In order to study the viability of the seeds and to raise the seedlings in natural conditions initially their germination ability was determined. Mature dried pods collected from various locations in the same season i.e., summer, were soaked in tap water for 48 hrs and germinated in field conditions, i.e. *in vivo* seed germination studies. To break the dormancy of the seeds, pods collected from various locations were washed thoroughly with double distilled water and were treated with different dormancy breaking chemical agents like KNO_3, H_2O_2, KCl, HNO_3, H_2SO_4, GA_3 and KN (100 – 5000 ppm) for 1hr and also subjected to mechanical scarification apart from hot water and cold water treatments. Germination percentage was determined after 3 days.

5.3.2. Studies on *in vitro* seed germination

In order to enhance the seed germination capacity under aseptic conditions, mature dried pods collected from various locations were subjected to scarification to remove the tough seed coat (3 layers) covering the seeds. Scarification was performed by soaking the pods in tap water overnight or by using boiling water or sulphuric acid and the seed coat was removed mechanically. The seeds were pretreated with 2% bavastin for 30 minutes, surface sterilized with 70% (V/V) ethanol for 2 minutes and with 0.1% (W/V) mercuric chloride for 12 minutes followed by repeated washing with sterile double distilled water and were aseptically placed onto different strengths of MS (Murashige and Skoog 1962) medium - with different percentages of agar

(static medium) and without agar (liquid), full strength MS medium - with different percentages of phytagel, different concentrations of GA_3, in 3% sucrose and on 1% agar. Seeds treated with cold water were cultured on MS medium with 0.8% agar and percentage of germination was recorded.

5.4. Establishment of *in vitro* regeneration protocol

5.4.1. Selection and standardisation of media

To find out the influence of different types of media and its supplements on growth and differentiation of multiple shoots an attempt has been made by using MS, McCown and Gamborg's media (Murashige and Skoog, 1962; Gamborgs, 1968)

5.4.2. Explant evaluation

In view of selecting best explant capable of active proliferation of multiple shoots, different explants like nodes, leaves, shoot tips, hypocotyl, internodal segments, cotyledonary nodal meristem and seeds were used. The source of the explants like nodal segments, leaves and shoot tips was from Red sanders plantations of University of Hyderabad (UH) and the seeds used were collected from different forests of A.P. The morphogenetic responses of all the explants were studied under identical cultural conditions and a comparitive account of the results were obtained.

5.4.3. Influence of agar, sucrose and orientation of the explant

Influence of different percentages of agar (0.5, 0.6, 0.7 and 0.8%), on MS medium was tested in order to standardize an efficient protocol for multiple shoot regeneration. Effect of both vertical and horizontal orientation of the explant was investigated to increase the proliferation of multiple shoots.

5.4.4. Role of cytokinins

Influence of different cytokinins like BAP, KN, TDZ alone and BAP in combination with TDZ (0.1-7.0 mgL^{-1}) in MS medium was checked for efficient multiple shoot regeneration from nodal explants and seeds. Although the optimum and best hormonal treatment for efficient shoot regeneration was analysed, the effect of coconut milk at 10% was also studied. The data was analysed using ANOVA.

5.4.5. Induction of axillary bud sprouting

For *in vitro* studies, nodal explants of 3 - 4 cm in length collected from the plantations of University of Hyderabed campus, were kept under running tap water for 1/2hr, washed thoroughly and surface sterilized in 70% ethanol for 2 min followed by 0.1% (W/V) of $HgCl_2$ for 15 min under sterile conditions. The explants were then rinsed 4 - 5 times in sterile double distilled water with duration of 5 min each. The sterilized explants were then cultured onto MS medium with different concentrations of phytohormones like BAP, KN, 2,4 D and TDZ (0.1-7.0 mgL^{-1}) individually and in combinations either vertically or horizontally in contact with the medium. Explants were also taken from the *in vitro* germinated seedlings, as there will not be requirement of sterilization. All the experiments were repeated thrice and 40 explants were used for each treatment.

5.4.6. Callus induction from leaves, shoot tip, hypocotyl and internodal segments

Leaves with or without petiole 1- 4 cm in length derived from both *in vitro* germinated seedlings of around 5 months age were collected from plantations in UH campus and used. They were collected from different positions of the shoots i.e., both young and mature leaves. Leaves were initially washed in tap water for 15 min and

surface sterilized with 70% ethanol for 1min followed by treatment with 0.1% (W/V) $HgCl_2$ for 6 min under sterile conditions. The explants were then rinsed 4 - 5 times in sterile double distilled water with duration of 5 min each. The sterilized explants were then cultured onto medium with different concentrations of phytohormones like BAP, TDZ (0.1- 7.0 mg L^{-1})· individually and in combinations, with either adaxial or abaxial side in contact with the medium.

Shoot tip, hypocotyl and internodal segments of 1 - 2 cm in length derived from both *in vitro* germinated seedlings and also collected outside from plantations in UH campus were used as explants. The explants were initially washed in tap water for 15 mins and surface sterilized with 70% ethanol for 30 sec followed by treatment with 0.1% (W/V) $HgCl_2$ for 5 mins under sterile conditions. The explants were then rinsed thoroughly 4-5 times in sterile double distilled water for 5 min each. The sterilized explants were then cultured onto medium with different concentrations of phytohormones like BAP, KN, 2,4 D and TDZ (0.1-7.0 mg L^{-1}) individually and in combinations either vertically or horizontally in contact with the medium.

5.4.7. Regeneration of multiple shoots from seeds as explants

For multiple shoot regeneration from seeds, mature dried pods collected from various locations were subjected to mechanical scarification by using scalpel and scissors followed by subjecting the seeds to sterilization by using (2% Bavastin, 30 min) followed by (70% ethanol for 2 min, 0.1% $HgCl_2$ - 12 min) and aseptically placed on MS medium with different concentrations of various hormones. Subculturing of single node from multiple shoots raised on MS medium with 1.0

mgL^{-1} BAP and 2.0 mgL^{-1} KN was carried out on MS medium supplemented with 1.0 mgL^{-1} BAP.

5.4.8. Root induction from shoots

Since it is woody and a highly recalcitrant species rooting for individual shoots was found to be highly difficult for individual shoots and hence *in vitro* differentiated healthy shoots measuring 5-6 cm, in bunches derived from the seeds were excised and subjected to *in vitro* rooting. For rooting, different strengths of MS medium alone in combination with various hormones like IAA, IBA and NAA (0.1-10 mgL^{-1}) were used. For all the experiments on induction of axillary bud sprouting, shoot multiplication and root induction from shoots, the cultures were maintained at 25 ± 2^0C under a 16 hr photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 $\mu Em^{-2}S^{-1}$ provided by white fluorescent tubes. All the experiments were repeated thrice and 20 replicates per treatment were taken. The data was analyzed statistically using one way analysis of variance (ANOVA) in case of multiple shoot regeneration from seeds.

5.4.9. Acclimatization of regenerated plants

In vitro regenerated plantlets raised from single seed, having well developed roots were removed from culture bottles carefully without destruction of the root system, and washed properly to free sucrose and agar. In the first week, they were transferred to perforated plastic cups with soilrite (a white granular chemically inert supporting powder) for gradual acclimatization and nurtured initially with MS liquid (Basal) solution without sucrose, every alternate day followed by irrigation with water after 15 days. The cups were covered with polythene bags to maintain the high

humidity around the plants and were maintained in growth chamber. After 15-20 days of acclimatization, the polythene bags were removed and the plantlets were transferred into magenta boxes with soilrite, manure and sand in 1:1:1 ratio by volume and were acclimatized. The complete hardening process was carried out in the glass house. The percentage survival was recorded at this stage.

5.5. Morphological and molecular diversity analysis in *P. santalinus*

P. santalinus diversity was studied in terms of morphology of mature pods and few characteristic features of the 5 months old germinated seedlings and at the molecular level using RAPD markers.

5.5.1. Morphological diversity using phenotypic parameters

Some of the diagnostic morphological traits (pod weight, pod length, pod width, leaf length, leaf width, shoot length and number of axillary buds) were studied by considering mature pods and germinated seedlings (5 months old) of 16 accessions collected from various locations. The pod width and pod length were measured using a metre scale and the mean was calculated and later analysis was done by ANOVA. The experiments were carried out with 3 replicates of 25 each. For data to be analyzed in case of germinated seedlings seeds of each accession were sown on a square bed with an inter plant spacing and inter row spacing of 10 cm. The bed was irrigated properly and minimal quantities of insecticides were sprayed to reduce the plant damage and to capture the maximum diversity.

5.5.2. Molecular Diversity using RAPD markers

For analyzing molecular diversity in *P. santalinus* 15 accessions collected from different areas including the two accessions were collected from nurseries for reference to compare with germplasm collected from A.P, (supposed to be naturally grown in forests in Kerala and Karnataka) which were assessed using RAPD markers. Details regarding accessions used in molecular analysis are shown in Table 16. The primers used were Operon primers from Kits OPA and OPC.

5.5.2.1. Isolation of genomic DNA

DNA isolation from young and old leaves of *P. santalinus* was very difficult by normal conventional methods like the CTAB protocol developed by (Doyle and Doyle; 1960), SDS method (Dellaporta, 1983) and by using other chemicals like lithium chloride and sorbitol. Due to the presence of secondary metabolites, tannins, polyphenols and polysaccharides, discrete and proper amplification was not observed and thus the DNA was isolated using Plant DNA Zol isolation Kit. (Invitrogen, Life technologies, India). The only modification done was, before extraction, plants were kept in dark for 3 days to reduce the amount of polysaccharides, which interfere in the purification steps of DNA. DNA was isolated from similar age group plants (1 Year).

5.5.2.2. Qualitative and quantitative extraction of DNA

To test the quality of DNA, the OD values were recorded at 260 and 280 nm and the ratio of OD 260 to OD 280 was calculated to check the purity of each DNA sample. Pure DNA preparations showed the values of ratio OD 260 to OD 280 between 1.7 and 1.8. Further purity of DNA, was tested by subjecting to gel

electrophoresis, using 0.8% TBE- agarose. Gels were stained with ethidium bromide and viewed on a UV transilluminator, photographed with the help of a gel documentation system (LTF Labor technik). Based on the gel and the intensity of the band the DNA quality and quantity was identified by using Lambda DNA marker. DNA was quantified based on the spectrophotometer measurements of UV absorption at 260 nm, assuming 1 OD at 260 nm is equal to 50 ng of DNA (Sambrook *et al.*, 1982) and the concentrated DNA was diluted with ultrapure Milli Q (Milli Q academic) sterile water to 50 ng/µl.

5.5.2.3. RAPD Analysis

Forty decamer primers of arbitrary sequence (Kits A and C provided by Operon Technologies Inc, Alameda, CA) were tested for PCR amplification among 15 accessions of *P. santalinus*. The analysis involved 2 steps a) PCR amplification and annealing of single arbitrary primer at random on the total genome. b) Agarose gel electrophoresis.

PCR reactions were carried out in a DNA Thermocycler (MJ Research Inc. USA.) with a heated lid. Each 15 µl reaction volume contained about 50 ng of template DNA, 1X PCR Buffer (10 mM Tris Hcl pH 5.3, 50 mM Kcl), 3 mM $MgCl_2$ (Invitrogen Life Technologies, India), 0.2 mM dNTP Mix (Genetix company, India), 0.5µM of single primer (Genetics, Delhi, India) and 1U of *Taq* DNA *polymerase* (Invitrogen Life Technologies, India) and programmed for an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 45sec at 94 ^{0}C, 1 min at 37 ^{0}C, extension was carried out at 72^{0}C for 1 min and final extension at 72 ^{0}C for 7 min and a hold

temperature of 4°C at the end. Negative controls were also run without template DNA to ensure the amplification.

PCR products were electrophoresed on 2% (w/v) agarose gels, in 1X TBE Buffer at 50V for 3 hrs and then stained with Ethidium bromide (0.5µg/ml). Gels with amplification fragments were visualized and photographed under UV light. Lambda DNA *EcoR1 Hind111* double digest was used as molecular marker (Genetics, New Delhi, India) to know the size of the fragments

5.5.2.4. Data scoring and analysis

PCR reactions and electrophoresis were repeated at least twice to ascertain the reproducibility of the bands. For each accession, polymorphism was scored as 1 for the presence and 0 for absence of a band RAPD data generated with forty primers, were used to compile a binary matrix for cluster analysis using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Biosatatistics, New York, USA, software version 2.02j package). Genetic similarity among accessions was calculated according to Dice similarity coefficient **(Dice, 1945).** The similarity coefficients were then used to construct a dendrogram using the UPGMA (Unweighted Pair-Group Method with Arithmetical averages) through NTSYS-pc package (Rohlf, 1998).

5.6 Molecular diversity and similarity in *R. serpentina* and *R. tetraphylla*

Morphologically accessions of *R. serpentina* and *R. tetraphylla* are collected from various locations show close semblance and hence RAPD markers were used to assess the genetic diversity and similarity among the accessions of both the species.

Analysis of interpopulation variations in *R. serpentina* and *R. tetraphylla* was carried out by screening with 40 primers from OPA and OPC Kits. Intrapopulation variations in *R. serpentina* among the eight plants collected from (Dulapally) was also analyzed. Studies on interspecific variations and assessment of the genetic similarity between *R. serpentina* and *R. tetraphylla* was carried out by screening with 40 primers from OPA and OPC Kits.

5.6.1. Isolation of genomic DNA

DNA was isolated by using CTAB protocol developed by Doyle and Doyle (1962) with slight modifications. Young leaf tissue (3 gm) was ground into a fine powder in liquid Nitrogen along with 0.1 gm of PVPP and transferred to preheated 2% CTAB extraction buffer (2% CTAB, 100 mM Tris HCl, pH 8, 20 mM EDTA, 1.4 M NaCl) containing 10 mM beta mercaptoethanol per gram of tissue. The slurry was incubated for 90 min at 65°C in a water bath. An equal volume of chloroform: isoamylalcohol (24:1) was added to the extract prior to centrifugation at 12000x *g* for 15 min at 4°C. To the supernatant equal volumes of ice cold isopropanol was added and incubated at –20°C for a period of 30 min or overnight followed by centrifugation at 12000x *g* for 15 min at 4°C. The pellet was collected and washed with 70% ethanol, centrifuged at 10000x *g* for 8 min. The pellet was dried and redissolved in 100µl of TE buffer. In order to eliminate RNA contamination the sample was treated with 5µl of RNase A (10 µg/µl), incubated at 37°C overnight. This was followed by phenol chloroform: isoamylalcohol (1:1) extraction followed by centrifugation at 8000x *g* for 15 min. To the supernatant equal volumes of chloroform: isoamylalcohol (24:1) was added and centrifuged at 12000x *g* for 15 mins. To the supernatant 1/10th

volume of 3 M sodium acetate and equal volumes of ice-cold isopropanol was added and left for 30 min or overnight at –20°C to precipitate DNA followed by centrifugation at 12000g for15 min. The DNA was washed by adding 70% ethanol and centrifuged at 10000g for 10 min at 4^0C. (Sambrook *et.al* 1989). After complete drying the pellet was dissolved in TE buffer depending on the pelletand was stored at –20^0C for future use. DNA quality and concentration were evaluated spectrophometrically at 260 nm and 280 nm respectively and the DNA concentrations were rechecked by visual assessment of band intensities on 0.8% agarose gel. DNA purified from each accession was diluted to 50 ng/µl for PCR reactions same as mentioned earlier in case of *P. santalinus*. RAPD analysis and data scoring was done as stated earlier for *P. santalinus* following similar methodology (Please refer sections 5.5.2.3 and 5.5.2.4)

6. Results

6.1. Collection of different accessions of chosen species

A total of 27 accessions of *Pterocarpus santalinus* were collected from various locations of Andhra Pradesh, India and also from the local nurseries in Hyderabad (A.P., India). The plants collected from local nurseries were supposed to be grown naturally in forests of Kerala and Karnataka (India) were used as reference material for genetic diversity analysis to compare with accessions collected from A.P. Natural stand of *P. santalinus* with flowers in the forests of Tirupathi and Talakona

Figure 6a and b shows (Chittoor District, A.P India) locations from where the germplasm was collected. The characteristic features, which are the key characters, used for identification of germplasm of *P. santalinus*, *R. serpentina* and *R. tetraphylla* are listed in Table 5. Field observations of *P. santalinus* i.e., characteristic bark with rectangular ridges, sprouting of shoot on the stem bole and occurrence of inflorescence during the month of April, *P. santalinus* in the forests in Kadapa (Cuddapah, District, A.P) (Figure 7 a-d). *P. santalinus* was introduced to the UH campus (Hyderabad, A.P) is (Figure. 8a). The germplasm in the form of plants (300) is maintained in the experimental site (Figure 8 b and c) and field gene bank and seeds are maintained in seed bank at appropriate temperature and moisture (Figure. 9) . Details of germplasm collections of *P. santalinus* are enlisted in Table 6. Among 27 collected accessions 15 accessions were used for molecular analysis.

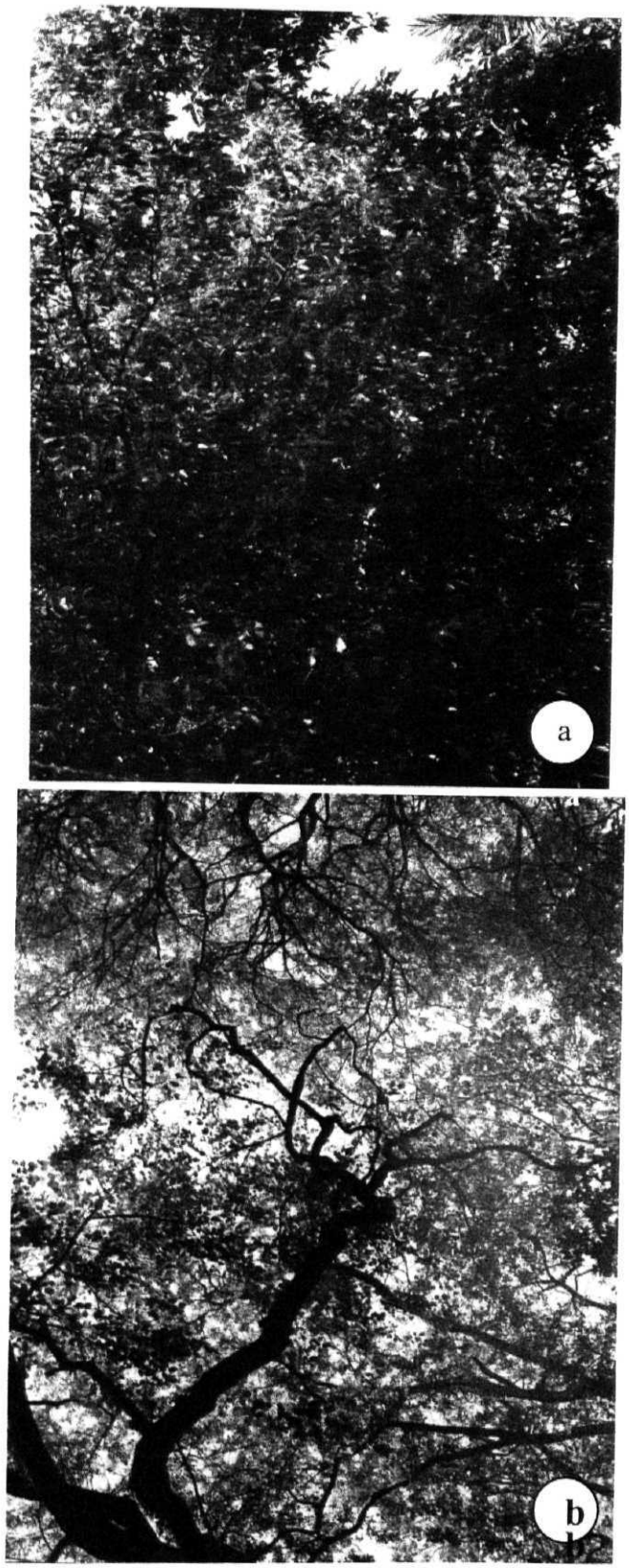

Figure 6: Natural distrubution and flowering in *P. santalinus*

a) Natural stand of *P.santalinus* in the forests of Tirupathi.

b) Natural stand of *P.santalinus* in the forests of Talakona

Table 5: Salient investigated medicinal characters of plants

Taxon name / Important key characters
***Pterocarpus santalinus* L.f.** Endemic trees, blackish brown bark with rectangular clefts, trifoliate broadly egg shaped, orbicular leaves, margin shiny, leathery and entire. Flowers bisexual, yellow, simple sparingly branched racemes, orbicular, winged pods, reddish brown kidney shaped seeds.
***Rauvolfia serpentina* Benth. Ex. Kurz** Herbs and under shrubs, stems woody at base, rootstock thick and woody, bark ashy white, thin, watery latex, leaves 3-4 sometimes opposite, elliptic, oblanceolate, margin entire, papery, with distinct stalk, flowers bisexual, terminal or axillary umbellate cymes fruits – drupes, fleshy, ovoid purplish black when ripe, seeds ovoid, purplish black when ripe, seeds ovoid.
***R. tetraphylla* L.f** Small branched woody shrub, leaves whorled, ovate elliptic, flowers greenish creamy white in umbellate cymes. Fruits are drupes ovoid, deep red purple when ripe. Seeds oblong and rugose.

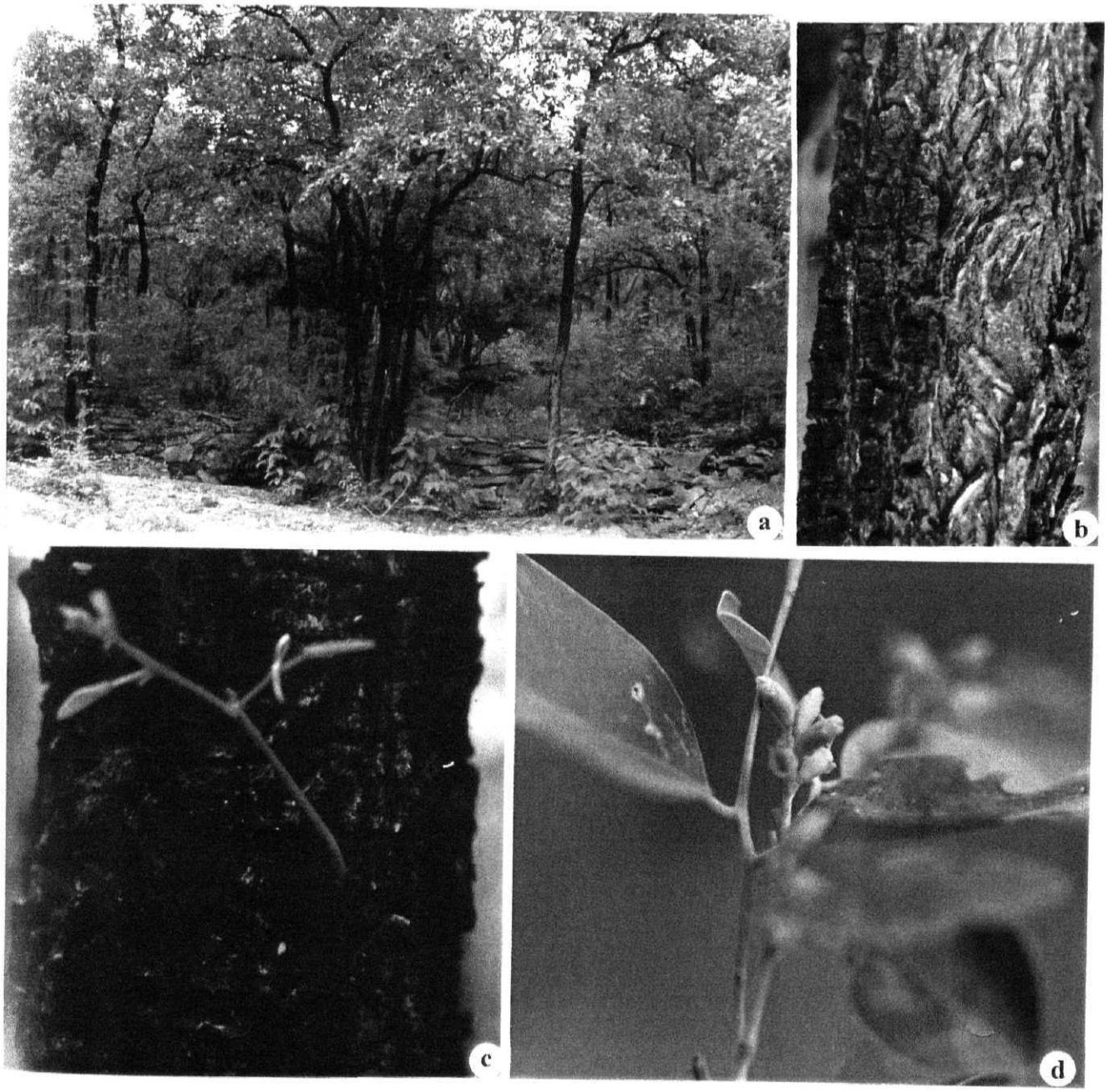

Figure 7: Field observations of *Pterocrapus santalinus* L.f in natural conditions.

a) *P. santalinus* in Lankamalai forests in Kadapa (Cuddapah district, A.P)
b) Characteristic bark of *P. santalinus* with rectangular ridges.
c) Sprouting of shoot from the stem bole of *P. santalinus* on UH campus
d) Inflorescence of *P. santalinus* during April in forests of Tirupathi.

Figure 8:*Ex situ* conservation of *P. santalinus* in University of Hyderabad (UH) campus

a) Plantations of *P. santalinus* in UH campus.
b) *P. santalinus* seedling from Lankamalai forests in Kadapa (Cuddapah district, A.P) of introduced to experimental site
c) *In vivo* seed germination of *P. santalinus* in experimental site.

Figure 9 : Storage of pods of *P. santalinus* in seed bank

Table 6: Germplasm collection details of *Pterocarpus santalinus* from various locations of Andhra Pradesh

District in A.P	**Location**	**Nature of germplasm**
Hyderbad	UH campus Durgamcheruvu	Leaves
Cuddapah	Siddhavotam, Sanipaya, Gadela, Cuddapah, Balpally, Papireddypally, V.Rajampet Rayachoti, Rajampet, Kodur	Seeds/seedlings
Chittoor	Seshachalam (Tirumala Hills), Tirupathi, S.V.Campus, Bhakrapet, Papavinasanam, Talakona, Ramachandrapuram, Sorakaipalem, Narsingapuram	Seeds/ seedlings
Nellore	Tada, Rapur, Veligonda Hills	Seeds
Ananthapur	Kalasamudram	Leaves
Vishakapatnam	Araku valley, A.U campus	Seeds

A total of 6 accessions of *R. serpentina* and 7 accessions of *R. tetraphylla* were collected from different locations of A.P in the form of both plants, stem cuttings and seeds which were maintained in the experimental site and seed bank. A total of 30 plants of both the species were collected from various forests of A.P. and are being maintained in field gene bank (Figures. 10 and 11). Details of germplasm collections of *R. serpentina* and *R. tetraphylla* are shown in tables 7 and 8.

6.2. *In vivo* seed germination – *P. santalinus*

In *in vivo* conditions, maximum seed germination (50-60%) was observed from pods collected from Papireddypally and Balpally (Cuddapah/Kadapa District, A.P) and least percentage (5%) of germination was observed from the pods collected from Vishakapatnam (A.P, India) The pods when treated with different dormancy breaking agents like H_2SO_4, KNO_3, KCl, HCl etc., in, *in vivo* conditions for at different time intervals, there was no seed germination observed except in pods treated with potassium nitrate (100 ppm), were able germinate but the percentage of germination was very less (2%) (Figure. 12 a and b). Details are shown in Table 9.

6.3. *In vitro* seed germination – *P. santalinus*

Overnight soaking in tap water followed by mechanical scarification was most effective. In *in vitro* conditions, 100% seed germination was observed from seeds collected from Balpally (Cuddapah/ Kadapa District, A.P) on MS (liquid), ½ MS (liquid), 3% sucrose, 1% agar and MS with 0.1% phytagel. Seed germination details of seeds

Fi ure 10: *Ex situ* conservation of *R.serpentina* in experimental site collected from various lo tions a) Plants collected from Dulapally with flowering (Ranga reddy District) b) Maredumilli (East Godavari) c) Araku (Vishakapatnam) d) Hyderabad (Hyderabad) e) Ri ned fruits collected from plants grown in field experimental site f) Storage of ge plasm in the seed bank.

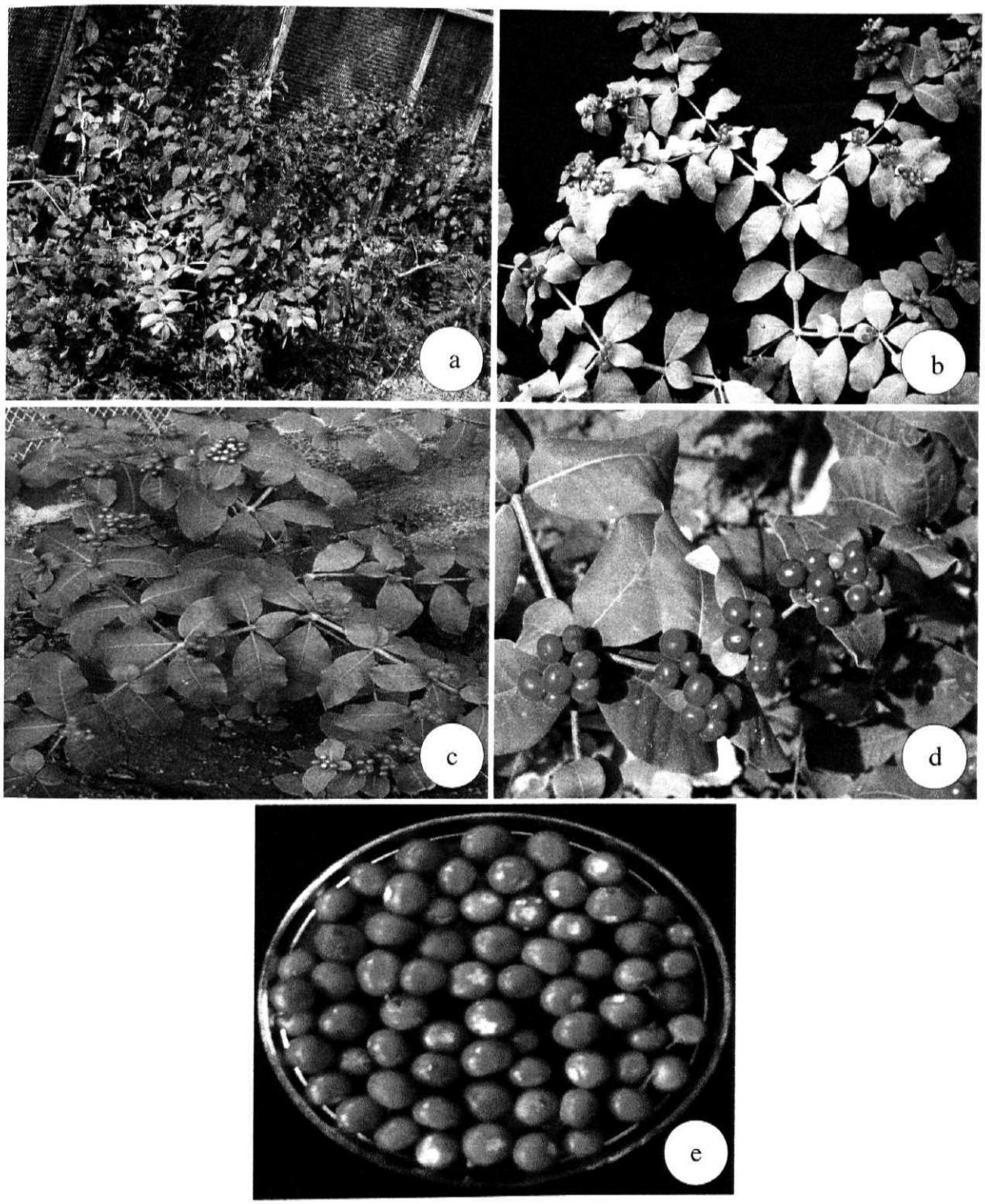

Figure 11: *Ex situ* conservation of *R.tetraphylla* in experimental site collected from various locations.
a) Plants collected from Tirupathi (Chittoor District) b) Mallur (Warangal) c) Balpally (Cuddapah) d) Profuse fruiting in August e) Ripened fruits of *R.tetraphylla*

Table 7: Germplasm collection details of *R. serpentina* from different locations of Andhra Pradesh

District in A.P	Location	Nature of germplasm
Warangal	Mallur	Plants
Visakhapatnam	Araku	Plants
Hyderabad	Hyderabad	Seedlings/seeds
Medak	Dulapally	Stem cuttings
Khammam	Sukumamidi	Plants
E.Godavari	Maredumilli	Plants

Table 8: Germplasm collection details of *R. tetraphylla* from different locations of Andhra Pradesh

District in A.P	Location	Nature of germplasm
Warangal	Mallur	Plants
Chittoor	Tirupathi	Plants
Cuddapah	Balpally	Plants/Seeds
Hyderabad	Hyderabad	Seedlings
Ranga reddy	Dulapally	Stem cuttings
Krishna	Vijayawada	Plants
Khammam	Maredumilli	Plants

Table 9: *In vivo* seed germination in seeds of *P.santalinus* collected from various locations Andhra Pradesh

Location	Avg % of germination
Balpally	50
Papireddypally	60
Cuddapah	40
Visakhapatnam	5
Rapur	25
Veligonda Hills	20
Talakona	15
Tirupathi	10
Papavinasanam	10

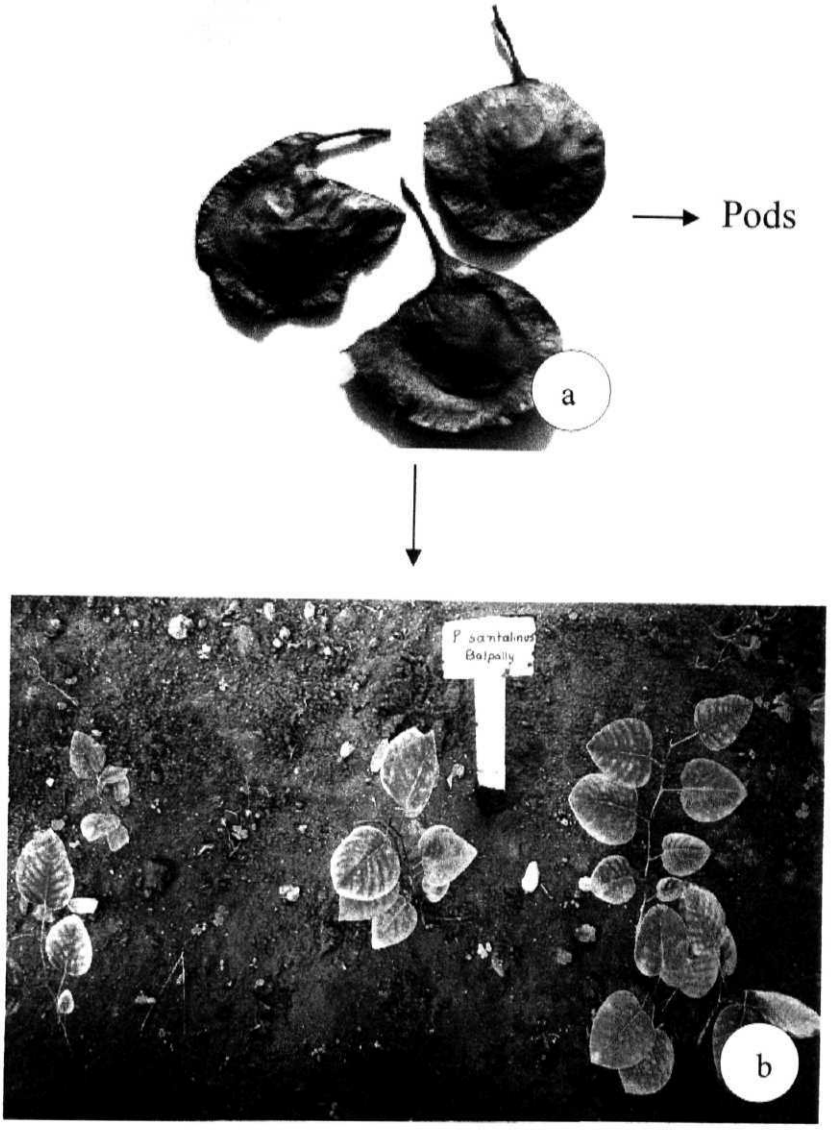

Figure 12 a and b: ***In vivo*** seed germination of ***P. santalinus.*** Five months old seedlings of of Balpally accession in our experimental site

collected from various locations on MS basal medium (Figure 13 a-e). Details of different dormancy breaking agents are shown in Table 10.

6.4. *In vitro* regeneration – *P. santalinus*

Initially different types of media (table 11) and media with different hormones were used for multiple shoot regeneration (table 12). The explant source and the morphogenetic response observed from different explants is shown in table 13 which will be explained further in detail. Horizontal orientation of the explant and 0.6% agar were found to be most effective for multiple shoot regeneration (Figure 14 a-c, tables 14 and 15). Cotyledonary nodal meristem excised from the *in vitro* germinated seed on MS basal medium when cultured on MS, with different cytokinins at various concentrations (0.1-7.0 mgL^{-1}) both singly and in combinations, produced multiple shoots on MS medium with 1.0 mgL^{-1} BAP and later did not show any further elongation in the same medium as well as when transferred onto other shoot elongation medium i.e., MS+GA_3 (0.2-7.0 mgL^{-1}) as shown in figure 14 d-g. Nodal explants when cultured on MS medium supplemented with 3.0 mgL^{-1} BAP and 5.0 mgL^{-1} KN resulted in maximum number of multiple

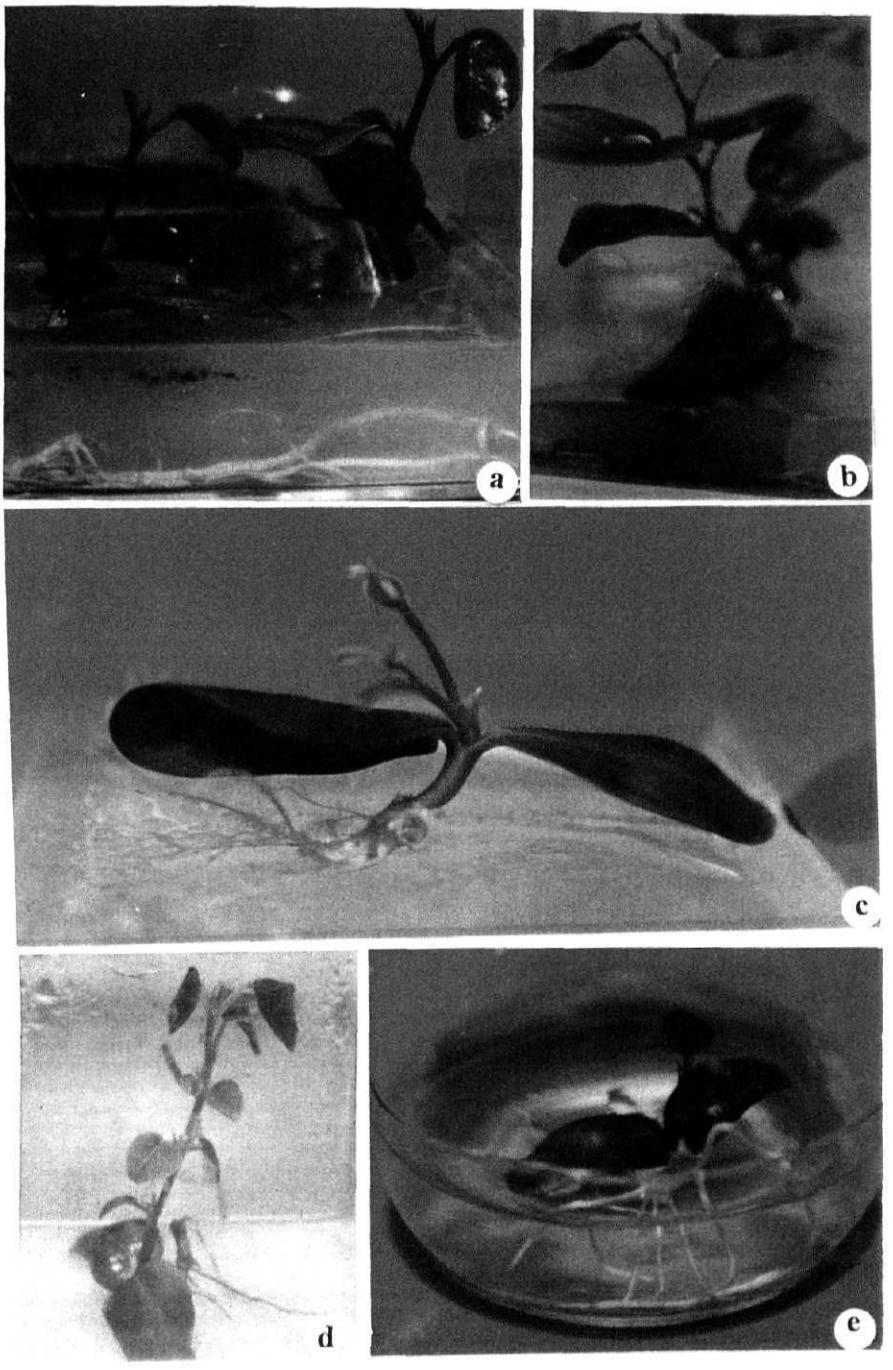

Figure 13: *In vitro* seed germination of *P. santalinus* collected from various locations on MS Basal medium

a) Balpally
b) Rapur
c) Papireddypally
d) Rajampet
e) Vishakapatnam

Table 10: *In vitro* seed germination of *P.santalinus* (Balpally) by using different treatments

Medium	% response
MS + 0.8% Agar	60
MS + 0.7 % Agar	50
MS + 0.6% Agar	80
MS + 0.5% Agar	70
½ MS + 0.8% Agar	70
¼ MS + 0.8% Agar	-
¾ MS + 0.8% Agar	-
MS (liquid)	100
¾ MS (liquid)	100
½ MS (liquid)	-
¼ MS (liquid)	-
MS +1.0 mgL^{-1} GA_3	-
MS +2.0 mgL^{-1} GA_3	-
MS +3.0 mgL^{-1} GA_3	-
MS + Cold water treatment	50
MS + Hot water treatment	90
MS + 0.1% Phytagel	100
1% Agar	100
3% Sucrose	100

Table 11: Influence of different media and growth regulators on multiplication from the nodal explants

Media + hormone used (mgL^{-1})	% response
MS + BAP (0.1-7.0)	60
MS + KN (0.1-7.0)	60
MS + TDZ (0.1 -7.0)	20
MS + 10 % Coconut water	20
MS + 2,4 -D (0.1-7.0)	-
Mc Cown + BAP (0.1-7.0)	60
Mc Cown + KN (0.1-7.0)	20
B5 (Gamborg's)	20
B5 + BAP (0.1-7.0)	60
B5 + KN (0.1-7.0)	60
B5 + TDZ (0.1-7.0)	40

Table 12: Different combinations and concentrations of hormones used for multiple shoot regeneration from nodal explants, seeds and leaf tissue.

Media	Conc. (mgL^{-1})
MS + BAP	0.1-7.0
MS + KN	0.1-7.0
MS + AH	0.1-7.0
MS + TDZ	0.1-7.0
MS + TDZ + AH	1.0+0.1
MS + CW	10%
MS + BAP + KN	2.0 + 1.5
MS + 2,4 D	0.1-7.0
MS + 2,4 D + BAP	2.0+ 2.0
MS + ZN + KN + TDZ	2.0 + 2.0 + 2.0
Mc Cown Basal	-
Mc Cown + BAP	0.5 – 4.0
Mc Cown + KN	0.5 – 4.0
Mc Cown + BAP + KN	2.0 + 1.5
B-5 Basal	-
B-5 + BAP	0.5 – 4.0
B-5 + KN	0.5 – 4.0
B-5 + BAP + KN	2.0 + 1.5
MS Basal	-

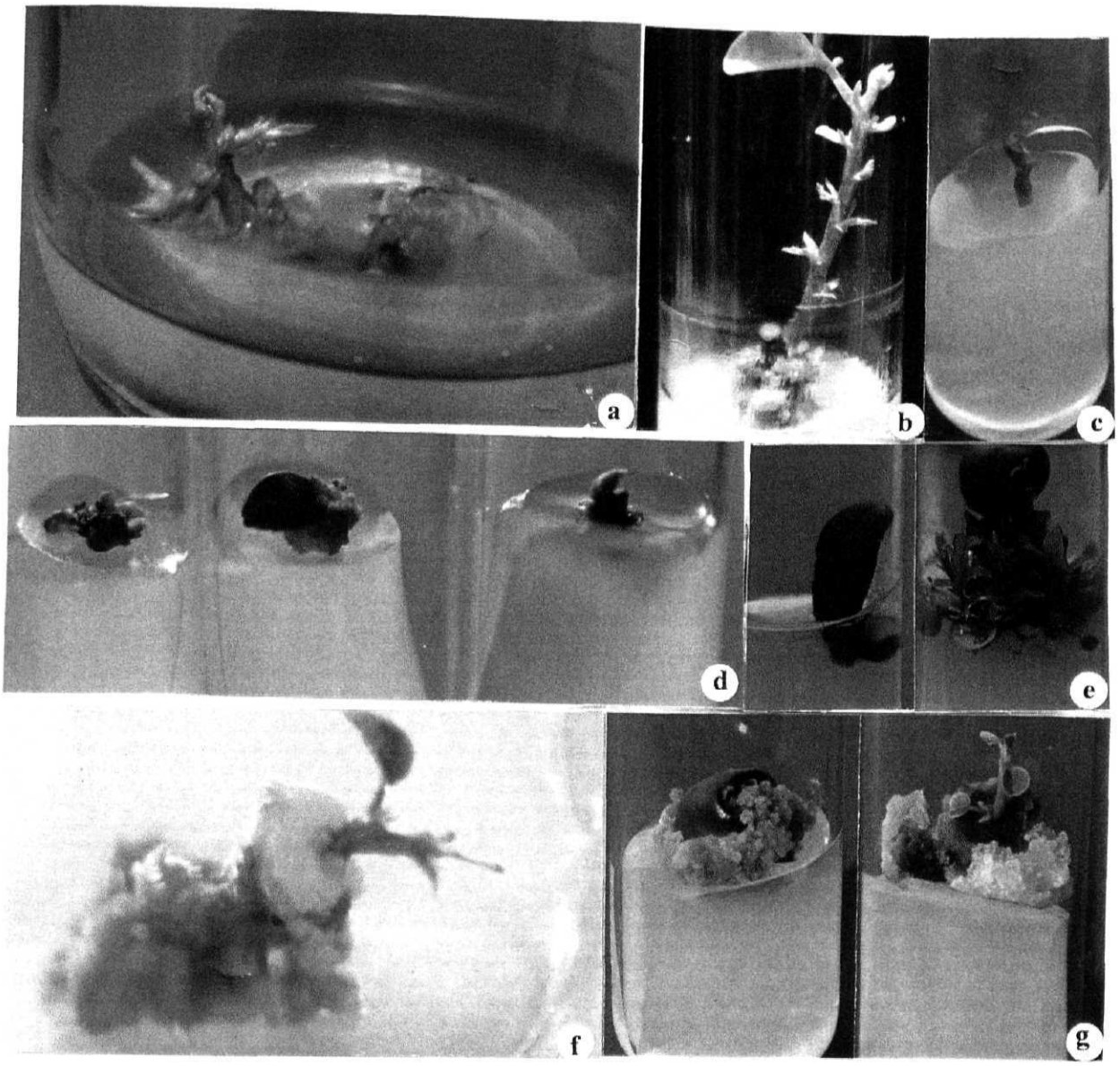

Figure 14: Advantage of orientation and response of various explants on MS medium Supplemented with different growth regulators

a) Horizontal orientated nodal explant with hormones cultured on semisolid (0.6% Agar)
b) MS medium with $1mgL^{-1}$ BAP after 3 weeks, vertical orientation
c) Shoot tip from *in vitro* raised germinated seedling cultured on MS medium with 0.5 mgL^{-1} BAP after 1 week.
d) Leaf explants on MS medium supplemented with $1mgL^{-1}$ KN after 2 weeks.
e) Initiation of multiple shoots from cotyledonary nodal meristem cultured on MS medium with $1mgL^{-1}$ BAP after 4 weeks.
f) Development of non-embryogenic callus from shoot tip on MS with $1mgL^{-1}$ TDZ after 5 weeks.
g) Friable callus from cotyledonary leaf and shoot tip cultured on MS medium with $1mgL^{-1}$ TDZ after 3 weeks.

Table 13: Morphogenetic response of different explants with different media combinations

Source of the explant	Medium / Conc. mg L^{-1}	Morphogenetic response
1. Node		
UH campus, i*n vitro* raised seedlings from seeds collected from Balpally, Rapur, Papireddypally, Rajampet and Vishakapatnam	MS + BAP MS + KN MS + BAP+ KN MS + TDZ MS + BAP + TDZ	6-7 multiple shoots observed.
2. Seeds		
Balpally, Rapur, Papireddypally, Rajampet, Vishakapatnam	MS Basal MS + 2KN + 1BAP MS + 1BAP	Multiple shoot regeneration observed from seeds collected from Balpally
3. Hypocotyl		
UH campus, i*n vitro* raised seedling from seeds collected from Balpally, Rapur, Papireddypally, Rajampet and Vishakapatnam	MS + BAP MS + KN MS + BAP+ KN MS + TDZ MS + BAP + TDZ	Development of non -embryogenic callus
4. Shoot tip		
UH campus, *In vitro* raised seedling from seeds collected from Balpally, Rapur, Papireddypally, Rajampet and Vishakapatnam	MS + BAP MS + KN MS + BAP+ KN MS + TDZ MS + BAP + TDZ	Development of non-embryogenic callus
5. Cotyledonary nodal meristem		
Balpally	MS + BAP MS + KN MS + BAP+ KN MS + TDZ MS + BAP	Initiation of 6-7 multiple shoots.
6. Internodal segments		
UH campus *In vitro* raised seedling from seeds collected from Balpally, Rapur, Papireddypally, Rajampet and Vishakapatnam	MS + BAP MS + KN MS + BAP+ KN MS + TDZ MS + BAP MS + 2,4 D	Development of non embryogenic callus
7. Leaves		
UH campus	MS + BAP MS + KN MS + BAP+ KN MS + TDZ	Development of non embryogenic of callus

Table 14: Influence of different concentrations of agar on multiple shoot regeneration

Media + percentage of agar	**No. of explants**	**% response**
MS + 0.5%	40	-
MS + 0.6%	40	80
MS + 0.7%	40	60
MS + 0.8%	40	60

Table 15: Advantage of explant orientation and percentage of agar in glass bottle

Medium	**No. of explants**	**% response**
MS + 0.6% agar + 0.1mgL^{-1} BAP (Vertical)	30	Growth of only one single branch with 7-8 axillary buds
MS + 0.6% agar + 0.1mgL^{-1} BAP (Horizontal)	30	Growth of different branches with profuse axillary bud proliferation

shoots (6.0) whereas MS medium fortified with different concentrations of TDZ and a combination of TDZ and BAP given in tables 16-19. Shoot tips and leaves when considered as explants resulted in development of non-embryogenic callus. Seeds when cultured on MS medium with 2.0 mgL^{-1} KN and 1.0 mgL^{-1} BAP, after 15 days an average of 19-20 multiple shoots were observed, each shoot with 8-9 axillary buds as (figures 15-19). The details are shown in table 20. Each axillary bud (node) when cultured onto MS medium with 1.0 mgL^{-1} BAP, 2-3 multiple shoots were observed. Since it is a woody species acclimatized in the glass house but the percentage survival was very low (20%) (figures 20 and 21).

6.5. Morphological diversity analysis in *P. santalinus*

A total of 16 accessions collected from various locations could be clearly identified based on diagnostic morphological traits and the differences in pod characteristics. Distinct morphological variations were observed in pod characteristics like pod (weight, width, and length) and in few parameters like leaf length, leaf width, shoot length and number of nodes in five months old germinated seedlings which could be due to environmental and edaphic factors. Wide ranges of morphological variations were observed among all the accessions collected from various locations (figure 22 a-h). Longer leaves were observed in plants

Table 16: Influence of 6- Benzyl amino purine (BAP) on multiple shoot induction from nodal explants

Medium used (mgL^{-1})	No.of explants	Dev. of callus	Av.no.of shoots
MS Basal	20	+	2.5
MS + 0.1	20	+	4.0
MS + 0.5	20	+	4.0
MS + 1.0	20	+	.5.0
MS + 2.0	20	++	5.2
MS + 3.0	20	++	6.5
MS + 4.0	20	++	5.0
MS + 5.0	20	+++	5.0
MS + 6.0	20	+	2.5
MS + 7.0	20	+	2.0

Table 17: Influence of Kinetin (KN) on multiple shoot induction from nodal explants

Medium used (mgL^{-1})	No. of explants	Dev. of callus	Av.no.of shoots
MS Basal	20	1	-
MS + 0.1	20	++	3.0
MS + 0.5	20	+++	3.5
MS + 1.0	20	-	4.0
MS + 2.0	20	-	4.5
MS + 3.0	20	-	6.5
MS + 4.0	20	-	3.5
MS + 5.0	20	-	2.0
MS + 6.0	20	-	-
MS + 7.0	20	-	-

Table 18: Influence of Thiadiazuran (TDZ) on multiple shoot induction from nodal explants

Medium used(mgL^{-1})	No. of explants	Dev. of callus	Av.no.of shoots
MS Basal	20	Not much	1.6
MS + 0.1	20	"	2.0
MS + 0.5	20	"	2.5
MS + 1.0	20	"	4.5
MS + 2.0	20	"	4.8
MS + 3.0	20	"	4.5
MS + 4.0	20	"	4.0
MS + 5.0	20	"	4.0
MS + 6.0	20	"	2.5
MS + 7.0	20	"	2.0

Table 19: Influence of BAP and TDZ on multiple shoot induction from nodal explants.

Medium used (mgL^{-1})	No. of explants	Dev. of callus	Av.no.of shoots
MS Basal	20	Not observed	2.0
MS + 0.1	20	"	2.0
MS + 0.5	20	"	2.5
MS + 1.	20	"	2.8
MS + 2.0	20	"	4.0
MS + 3.0	20	"	6.0
MS + 4.0	20	"	2.0
MS + 5.0	20	"	2.0
MS + 6.0	20	"	2.0
MS + 7.0	20	"	-.

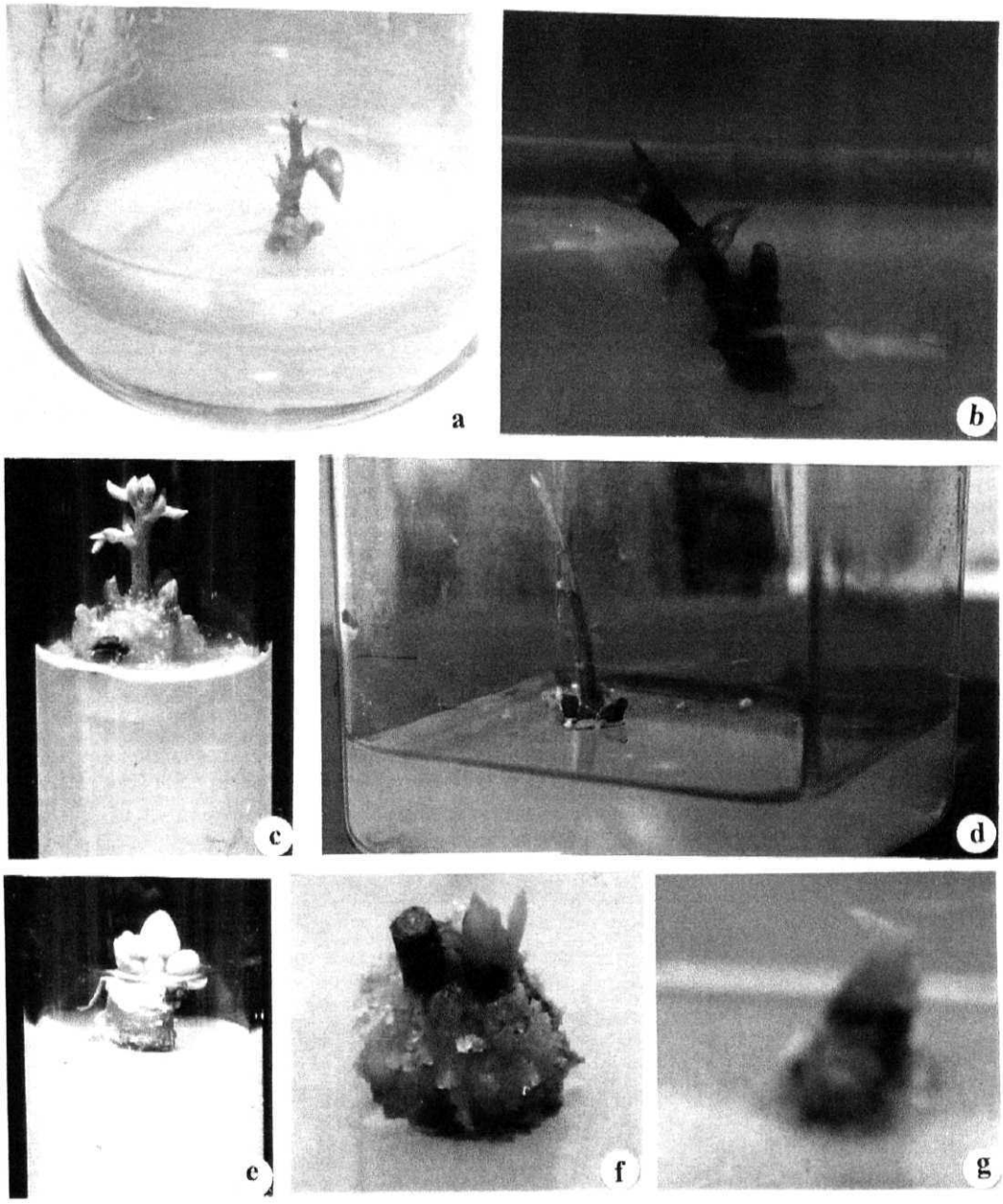

Figure 15: Shoot regeneration by using nodal explants collected from plantations of *P.santalinus* in UH campus on

a) MS Basal medium
b) MS medium supplemented with 0.5 mgL^{-1} BAP after 1 week
c) MS medium supplemented with $1mgL^{-1}$ BAP after 1 week
d) Elongation on MS medium with 1mg L^{-1} BAP after 2 weeks
e) MS medium supplemented with $1mgL^{-1}$ TDZ after 4 weeks
f) MS medium with 0.5 mgL^{-1} KN after 1 week
g) MS medium with 1 mgL^{-1} KN after 1 week.

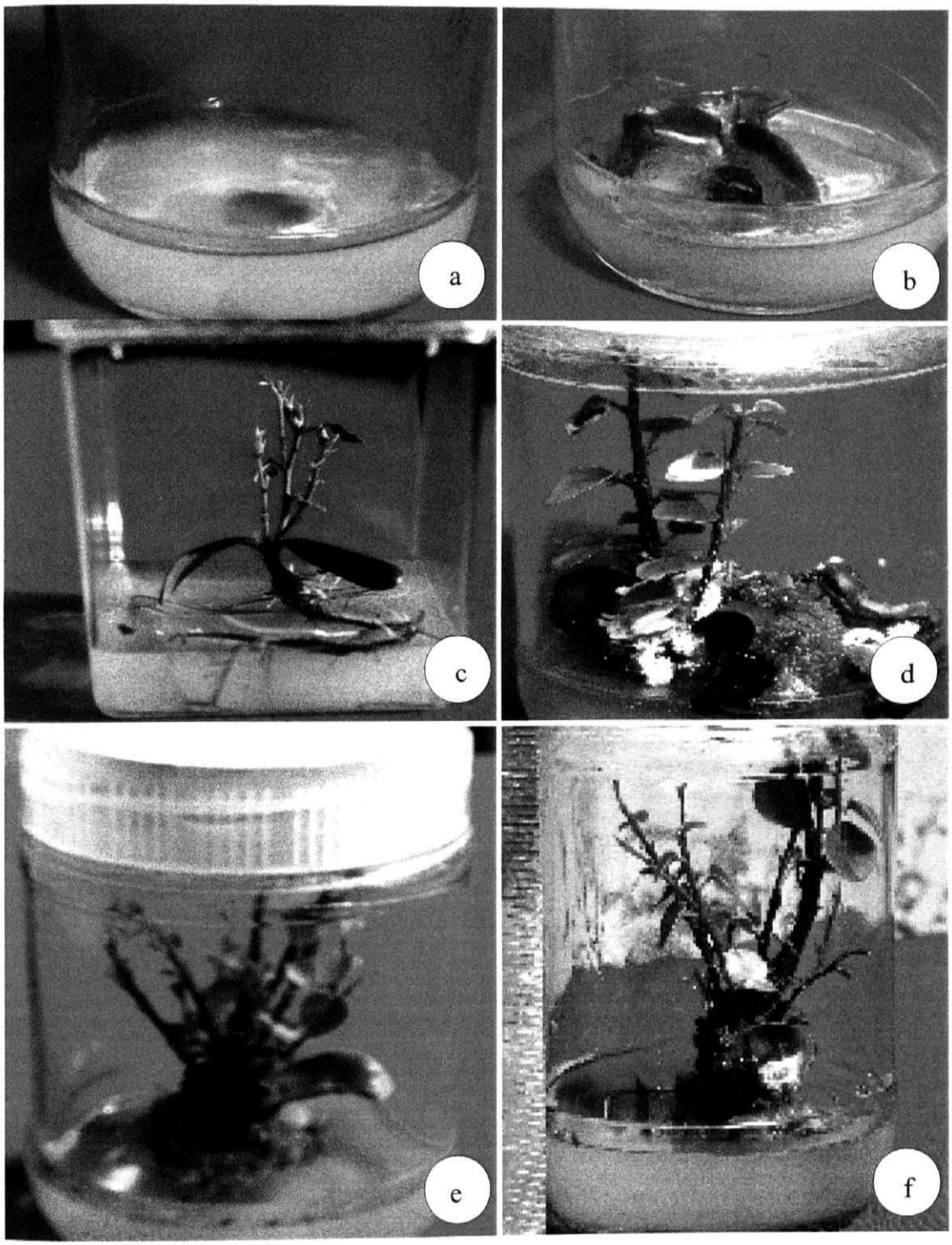

Figure 16: Sequential stages during multiple shoot regeneration from seed explants of *P.santalinus*. a) Micropylar end of the seed in contact with MS Basal medium. b) Seed germination on MS Basal medium after 1 week of inoculation. c) Mutiple shoot regeneration on MS medium supplemented with 1 mgL^{-1} BAP and 2 mgL^{-1} KN after 2 weeks. d) Elongation of multiple shoots on MS medium supplemented with mgL^{-1} BAP and 2 mgL^{-1} KN after 3 weeks. e & f) Multiple shoot regeneration along with rooting on MS medium supplemented with 1 mgL^{-1} BAP and 2 mgL^{-1} KN after 5 weeks.

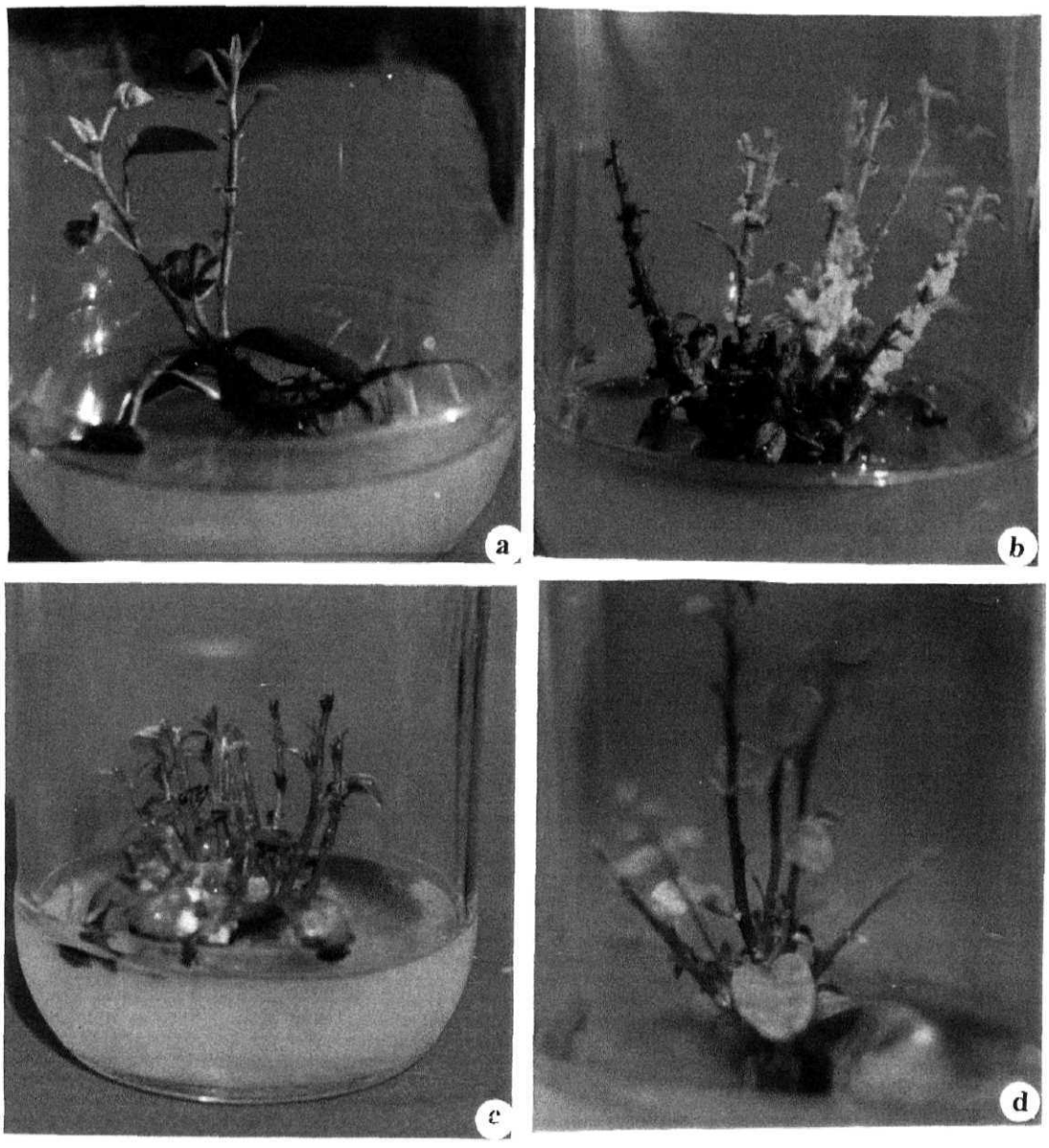

Figure 17: Multiple shoot regeneration in *P. santalinus* from seeds.

a. Seed germination on MS Basal medium after 2 weeks.
b. Multiple shoot regeneration on MS medium supplemented with 1mgL^{-1} BAP and 2 mgL^{-1} KN after 2 weeks.
c. Multiple shoot regeneration on MS medium supplemented with 1mgL^{-1} BAP and 2 mgL^{-1} KN after 3 weeks.
b) Elongation and rooting of multiple shoots and on MS medium supplemented with 1mgL^{-1} BAP and 2 mgL^{-1} KN after 5 weeks.

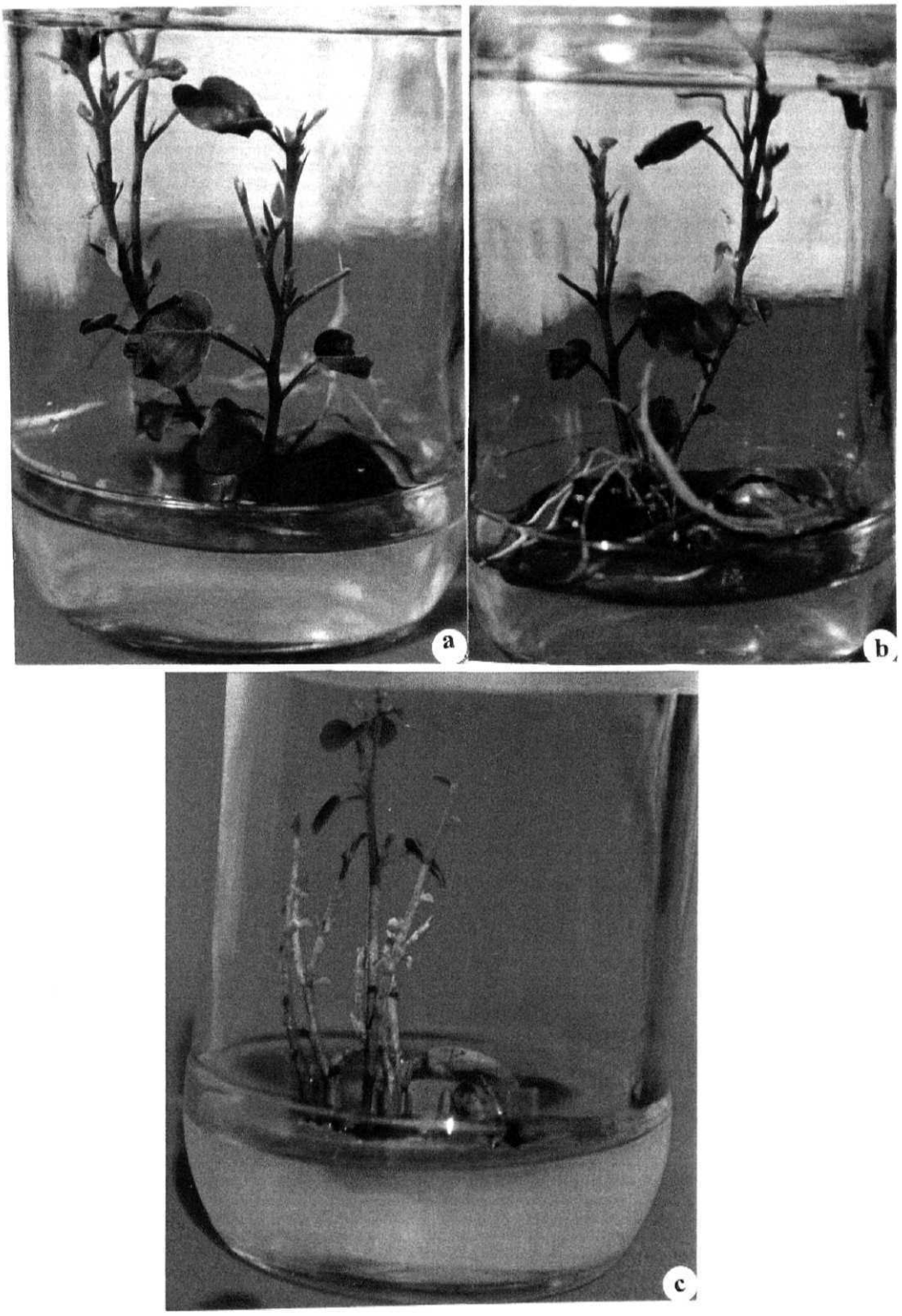

Figure 18: (a - c) Multiple shoot regeneration and rooting in *P. santalinus* from seeds on MS medium supplemented with 1mgL^{-1} BAP + 2 mgL^{-1} KN after 5 weeks.

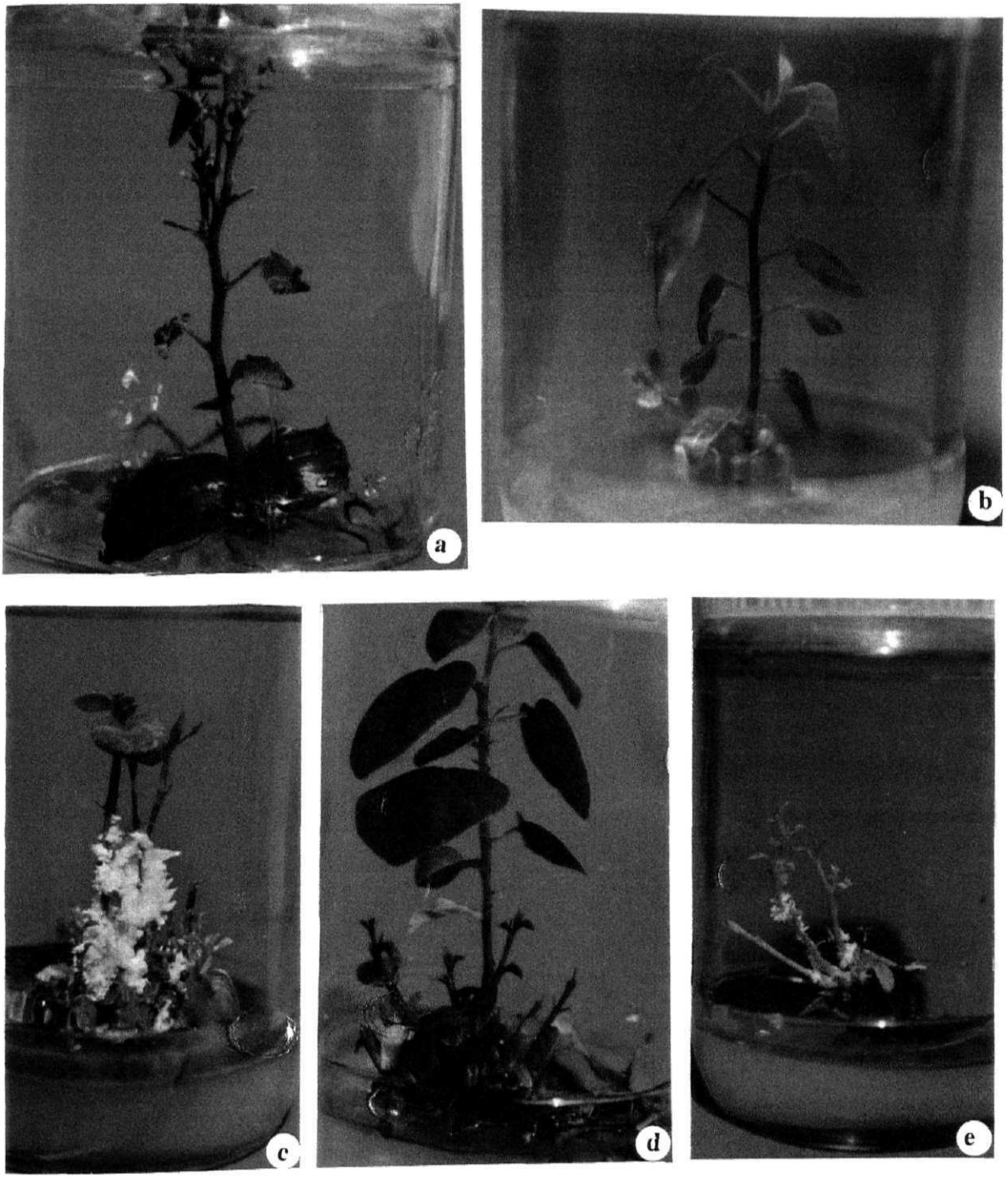

Figure 19: (a - e) Elongation, axillary branching and rooting in *P. santalinus* from seeds collected from Balpally on MS medium supplemented with 1mgL^{-1} BAP + 2 mgL^{-1} KN after 5 weeks.

Table 20: Effect of different hormones on regeneration of multiple shoots from seeds as eplants in *P.santalinus*.

Medium (mg L^{-1})	No. of shoots	Shoot length	No. of nodes	% responded
	Mean ± SE		Mean ± SE	
MS Basal	2.33 ± 0.66	4.72 ± 0.65	4.88 ± 0.95	90
MS + 1 BAP	3.62 ± 0.46	4.09 ± 1.27	4.75 ± 1.38	80
MS + 1 KN + 1 BAP	3.66 ±1.13	2.42 ± 0.59	3.14 ± 0.8	60
MS + 2 K + 1 BAP	17 ± 2.36	5.04 ± 1.13	6.7 ± 1.5	90
MS + 1 TDZ	1.6 ± 2.77	1.8 ± 0.34	2.2 ± 0.0	60
MS + 1 TDZ+ 1 AH	1.0 ± 0.0	3.4 ± 0.23	6.0 ± 0.81	50
MS + 2 KN	2.5 ± 0.28	3 .0 ± 0.46	4.6 ± 0.69	60

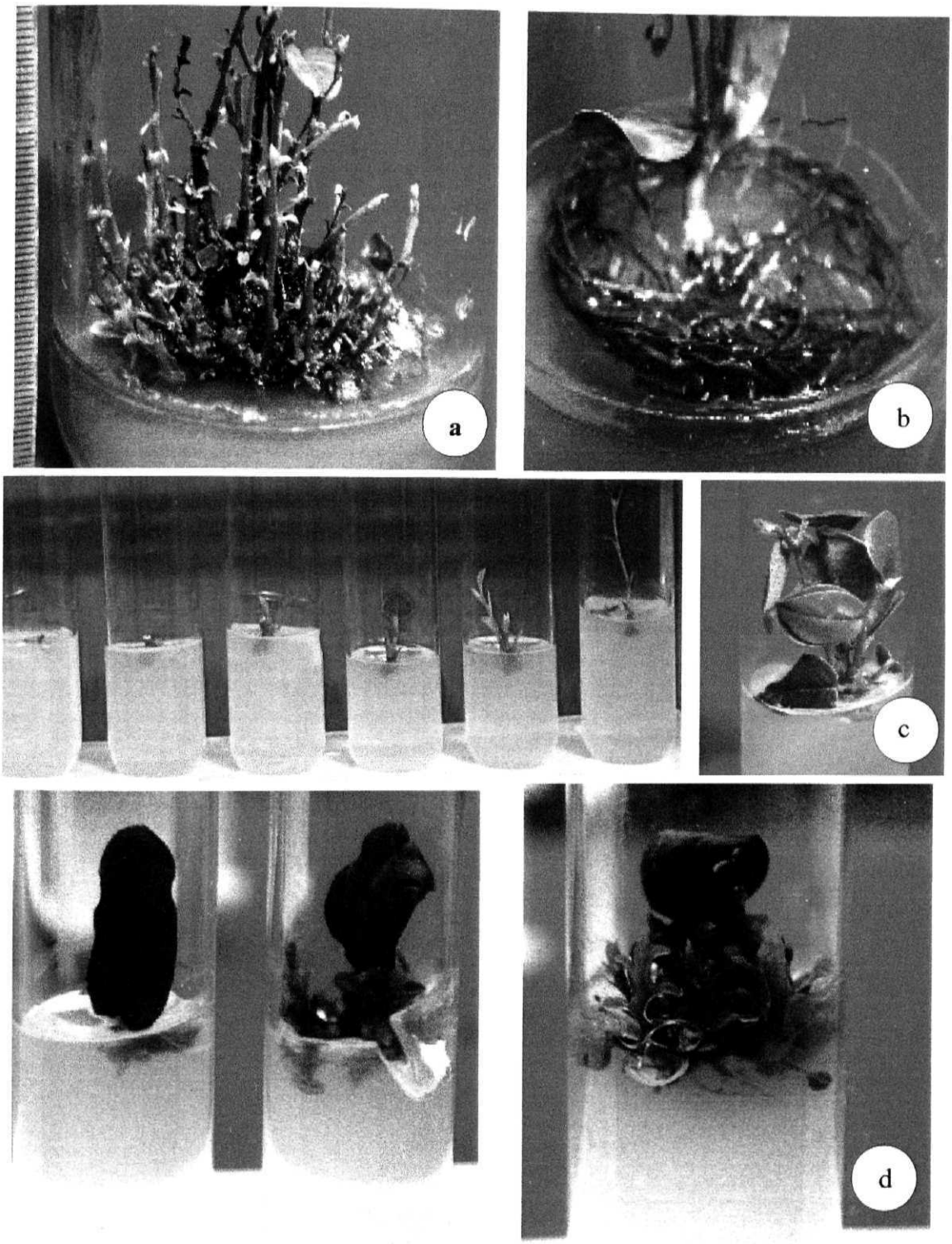

Figure 20: a) Multiple shoot regeneration on MS +1mgL^{-1} BAP and 2mgL^{-1} KN. b) Rooting in phytagel. c) Different stages of multiple shoot regeneration from node on MS with 1mg/L^{-1} BAP. d) Multiple shoot initiation from cotyledonary nodal meristem on MS with 1mg/L^{-1} BAP.

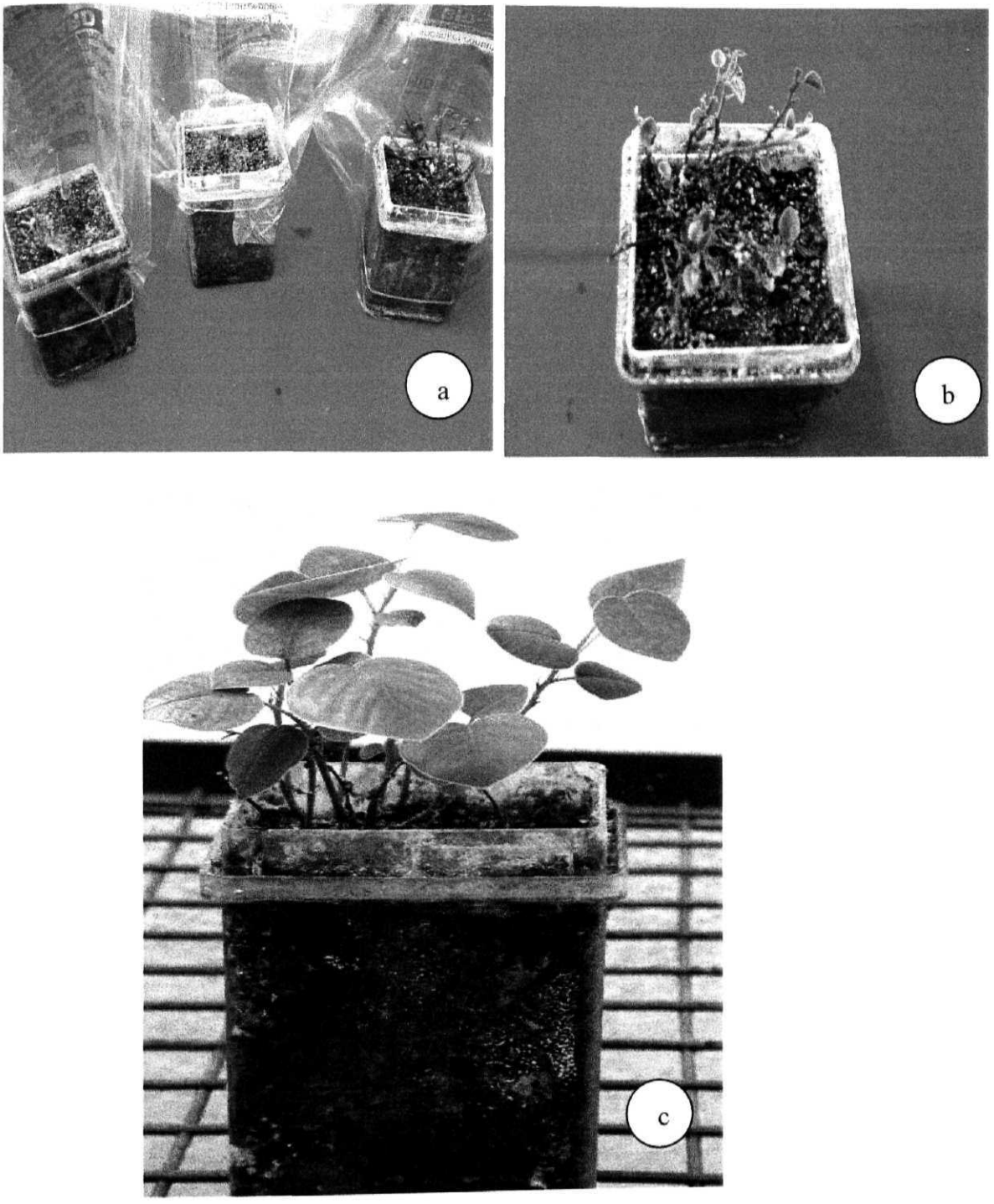

Figure 21: Acclimatisation of *P. santalinus* plants in glass house

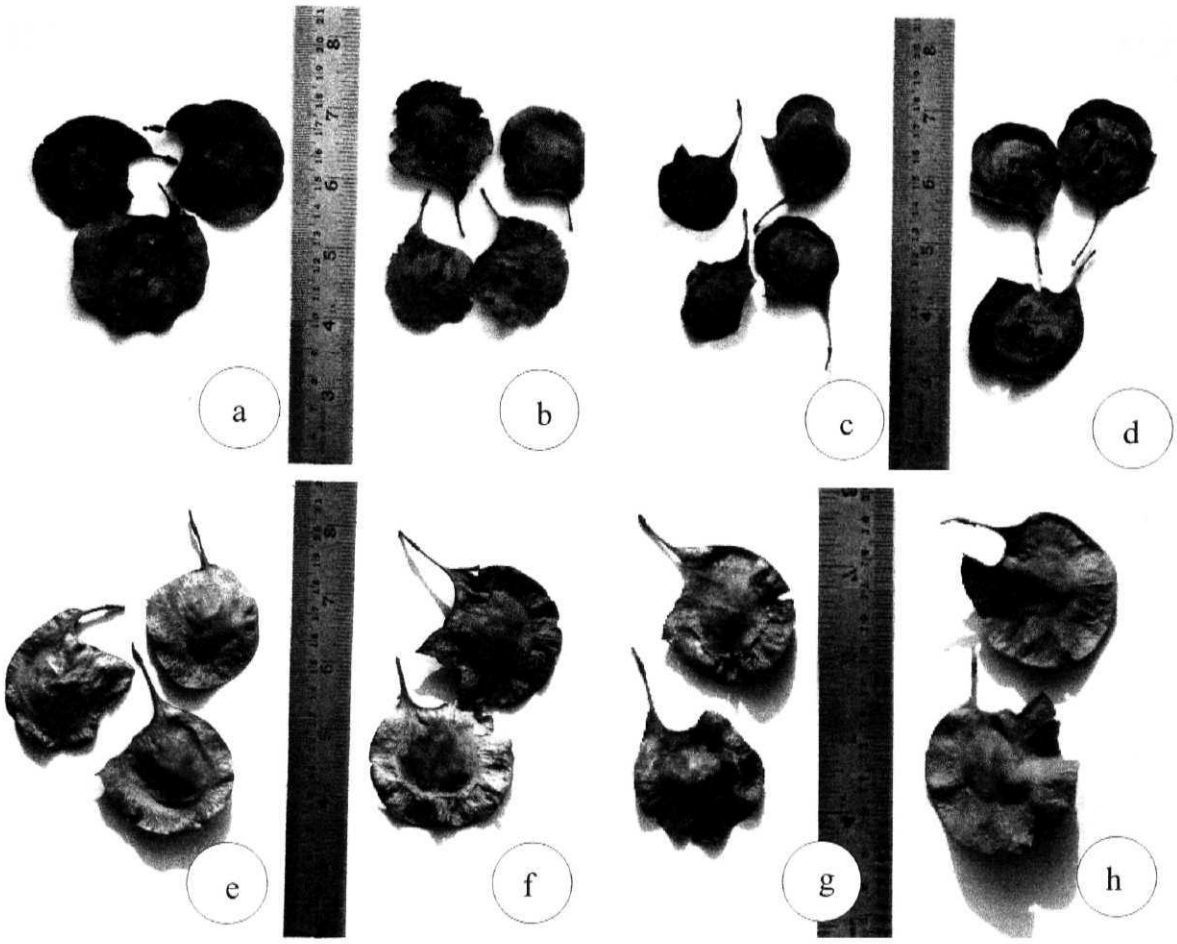

Figure 22: Morphological variations in pod characteristics (length, width and shape) collected from various locations of Andhra Pradesh (India)

a) Papireddypally b) Raichoti c) Tirumala d) Rajampet e) Cuddapah f) Vishakapatnam g) Talakona and h) V..Rajampet

collected from Papireddypally (Kadapa) and Vishakapatnam (5cm) and short leaves in plants from Sorakaipalem and Tada (2.8%) of Chittoor district._Pod weight was observed to be highest in the pods collected from Araku, Vishakapatnam district (2.9 gm) and lowest from Papavinasanam, Chittoor district (1.9gm) (figure 23). Similarly longer pods were seen in pod lots from Vishakapatnam and Rapur (Nellore) and short pods collected from Balpally (Kadapa) (figure 24). Pod width was highest from pods collected from Rapur and Araku and lowest in pods of Papavinasanam (figure 24). Number of axillary buds were more in plants from Bangalore (Karnataka state) and least in plants from Talakona (Chittoor) (figures 26 and 27).

6.6. RAPD analysis of *P. santalinus*

The accessions used for molecular analysis along with the latitude and longitude of area of collection are shown and stated in the table 21. Spectrophotometric measurements of DNA at 260 nm and 280 nm revealed higher concentration of DNA, which was diluted to 50 ng/µl with sterile ultrapure Milli Q water for further RAPD analysis (figure 28). A total of 40 primers when screened with OPA and OPC series for appropriate amplification and pattern formation out of which 26 primers were selected for tests of the repeatability of the method. Analysis of 15 accessions of *P. santalinus* revealed 100% of polymorphism (figures 29-52). Screening of the entire set of samples was done thrice to assess repeatability of the RAPD profiles, and identical RAPD patterns were obtained. The number of scorable polymorphic markers generated are 217 similar to that of the total number of markers

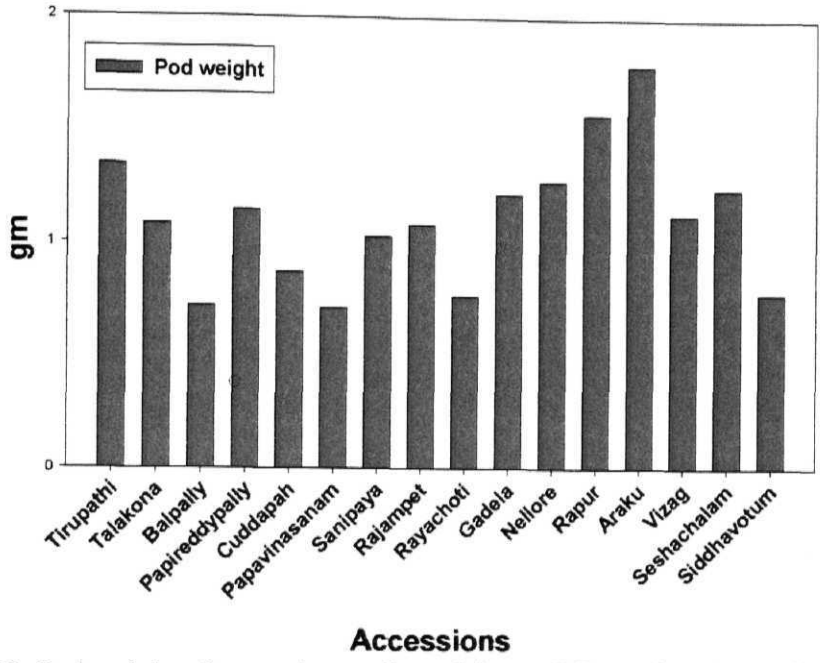

Figure 23. Pod weight of accessions collected from different locations of AP. SE of treatment means after analysis with one way ANOVA is 0.095

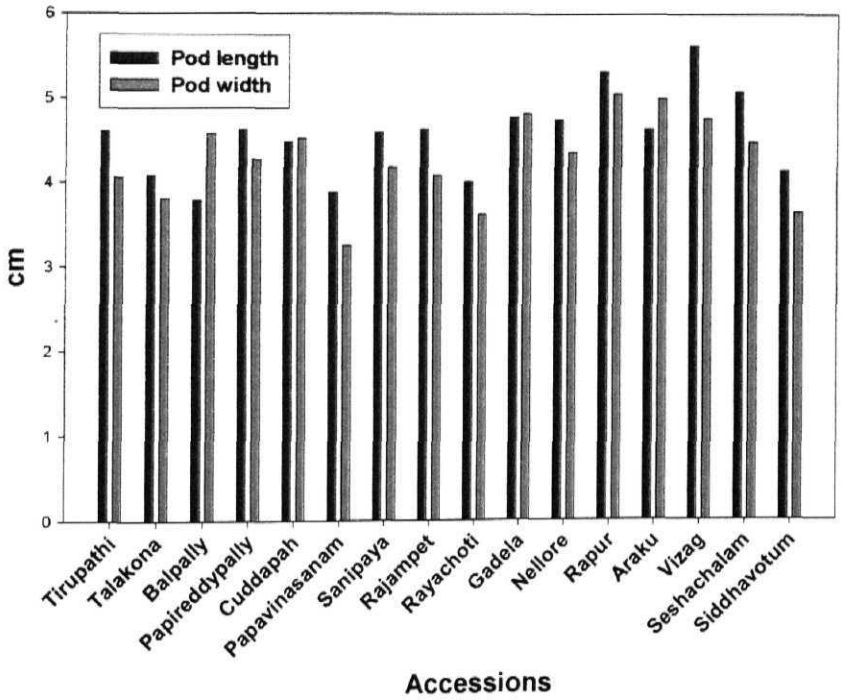

Figure 24. Pod width and length of accessions collected from different locations of AP. SE of treatment means after analysis with one way ANOVA is 0.125 for leaf length and 0.095 for leaf width

Table 21: Represents the accessions of *P. santalinus* collected from different locations from Andhra Pradesh used for molecular diversity studies.

Acc. No	Location	State/Dist	Latitude	Longitude
PSTI	Tirupathi	Chittoor	12° 08' N	75° 13'E
PSBY	Balpally	Kadapa (Cuddapah)	14° 25' N	79° 18'E
PSNA	Narsingapuram	Chittoor	14° 08' N	76° 20'E
PSSA	Sanipaya	Cuddapah	14° 07' N	78° 58'E
PSRM	Ramachandrapuram	Chittoor	13° 10' N	77° 20'E
PSSM	Siddhavotum	Cuddapah	04° 25' N	78° 58'E
PSBE	Bangalore	Karnataka	13° 12' N	77° 20'E
PSGA	Gadela	Cuddapah	14° 10' N	79° 30'E
PSTN	Talakona	Chittoor	13° 30' N	79° 08'E
PSTA	Tada	Nellore	14° 00' N	80° 05'E
PSKA	Kerala	Kerala	08 °15' N	77° 04'E
PSRY	Rajamundry	East Godavari	17° 08' N	81° 20'E
PSSP	Sorakaipalem	Chittor	12° 04' N	74° 18'E
PSKR	Kodur	Cuddapah	14° 52' N	78° 01'E
PSHC	Gachibowli	Hyderabad	17° 38' N	28° 12'E

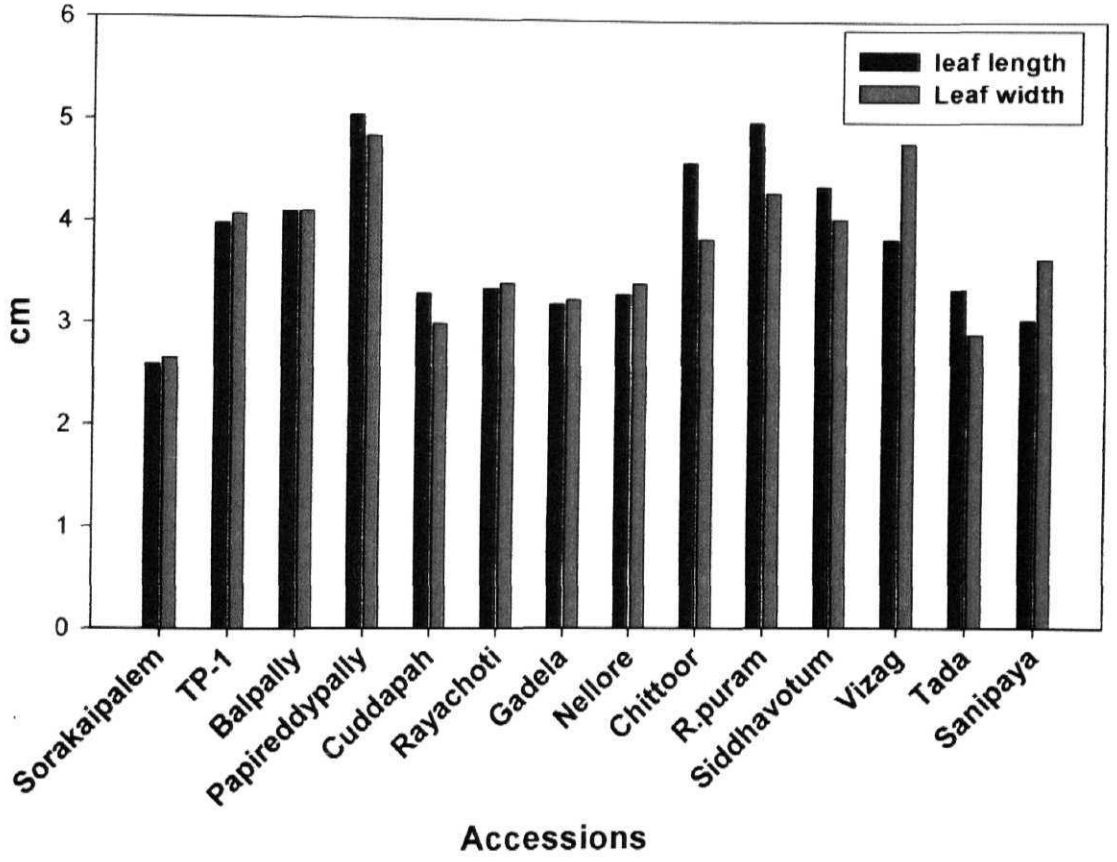

Figure 25. Leaf characteristics (length and width) in accessions of *P. santalinus* collected from different locations of AP. SE of treatment means obtained after analysis with one way ANOVA is 0.125 for leaf length and 0.095 for leaf width

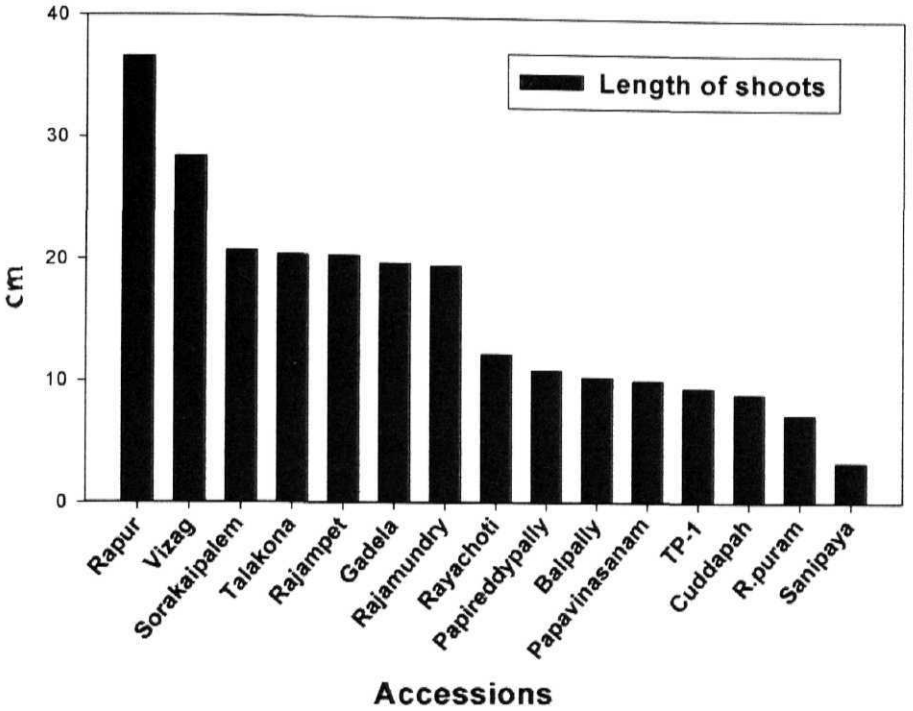

Figure 26. Number of shoots in accessions of *P. santalinus* collected from different locations of AP. SE of treatment means obtained after analysis with one way ANOVA is 1.036

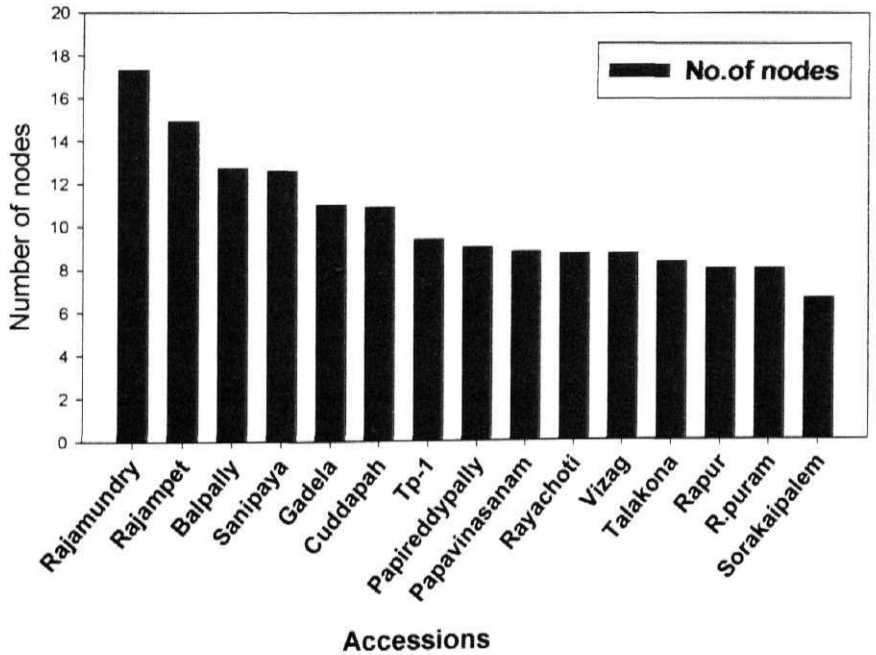

Figure 27. Number of nodes in accessions of *P. santalinus* collected from different locations of AP. SE of treatment means obtained after analysis with one-way ANOVA is 0.667

Pterocarpus santalinus

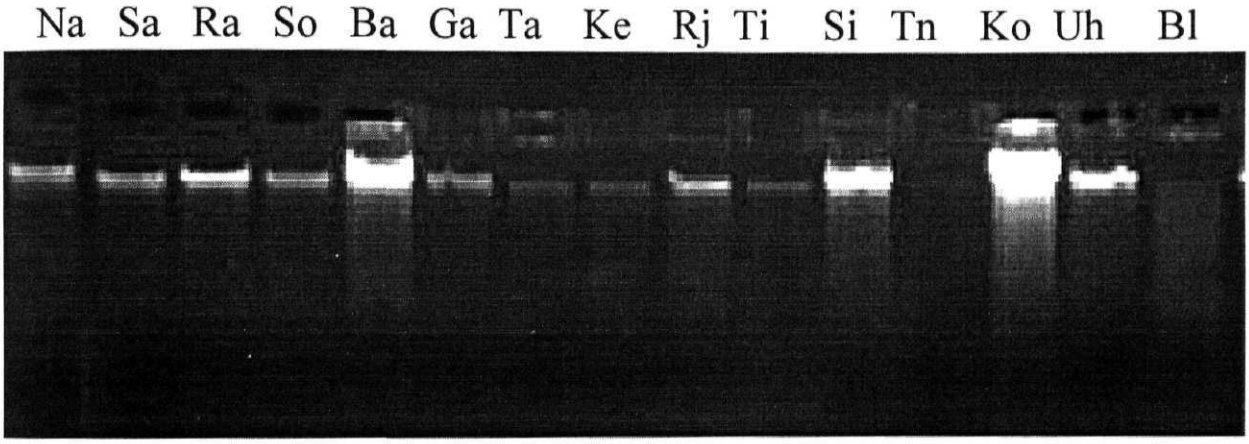

Figure 28: Genomic DNA of various accessions of *P. santalinus* collected from different locations of Andhra Pradesh, Karnataka and Kerala (India). (Legends of the lanes according to the Table 23)

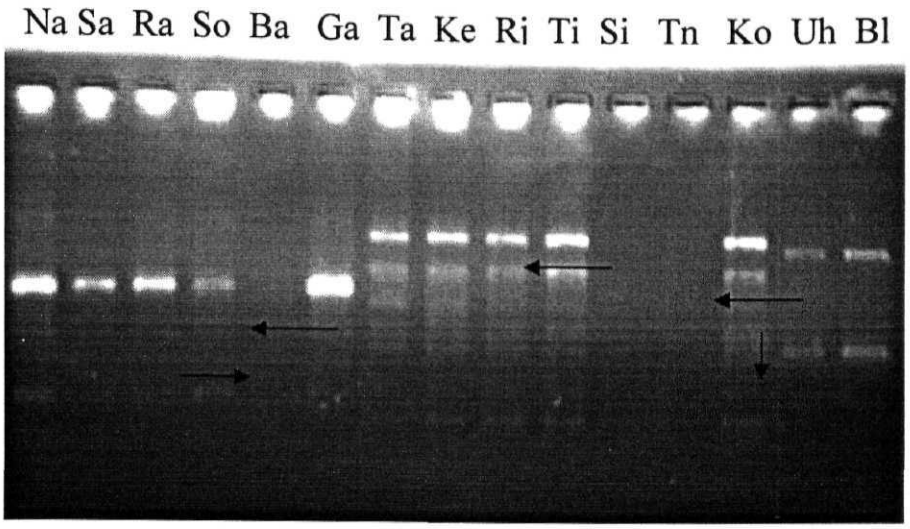

Figure 29:RAPD profile of 15 accessions of individual DNAs each of *P. santalinus* accessions generated on 2% agarose gels using primer OPC-01(5'TTCGAGCCAG3'). Arrows indicate some of the polymorphic bands scored which are putative accessions specific markers. Lanes "Ba, Si and Tn" represents the absence of amplified products.

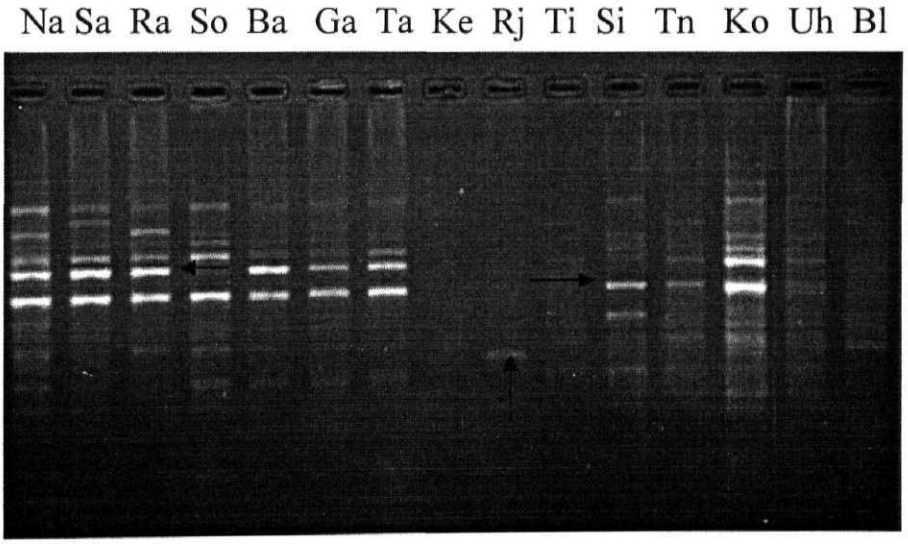

Figure 30:RAPD profile of 15 accessions of individual DNAs each of *P. santalinus* accessions generated on 2% agarose gels using primer OPC-02 (5'GTGAGGCGTC3'). Lane "Ke" indicates the absence of amplification . Lanes "So,Tn and Uh" indicates the accession specific markers and lane "Ko" represents the highest number of markers generated among all the accessions.

Pterocarpus santalinus

Na Sa Ra So Ba Ga Ta Ke Rj Ti Si Tn Ko Uh Bl

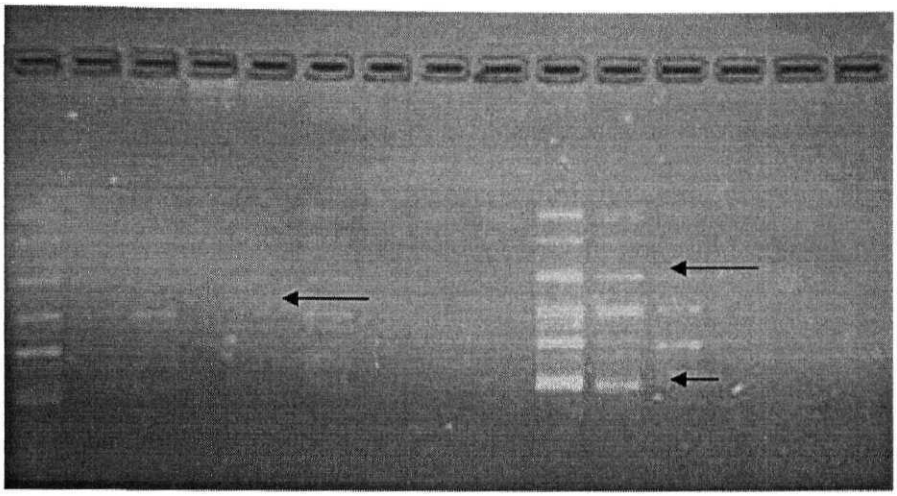

Figure 31:RAPD profile using primer OPC-03 (5'GGGGGTCTTT3'). Arrows indicate the lanes where there is no amplification restricted to some of the accessions, and polymorphic markers generated.

Na Sa Ra So Ba Ga Ta Ke Rj Ti Si Tn Ko Uh Bl

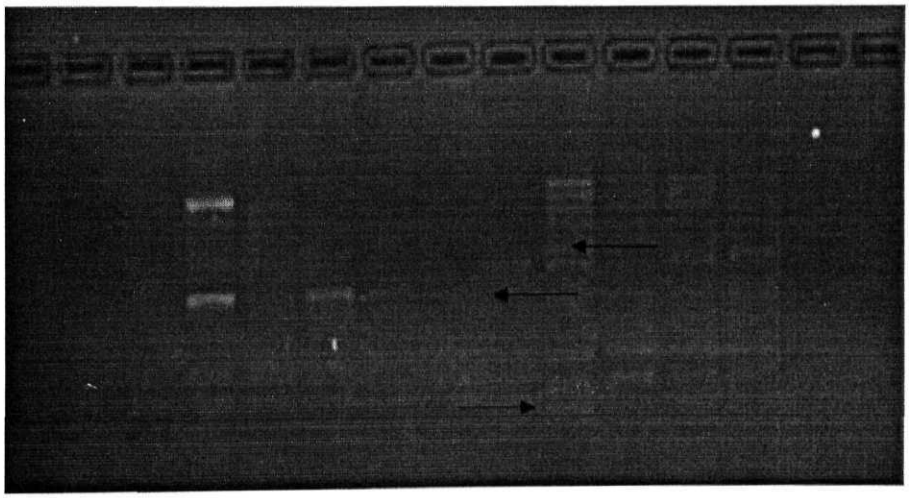

Figure 32:RAPD profile using primer OPC-04 (5'CCGCATCTAC 3'). Arrows indicate the presence of accession specific markers. Lanes "Na, Sa, Ra, Ke, Uh and Bl" represents the absence of amplified products. A unique accession specific markers is generated in lane Ti.

Na Sa Ra So Ba Ga Ta Ke Rj Ti Si Tn Ko Uh Bl

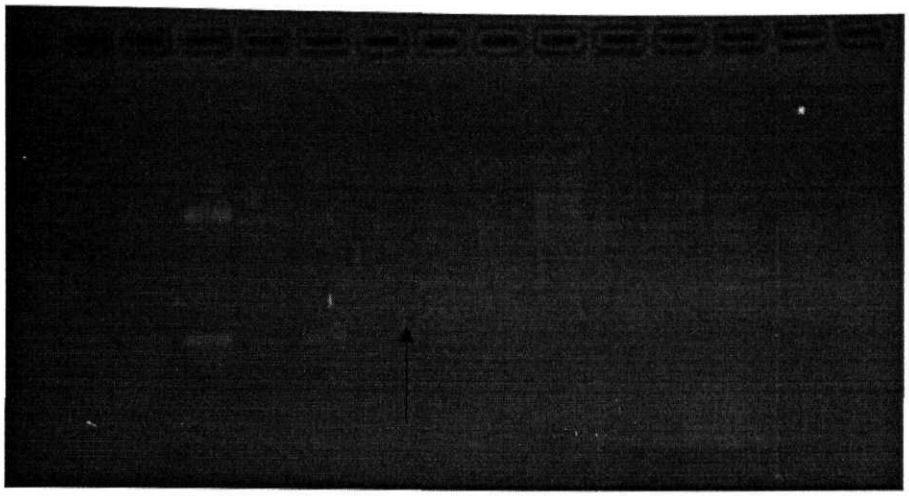

Figure 33: RAPD profile using primer OPC-05 (5' GATGACCGCC3'). Lane "So" represents the presence of two accession specific markers whereas absence of amplified products with other accessions is represented by an arrow.

Na Sa Ra So Ba Ga Ta Ke Rj Ti Si Tn Ko Uh Bl

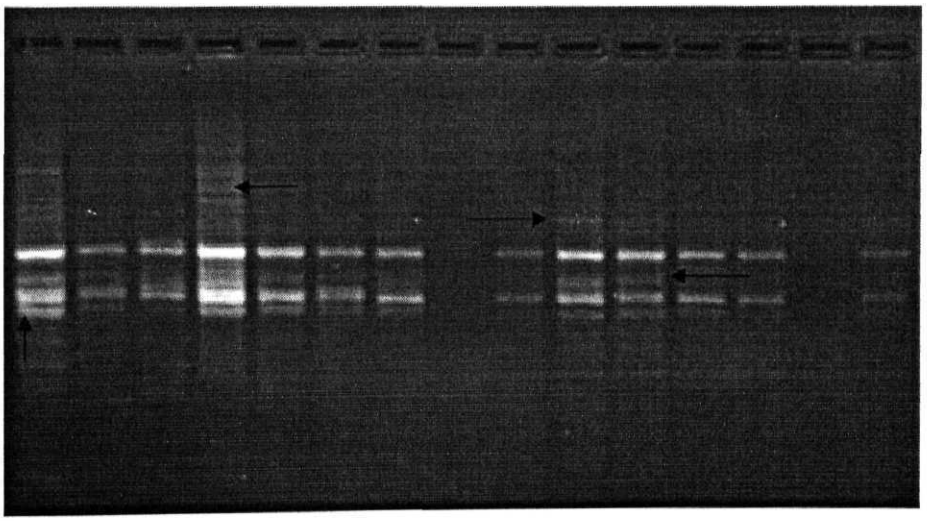

Figure 34: RAPD profile using primer OPC-06 (5' GAACGGACTC 3'). Arrows indicate the presence of putative accession specific markers in lanes "So and Ti" inspite of the generation of highest number of bands whereas in other lanes maximum monomorphism is observed as indicated by arrows.

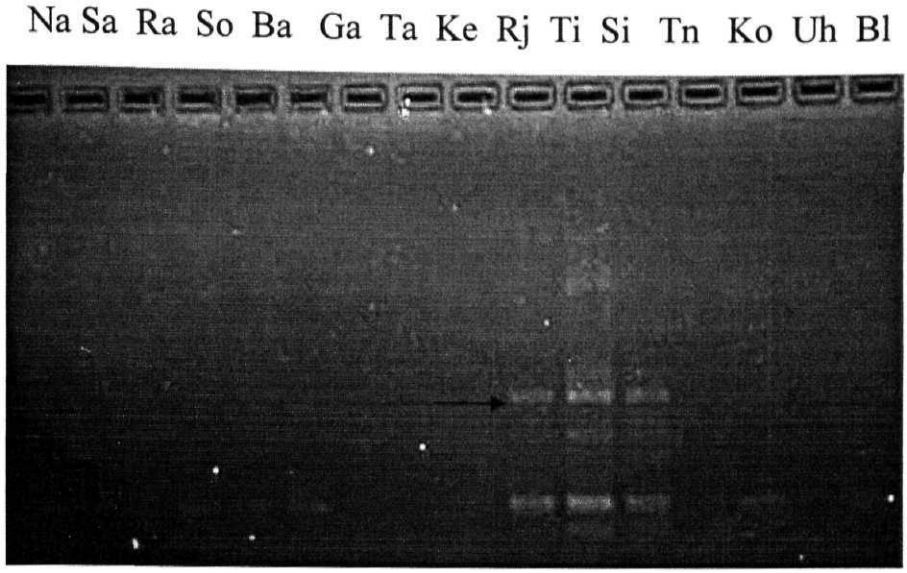

Figure 35: RAPD profile using primer OPC-07 (5' GTCCCGACGA 3'). Arrows represent the presence of polymorphic markers in lanes "Ti, Si and Tn" whereas in other lanes no amplified products were generated which may be due to absence of priming site.

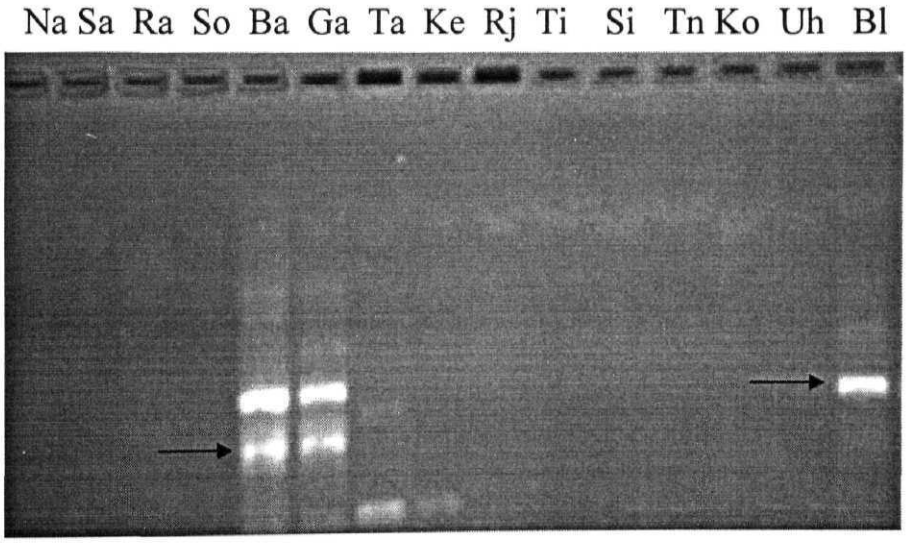

Figure 36:RAPD profile using primer OPC-08 (5' TGGACCGGTG 3'). Arrows represents the presence of putative accessions specific polymorphic markers and higher intensity of the band may indicate the presence of more copy number of the particular sequence. There is no amplification observed with accessions in lanes "Na, Sa, Ra,So , Rj, Ti, Si, Tn, Ko and Uh" respectively.

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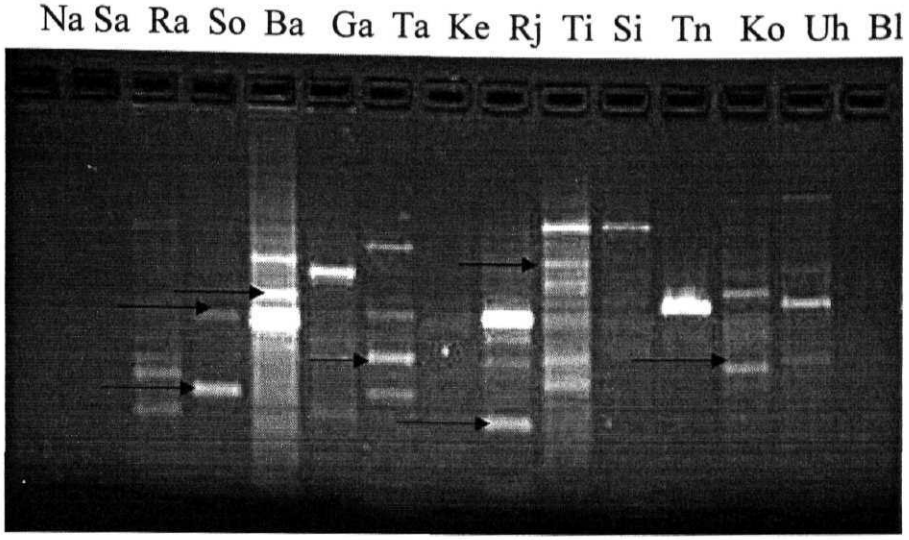


Figure 37: RAPD profile using primer OPC-09 (5' CTCACCGTCC 3'). Putative accessions specific markers which are polymorphic are represented by arrows. Amplification was not observed in accessions " Na and Sa", which may be due to the absence of priming site.

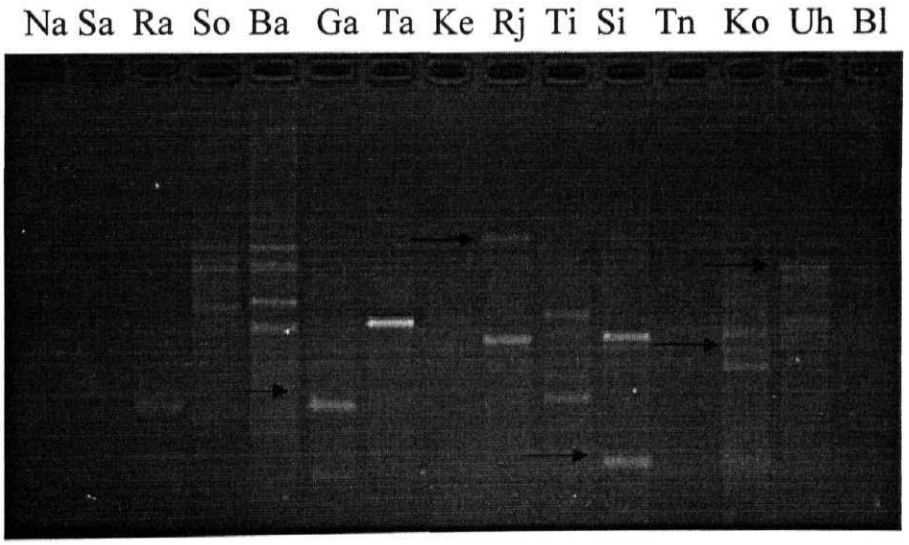


Figure 38: RAPD profile using primer OPC-10 (5' TGTCTGGGTG 3'). In lanes "Rj and Tn" the arrow represents the accessions specific markers whereas lot of polymorphic bands are generated with other accessions. In lanes "Na and Sa" there is no amplification observed

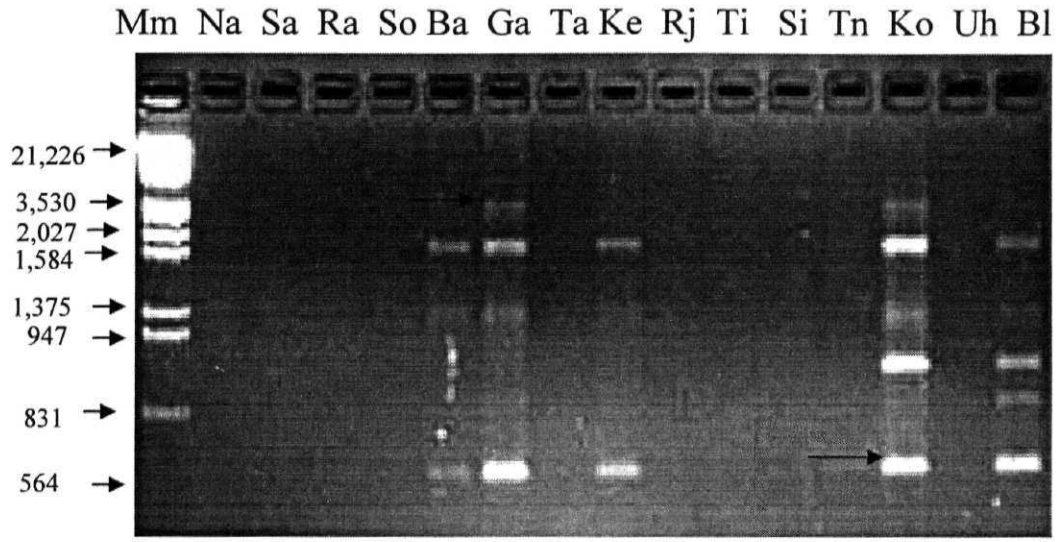


Figure 39 :RAPD profile using primer OPC-13 (5' AAGCCTCGTC 3'). Arrow indicates the presence of specific polymorphic bands in lanes "Ba ,Ga, Ke,Tn,Ko and Bl" whereas there are no amplified products generated with other accessions. Lane Mm represents the Lambda DNA *Hind 111 EcoR1* Double digest molecular markers

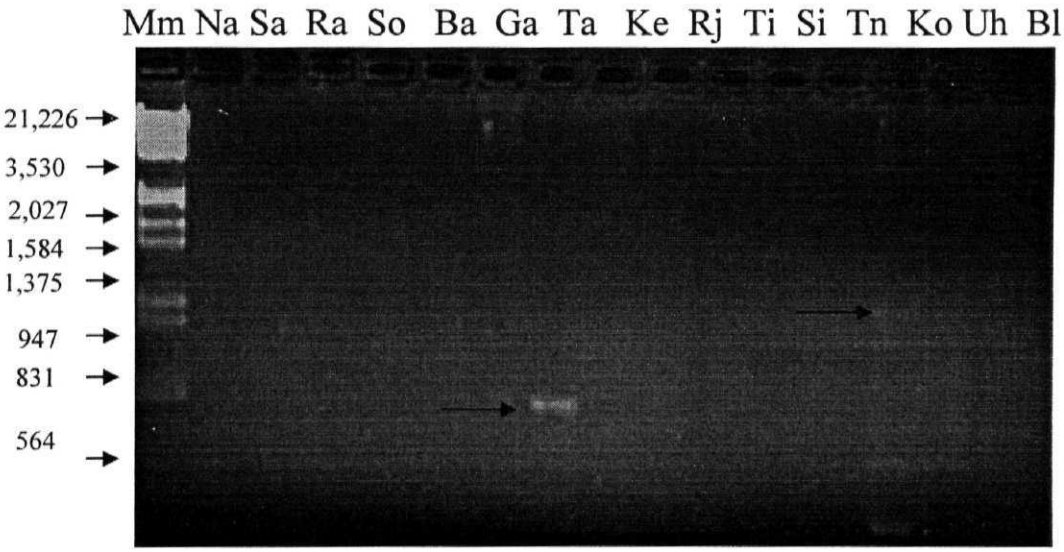


Figure 40: RAPD profile using primer OPC-14 (5' TGCGTGCTTG 3'). No amplification was observed among all the accessions may be due to the absence of primimg sites, except in lane "Ta" where one accession specific putative marker was detected. Lane Mm represents the Lambda DNA *Hind 111 EcoR1* Double digest molecular marker.

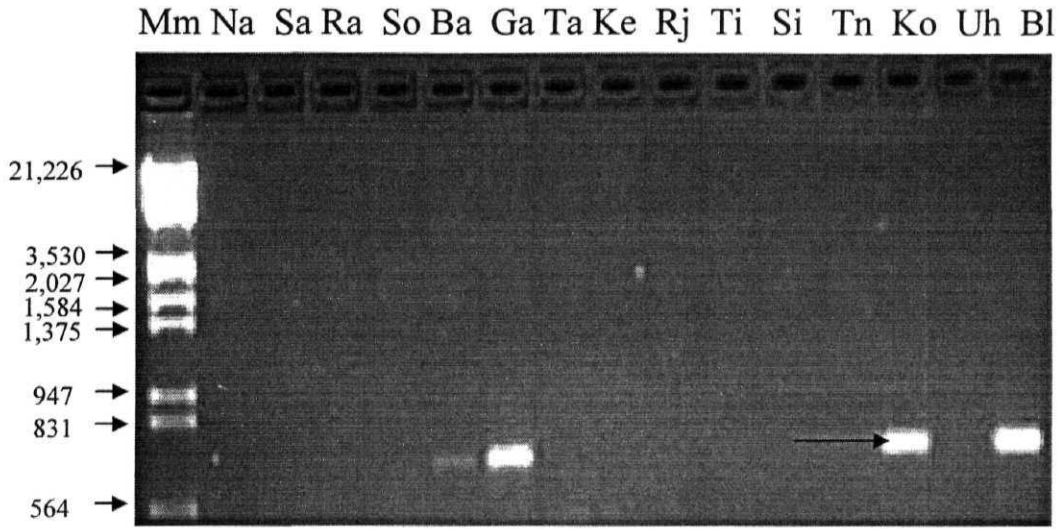


Figure 41: RAPD profile using primer OPC-15 (5' GACGGATCAG 3'). A single accessions specific marker is observed in lanes "Ba, Ga, Ko and Bl" where the increased intensity of the bands indicates the presence of higher copy number of the sequence represented by an arrow Lane Mm represents the Lambda DNA *Hind 111 EcoR1* Double digest

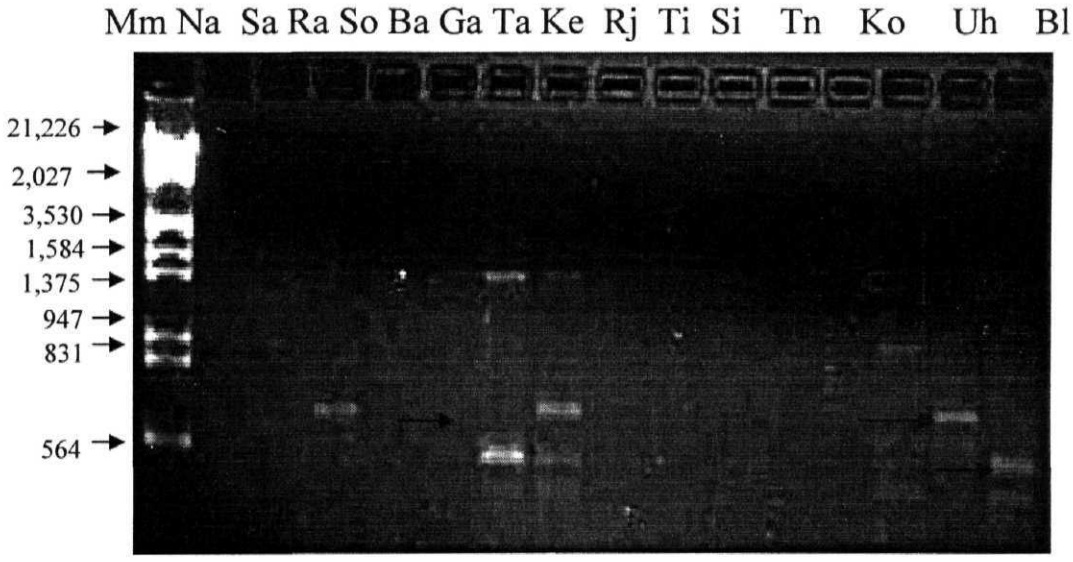


Figure 42: RAPD profile using primer OPC-16 (5' CACACTCCAG3'). Polymorphic bands are generated with most of the accessions indicated by arrows where some of them are accession specific. Lane Mm represents the Lambda DNA *Hind 111 EcoR1* Double digest

Pterocarpus santalinus

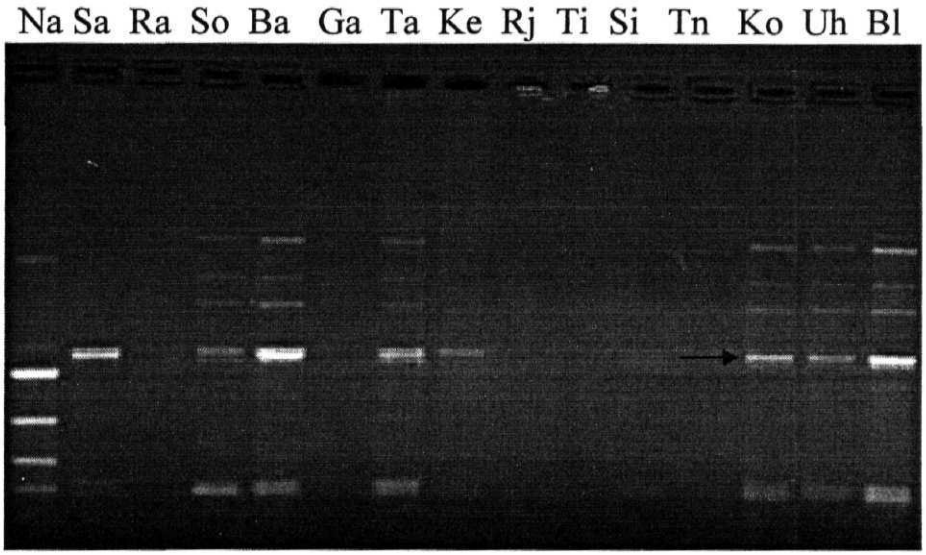


Figure 43: RAPD profile using primer OPA-01 (5'CAGGCCCTTC3'). Arrows indicate the presence of polymorphic markers which are accession specific. In lanes "Ra, Ga, Rj, Ti, Si and Tn" amplified fragments are absent.

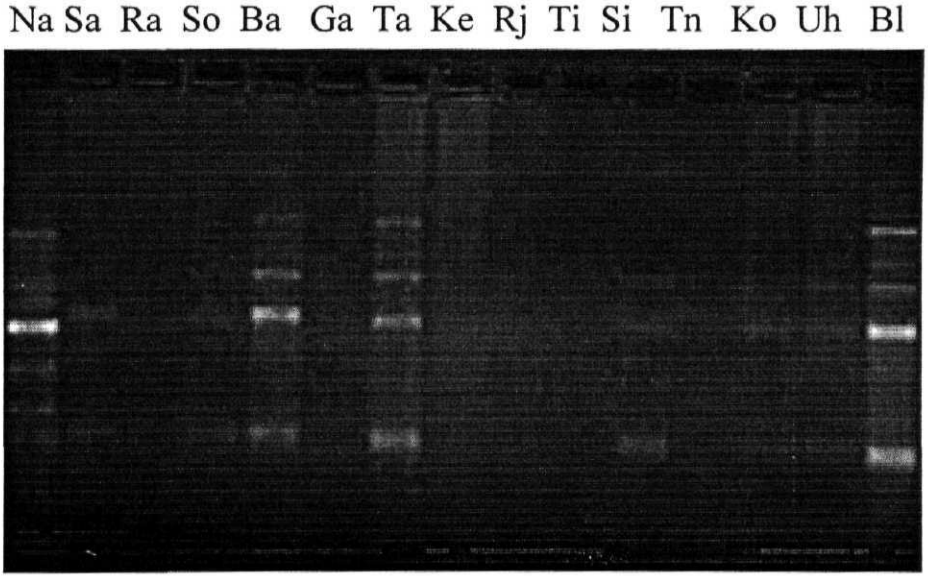


Figure 44: RAPD profile using primer OPA-02 (5'TGCCGAGCTG3'). Amplified products are generated with accessions in lane "Na, Sa, Ta, Si and Bl" and amplification is not observed with remaining accessions in other lanes

Pterocarpus santalinus

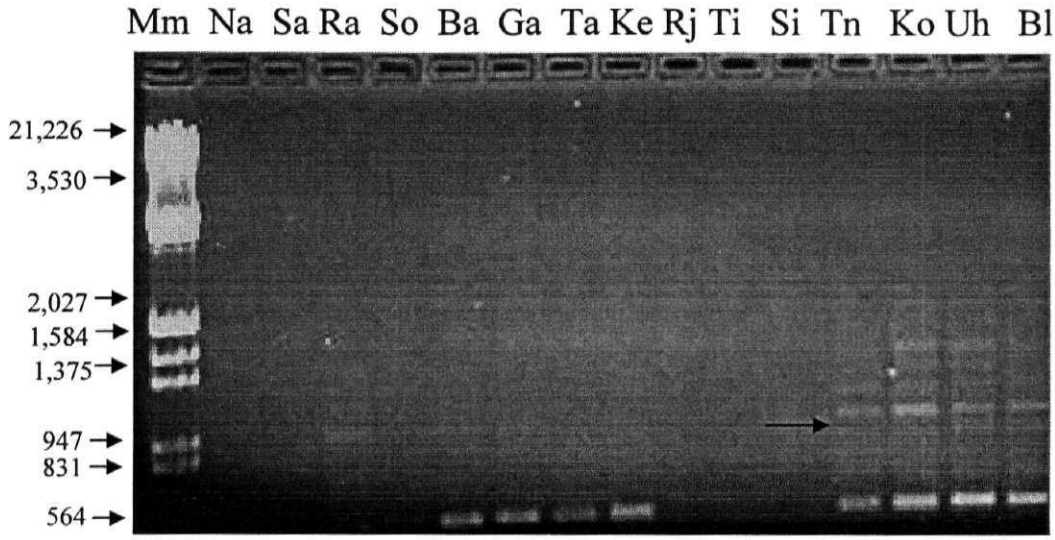


Figure 45: RAPD profile using primer OPC-19 (5' GTTGCCAGCC 3'). Lanes "Tn, Ko, Uh and Bl" represents the presence of polymorphic bands generated which are accessions specific putative markers. There is no amplification in lanes "Rj, Ti and Si" Lane Mm represents the Lambda DNA *Hind 111 EcoR1* Double digest.

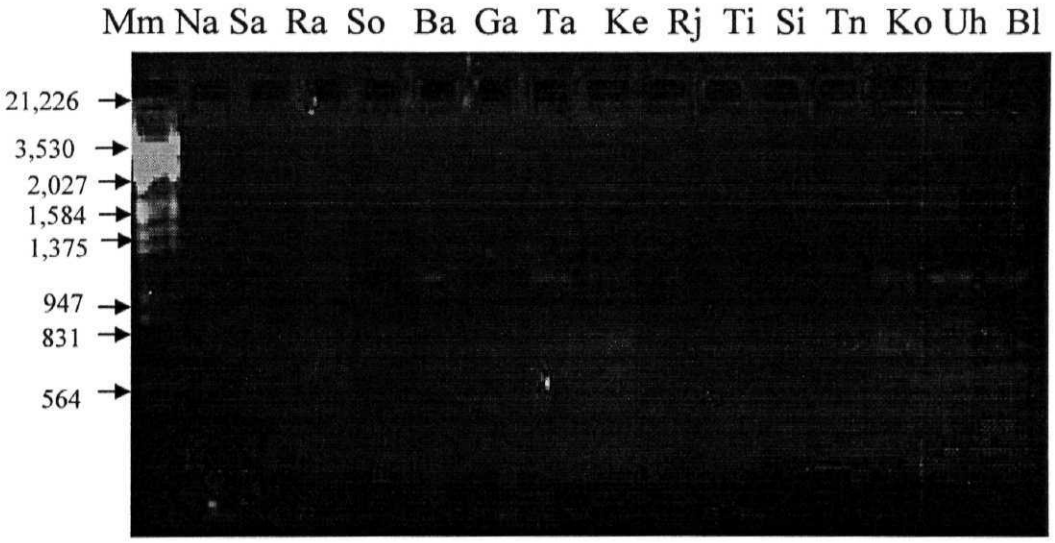


Figure 46: RAPD profile using primer OPC-20 (5'ACTTCGCCAC3'). No amplification observed with any of the accessions, may be due to the absence of priming sites or due to the experimental error at a negligible rate.

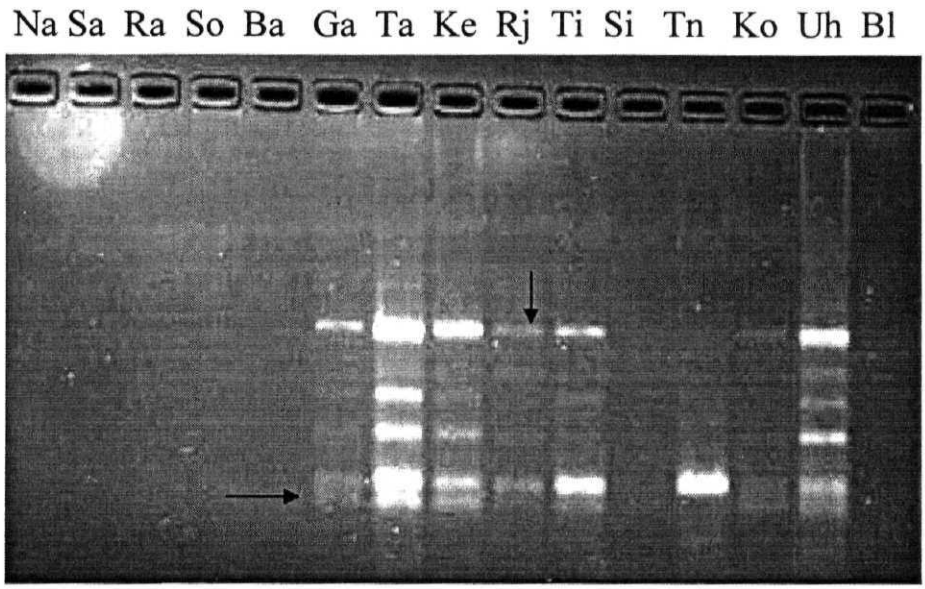


Figure 47: RAPD profile using primer OPA-03 (5' AGTCAGCCAC3'). Arrows indicate the presence of polymorphic bands in few accessions. Lanes "Na, Sa, Ra, So, Ba, Si and Bl" represents the absence of amplified fragments which may indicate the absence of priming sites of the sequence .

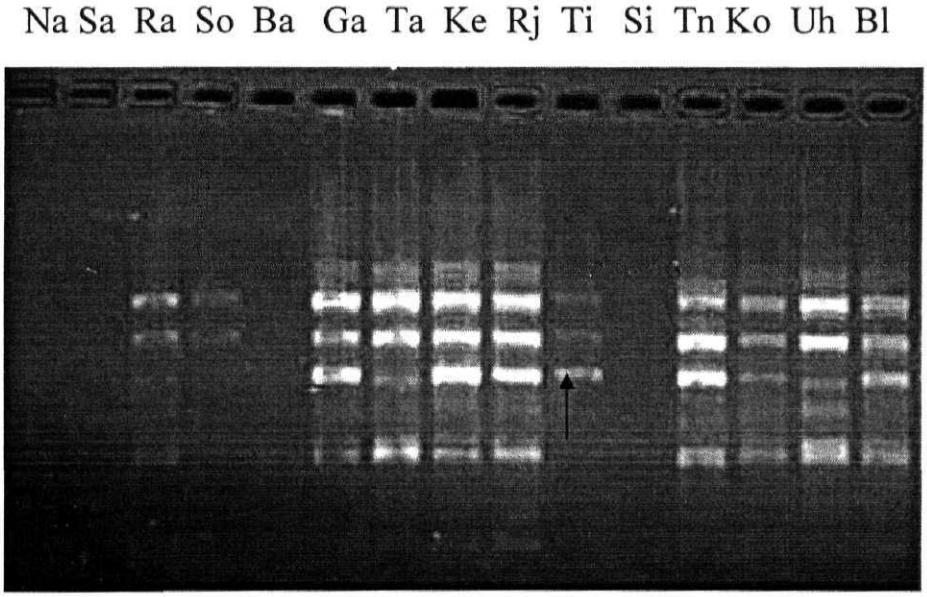


Figure 48: RAPD profile using primer OPA-04 (5'AATCGGGCTG3'). An arrow indicates the presence of polymorphic markers in most of the accessions and absence of amplification is observed in accessions in lanes "Na, Sa, Ba and Si".

Pterocarpus santalinus

Na Sa Ra So Ba Ga Ta Ke Rj Ti Si Tn Ko Uh Bl

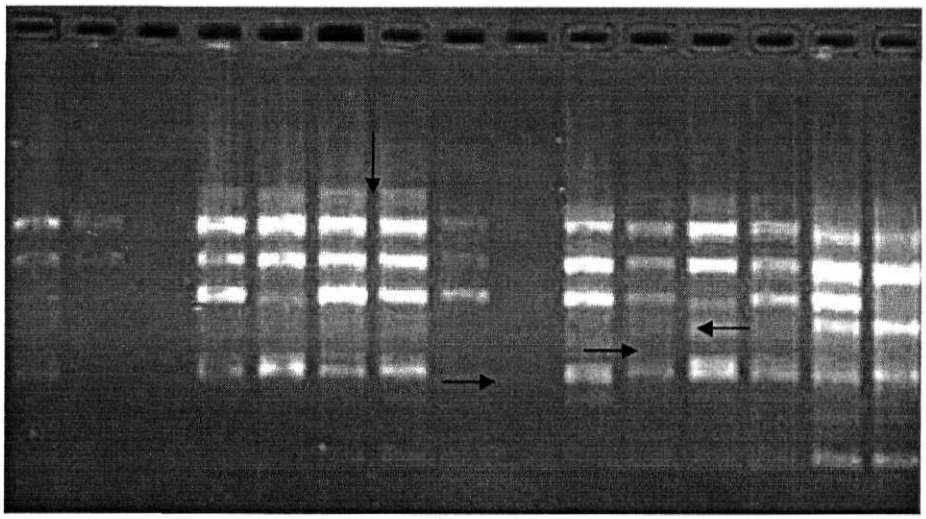
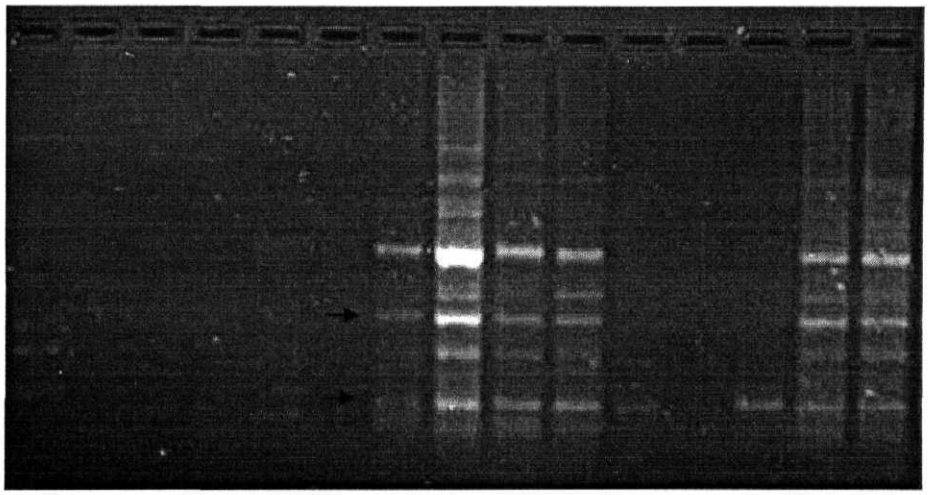


Figure 49: RAPD profile using primer OPA-05 (5'AGGGGTCTTG3').Arrows indicate the presence of unique accession specific markers . Lanes "Ra and Rj" indicate the absence of amplification products

Na Sa Ra So Ba Ga Ta Ke Rj Ti Si Tn Ko Uh Bl



OPA-6

Figure 50: RAPD profile using primer OPA-06 (5' GGTCCCTGAC 3').Presence of polymorphic bands is observed in few accessions represented in the gel with arrows and amplification is not observed with some of the accessions in lanes "Na, Sa, Ra, So, Ba, Ga, Si, Tn and Ko".

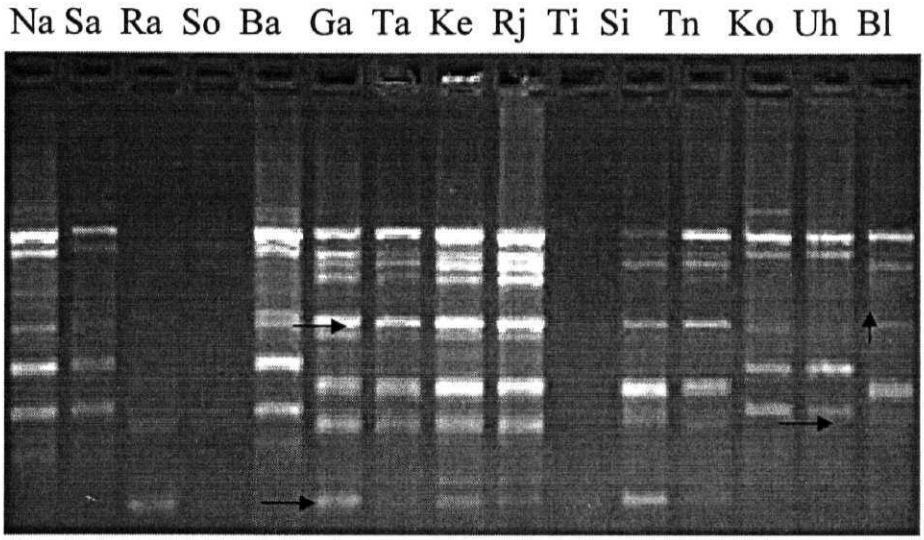


Figure 51: RAPD profile using primer OPA-18 (5'AGGTGACCGT3'). Arrows indicate the presence of polymorphic markers, very faint amplification is observed with accessions in lane "Ra, So and Ti" Maximum number of polymorphic bands are generated with this primer

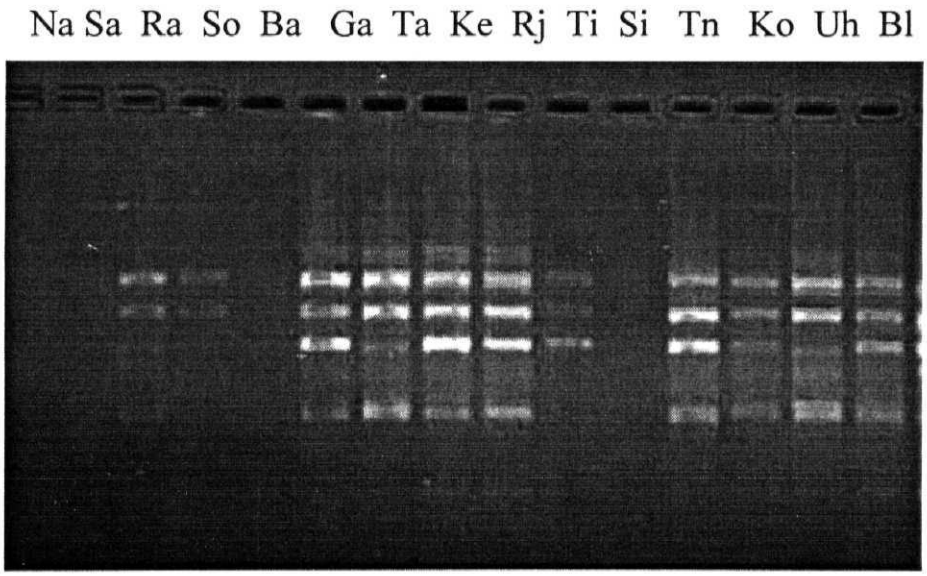


Figure 52: RAPD profile using primer OPA-19 (5' CAAACGTCGG 3'). Presence of polymorphic bands is observed with most of the accessions represented in the gel. There is no amplification observed in some of the accessions represented in lanes "Na, Sa, Ba and Si" may be due to the absence of priming sites

(Table 22). The number of scorable polymorphic markers generated for accessions are shown in tables 23, 24. Levels of genetic diversity within accessions i.e., the genetic distance ranged from 0.14 to 0.76. The minimum genetic distance of 0.14, exhibited between the accessions collected from Narsingapuram (Chittoor) and Raichoti (Kadapa) whereas the accessions that exhibited a maximum genetic distance exhibited between the accessions from Narsingapuram and Raichoti whereas the accessions that exhibited a maximum genetic distance of 0.76 belongs to plants collected from Kerala and Raichoti. The mean value of genetic distance among all the accessions is 3.168. The values of genetic distances calculated in plant species which are grouped together in cluster analysis irrespective of the geographical distances are from the accessions collected from Kerala and Hyderabad, exhibited a genetic distance of 0.55, the accessions collected from Kodur (Kadapa) and Raichoti exhibited a genetic distance of 0.76, the accessions grouped together i.e., collected from Gadela (Kadapa) and Talakona (Chittoor) showed a genetic distance of 0.72, those collected from Ramachandrapuram (Chittoor, A.P) and Siddhavotum (Kadapa) exhibited a genetic distance of 0.59 and those collected from Narsingapuram (Chittoor) and Sanipaya (Kadapa) exhibited a genetic distance of 0.57 (figures 53 and 54). The number of accession specific unique markers observed belonging to particular location, the primer used for screening with less amount of monomorphism and some of the primers did not exhibit any amplification (table 25).

Table 22: Information regarding primer code, sequence, percentage polymorphism among 15 accessions of *P. santalinus.* collected from different locations

Primer Code	Primer sequence 5' – 3'	TB	PB	% polymorphism
OPA-01	CAGGCCCTTC	8	8	100
OPA-02	TGCCGAGCTG	8	8	100
OPA-03	AGTCAGCCAC	9	9	100
OPA-04	AATCGGGCTG	8	8	100
OPA-05	AGGGGTCTTG	8	8	100
OPA-06	GGTCCCTGAC	13	13	100
OPA-18	AGGTGACCGT	15	15	100
OPA-19	CAAACGTCGG	12	12	100
OPA-20	GTTGCGATCC	6	6	100
OPC-1	TTCGAGCCAG	8	8	100
OPC-2	GTGAGGCGTC	9	9	100
OPC-3	GGGGGTCTTT	14	14	100
OPC-4	CCGCATCTAC	6	6	100
OPC-5	GATGACCGCC	7	7	100
OPC-6	GAACGGACTC	13	13	100
OPC-7	GTCCCGACGA	3	3	100
OPC-8	TGGACCGGTG	5	5	100
OPC-9	CTCACCGTCC	10	10	100
OPC-10	TGTCTGGGTG	6	6	100
OPC-11	AAAGCTGCGG	15	15	100
OPC-13	AAGCCTCGTC	6	6	100
OPC-14	TGCGTGCTTG	6	6	100
OPC-15	GACGGATCAG	3	3	100
OPC-16	CACACTCCAG	9	9	100
OPC-18	TGAGTGGGTG	4	4	100
OPC-20	ACTTCGCCAC	6	6	100

Table 23: Accession wise polymorhic bands in both *P.santalinus7* accessions with both A and C series of Operon primers

Primer	1		2		3		4		5		6		7	
Code	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB
OPA1	5	5	1	1	2	2	4	4	2	2	1	1	2	2
OPA2	0	0	2	2	5	5	0	0	2	2	6	6	6	6
OPA3	1	1	2	2	0	0	6	6	6	6	0	0	6	6
OPA4	0	0	0	0	2	2	2	2	0	0	5	5	5	5
OPA5	0	0	0	0	3	3	3	3	0	0	5	5	5	5
OPA6	0	0	0	0	0	0	0	0	0	0	5	5	13	13
OPA18	8	8	3	3	2	2	3	3	9	9	9	9	9	9
OPA19	6	6	6	6	2	2	1	1	7	7	9	9	9	9
OPA20	4	4	2	2	0	0	2	2	3	3	1	1	1	1
OPC1	2	2	2	2	3	3	3	3	0	0	4	4	6	6
OPC2	5	5	1	1	1	1	1	1	4	4	3	3	2	2
OPC3	0	0	0	0	6	6	6	6	10	10	11	11	11	11
OPC4	0	0	0	0	6	6	6	6	5	5	5	5	4	4
OPC5	0	0	0	0	0	0	4	4	0	0	1	1	0	0
OPC6	13	13	6	6	5	5	11	11	6	6	4	4	4	4
OPC7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPC8	0	0	0	0	0	0	2	2	4	4	3	3	1	1
OPC9	0	0	0	0	3	3	2	2	6	6	2	2	3	3
OPC10	6	6	2	2	4	4	0	0	0	0	4	4	0	0
OPC11	14	14	6	6	6	6	5	5	5	5	6	6	4	4
OPC13	0	0	0	0	0	0	0	0	4	4	4	4	0	0
OPC14	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPC15	0	0	0	0	0	0	0	0	2	2	2	2	1	1
OPC16	0	0	0	0	1	1	0	0	0	0	3	3	4	4
OPC19	0	0	0	0	0	0	1	1	1	1	1	1	1	1
OPC20	0	0	0	0	2	2	0	0	2	2	0	0	2	2

Table 24: Accession wise polymorhic bands in both *P.santalinus 8* accessions with both A and C series of Operon primers

Primer	8		9		10		11		12		13		14		15	
Code	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB
OPA1	1	1	0	0	0	0	1	1	2	2	4	4	4	4	4	4
OPA2	7	7	7	7	1	1	2	2	8	8	7	7	7	7	7	7
OPA3	1	1	0	0	0	0	1	1	0	0	7	7	7	7	9	9
OPA4	5	5	5	5	3	3	0	0	5	5	6	6	6	6	6	6
OPA5	5	5	5	5	3	3	1	1	5	5	5	5	7	7	7	7
OPA6	8	8	9	9	1	1	0	0	1	1	6	6	6	6	6	6
OPA18	9	9	9	9	3	3	10	10	8	8	5	5	4	4	7	7
OPA19	9	9	9	9	0	0	8	8	9	9	7	7	6	6	7	7
OPA20	1	1	0	0	0	0	3	3	0	0	5	5	5	5	6	6
OPC1	6	6	5	5	5	5	0	0	0	0	4	4	2	2	2	2
OPC2	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0
OPC3	8	8	1	1	0	0	1	1	8	8	9	9	9	9	0	0
OPC4	5	5	6	6	6	6	6	6	0	0	0	0	0	0	0	0
OPC5	0	0	0	0	7	7	1	1	1	1	1	1	1	1	0	0
OPC6	2	2	1	1	6	6	7	7	6	6	4	4	2	2	2	2
OPC7	0	0	3	3	3	3	3	3	0	0	2	2	0	0	0	0
OPC8	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	1
OPC9	2	2	4	4	4	4	2	2	2	2	3	3	1	1	3	3
OPC10	0	0	0	0	6	6	6	6	3	3	0	0	0	0	0	0
OPC11	0	0	1	1	1	1	6	6	6	6	11	11	8	8	7	7
OPC13	2	2	3	3	0	0	0	0	0	0	4	4	2	2	3	3
OPC14	2	2	0	0	0	0	0	0	0	0	6	6	0	0	2	2
OPC15	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
OPC16	0	0	1	1	0	0	0	0	0	0	1	1	8	8	2	2
OPC19	1	1	1	1	0	0	0	0	0	0	6	6	6	6	6	6
OPC20	1	1	1	1	0	0	0	0	1	1	2	2	3	3	2	2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
PSNR	1.00														
PSSA	0.57	1.00													
PSRM	0.38	0.39	1.00												
PSSM	0.39	0.39	0.59	1.00											
PSBE	0.38	0.48	0.44	0.56	1.00										
PSGA	0.33	0.33	0.55	0.46	0.54	1.00									
PSTN	0.22	0.30	0.42	0.47	0.55	0.72	1.00								
PSKA	0.20	0.33	0.38	0.42	0.46	0.58	0.74	1.00							
PSRY	0.14	0.22	0.34	0.30	0.35	0.62	0.71	0.76	1.00						
PSTI	0.21	0.17	0.39	0.36	0.19	0.42	0.48	0.41	0.55	1.00					
PSSM	0.42	0.46	0.48	0.45	0.47	0.49	0.46	0.50	0.52	0.49	1.00				
PSTA	0.35	0.40	0.54	0.44	0.47	0.66	0.61	0.58	0.62	0.39	0.62	1.00			
PSKR	0.34	0.34	0.40	0.38	0.41	0.55	0.52	0.50	0.42	0.27	0.41	0.60	1.00		
PSHC	0.28	0.33	0.39	0.36	0.43	0.55	0.54	0.43	0.44	0.24	0.34	0.55	0.72	1.00	
PSBI	0.28	0.32	0.32	0.38	0.43	0.50	0.52	0.62	0.45	0.17	0.37	0.53	0.67	0.62	1.00

Figure 53: Similarity matrix of *P. santalinus* generated from Dice estimate similarity based on the number of shared fragments

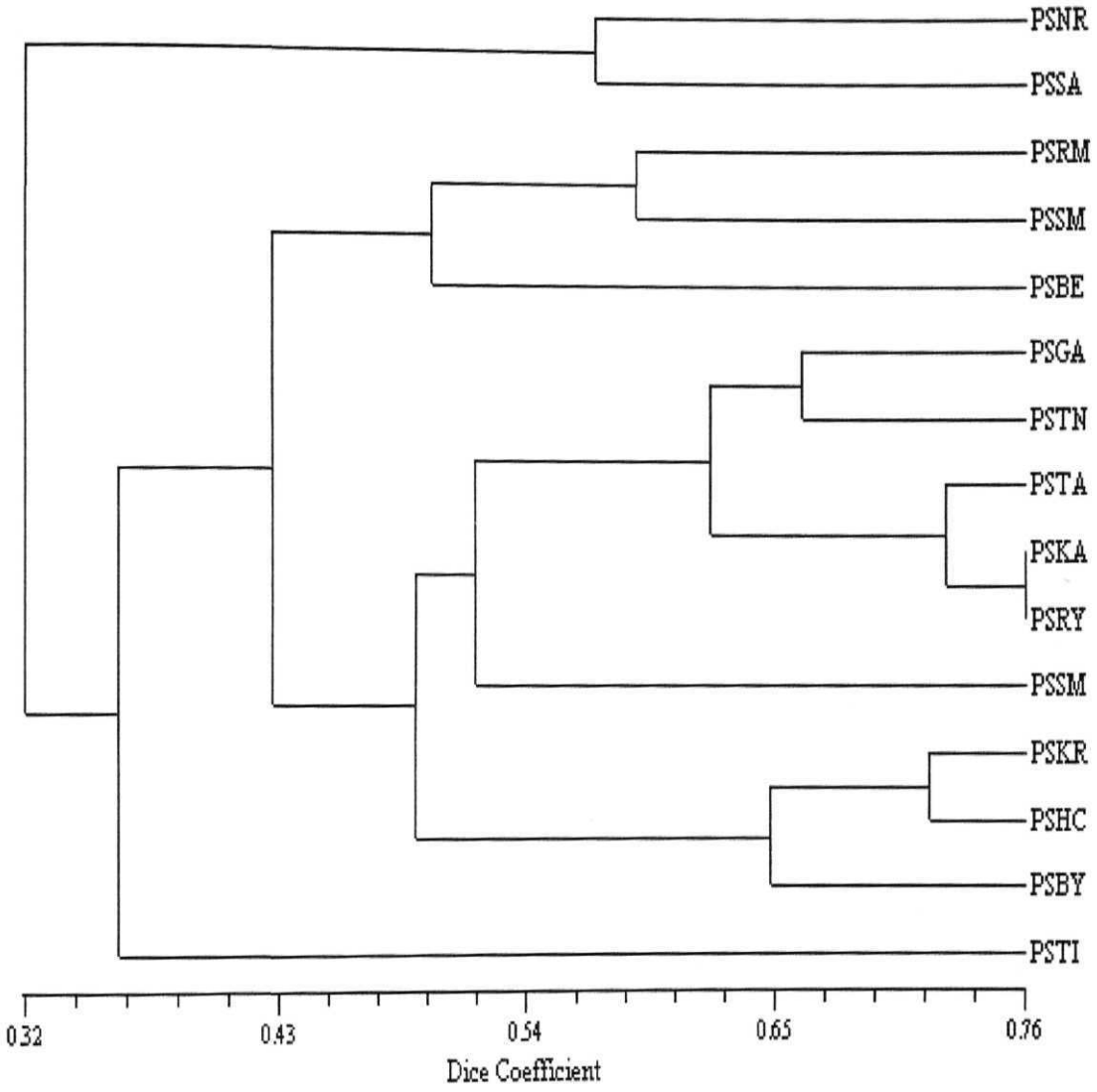


Figure 54: Cluster analysis of 15 accessions of *P. santalinus* using Dice similarity coefficient

Table 25: Depicts the accessions of *P. santalinus* that showed unique markers and where there is no amplification.

No. of unique bands/No mplification	Primer code	Location from where collected
1	OPC-1	Talakona
1	OPC-1	Kodur
No amplification	OPC-1	Balpally, Siddhavotum and Talakona
1	OPC-2	Sorakaipalem
1	OPC-2	Siddhavotum
1	OPC-2	Rajampet
No amplification	OPC-2	Kerala
1	OPC-3	Gadela
1	OPC-3	Kodur
No amplification	OPC-3	Sorakaipalem, Bangalore, UH campus, Balpally, Talakona, Rajampet and Kerala.
1	OPC4	Tirupathi
No amplification	OPC-4	Narsingapuram, sanipaya, UH campus, Balpally
2	OPC-5	Sorakaipalem
No amplification	OPC-5	All other accessions
1	OPC-6	Sorakaipalem
No amplification	OPC-6	Kerala and UH campus
1	OPC-6	Tirupathi
3	OPC-7	Tirupathi, Siddhavotum and Talakona
No amplification	OPC-7	Narsingapuram, Sanipaya, Rajampet, Sorakaipalem, Balpally, Gadela, Talakona, Kerala, Kodur, UH campus, Balpally
2	OPC-8	Balpally,Gadela, Bangalore
2	OPC-8	Talakona and Kerala
No amplification	OPC-8	Narsingapuram, Sanipaya, Rajampet, Sorakaipalem, Rajampet, Tirupathi, Siddhavotum, Kodur and UH campus.
2	OPC-9	Tirupathi, Siddhavotum
2	OPC-9	Gadela, Tirupathi
2	OPC-9	Bangalore, Talakona
1	OPC-9	Talakona
2	OPC-9	Rajampet, Gadela
1	OPC-9	Rajampet

No. of unique bands/No mplification	Primer code	Location from where collected
No amplification	OPC-9	Narsingapuram, Sanipaya, Balpally
1	OPC-10	Siddhavotum
No amplification	OPC-10	Narsingapuram, Sanipaya, rajampet, Kerala, Talakona, balpally
No amplification	OPC-13	Narsingapuram, Sanipaya, rajampet,Sorakaipalem, Rajampet, Tirupathi and UH campus.
No amplification	OPC-14	Except in Talakona, amplification not seen in other locations
2	OPC-15	Kodur and Balpally
2	OPC-15	Balpallly and Gadela
No amplification	OPC-15	No amplification in remaining accessions
2	OPC-16	Gadela, Talakona
2	OPC-16	Rajampet, Talakona
3	OPC-16	Rajampet, Talakona and UH campus
3	OPC-16	Gadela, Talakona and Balpally
No amplification	OPC-19	Narsingapuram, Sanipaya, Sorakaipalem, Rajampet, Tirupathi and Siddhavotum
No amplification	OPA-1	Rajampet, gadela, Rajamundry, Tirupathi, Siddhavotum and Talakona
No amplication	OPA-2	Sanipaya, Rajampet, Sorakaipalem, Gadela, Kerala, Rajampet, Tirupathi, Talakona, Kodur and UH campus
No amplification	OPA-3	Narsingapuram, Sanipaya, Rajampet, Sorakaipalem, Balpally, Siddhavotum and Banglore
No amplication	OPA-4	Narsingapuram, Sanipaya, Balpally and Siddhavotum
1	OPA-5	Rajampet and Rajamundry
1	OPA-5	Talakona
1	OPA-5	UH campus
No amplification	OPA -5	Narsingapuram, Sanipaya, Rajampet, Sorakaipalem, Balpally, Gadela, Talakona
2	OPA-18	Rajampet, Sorakaipalem
6	OPA-18	Kodur and UH campus
No amplification	OPA-18	Tirupathi
No amplification	OPA-19	Narsingapuram, Sanipaya, Bangalore, Siddhavotum

The percentage of polymorphism observed for each accession with all the primers is calculated which is 100% for all the accessions individually. The primers with maximum number of polymorphic bands are OPA-18 and OPC-11 with 15 bands and the primers with minimum number of polymorphic bands are OPC-15 and OPC-7 with 3 bands and hence the number of bands generated with all the primers ranged in between 3 and 15.The GC % of all the primers was ranged from 60-70. The amplified fragments ranged from 300-3,500 bp. The number of loci and polymorphic loci varies widely between primers and populations. The highest within genetic variation was detected in the accession collected from of Tirupathi (Chittoor, A.P) which was completely grouped as a separate entity in the cluster diagram.

In case of *P. santalinus* (54), which are accession specific, number of unique markers is generated as mentioned in table 25. Unique markers (23) are also accession specific, were identified in *R. serpentina* and in 4 unique markers, which are accession specific, were identified in *R. tetraphylla* which may be useful for carrying out further analysis by cloning, sequencing and homology studies.

RAPD analysis in R. serpentina and *R. tetraphylla*

Spectrophotometric measurements of DNA at 260 nm and 280 nm revealed higher concentration of DNA which was diluted to 50 ng/µl for further RAPD analysis for *R. serpentina* and *R. tetraphylla*. Genomic DNA was isolated from various accessions collected from different locations (figures 55 and 56). The latitude and longitude of the accessions collected for both *R. serpentina* and *R. tetraphylla* are shown in the tables 26 and 27.

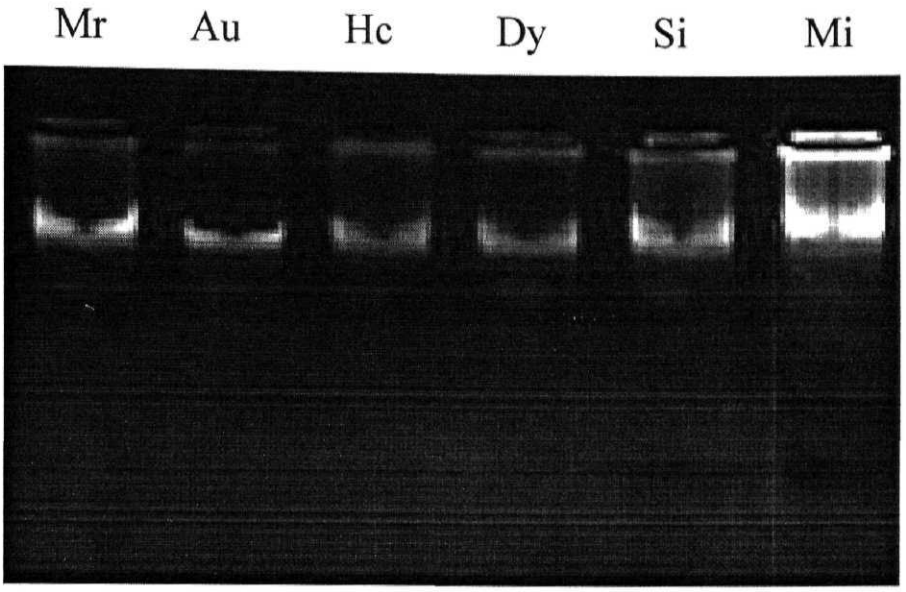


Figure 55: Genomic DNA of six accessions of *R. serpentina* collected from different locations of Andhra Pradesh, India . Details of accessions are mentioned in Table 26.

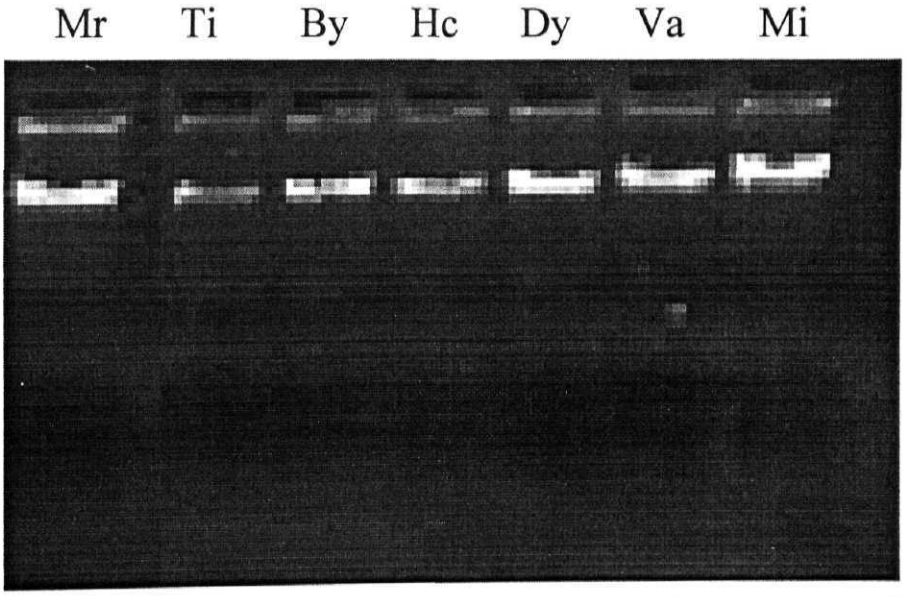


Figure 56: Genomic DNA of six accessions of *R. tetraphylla* collected from different locations of Andhra Pradesh, India. Details of accessions are mentioned in Table 27.

Table 26: *R. serpentina* germplasm accessions collected from different locations of A.P used for molecular diversity analysis.

Acc.No	Location	District	Latitude	Longitude
RSMR	Mallur	Warangal	18 ^{0}15'	80 ^{0}31'
RSAU	Araku	Vizag	19^0 08'	19^0 08'
RSHC	Hyderabad	Hyderabad	27^0 20'	78^0 20'
RSDY	Dulapally	Medak	27 ^{0}20'	78 ^{0}20'
RSSI	Sukumamidi	Khammam	17^0 45'	81 ^{0}50'
RSMI	Maredumilli	E.Godavari	19^0 35'	80 ^{0}48'

Table 27: *R. tetraphylla* germplasm accessions collected from different locations of A.P used for molecular diversity analysis.

Acc.No	Location	District	Latitude	Longitude
RTMR	Mallur	Warangal	18 ^{0}15'	80 ^{0}31'
RTTI	Tirupathi	Chittoor	13 ^{0}10'	77 ^{0}20'
RTBY	Balpally	Kadapa	14 ^{0}25'	79 ^{0}18'
RTHC	Hyderabad	Hyderabad	27^0 20'	78^0 20'
RTDY	Dulapally	Ranga reddy	27 ^{0}20'	78 ^{0}20'
RTVA	Vijayawada	Krishna	15 ^{0}09'	75^0 14'
RTMI	Maredumilli	East Godavari	19^0 35'	80 ^{0}48'

6.7. RAPD analysis of *R. serpentina*

A total of 40 primers when screened from OPA and OPC series for appropriate amplification and pattern formation out of which all them were selected for tests of the repeatability of the method. Analysis of 6 accessions of *R. serpentina* revealed 69.3 i.e., ~ 70% of polymorphism. Screening of the entire set of samples was repeated thrice to assess repeatability of the RAPD profiles, and identical RAPD patterns were obtained. The molecular diversity between six accessions of *R. serpentina* when compared, showed lot of polymorphism with many primers and monomorphism with few primers except in the accession collected from Sukumamidi (Khammam), which was found to be a variant when compared to the other accessions. The number of scorable polymorphic markers generated is 263 out of 379 total markers generated (table 28, figures 57-96). The polymorphism exhibited by accessions in *R. serpentina* is shown in table 29 Unique markers (23) are represented in table 30. Levels of genetic diversity within accessions i.e., the genetic distance ranged from 0.596 to 0.928. The minimum genetic distance of 0.596 is exhibited between the accessions collected from Sukumamidi (Khammam) and Maredumilli (East Godavari), whereas the accessions that exhibited a maximum genetic distance of 0.928 belongs to plants collected from Dulapally (Medak) and Hyderabad. The mean value of genetic distance among the accessions is 2.167. The plant species (figure 97) which are grouped together in Cluster analysis irrespective of the geographical distances are the accessions collected from Araku (Vishakapatnam) and Hyderabad, showed a genetic distance of 0.905 which are grouped together in the dendrogram (figure 98).

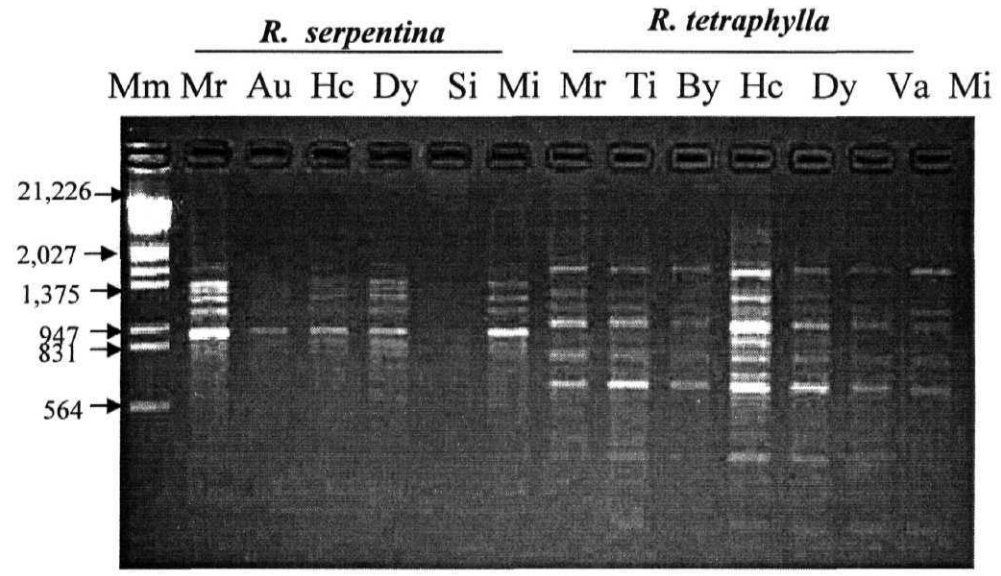


Figure 57: RAPD profile for the genetic difference and similarity of six accessions of *R. serpentina* and seven accessions of *R. tetraphylla* on 2% agarose gel using primer OPA-01 (5' CAGGCCCTTC3'). Monomorphism is observed with all the accessions of *R. serpentina* except in lane Si, where there are no amplified products generated and less polymorphism is observed among the accessions of *R. tetraphylla* but when both the species are compared there are highly polymorphic markers generated. Lane Mm represents molecular marker i.e., Lambda DNA *Hind 111* and *EcoR1* Double digest.

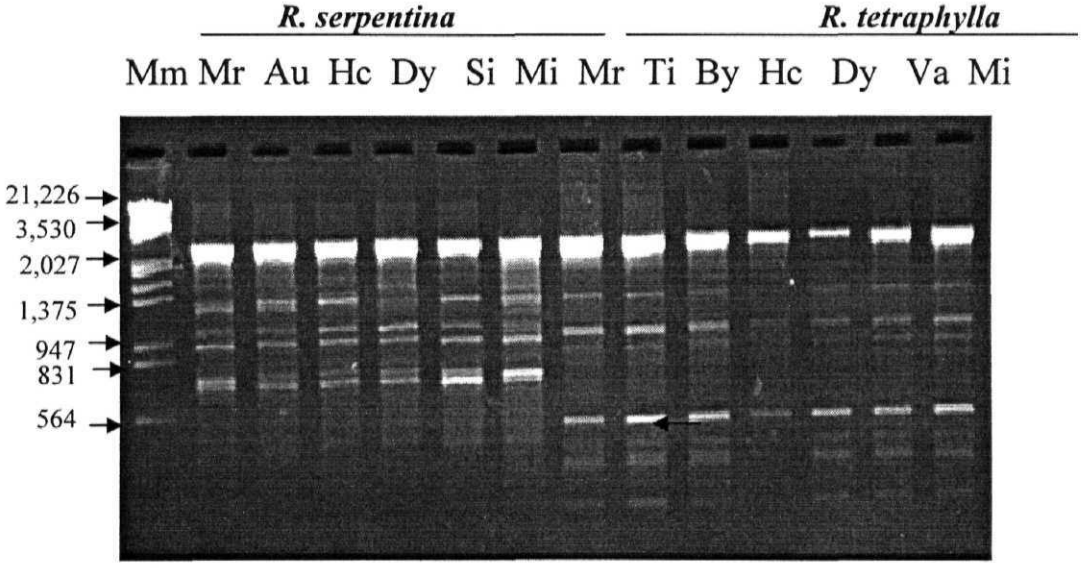


Figure 58: RAPD profile for the genetic difference and similarity of six accessions of *R. serpentina* and seven accessions of *R. tetraphylla* on 2% agarose gel using primer OPA-02 (5' TGCCGAGCTG 3').Monomorphism is observed with all the accessions of *R. serpentina* and also among the accessions of *R. tetraphylla* but when both the species are compared there are very few polymorphic markers. Arrow indicate the presence of polymorphic markers of *R. serpentina* to *R. tetraphylla* Lane Mm represents molecular marker i.e., Lambda DNA *Hind 111* and *EcoR1* Double digest.

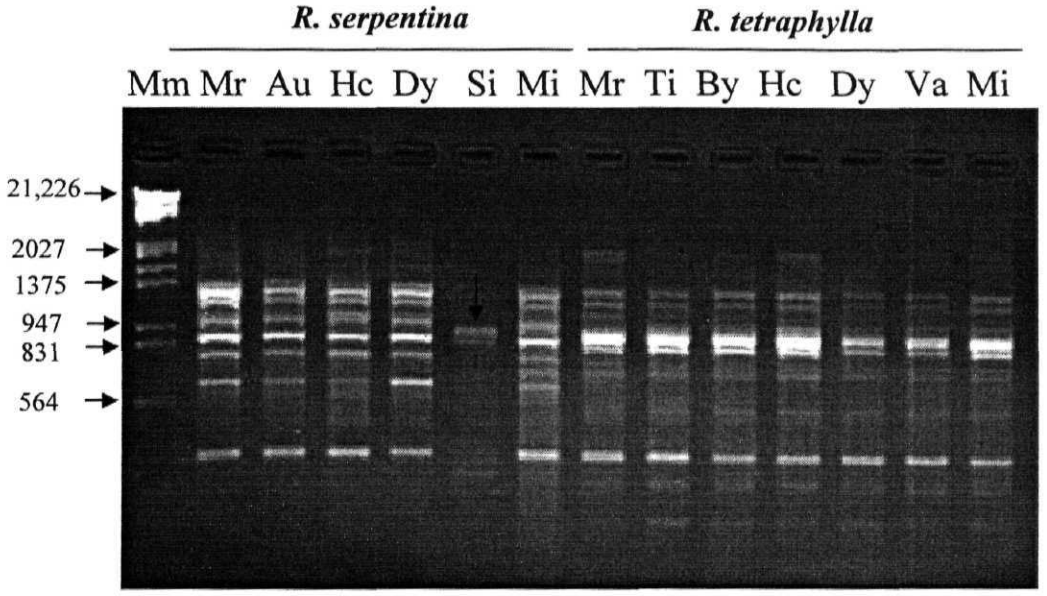


Figure 59: RAPD profile using primer OPA-03 (5'AGTCAGCCAC3'). Monomorphic markers are generated with all the accessions of *R. serpentina* except in the lane Si, and *R. tetraphylla* where a species specific diagnostic accession specific marker was generated indicated by an arrow in case of *R. serpentina* but when both the species are compared highest polymorphism was recorded.

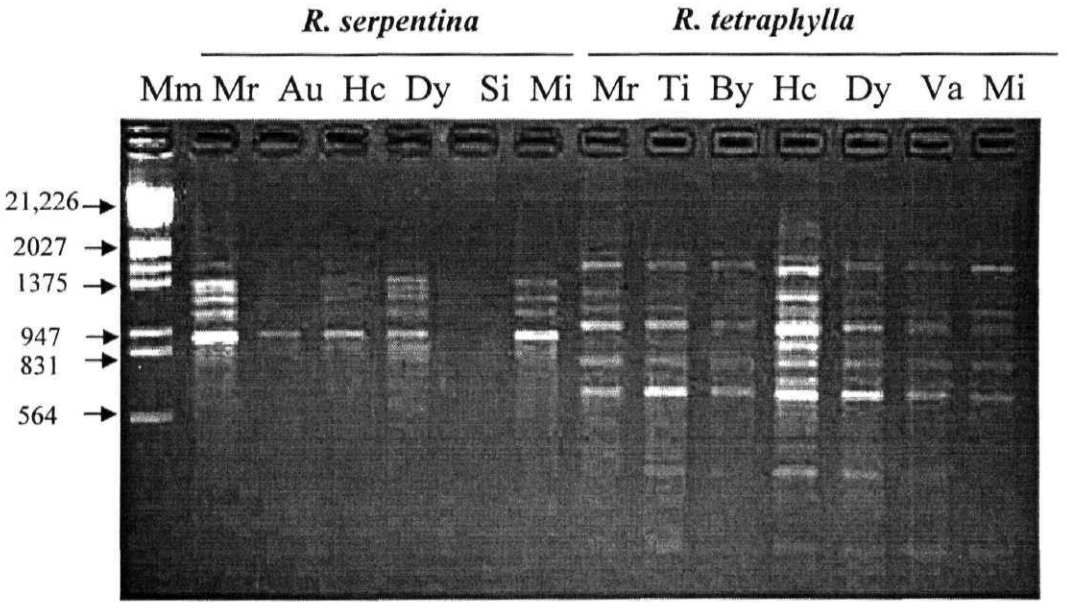


Figure 60: RAPD profile using primer OPA-04 (5'AATCGGGCTG3'). Monomorphism was observed among the accessions of *R. serpentina* and *R. tetraphylla* except in lane Si, where there is no amplification. Markers, which are monomorphic among the accessions of *R. tetraphylla* are polymorphic in *R. serpentina.*

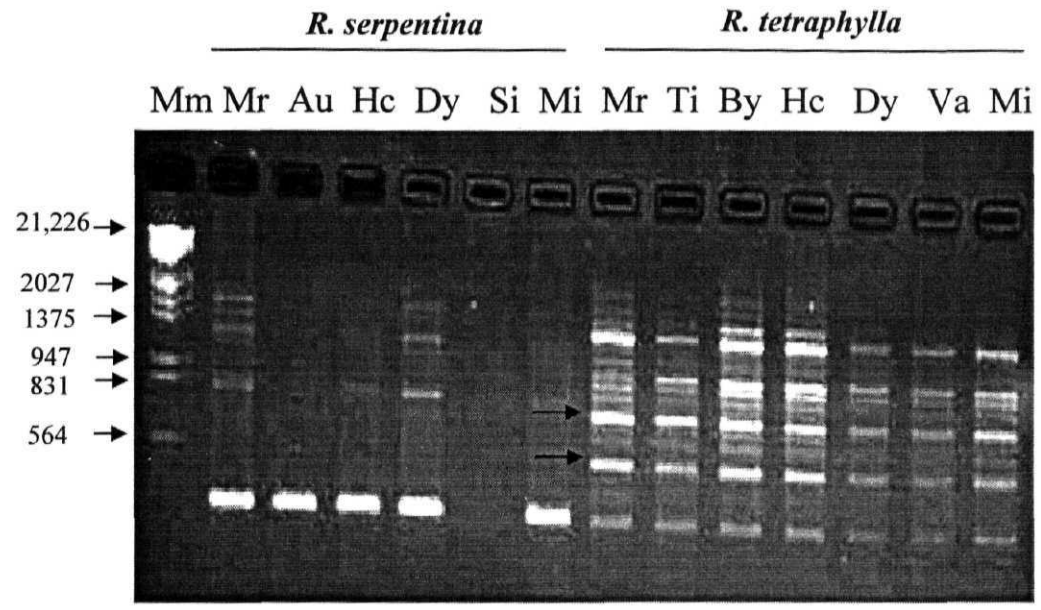


Figure 61: RAPD profile using primer OPA-05 (5'AGGGGTCTTG3'). Monomorphic markers are generated with all the accessions of *R. serpentina* and *R. tetraphylla* except in the lane Si where there is no amplification. Some of the markers of *R. tetraphylla* are polymorphic to that of *R. serpentina* , indicated by arrows

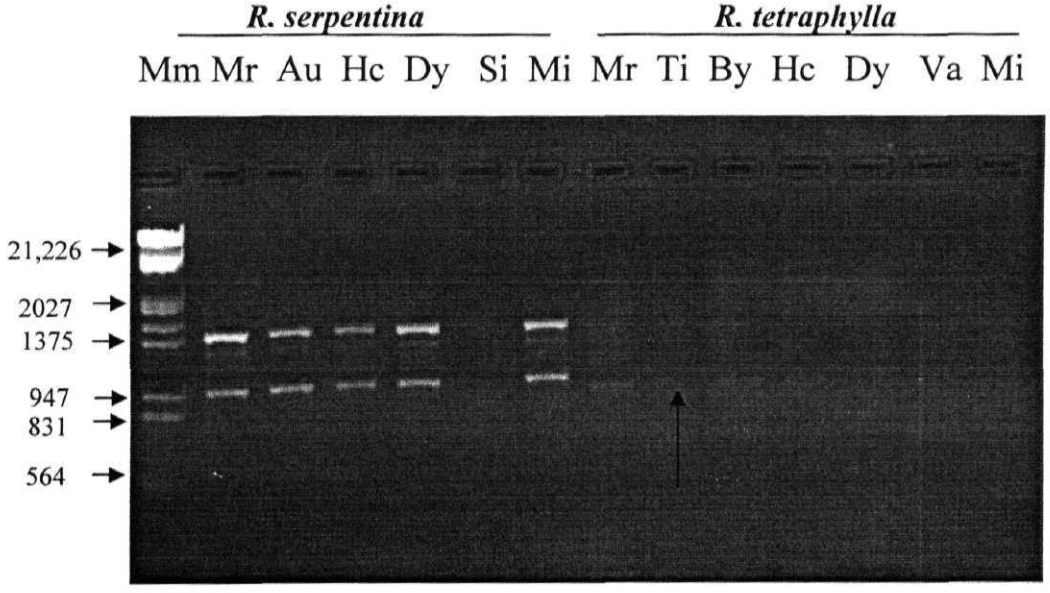


Figure 62: RAPD profile using primer OPA-06 (5'GGTCCCTGAC3'). Monomorphic markers were generated among all the accessions of *R. serpentina* except in Lane Si where there is was no amplification. Arrow indicates the absence of polymorphism among the accessions of *R. tetraphylla*

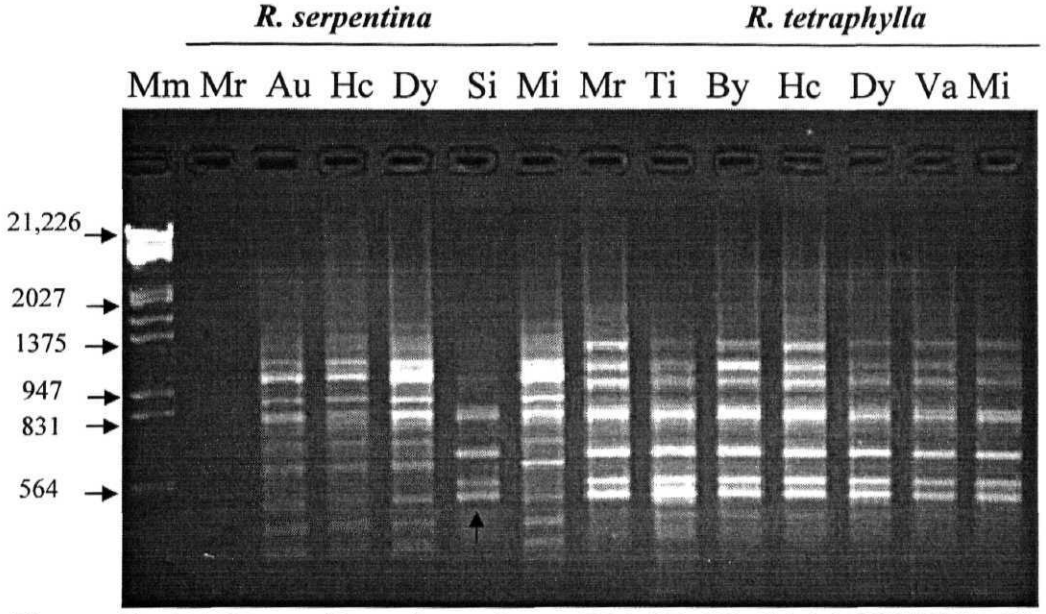


Figure 63: RAPD profile using primer OPA-07 (5' GAAACGGGTG 3'). Monomorphic markers are generated among all the accessions of *R. serpentina* whereas in lane Si polymorphic markers are generated as indicated with an arrow and in lane Mr there is no amplification. Monomorphic markers are generated among all the accessions of *R. tetraphylla* which were similar to markers among few accessions of *R. serpentina*.

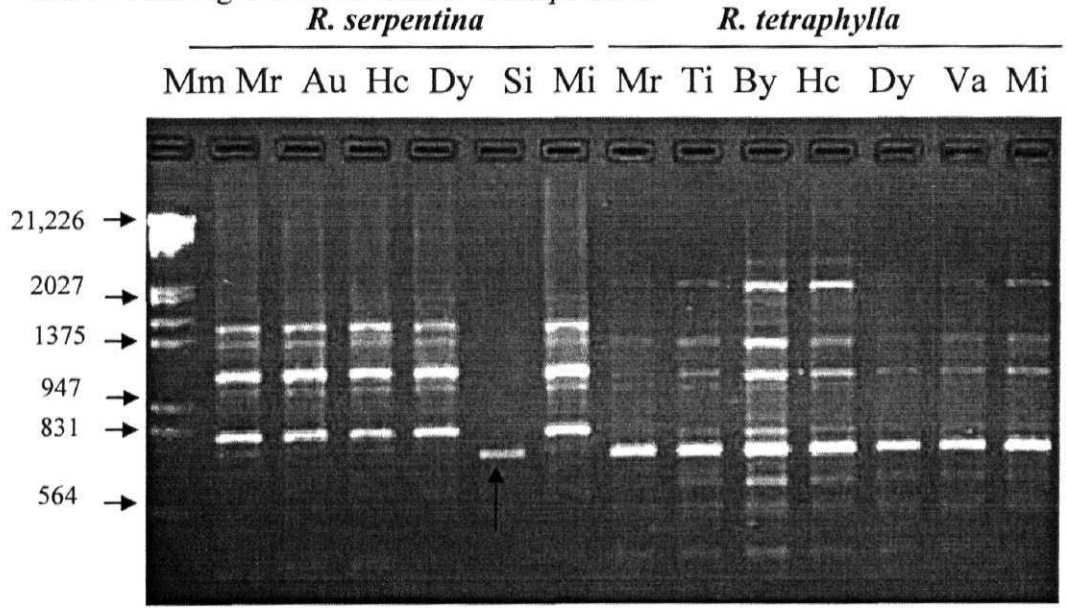


Figure 64: RAPD profile using primer OPA-08 (5'GTGACGTAGG3'). Monomorphic markers are generated among all the accessions of *R. serpentina* except in lane Si where a species and accession specific diagnostic marker was generated as represented with an arrow. Similarly monomorphic markers are generated among all the accessions of *R. tetraphylla* where few are polymorphic to that of accessions of *R. serpentina*.

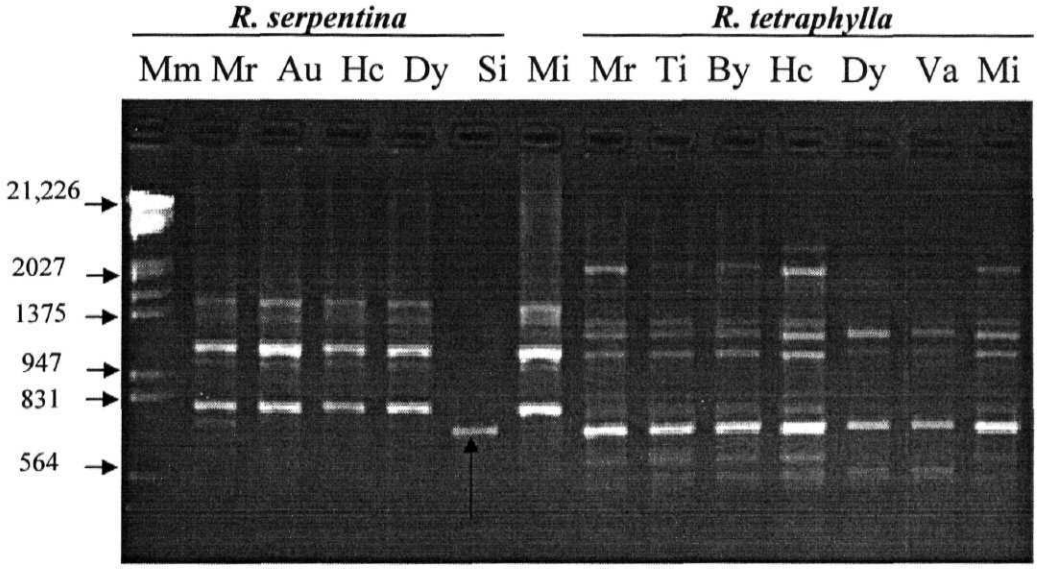


Figure 65: RAPD profile using primer OPA-09 (5' GGGTAACGCC 3'). Monomorphic markers are generated among all the accessions of *R. serpentina* except in lane Si where a species and accession specific diagnostic marker was generated as represented with an arrow. Polymorphic markers are generated among accessions of *R. tetraphylla* which are also represented with arrows which are absent in *R. serpentina*

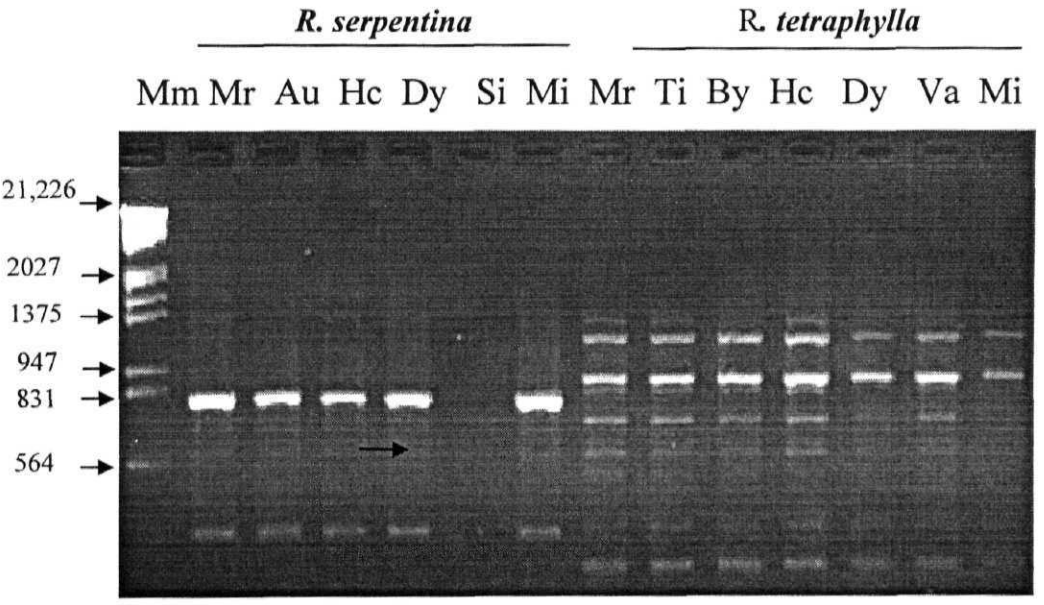


Figure 66: RAPD profile using primer OPA-10 (5' GTGATCGCAG3'). Monomorphism is observed among the accessions of both *R. serpentina* and *R. tetraphylla* but there is a dissimilarity of distribution of markers observed among the two species.

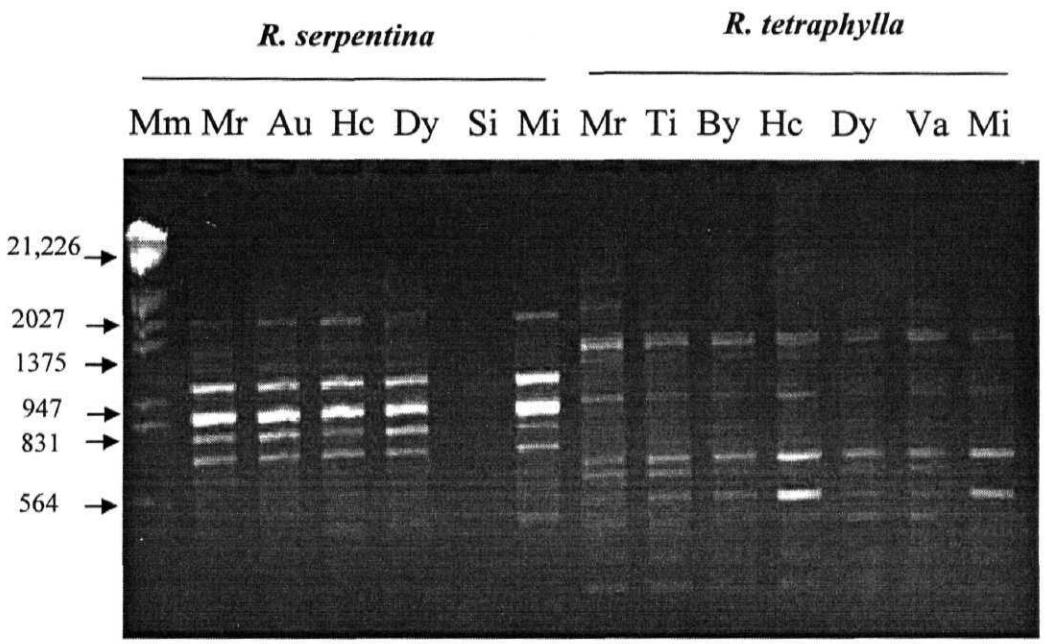


Figure 67: RAPD profile using primer OPA-11 (5' CAATCGCCGT 3'). Monomorphic markers are generated among all the accessions of *R. serpentina* and *R. tetraphylla* except in lane Si, where there is no amplification. There is no similarity of the markers between both the species. .

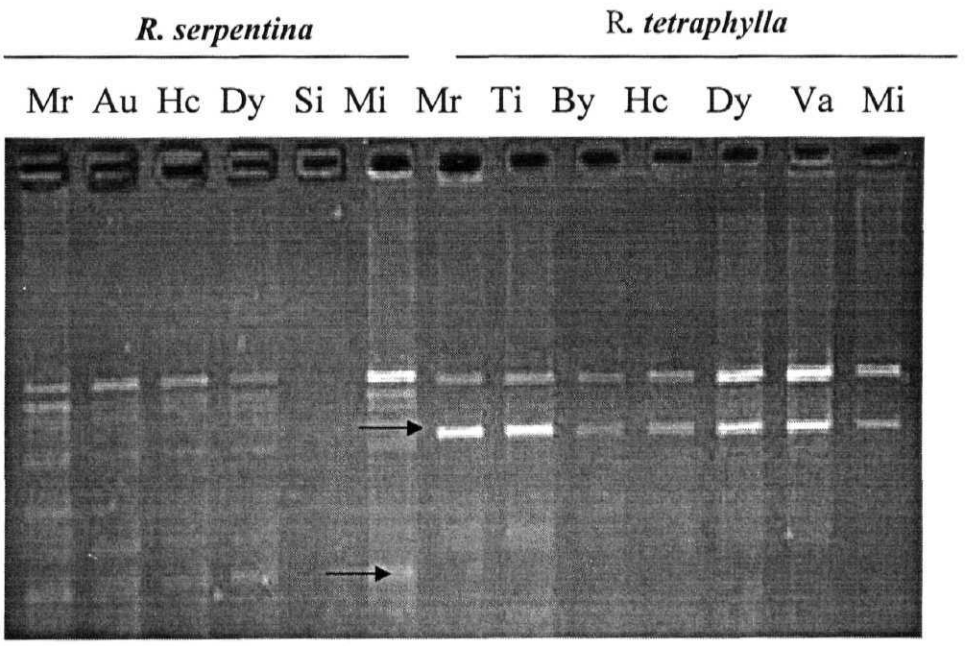


Figure 68: RAPD profile using primer OPA-12 (5' TCGGCGATAG 3').Arrows represents the presence of polymorphic markers among the accessions of *R. serpentina* but in lane Si, there are no amplified fragments. Monomorphism is observed among the accessions of *R. tetraphylla*.

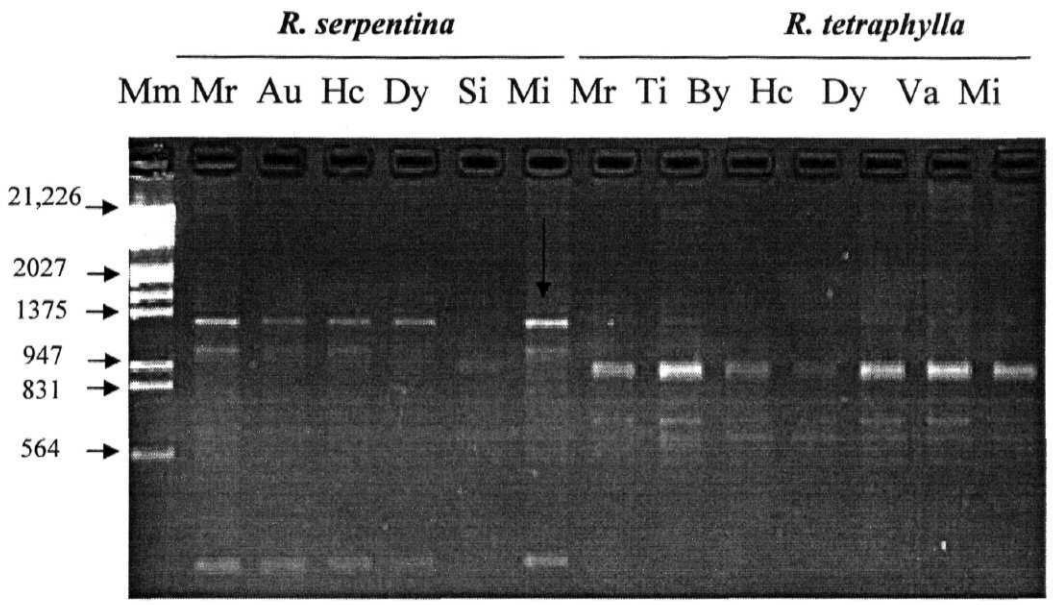


Figure 69: RAPD profile for using primer OPA-13 (5' CAGCACCCAC 3') In *R. serpentina* monomorphism is observed among all the accessions except in lane Si, a single species and accession specific diagnostic marker is generated which is indicated with an arrow. Few polymorphic markers are generated among all the accessions in *R. tetraphylla* Very few markers coincide with both the accessions of *R. serpentina* and *R. tetraphylla.*

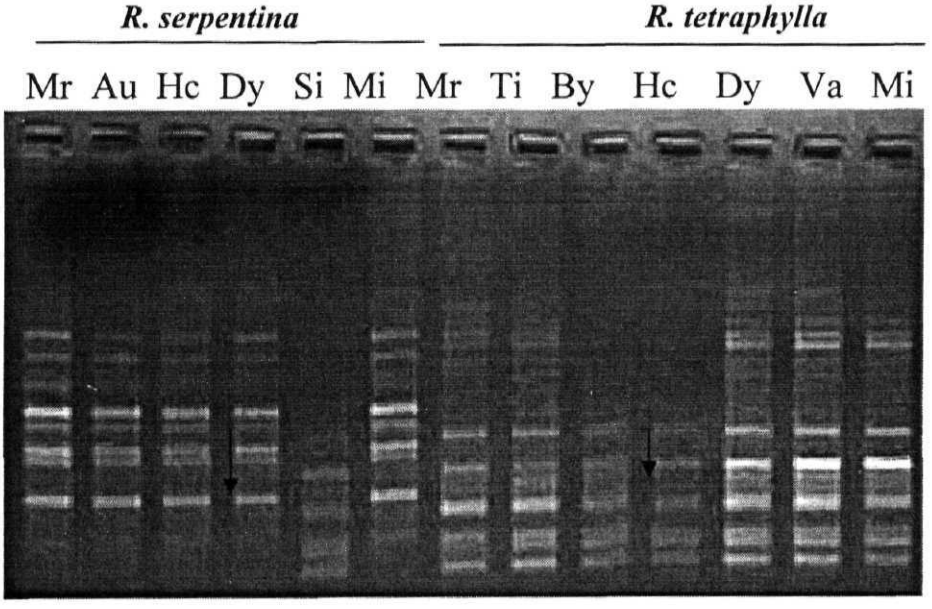


Figure 70: RAPD profile using primer OPA-14 (5' TCTGTGCTGG 3') Monomophic markers are generated among all the accessions of *R. serpentina* except in lane Si, where polymorphic markers are depicted as indicated by the arrow. Mostly monomorphism is observed among the accessions of *R. tetraphylla* among all the accessions except in lane Hc which corresponds to the similarity as in lane Si.

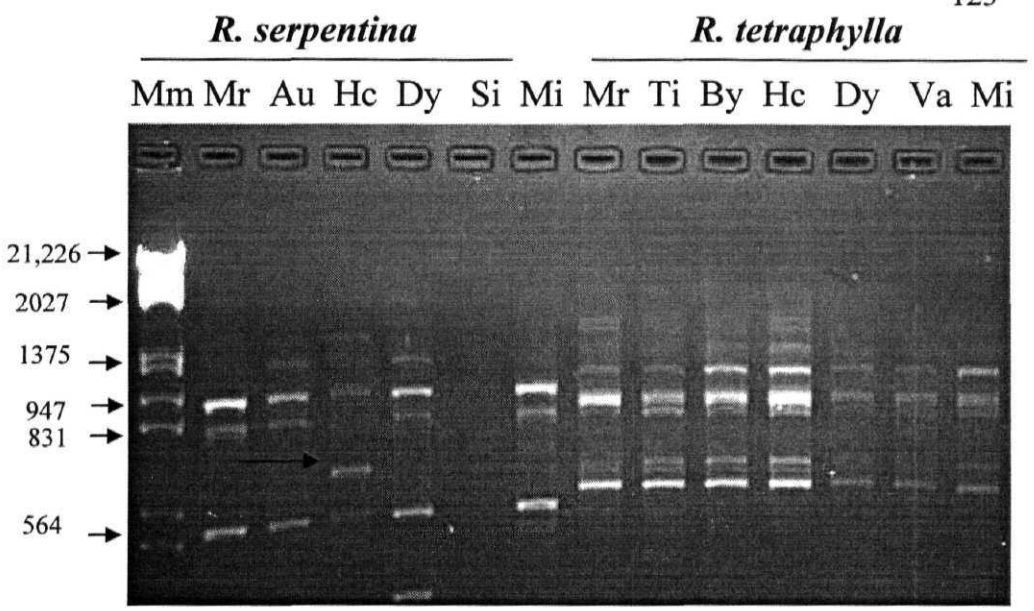


Figure 71: RAPD profile using primer OPA-15 (5' TTCCGAACCC 3'). Polymorphic markers are generated among all the accessions of *R. serpentina* except in lane Si, where there is no amplification. In lane Hc a species specific unique marker is generated which is represented by the arrow. In *R. tetraphylla* polymorphic markers are generated out of which few are similar to that of *R. serpentina*.

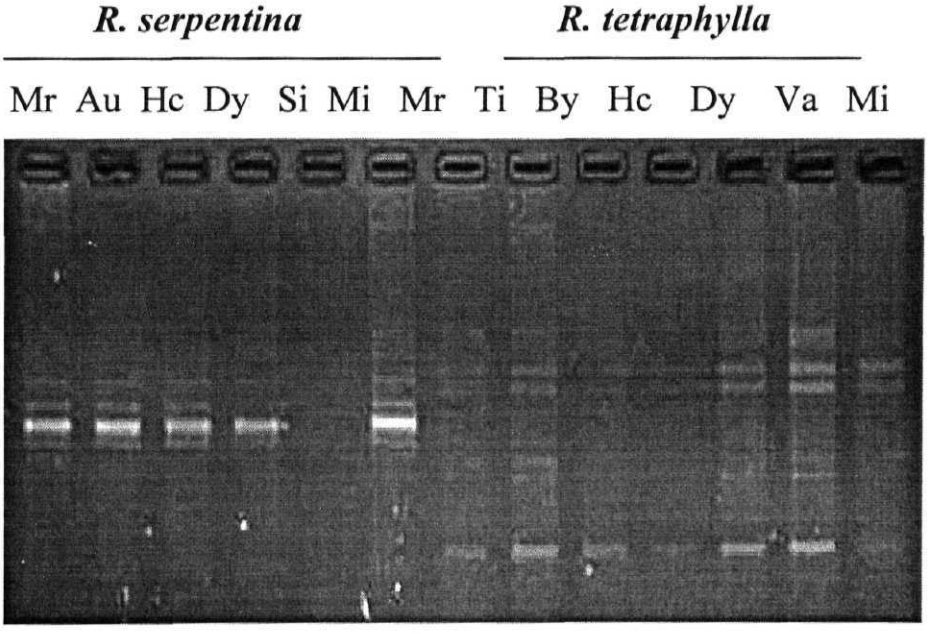


Figure 72: RAPD profile using primer OPA-16 (5'AGCCAGCGAA 3'). Polymorphic markers are generated among all the accessions of *R. serpentina* except in lane Si where there is no amplification. Polymorphic markers are generated among all the accessions of *R. tetraphylla* which are not similar to those of *R.serpentina*

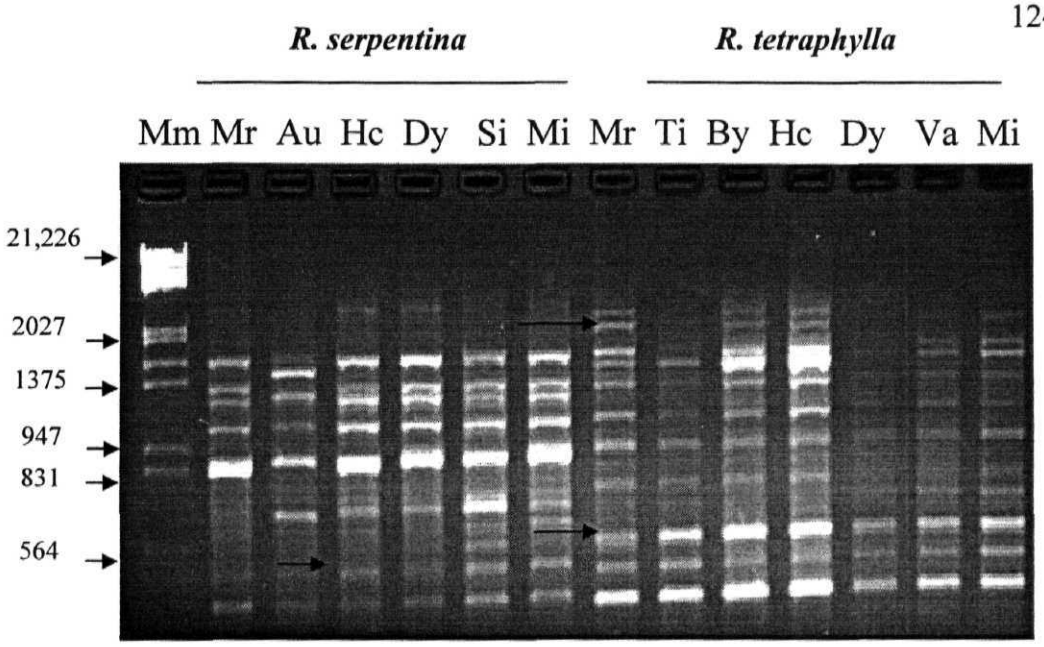


Figure 73: RAPD profile using primer OPA-17 (5 GACCGCTTGT 3'). Polymorphic markers are generated in case of accessions in *R. serpentina* and *R. tetraphylla.* Arrows represent the diagnostic species specific markers in both the species

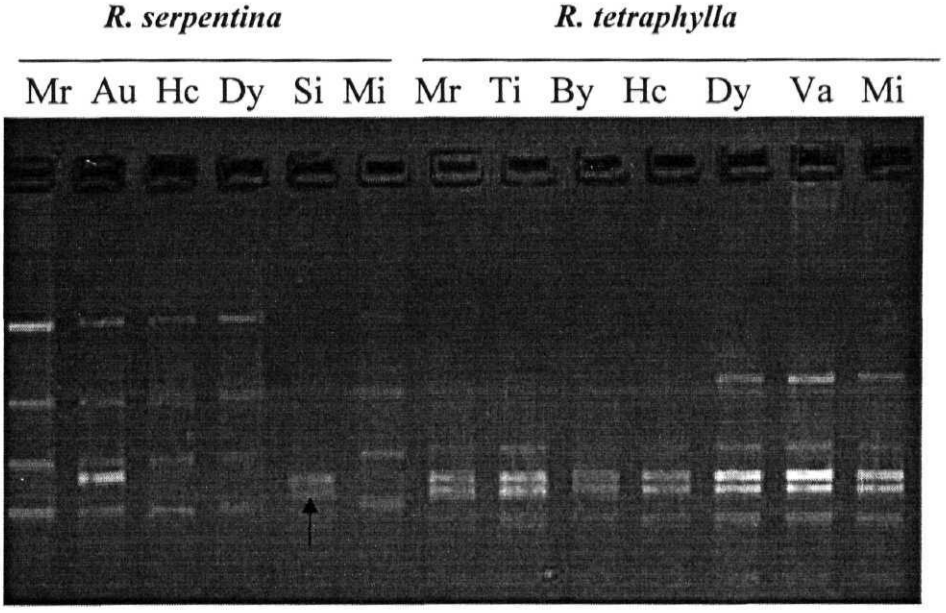


Figure 74: RAPD profile using primer OPA-18 (5'AGGTGACCGT 3'). In *R. serpentina* and in *R. tetraphylla* polymorphic markers are depicted. In lane Si arrow indicates the presence of two accession specific markers in *R. serpenina* which shows similarity with that of the monomorphic markers in *R. tetraphylla*.

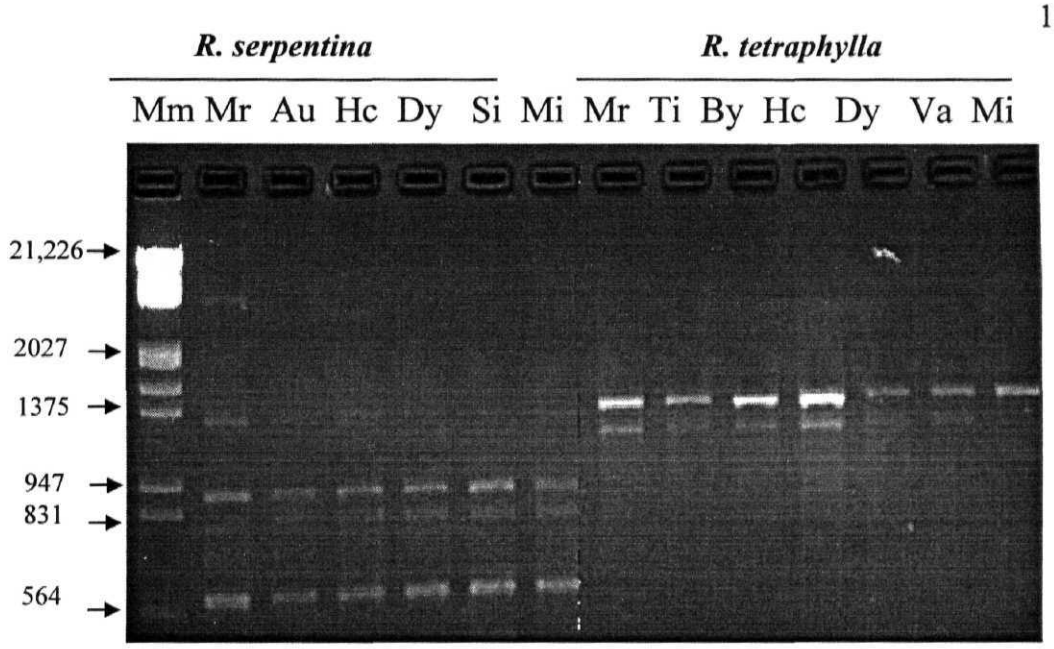


Figure 75: RAPD profile using primer OPA-19 (5' CAAACGTCGG 3').Mostly monomorphic bands are generated in among accessions of both the species which do not coincide between both the species.

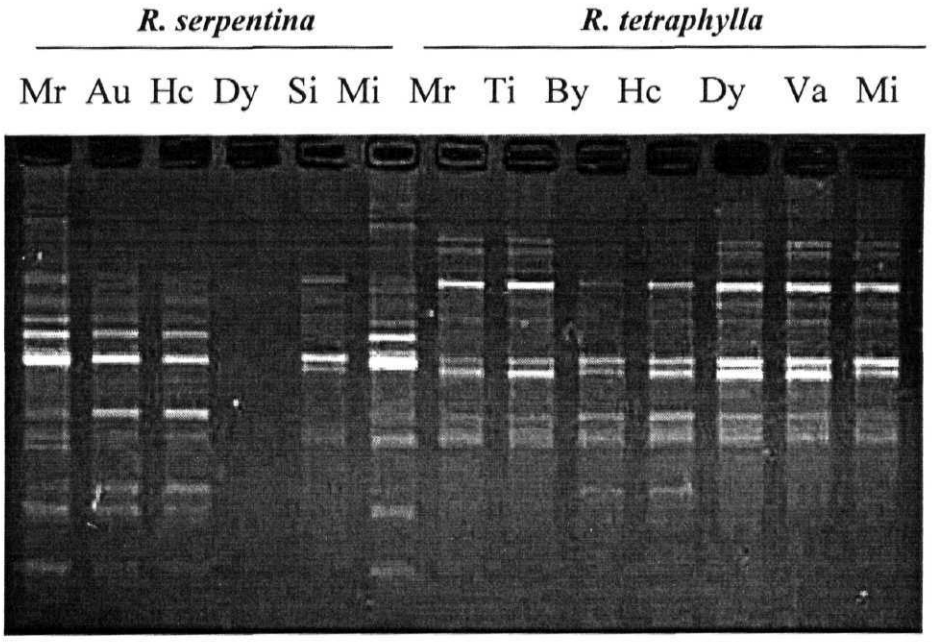


Figure 76: RAPD profile using primer OPA-20 (5' GTTGCGATCC 3'). Monomorphism is observed with all the accessions of *R. serpentina* except in lane Dy, where there is absence of amplified fragments. Among *R. tetraphylla* accessions polymorphic markers are generated as indicated with arrows. Many markers are in common in both the species as shown with arrows.

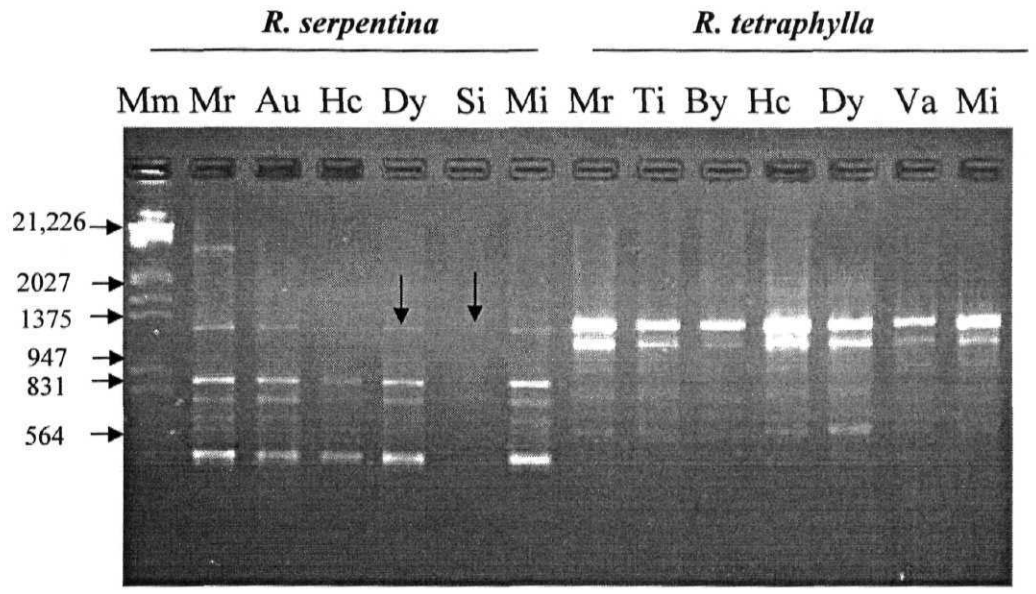


Figure 77: RAPD profile using primer OPC-01 (5'TTCGAGCCAG3'). Polymorphic markers are generated in both the species apart from which in lane Si, a unique band is generated which is represented with an arrow. In *R. teraphylla* . Unique markers for 2 accessions are generated as with arrows in lane Hc and Dy.

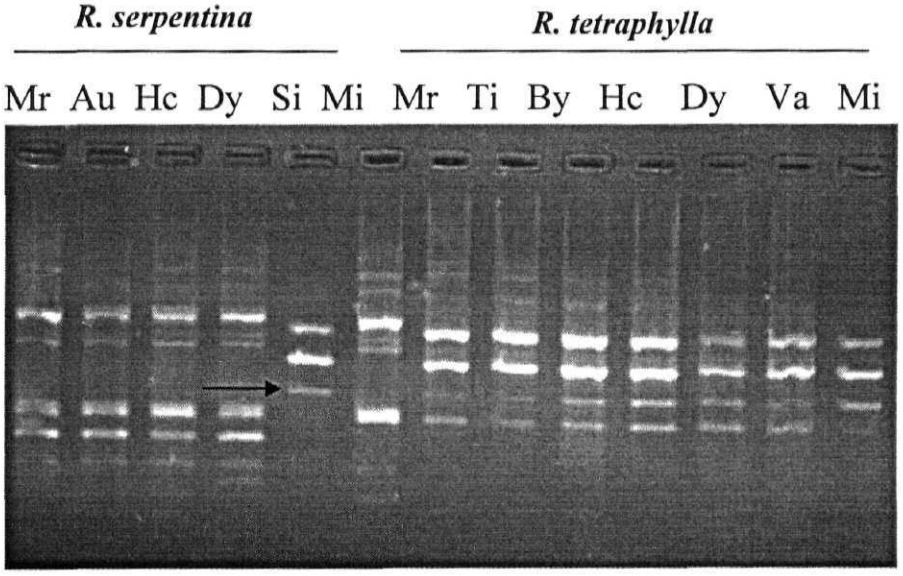


Figure 78: RAPD profile using primer OPC-02 (5' GTGAGGCGTC 3').In case of accessions in *R. serpentina* polymorphic markers are generated along with a unique marker in lane Si, as represented with an arrow which are similar to that of the markers among accessions of *R. tetraphylla.*

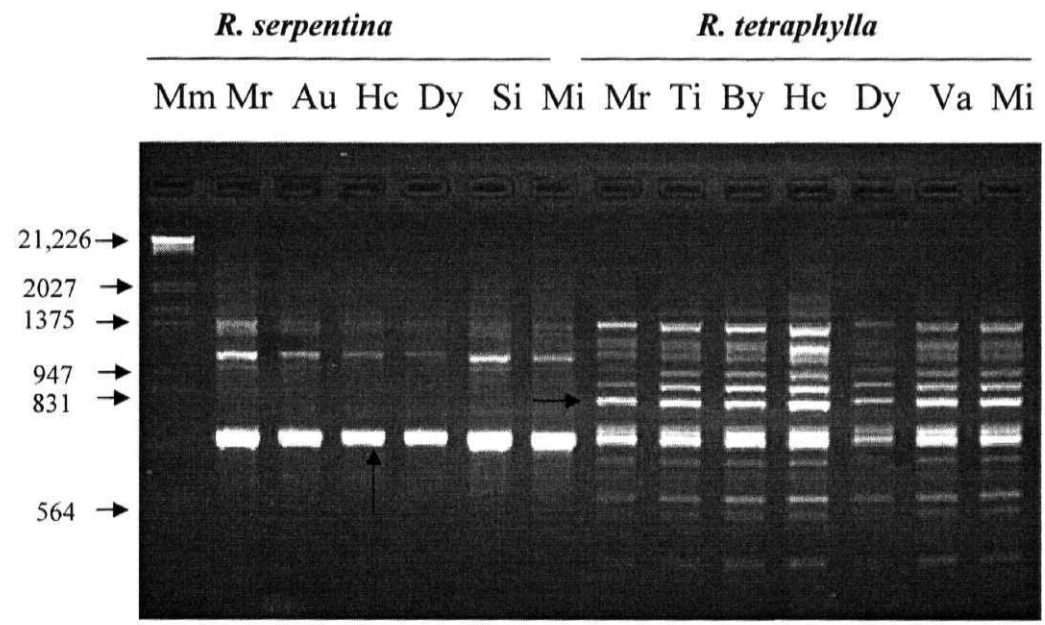


Figure 79: RAPD profile using primer OPC-03(5' GGGGGTCTTT 3').Mostly monomorphic markers are generated among the accessions of *R. serpentina* and *R. tetraphylla* but unique species specific polymorphic bands are generated in *R. tetraphylls*

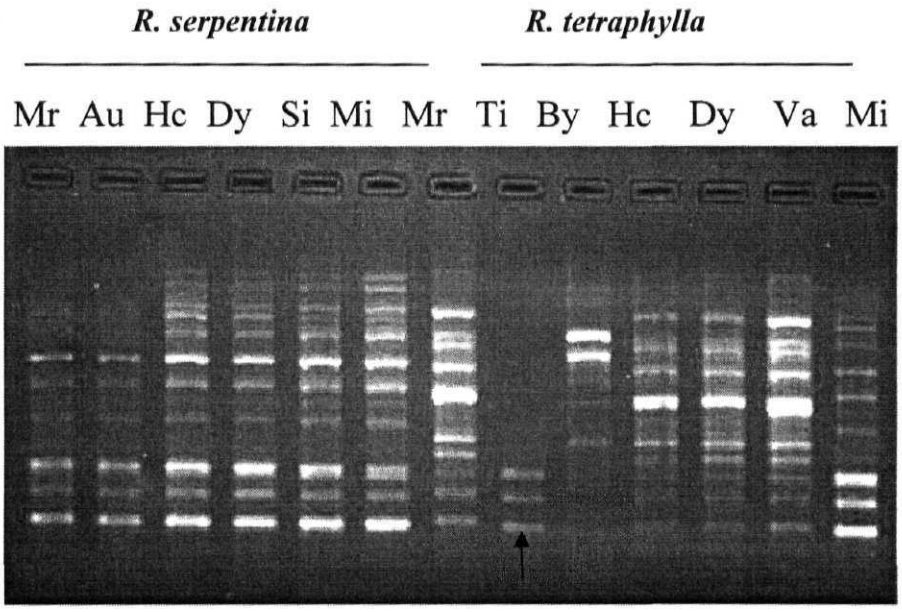


Figure 80: RAPD profile using primer OPC-04 (5' CCGCATCTAC 3').Many polymorphic markers are generated among both *R. serpentina* and *R. tetraphylla* accessions except in lane Ti only three markers are generated which are mostly monomorphic represented by an arrow.

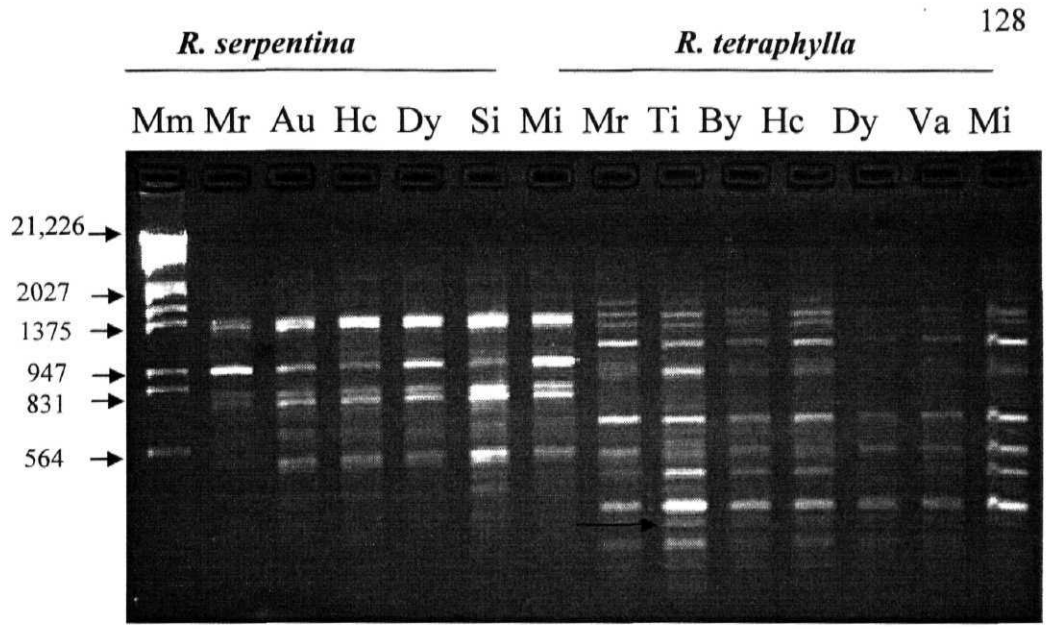


Figure 81: RAPD profile using primer OPC-05 (5'GATGACCGCC 3').Many polymorphic markers are generated among the accessions of *R. serpentina* and *R. tetraphylla* some of which are specific to *R. tetraphylla* which are represented with an arrow.

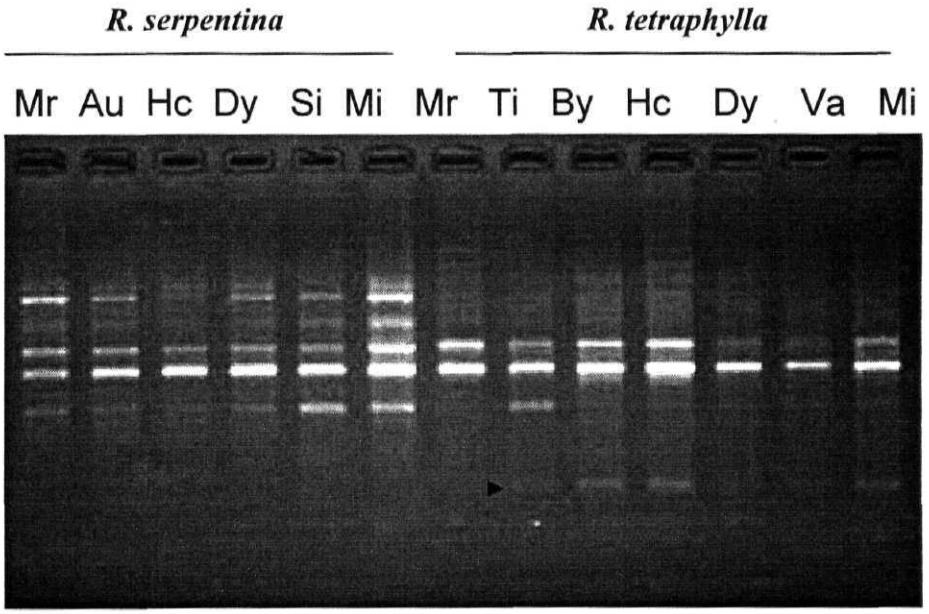


Figure 82: RAPD profile using primer OPC-06 (5'GAACGGACTC3'). Monomorphism was observed among all the accessions of *R. serpentina* and polymorphism was observed among the accessions of *R. tetraphylla* where, in few accessions, species specific diagnostic markers are generated which are represented with an arrow.

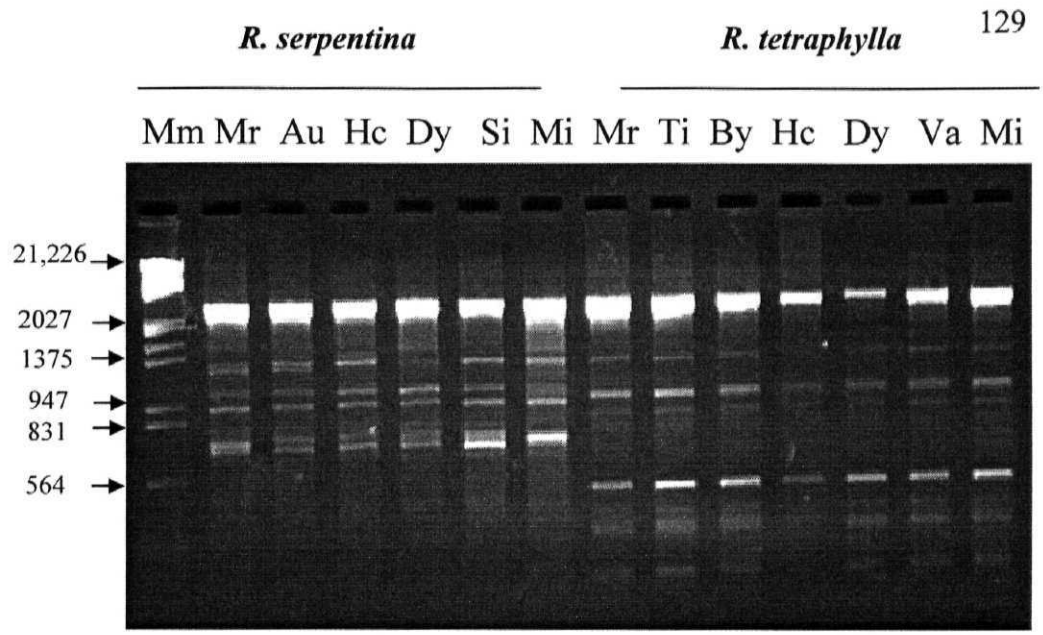


Figure 83: RAPD profile using primer OPC-07 (5'GTCCCGACGA3') no polymorphic markers are generated in *R. serpentina* whereas in *R. tetraphylla* few polymorphic markers are generated. Some common markers are generated between both the species of *Rauvolfia* whereas some are specific to the particular species.

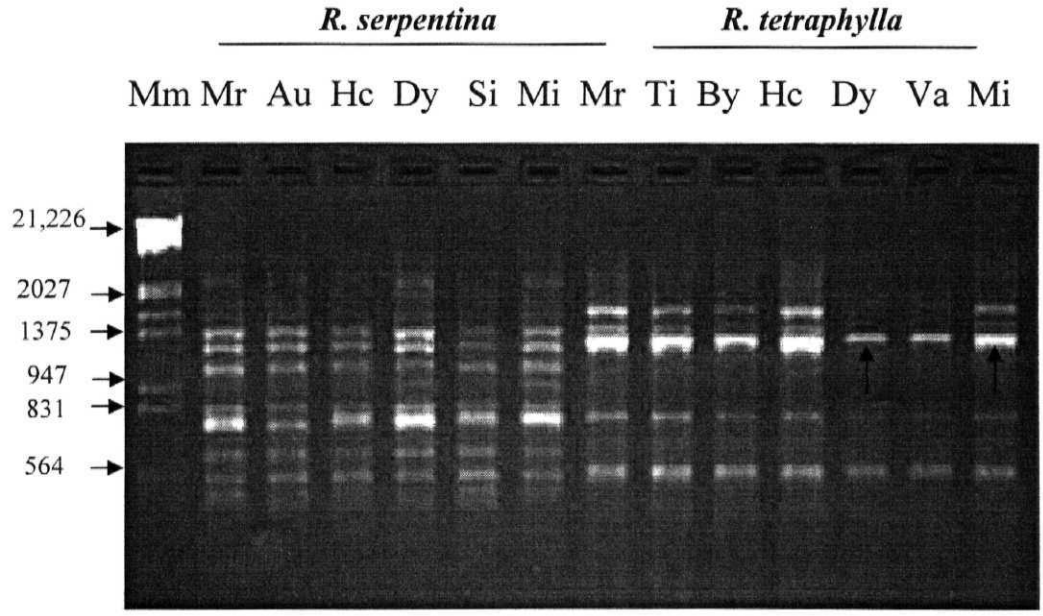


Figure 84: RAPD profile using primer OPC-08 (5'TGGACCGGTG3'). Monomorphic markers are generated among the accessions of *R. serpentina* and polymorphic markers are generated among the accessions of *R. tetraphylla* where some of the markers are specific to few accessions among the species which are represented with arrows.

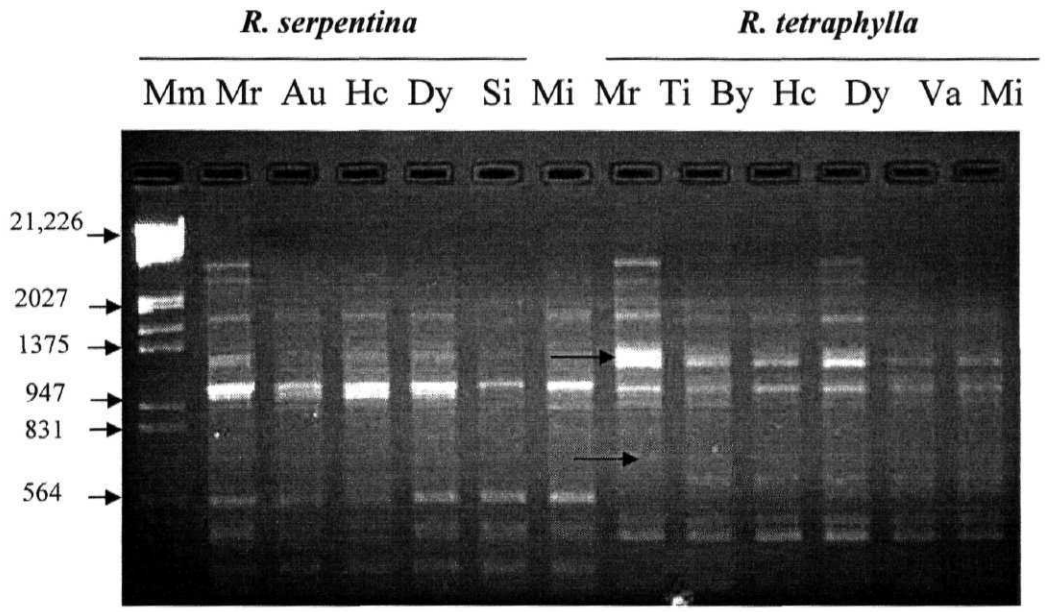


Figure 85: RAPD profile using primer OPC-09 (5'CTCACCGTCC 3').Both mono and polymorphism bands are generated among the accessions of *R. serpentina* and *R. tetraphylla* whereas some of the markers as indicated with arrows in lane Dy,Mr and Hc are similar to each other. In lane Mi a unique accession and species-specific marker is generated which is represented with an arrow. .

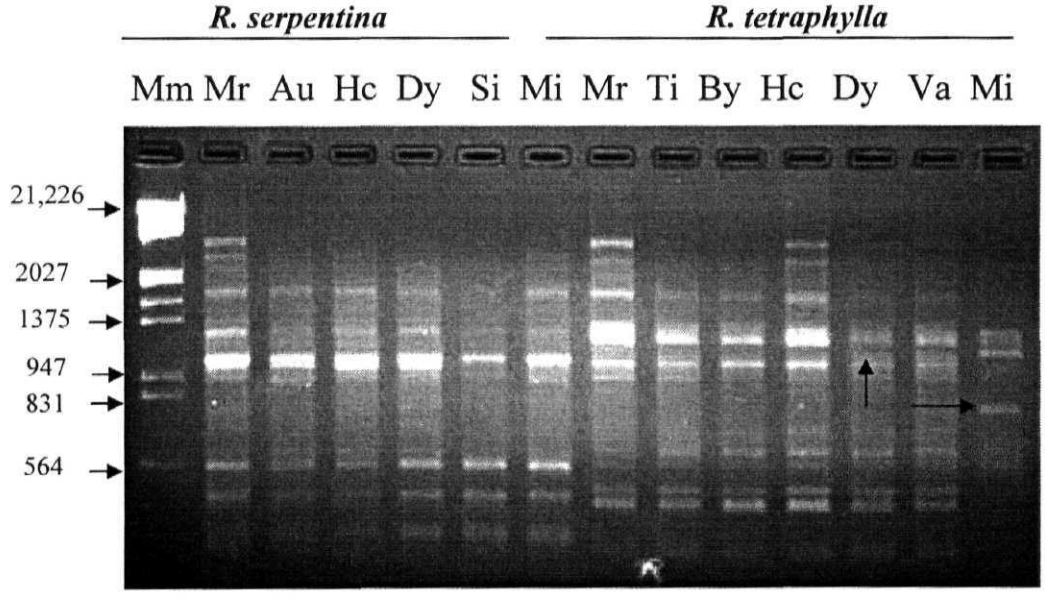


Figure 86: RAPD profile using primer OPC-10 (5'TGTCTGGGTG 3').Both mono and polymorphic bands are generated among the accessions of *R. serpentina* and *R. tetraphylla* whereas some of the markers as indicated with arrows in lane Dy,Mr and Hc are similar to each other. In lane Mi a unique accession and species-specific marker is generated which is represented with an arrow.

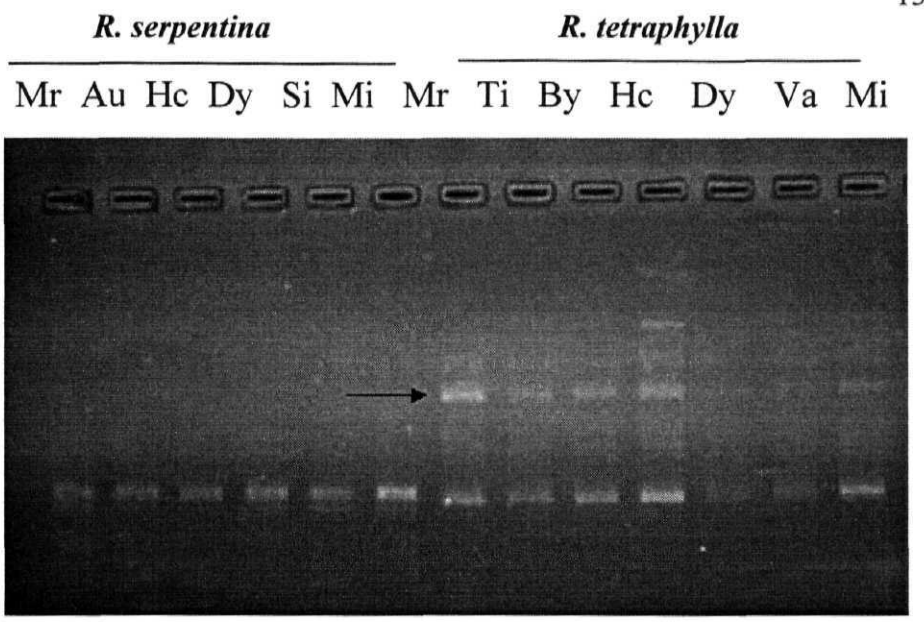


Figure 87: RAPD profile using primer OPC-11 (5'AAAGCTGCGG 3'). Monomorphic markers are generated among all the accessions of *R. serpentina* which are similar to that of markers in *R. tetraphylla* apart from which few polymporphic markers also are regenerated which are indicated with arrows.

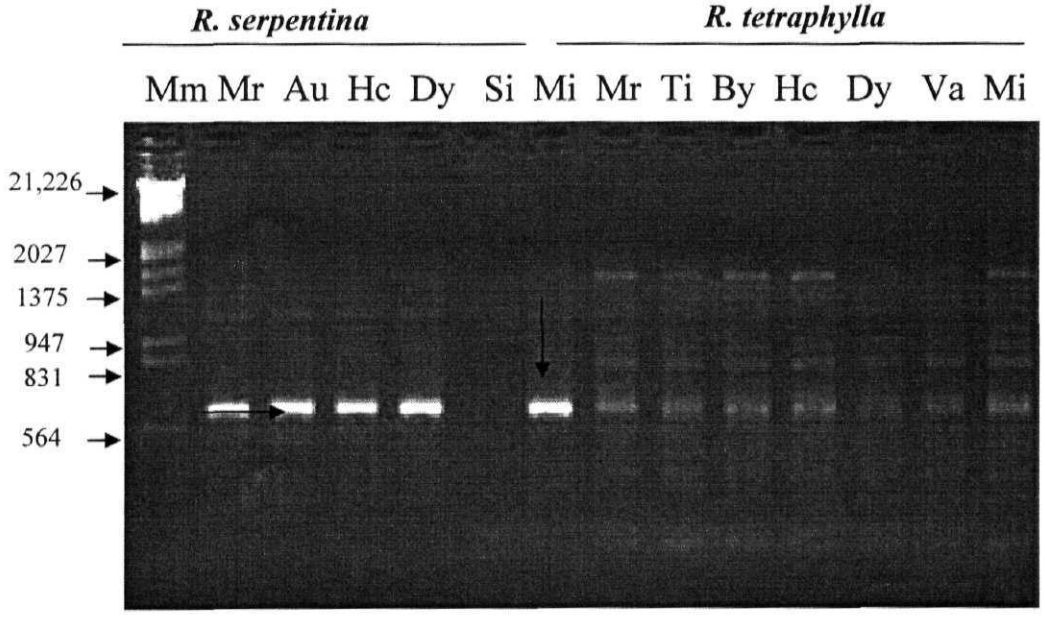


Figure 88: RAPD profile using primer OPC-12 (5'TGTCATCCCC 3').A single marker is generated among all the accessions in *R. serpentina* but in lane Si, there is no amplification which is indicated with arrows. Few polymorphic bands are generated among accessions in *R. tetraphylla* mostly of which are monomorphic.

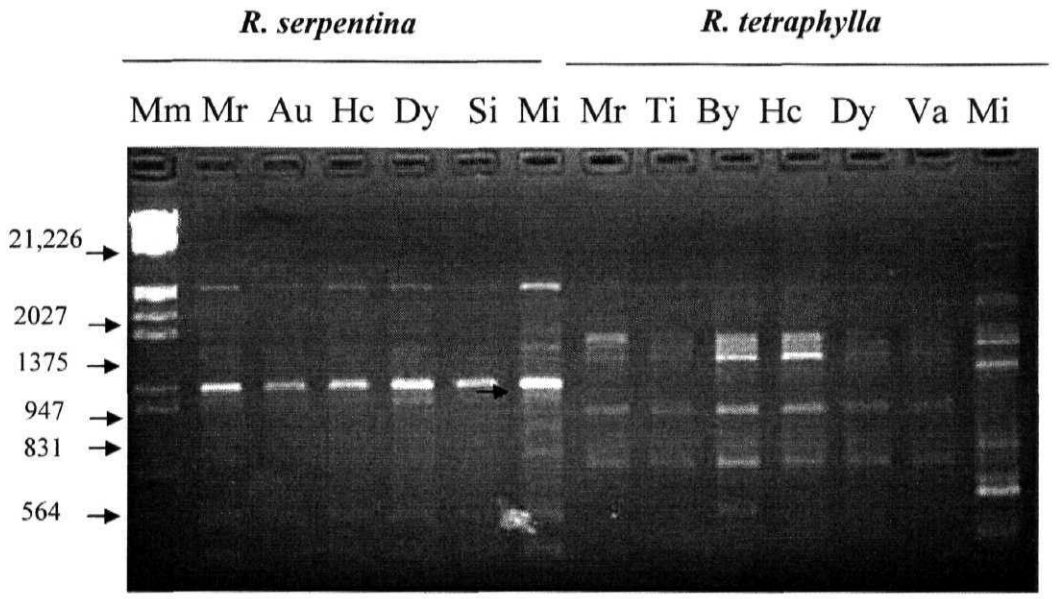


Figure 89:RAPD profile using primer OPC-13 (5'AAGCCTCGTC3'). Polymorphism is noted in *R. serpentina* to a lesser extent than *R. teraphylla* where numerous polymorphic bands are observed. In lane Mi, a unique species-specific diagnostic marker is generated in *R. serpentina* .

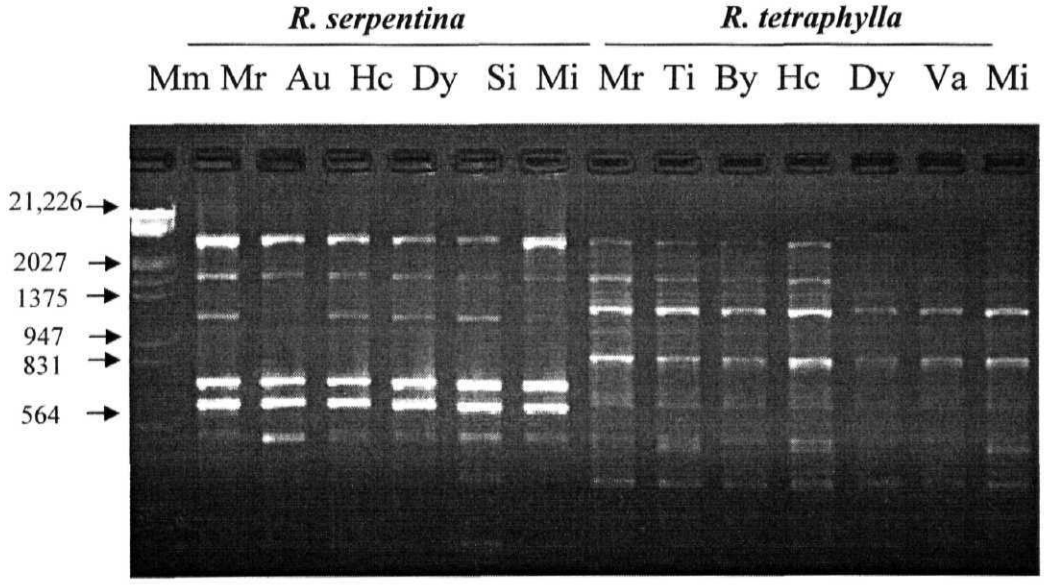


Figure 90: RAPD profile using primer OPC-14 (5'TGCGTGCTTG3'). Polymorphic markers are generated among accessions of both the species where most of the markers are similar between both the accessions, which are indicated with arrows.

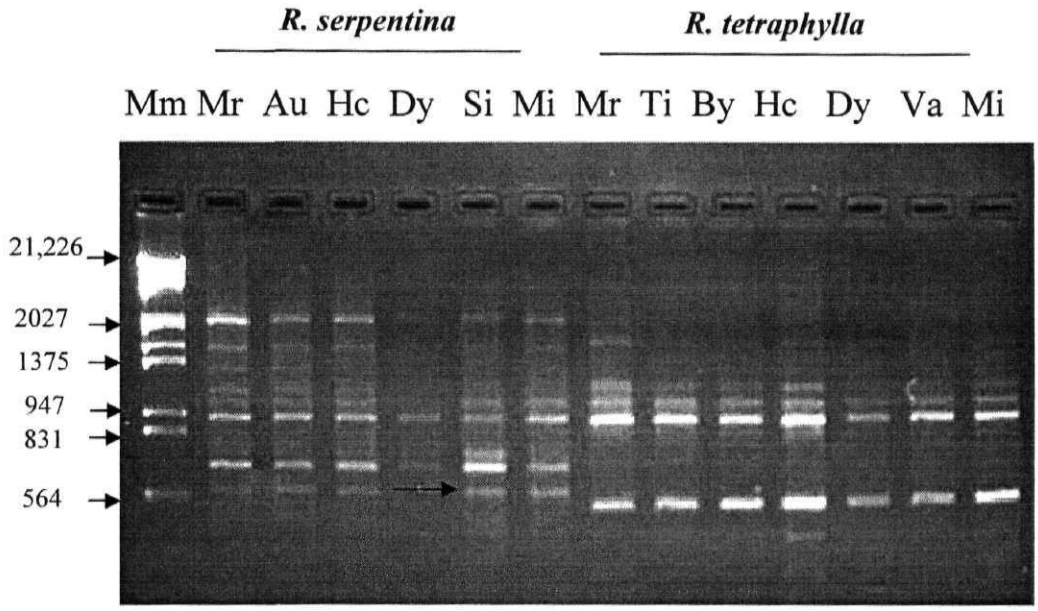


Figure 91: RAPD profile using primer OPC-15 (5' GACGGATCAG 3'). Few poly and many monomorphic bands are represented among both the species but a unique accession specific marker is represented in lane Si, indicated withan arrow.

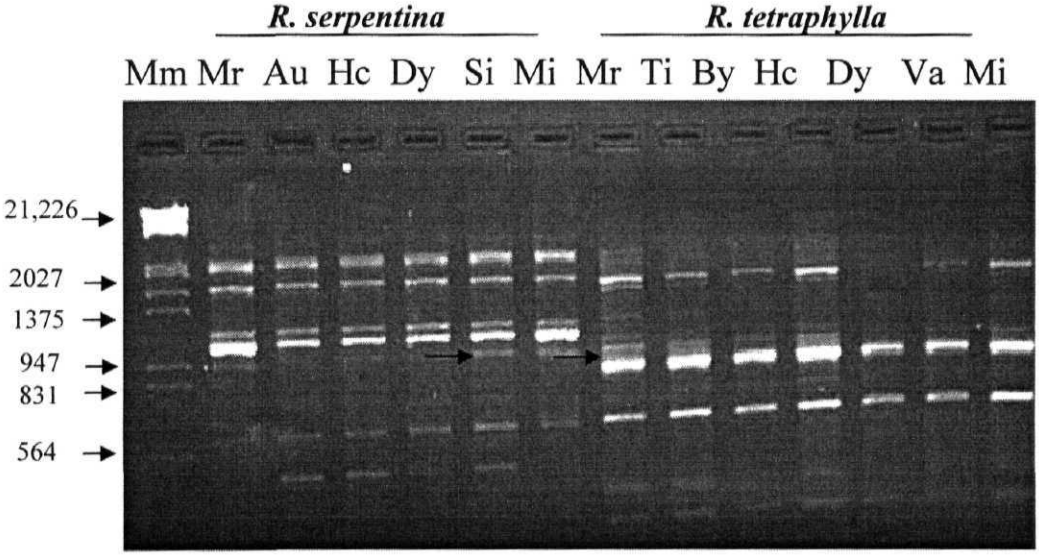


Figure 92: RAPD profile using primer OPC-16 (5'GACGGATCAG3').Mostly mono and polymorphic markers are generated between both the species and in lane Si and lane Mi accessions specific diagnostic markers are generated which are represented with an arrow.

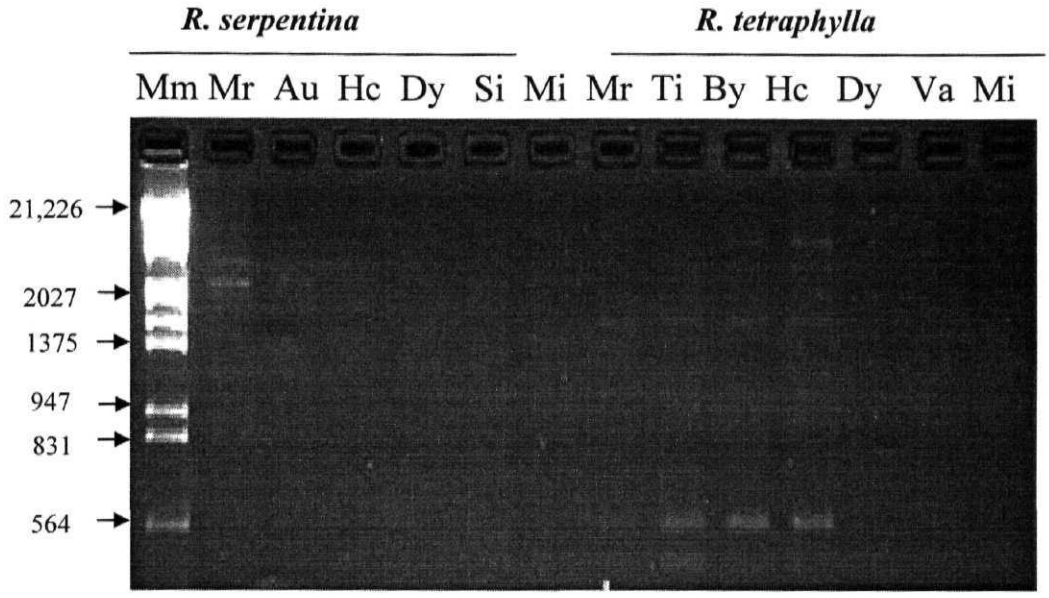


Figure 93: RAPD profile using primer OPC-17 (5'TTCCCCCCAG 3').Very few and faint bands are revealed which are represented lanes Mr, Au, Ti, By and Hc, may be due to the absence of priming site or due to experimental error.

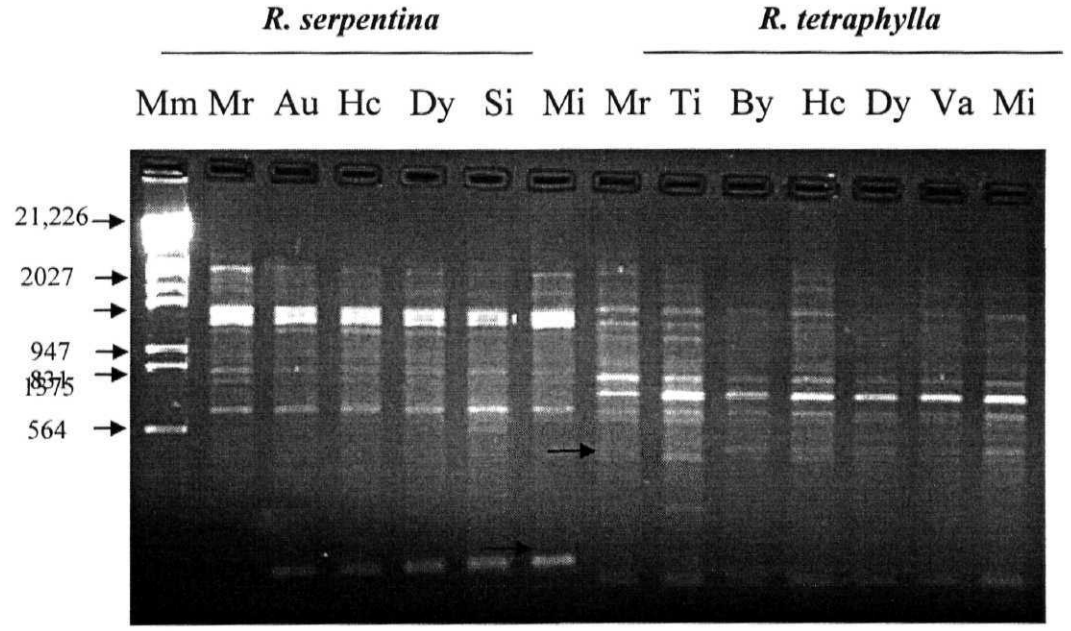


Figure 94: RAPD profile using primer OPC-18 (5' TGAGTGGGTG 3'). Polymorphic markers are generated among both species of *Rauvolfia,* arrows indicate the presence of unique markers which are accessions specific and indicated with arrows.

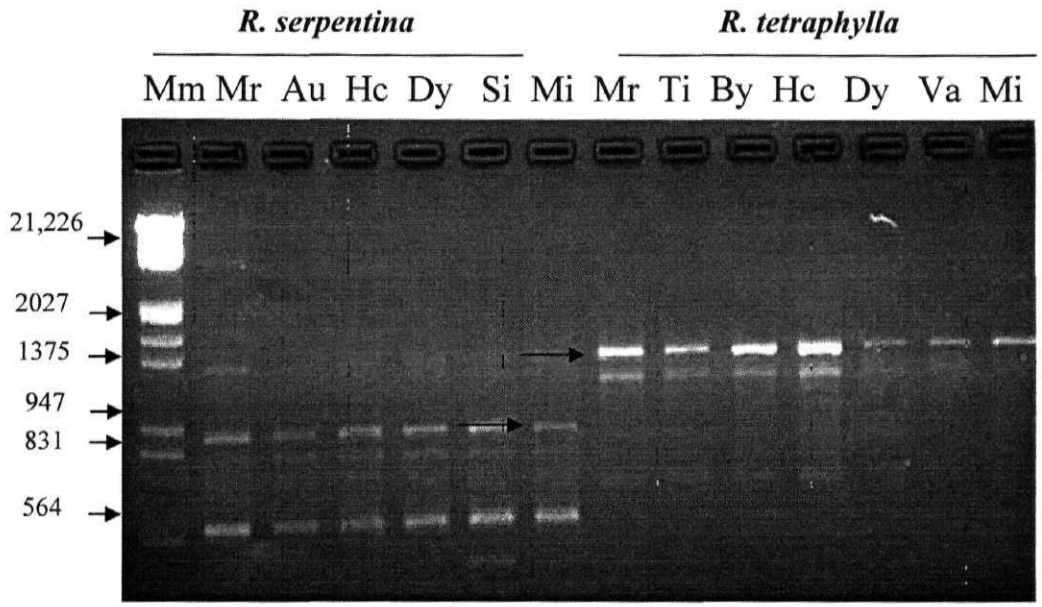


Figure 95: RAPD profile using primer OPC-19 (5' GTTGCCAGCC 3'). Monomorphic markers are generated among all the accessions of *R. serpentina* and *R. tetraphylla* which are represented in the form of arrows but which are highly polymorphic in between both the species.

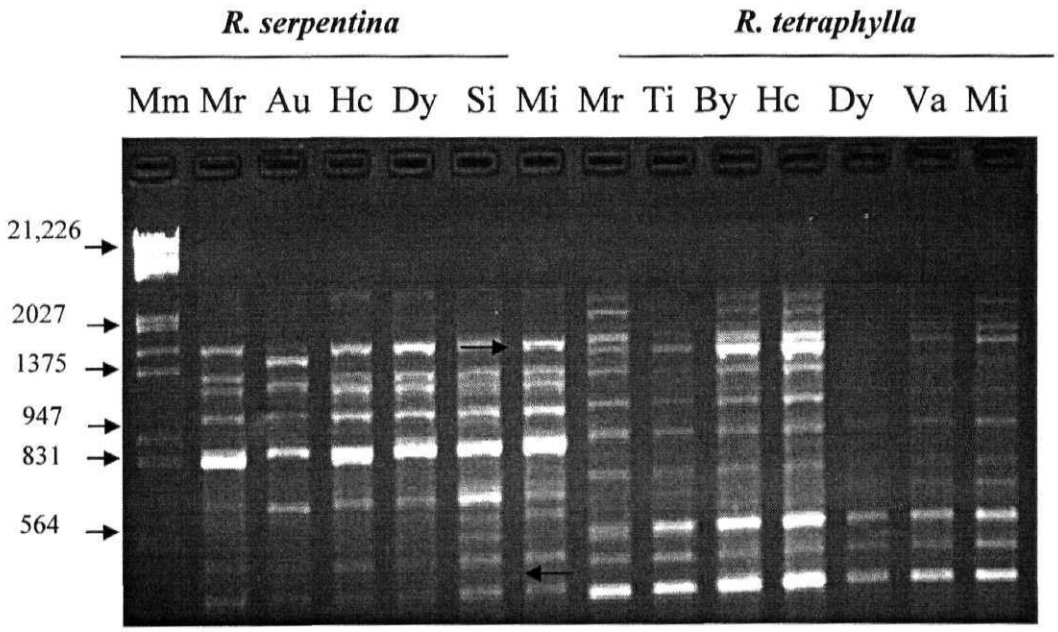


Figure 96: RAPD profile using primer OPC-20 (5'ACTTCGCCAC3'). Polymorphic markers are generated in *R. serpentina* and monomorphic bands are observed among all the accessions of *R. tetraphylla*

Table 28: Represents polymorphism among six accessions of *R. serpentina*

Primer Code	Primer sequence 5' –3'	TB	PB	% of polymorphism
OPA-01	CAGGCCCTTC	6	6	100
OPA-02	TGCCGAGCTG	2	2	100
OPA-03	AGTCAGCCAC	16	16	100
OPA-04	AATCGGGCTG	9	9	100
OPA-05	AGGGGTCTTG	12	12	100
OPA-06	GGTCCCTGAC	12	5	41.6
OPA-07	GAAACGGGTG	18	16	88.8
OPA-08	GTGACGTAGG	11	11	100
OPA-09	GGGTAACGCC	11	11	100
OPA-10	GTGATCGCAG	7	7	100
OPA-11	CAATCGCCGT	8	8	100
OPA-12	TCGGCGATAG	8	8	100
OPA-13	CAGCACCCAC	14	13	92.8
OPA-14	TCTGTGCTGG	11	11	100
OPA-15	TTCCGAACCC	7	7	100
OPA-16	AGCCAGCGAA	6	6	100
OPA-17	GACCGCTTGT	8	8	100
OPA-18	AGGTGACCGT	17	17	100
OPA-19	CAAACGTCGG	7	1	14.2
OPA-20	GTTGCGATCC	11	2	18.1
OPC-01	TTCGAGCCAG	8	7	87.5
OPC-02	GTGAGGCGTC	14	13	92.8
OPC-03	GGGGGTCTTT	12	4	33.3
OPC-04	CCGCATCTAC	6	3	50
OPC-05	GATGACCGCC	11	7	63.6
OPC-06	GAACGGACTC	11	4	36.3
OPC-07	GTCCCGACGA	6	0	0
OPC-08	TGGACCGGTG	13	4	30.7
OPC-09	CTCACCGTCC	5	3	60.0
OPC-10	TGTCTGGGTG	6	6	100
OPC-11	AAAGCTGCGG	12	6	50.0
OPC-12	TGTCATCCCC	9	4	44.4
OPC-13	AAGCCTCGTC	9	4	44.4
OPC-14	TGCGTGCTTG	11	7	63.6
OPC-15	GACGGATCAG	8	3	37.5
OPC-16	CACACTCCAG	7	2	28.5
OPC-17	TTCCCCCCAG	2	0	0
OPC-18	TGAGTGGGTG	8	3	37.5
OPC-19	GTTGCCAGCC	7	2	28.5
OPC-20	ACTTCGCCAC	13	7	53.8

Table 29: Accession wise polymorhic bands in *R. serpentina* with both A and C series of operon primers

Primer code	RS1		RS2		RS3		RS4		RS5		RS6	
	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB
OPA1	6	6	1	1	6	6	6	6	0	0	5	5
OPA2	2	2	2	2	2	2	2	2	0	0	2	2
OPA3	0	0	14	14	14	14	14	14	8	8	14	14
OPA4	9	9	2	2	5	8	8	8	0	0	6	6
OPA5	12	12	12	12	12	12	12	12	0	0	12	12
OPA6	10	3	10	3	10	3	11	4	10	3	9	2
OPA7	11	9	11	9	13	11	11	9	5	3	13	11
OPA8	6	6	7	7	7	7	7	7	1	1	10	10
OPA9	7	6	7	6	7	6	7	6	3	2	9	8
OPA10	7	7	7	7	7	7	7	7	0	0	7	7
OPA11	6	6	6	6	7	7	8	8	0	0	8	8
OPA12	7	7	7	7	7	7	7	7	0	0	8	8
OPA13	11	10	11	10	11	10	11	10	4	3	11	10
OPA14	3	3	4	4	5	5	7	7	1	1	8	8
OPA15	3	3	3	3	3	3	2	2	1	1	6	6
OPA16	6	6	6	6	6	6	6	6	0	0	6	6
OPA17	5	4	6	5	5	4	5	4	4	3	5	4
OPA18	16	16	12	12	14	14	1	1	8	8	13	13
OPA19	7	1	6	0	6	0	6	0	6	0	6	0
OPA20	10	1	10	1	10	1	11	1	9	0	9	0
OPC1	8	7	7	6	3	2	5	4	1	0	7	6
OPC2	9	8	9	8	9	8	10	9	4	3	9	8
OPC3	8	0	8	0	11	3	11	3	12	3	11	3
OPC4	6	3	3	0	3	0	3	0	3	0	3	0
OPC5	5	1	8	4	8	4	8	4	11	7	6	2
OPC6	10	3	10	3	10	3	10	3	9	2	8	1
OPC7	6	0	6	0	6	0	6	0	6	0	6	0
OPC8	12	3	12	3	12	3	13	4	9	0	13	4
OPC9	2	0	2	0	2	0	2	0	5	3	2	0
OPC10	6	6	6	6	6	6	6	6	0	0	6	6
OPC11	11	5	10	4	10	4	9	3	7	1	8	2
OPC12	9	4	7	2	6	1	6	1	5	0	6	14
OPC13	9	4	7	2	6	1	6	1	5	0	9	3
OPC14	10	6	10	6	9	5	5	1	8	4	7	1
OPC15	6	1	5	0	6	1	6	1	8	4	6	1
OPC16	6	1	6	1	6	1	6	1	7	2	6	0
OPC17	2	0	2	0	2	0	2	0	2	0	2	2
OPC18	7	2	7	2	7	2	7	2	6	1	7	0
OPC19	6	1	5	0	5	0	5	0	6	1	5	0
OPC20	11	5	10	4	11	5	11	5	12	6	10	4

Table 30: Accessions of *R. serpentina* that showed unique markers and where there is no amplification.

Uniquemarker/No ampllification	**Primer Code**	**Location from where the accession is collected**
No amplification	OPA-1	Sukumamidi
1	OPA-3	Sukumamidi
No amplification	OPA-4	Sukumamidi
No amplification	OPA-5	Sukumamidi
No amplification	OPA-6	Sukumamidi
1	OPA-8	Sukumamidi
1	OPA-9	Sukumamidi
No amplification	OPA-10	Sukumamidi
No amplification	OPA-11	Sukumamidi
No amplification	OPA-12	Sukumamidi
1	OPA-13	Sukumamidi
5	OPA-14	Sukumamidi
1	OPA-15	Hyderabad,
No amplification	OPA-15	Sukumamidi
No amplification	OPA-16	Sukumamidi
2	OPA-18	Sukumamidi
No amplification	OPA-20	Dulapally
1	OPC-1	Sukumamidi
4	OPC-2	Sukumamidi
2	OPC-3	Sukumamidi, Maredumilli
1	OPC-9	Maredumilli
No amplification	OPC-12	Sukumamidi
1	OPC-15	Sukumamidi
2	OPC-16	Sukumamidi, Maredumilli
No amplification	OPC-17	In all the accessions

	1	2	3	4	5	6
RSMR	1.00					
RSAU	0.90	1.00				
RSHC	0.90	0.90	1.00			
RSDY	0.86	0.90	0.92	1.00		
RSSI	0.59	0.64	0.63	0.61	1.00	
RSMI	0.86	0.87	0.88	0.87	0.61	1.00

Figure 97: Similarity matrix of *R. serpentina* generated from Dice estimate of similarity based on the number of shared fragments

The percentage of polymorphism observed for each accession with all the primers is calculated which is 61% for accessions collected from Mallur (Warangal), 58%, Araku (Vishakapatnam), 60% Hyderabad, 60% Dulapally (Medak), 43% Sukumamidi (East Godavari) and 60% Maredumilli (East Godavari). The primer with maximum number of polymorphic bands is OPA-18 (17 bands) and the primer with minimum number of polymorphic bands is OPA-19 (1 band) and the primer, which did not show any polymorphism is OPC-7 i.e., which exhibited complete monomorphism. Hence the range of the bands generated for all the primers falls between 0-17. There is no primer, which did not exhibit amplification. The GC% of all the primers was ranging from 60-70%, which did not show any effect on amplification. The average number of polymorphic bands generated per primer is 6.5 out of the total number of bands of 9.4. The primers OPA-01, OPA-02, OPA-03, OPA-04, OPA-05 OPA-08, OPA-09, OPA-10, OPA-11, OPA-2, OPA-14, OPA-15, OPA-16, OPA-17, OPA-18 and OPC-10 exhibited 100 % polymorphism with all the accessions. The size of the amplified fragments ranged from 300-3,500 bp. The number of loci and polymorphic loci varies widely between primers and populations. The highest within genetic variation was detected in the accession collected from of Sukumamidi as it was falling under a separate group when compared to other accessions.

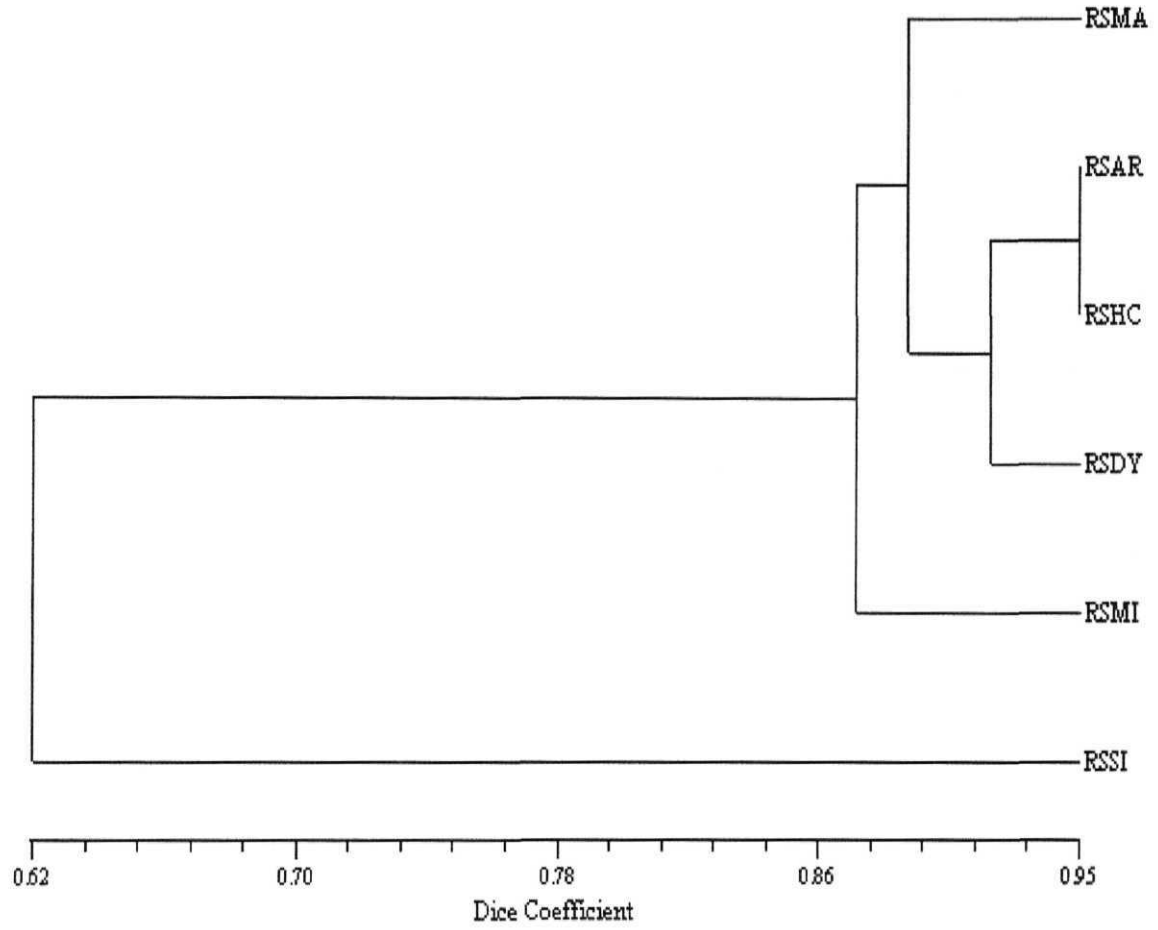


Figure 98: Cluster diagram of six accessions of *R. serpentina* based on Dice genetic identity

6.8. RAPD analysis of *R. tetraphylla*

A total of 40 primers were screened from OPA and OPC series for appropriate amplification and RAPD pattern formation. Analysis of 7 accessions of *R. tetraphylla* revealed 51.6% of polymorphism. Polymorphic markers are generated among the 7 accessions of *R. tetraphylla*, which were similar to some of the markers in *R. serpentina*. The polymorphism was less when compared to other plant species of study. A few unique markers (3) have been observed (Figures 57-96). The screening of the entire set of samples was repeated thrice to assess repeatability of the RAPD profiles and identical RAPD patterns were obtained. The number of scorable polymorphic markers generated is 205 out of 397 total markers generated (table 31). The polymorphism level with each accession is calculated and (table 32). Levels of genetic diversity within accessions i.e., the genetic distance ranged from 0.816 – 0.932. The minimum genetic distance of 0.816 is exhibited between the accessions collected Dulapally (Medak) and Hyderabad, whereas the accessions

Table 31: Polymorphism among seven accessions of *R. tetraphylla*

Primer Code	Primer sequence 5' – 3'	TB	PB	% polymorphism
OPA-01	CAGGCCCTTC	13	5	38.4
OPA-02	TGCCGAGCTG	4	4	100
OPA-03	AGTCAGCCAC	12	5	41.6
OPA-04	AATCGGGCTG	13	8	61.5
OPA-05	AGGGGTCTTG	9	4	44.4
OPA-06	GGTCCCTGAC	12	5	41.6
OPA-07	GAAACGGGTG	13	2	15.3
OPA-08	GTGACGTAGG	14	3	21.4
OPA-09	GGGTAACGCC	14	11	78.5
OPA-10	GTGATCGCAG	10	6	60.0
OPA-11	CAATCGCCGT	15	11	73.3
OPA-12	TCGGCGATAG	3	0	0
OPA-13	CAGCACCCAC	14	8	57.1
OPA-14	TCTGTGCTGG	7	2	28.5
OPA-15	TTCCGAACCC	5	2	40.0
OPA-16	AGCCAGCGAA	7	4	57.1
OPA-17	GACCGCTTGT	6	1	16.6
OPA-18	AGGTGACCGT	11	2	18.1
OPA-19	CAAACGTCGG	5	2	40.0
OPA-20	GTTGCGATCC	11	2	18.1
OPC-01	TTCGAGCCAG	5	5	100
OPC-02	GTGAGGCGTC	9	4	44.4
OPC-03	GGGGGTCTTT	14	14	100
OPC-04	CCGCATCTAC	14	6	42.8
OPC-05	GATGACCGCC	15	5	33.3
OPC-06	GAACGGACTC	12	5	41.6
OPC-07	GTCCCGACGA	10	7	70.0
OPC-08	TGGACCGGTG	12	7	58.3
OPC-09	CTCACCGTCC	6	3	50.0
OPC-10	TGTCTGGGTG	12	4	33.3
OPC-11	AAAGCTGCGG	15	13	86.6
OPC-12	TGTCATCCCC	10	3	30.0
OPC-13	AAGCCTCGTC	12	11	91.6
OPC-14	TGCGTGCTTG	7	3	42.8
OPC-15	GACGGATCAG	10	3	30.0
OPC-16	CACACTCCAG	10	5	50.0
OPC-17	TTCCCCCCAG	4	4	100
OPC-18	TGAGTGGGTG	17	11	64.0
OPC-19	GTTGCCAGCC	4	2	50.0
OPC-20	ACTTCGCCAC	11	3	27.2

Table 32: Depicts the accession wise polymorhic bands in *R. tetraphylla* with both A and C series of Operon primers

Primer code	RT1		RT2		RT3		RT4		RT5		RT6		RT7	
	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB
OPA1	13	5	10	2	13	5	13	5	8	0	8	0	8	0
OPA2	4	4	4	4	4	4	4	4	0	0	0	0	0	0
OPA3	9	1	11	3	10	2	11	3	11	3	10	2	10	2
OPA4	9	4	9	4	5	0	13	6	11	6	7	2	6	1
OPA5	8	0	9	1	9	1	9	1	9	1	9	1	9	1
OPA6	12	5	12	5	12	5	8	1	10	3	10	3	10	3
OPA7	13	2	13	2	13	2	13	2	11	0	12	1	13	2
OPA8	9	0	12	3	14	5	14	5	11	2	11	2	11	2
OPA9	11	8	12	9	11	8	11	8	4	1	6	3	10	7
OPA10	10	6	7	3	7	3	7	3	6	2	7	3	4	0
OPA11	11	7	6	2	5	1	9	5	5	1	5	1	4	0
OPA12	3	0	3	0	3	0	3	0	3	0	3	0	3	0
OPA13	13	7	13	7	6	0	6	0	13	7	13	7	11	5
OPA14	6	1	6	1	7	2	7	2	5	0	5	0	5	0
OPA15	5	2	4	1	3	0	3	0	4	1	4	1	3	0
OPA16	7	4	7	4	6	3	3	0	7	4	7	4	7	4
OPA17	6	1	6	1	5	0	5	0	6	1	6	1	6	1
OPA18	9	0	9	0	10	1	10	1	9	0	9	0	9	0
OPA19	3	0	3	0	3	0	5	2	3	0	3	0	3	0
OPA20	11	2	11	2	11	2	11	2	11	2	11	2	11	2
OPC1	5	3	9	3	2	0	5	3	5	3	4	1	4	1
OPC2	9	4	9	4	9	4	9	4	7	2	8	3	5	0
OPC3	9	9	3	3	7	7	10	10	9	9	10	10	10	10
OPC4	11	3	11	3	11	3	13	5	9	1	11	3	10	2
OPC5	12	2	14	4	11	1	12	2	11	1	11	1	11	1
OPC6	12	5	12	5	11	4	9	2	9	2	10	3	12	5
OPC7	8	5	7	4	9	6	9	6	7	4	4	1	5	2
OPC8	12	7	10	5	9	4	12	7	5	0	5	0	6	1
OPC9	3	0	3	0	3	0	6	3	3	0	3	0	3	0
OPC10	12	4	11	3	11	3	10	2	8	0	8	0	8	0
OPC11	11	9	11	9	10	8	13	11	10	8	13	11	5	8
OPC12	10	3	8	1	7	0	9	2	7	0	7	0	8	1
OPC13	4	2	4	2	7	5	7	5	5	3	4	2	8	6
OPC14	5	1	4	0	4	0	7	3	4	0	4	0	4	0
OPC15	10	3	9	2	9	2	9	2	7	0	9	2	0	0
OPC16	10	5	10	5	8	3	8	3	6	1	6	1	6	1
OPC17	3	3	4	4	3	3	3	3	0	0	0	0	0	0
OPC18	14	7	15	8	7	0	11	4	8	1	11	4	11	4
OPC19	3	2	3	2	3	2	4	3	3	2	3	2	2	1
OPC20	10	2	8	0	10	2	10	2	9	1	10	2	10	2

that exhibited a maximum genetic distance of 0.932 belongs to plants collected from Vijayawada and Dulapally (Medak). The mean value of genetic distance among the accessions is 2.733 (Figure 99). The mean values of genetic distances calculated in plant species which are grouped together in Cluster analysis irrespective of the geographical distances are the accessions collected from Dulapally and Vijayawada showed a genetic distance of 0.932, Balpally and Hyderabad with a genetic distance of 0.903, Mallur (warangal) and Tirupathi (Chittoor) with a genetic distance of 0.915 (Figure 100). Hyderabad, Balpally (Kadapa), Maredumilli (East Godavari) and Mallur (Warangal) accessions have charesteric bands. The percentage of polymorphism observed for each accession with all the primers is calculated which is 41% for accession from Mallur, 37% Tirupathi, 37% Balpally (Kadapa), 39% Hyderabad, 26% Dulapally, 28% Vijayawada and 28% Maredumilli (East Godavari). The primer with maximum number of polymorphic bands is OPC-3 (14 bands), with minimum number of polymorphic bands is OPA-17 (1 band) and the primer which did not show any polymorphism is OPA-12 i.e., exhibited complete monomorphism. Hence the range of the bands generated for all the primers falls between 0-14. All prmers exhibited amplification. The GC % of all the primers was ranging from 60-70%, did not show any effect on amplification. The average number of polymorphic bands per primer generated is 5.1 out of the total number of bands of 9.9. The primers OPA- 01, OPC-02, OPC-03 and OPC-17 exhibited 100 % polymorphism. The size of the amplified fragments ranged from 300-3,500 bp. The number of loci and polymorphic loci varied widely between primers and populations.

	1	2	3	4	5	6	7
RTMR	1.000						
RTTI	0.915	1.000					
RTBY	0.888	0.893	1.000				
RTHC	0.880	0.885	0.903	1.000			
RTDY	0.823	0.850	0.828	0.816	1.000		
RTVA	0.851	0.878	0.858	0.834	0.932	1.000	
RTMI	0.820	0.862	0.841	0.803	0.866	0.907	1.000

Figure 99: Similarity matrix of *R. tetraphylla* generated from Dice estimate of similarity based on the number of shared fragments.

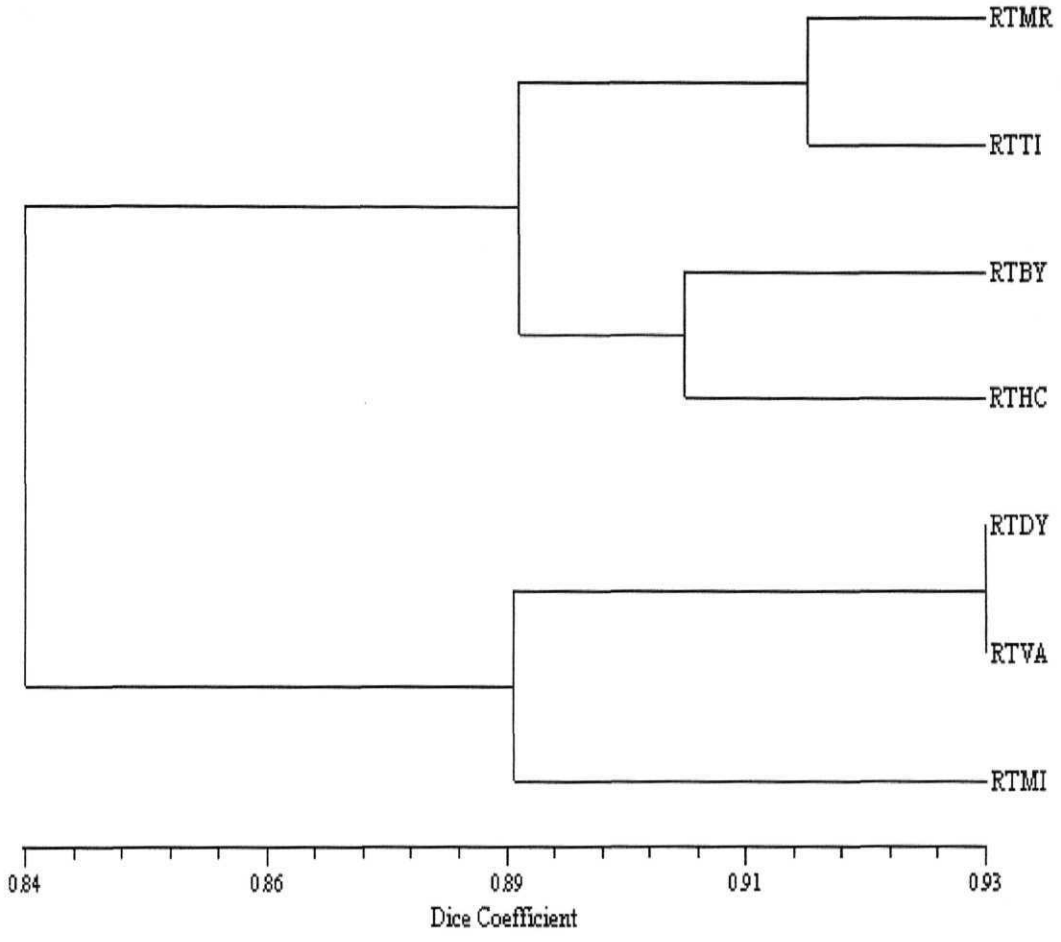


Figure 100: Cluster diagram of seven accessions of *R.tetraphylla* based on Dice genetic identity

6.9. Genetic similarity of *R. serpentina* and *R. tetraphylla* by RAPD analysis

Analysis of both *R. serpentina* (6) and *R. tetraphylla* (7) revealed 89.4% of polymorphism. RAPD profile for the genetic similarity of six accessions of *R. serpentina* and seven accessions *R. tetraphylla* on 2% agarose gels using 40 primers showed a similarity of 80% when the number of poly and monomorphic bands were scored. (Figure 57-96). The screening of the entire set of samples was repeated thrice to assess repeatability of the RAPD profiles, and identical RAPD patterns were obtained. The number of scorable polymorphic markers generated are 457 out of 511 total markers generated (Table 33) and the polymorphism in accessions (Tables 34 and 35). Levels of genetic diversity within accessions i.e., the genetic distance ranged from 0.407 – 0.955. The mimimum genetic distance of 0.407 (Figure 101) is exhibited between the accessions collected from Tirupathi (Chittoor) and Sukumamidi (Khamam) whereas the accessions that exhibited a maximum genetic distance of 0.955 belongs to plants collected from Dulapally and Hyderabad. The mean value of genetic distance among the accessions is 3.940. The mean values of genetic distances calculated in plant species which are grouped together in Cluster analysis irrespective of the geographical distances are the accessions collected from Dulapally (Medak) and Vijayawada showed a genetic distance of 0.499, Balpally (Kadapa) and Hyderabad showed a genetic distance of 0.943, Maredumilli (East Godavari) to Tirupathi showed a

Table 33: Polymorphism among *R. serpentina* (6 accessions) and *R. tetraphylla* (7 accessions).

Primer Code	Primer sequence 5' – 3'	TB	PB	% polymorphism
OPA-01	CAGGCCCTTC	12	12	100
OPA-02	TGCCGAGCTG	8	8	100
OPA-03	AGTCAGCCAC	14	14	100
OPA-04	AATCGGGCTG	12	12	100
OPA-05	AGGGGTCTTG	15	15	100
OPA-06	GGTCCCTGAC	18	15	83.3
OPA-07	GAAACGGGTG	16	11	68.75
OPA-08	GTGACGTAGG	13	13	100
OPA-09	GGGTAACGCC	15	15	100
OPA-10	GTGATCGCAG	11	11	100
OPA-11	CAATCGCCGT	16	16	100
OPA-12	TCGGCGATAG	8	8	100
OPA-13	CAGCACCCAC	19	19	100
OPA-14	TCTGTGCTGG	10	10	100
OPA-15	TTCCGAACCC	7	7	100
OPA-16	AGCCAGCGAA	13	13	100
OPA-17	GACCGCTTGT	11	10	90.9
OPA-18	AGGTGACCGT	13	13	100
OPA-19	CAAACGTCGG	8	7	87.5
OPA-20	GTTGCGATCC	14	7	50.0
OPC-01	TTCGAGCCAG	11	10	90.9
OPC-02	GTGAGGCGTC	14	10	71.4
OPC-03	GGGGGTCTTT	13	13	100
OPC-04	CCGCATCTAC	17	15	88.2
OPC-05	GATGACCGCC	20	18	90.0
OPC-06	GAACGGACTC	17	12	70.5
OPC-07	GTCCCGACGA	14	12	85.7
OPC-08	TGGACCGGTG	16	10	62.5
OPC-09	CTCACCGTCC	8	7	87.5
OPC-10	TGTCTGGGTG	8	8	100
OPC-11	AAAGCTGCGG	12	10	83.3
OPC-12	TGTCATCCCC	11	5	45.5
OPC-13	AAGCCTCGTC	13	13	100
OPC-14	TGCGTGCTTG	11	10	90
OPC-15	GACGGATCAG	15	14	93.3
OPC-16	CACACTCCAG	12	11	91.6
OPC-17	TTCCCCCCAG	7	7	100
OPC-18	TGAGTGGGTG	16	15	93.7
OPC-19	GTTGCCAGCC	9	8	88.8
OPC-20	ACTTCGCCAC	16	13	81.2

Table 34: Accession wise polymorhic bands in both *R. serpentina* and *R. tetraphylla* with A series of Operon primers (accessions 1-6: *R. serpentina*; accessions 1-7: *R. tetraphylla*).

Primer	**1**		**2**		**3**		**4**		**5**		**6**		**1**		**2**		**3**		**4**		**5**		**6**		**7**	
Code	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB
OPA1	5	5	1	1	4	4	4	4	0	0	2	2	11	11	9	9	11	11	11	11	7	7	7	7	7	7
OPA2	3	3	3	3	3	3	3	3	0	0	3	3	4	4	5	5	5	5	5	5	1	1	0	0	0	0
OPA3	0	0	10	10	10	10	10	10	7	7	11	11	10	10	10	10	11	11	11	11	10	10	9	9	9	9
OPA4	4	4	1	1	4	4	4	4	0	0	4	4	6	6	8	8	5	5	11	11	8	8	7	7	6	6
OPA5	9	9	9	9	9	9	9	9	0	0	10	10	11	11	10	10	10	10	10	10	10	10	10	10	10	10
OPA6	10	7	10	7	10	7	10	7	9	6	8	5	10	7	13	10	13	10	13	10	8	5	12	9	12	9
OPA7	9	7	9	7	12	10	10	8	4	2	8	6	10	8	10	8	10	8	10	8	9	7	10	8	10	8
OPA8	5	5	6	6	6	6	6	6	1	1	7	7	8	8	9	9	12	12	12	12	9	9	9	9	9	9
OPA9	7	7	7	7	7	7	7	7	1	1	9	9	12	12	10	10	11	11	9	9	5	5	6	6	8	8
OPA10	7	7	7	7	7	7	7	7	0	0	6	6	9	9	9	9	9	9	9	9	8	8	8	8	8	8
OPA11	8	8	8	8	8	8	8	8	0	0	7	7	11	11	10	10	9	9	12	12	10	10	10	10	10	10
OPA12	7	7	7	7	7	7	7	7	1	1	6	6	3	3	3	3	3	3	3	3	3	3	3	3	2	2
OPA13	9	9	9	9	9	9	9	9	4	4	10	10	13	13	13	13	4	4	4	4	15	15	15	15	13	13
OPA14	3	3	4	4	4	4	5	5	0	0	7	7	7	7	7	7	7	7	7	7	5	5	5	5	5	5
OPA15	3	3	3	3	3	3	2	2	1	1	4	4	3	3	4	4	3	3	3	3	4	4	4	4	4	4
OPA16	4	4	4	4	4	4	4	4	0	0	4	4	7	7	11	11	4	4	4	4	8	8	8	8	8	8
OPA17	5	4	6	5	4	3	4	3	8	7	7	6	8	7	8	7	6	5	6	5	6	5	8	7	7	6
OPA18	10	10	8	8	3	3	4	4	6	6	10	10	8	8	8	8	10	10	10	10	10	10	9	9	9	9
OPA19	6	5	5	4	5	4	5	4	5	4	5	4	3	2	3	2	3	2	3	2	3	2	3	2	3	2
OPA20	9	2	10	3	9	2	9	2	10	3	9	2	12	5	12	5	12	5	12	5	12	5	12	5	12	5

Table 35: Accession wise polymorhic bands in both *R. serpentina* and *R. tetraphylla* with C series of Operon primers (accessions 1-6: *R. serpentina*; accessions 1-7: *R. tetraphylla*).

Primer	**1**		**2**		**3**		**4**		**5**		**6**		**1**		**2**		**3**		**4**		**5**		**6**		**7**	
Code	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB
OPC1	9	9	7	7	5	5	6	6	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
OPC2	11	7	11	7	11	7	12	8	7	3	10	6	12	8	12	8	13	9	13	9	10	6	13	9	10	6
OPC3	8	8	8	8	10	10	10	10	10	10	10	12	12	3	3	7	7	10	10	10	10	10	10	10	11	11
OPC4	7	5	4	2	4	2	4	2	4	2	14	12	14	12	14	12	14	12	15	13	14	12	14	12	14	12
OPC5	6	4	9	7	9	7	8	6	8	6	6	4	12	10	13	11	12	10	12	10	12	10	12	10	12	10
OPC6	10	5	10	5	10	5	10	5	9	4	9	4	13	9	13	9	13	9	13	9	10	6	12	8	12	8
OPC7	10	3	10	3	10	3	12	5	9	2	12	10	12	10	11	9	11	9	11	9	7	5	7	5	7	5
OPC8	10	3	10	3	10	3	12	5	9	2	12	10	12	10	11	9	11	9	11	9	7	0	7	0	7	0
OPC9	1	0	1	0	1	0	1	0	1	0	1	0	5	4	5	4	5	4	5	4	8	7	5	4	8	7
OPC10	2	2	2	2	3	3	3	3	0	0	2	2	8	8	7	7	7	7	7	7	7	7	7	7	7	7
OPC11	8	6	6	4	6	4	6	4	6	4	7	5	9	7	8	6	8	6	10	8	7	5	10	8	5	3
OPC12	8	2	6	0	6	0	6	0	5	0	6	0	8	2	9	3	9	3	11	5	9	3	9	3	9	3
OPC13	6	6	5	5	5	5	7	7	3	3	9	9	4	4	4	4	6	6	6	6	4	4	4	4	7	7
OPC14	6	5	6	5	6	5	4	3	7	6	6	5	5	4	4	3	4	3	7	6	3	2	3	2	4	3
OPC15	7	6	5	4	6	5	6	5	7	6	8	7	11	10	11	10	11	10	11	10	9	8	11	10	11	10
OPC16	7	6	7	6	7	6	7	6	7	6	6	5	8	7	8	7	6	5	7	6	4	3	5	4	6	5
OPC17	6	5	6	5	6	5	6	5	6	5	7	6	12	11	13	12	8	7	10	9	8	7	10	9	10	9
OPC18	6	5	6	5	6	5	6	5	6	5	7	6	12	11	13	12	8	7	10	9	8	7	10	9	10	9
OPC19	6	5	5	4	5	4	5	4	6	5	5	4	3	2	3	2	3	2	3	2	3	2	3	2	3	2
OPC20	10	7	10	7	10	7	10	7	10	7	9	6	11	8	10	6	10	6	9	5	10	6	10	6	10	6

	1	2	3	4	5	6	7	8	9	10	11	12	13
RsMr	1.000												
RsAu	0.910	1.000											
RsHc	0.907	0.945	1.000										
RsDy	0.902	0.943	0.955	1.000									
RsSi	0.647	0.696	0.671	0.694	1.000								
RsMi	0.851	0.852	0.857	0.871	0.665	1.000							
RtMi	0.503	0.500	0.500	0.507	0.427	0.522	1.000						
RtMr	0.472	0.485	0.478	0.486	0.412	0.498	0.921	1.000					
RtTi	0.472	0.478	0.482	0.486	0.407	0.478	0.882	0.896	1.000				
RtBy	0.489	0.486	0.483	0.487	0.411	0.479	0.906	0.887	0.943	1.000			
RtHc	0.472	0.483	0.469	0.480	0.413	0.503	0.859	0.867	0.857	0.852	1.000		
RtDy	0.491	0.502	0.488	0.499	0.433	0.515	0.888	0.890	0.870	0.877	0.947	1.000	
RtVa	0.483	0.493	0.486	0.494	0.430	0.503	0.848	0.855	0.845	0.837	0.917	0.927	1.000

Figure 101: Similarity matrix of *R.serpentina and R.tetraphylla* generated from Dice estimate of similarity based on the number of shared fragments

of 0.921 which are grouped together in the dendrogram (figure 102. The percentage of polymorphism observed for each accession with all the primers is calculated which is 79.5% for *R. serpentina* (RS1) accession collected from Mallur, 79.4% (RS-Araku), 79.3% (RS-Hyderabad), 76.5%(RS-Dulapally), 71.2% (RS-Sukumamidi) and 82.8% (RS-Maredumilli), 86.2% (RT- Mallur), 85.8 (RT- Tirupathi), 87.8 (RT-Balpally), 86.0 (RT- Hyderabad), 82.2% (Dulapally), 83% (RT-Vijayawada) and 85.6% (RT-Maredumilli) The primer with maximum number of polymorphic bands are OPA-13 (19 bands) and the primer with minimum number of polymorphic bands are OPC-12 (5 bands) Hence the range of the bands generated for all the primers falls between 5-19. There is no primer, which did not exhibit amplification. The GC % of all the primers was ranging from 60-70%, which did not show any effect on amplification. The average number of polymorphic bands per primer generated is 11.4 out of the total number of bands of 12.7. The size of the amplified fragment ranged from 300-20,000bp. Amplified fragments ranged from 300 -3, 500 bp. The primers OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-08 OPA-09 to OPA-16, OPA-18, OPC-3, OPC-10, OPC-13, OPC-*17.*The number of loci and polymorphic loci varies widely between primers and populations.

6.11. Intrapopulation diversity of *R. serpentina* collected from Dulapally (Medak)

Spectrophotometric measurements of DNA at 260 and 280 nm revealed higher concentration of DNA, which was diluted to 50 ng/ul for further RAPD analysis as shown in figure 103. A total of 14 primers when screened from OPA and OPC series for appropriate amplification and pattern formation out of which all the 14

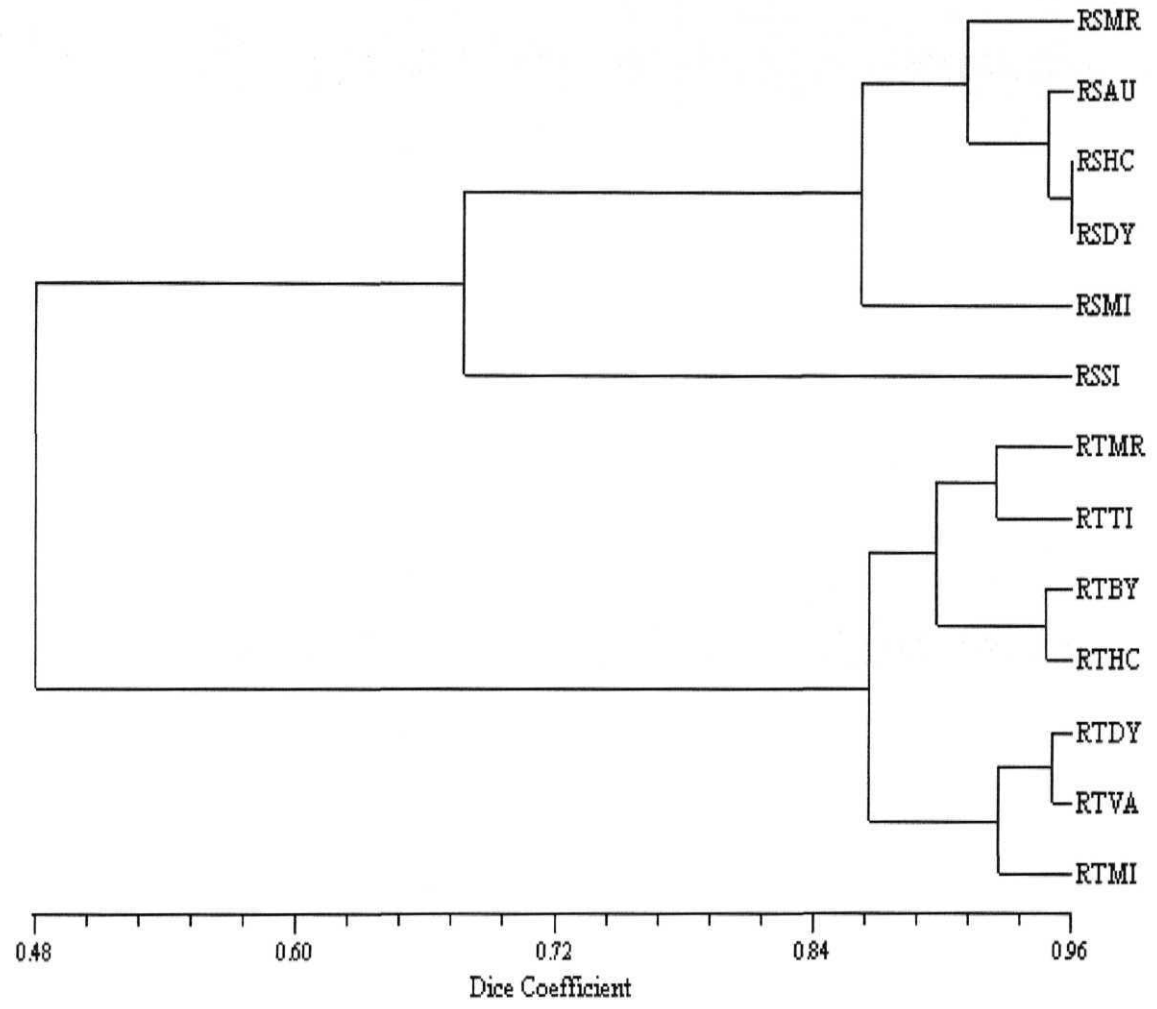


Figure 102: Cluster diagram of six accessions of *R. serpentina* and seven accessions of *R. tetraphylla* based on Dice genetic identity

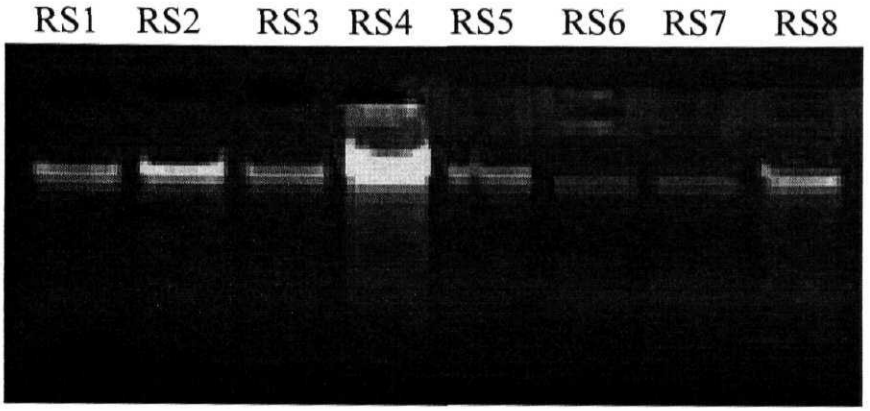


Figure103:Genomic DNA of various accessions of *R. serpentina* collected from Dulapally, location of Andhra Pradesh (India).

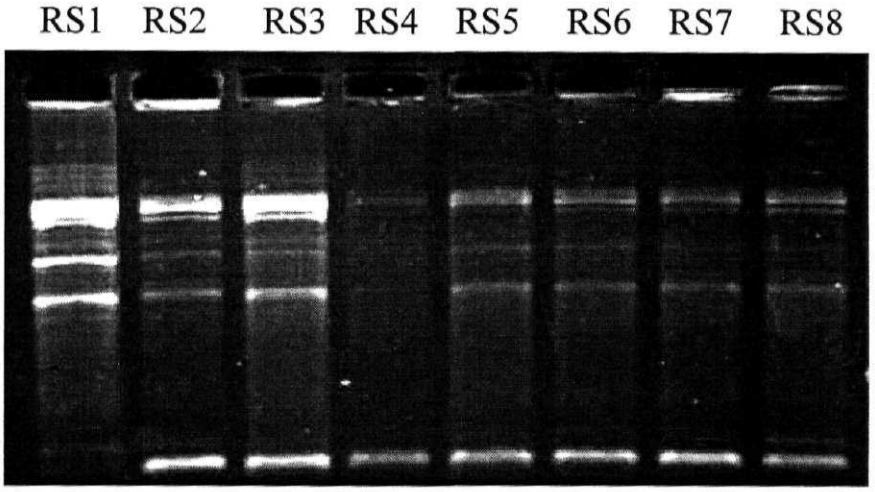


Figure 104: RAPD profile of *R. serpentina* collected from Dulapally area for intrapopulation studies where monomorphic bands are generated among all the accessions on 2% agarose gel using primer OPA-04 (5' AATCGGGCTG 3')

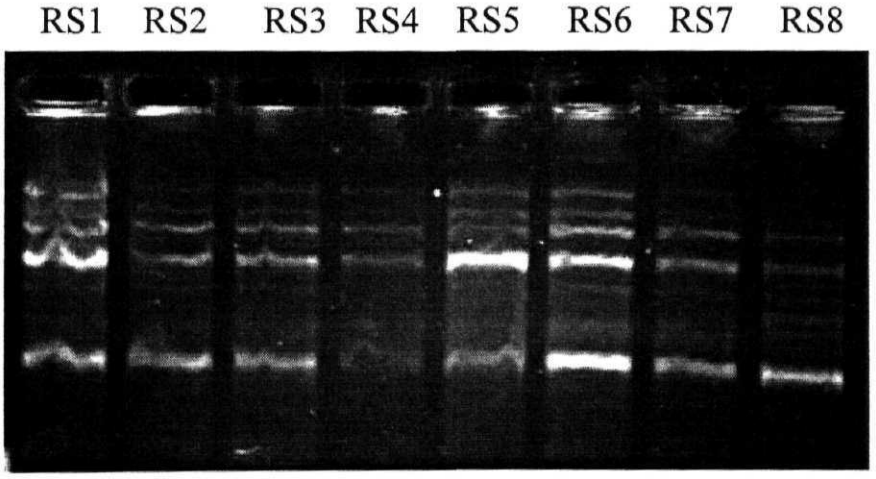


Figure 105:RAPD profile uusing primers OPA-06 (5' GGTCCCTGAC 3') Monomorphic bands are generated among all the accessions.

primers were selected for tests of the repeatability of the method. Analysis of eight plants of *R. serpentina* collected from similar location i.e., from Dulapally was revealed 77% of polymorphism. Screening of the entire set of samples was repeated thrice to assess repeatability of the RAPD profiles, and identical RAPD patterns were obtained. The number of scorable polymorphic markers generated are 86 out of 111 total markers generated as shown in table 36 and accession specific polymorphism is observed (table 37). Levels of genetic diversity within accessions i.e., the genetic distance ranged from 0.604 – 0.895. The minimum genetic distance of 0.604 is exhibited between the plants RS1 and RS4 collected from similar location, whereas the plants that exhibited a maximum genetic distance of 0.805 belongs to RS1 And RS3 collected from Dulapally. The mean value of genetic distance among the plants is 2.831. The percentage of polymorphism observed for each plant collected from the same location is 72.8% (RS1), 70.2% (RS2), 70.2% (RS3), 41.66% (RS4), 60.5% (RS5), 66.3% (RS6), 42.8% (RS7) and 61% (RS8) with the above mentioned primers. The primer with maximum number of polymorphic bands is OPA-10 (13 bands) and with minimum number of polymorphic bands is OPA-6 (3 bands) Hence the range of the bands generated for all the primers falls between 3-13. There is no primer, which did not exhibit amplification. The GC % of all the primers was ranging from 60-70%, which did not show any effect on amplification. The average number of polymorphic bands per primer generated is 6.1out of the total number of bands of 7.9. The primers that exhibit 100% polymorphism are OPC-4, OPA-14, OPA-13, OPC-19, OPA-10 and OPA-1. The size of the amplified fragments ranged from 300 bp -3, 500 bp. The

number of loci and polymorphic loci varies widely between primers and populations. (figures 104-113). Similarity matrix is shown in the figure 114 and the dendrogram is shown in the figure 115.

Table 36: Intrapopulation variation in eight plants of *R. serpentina* collected from Dulapally, Ranga Reddy District of Aandhra Pradesh.

Primer code	Primer sequence	Total bands	Polymorphic bands	% polymorphism
OPA-1	CAGGCCCTTC	7	7	100
OPA-2	TGCCGAGCTG	9	4	44.4
OPA-3	AGTCAGCCAC	9	4	44.4
OPA-6	GGTCCCTGAC	9	3	33.3
OPA-9	GGGTAACGCC	6	3	50
OPA-10	GTGATCGCAG	13	13	100
OPA-11	CAATCGCGT	6	5	83.3
OPA-13	CAGCACCCAC	12	12	100
OPA-14	TCTGTGCTGG	7	7	100
OPC-2	GTGAGGCGTC	2	0	0
OPC-3	GGGGGTCTTT	6	4	66.6
OPC-4	CCGCATCTAC	7	7	100
OPC-16	CACACTCCAG	7	6	85
OPC-19	GTTGCCAGCC	11	11	100

Table 37: Depicts the intrapopulation polymorphism in *R. serpentina* accession wise collected from Dulapally

Primer code	RS1		RS2		RS3		RS4		RS5		RS6		RS7		RS8	
	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB	TB	PB
OPA-1	7	7	6	6	7	7	0	0	1	1	2	2	1	1	1	1
OPA-2	9	4	9	4	7	2	6	1	7	2	9	4	6	1	7	2
OPA-3	9	4	9	4	9	4	5	0	5	0	9	4	9	4	9	4
OPA-6	8	2	8	2	8	2	6	0	7	1	7	1	7	1	9	3
OPA-9	5	2	5	2	5	2	4	1	5	2	6	3	4	1	5	2
OPA-10	13	13	8	8	7	7	7	7	10	10	6	6	8	8	5	5
OPA-11	6	5	6	5	6	5	1	0	5	4	6	5	6	5	6	5
OPA-13	11	8	10	7	10	7	3	0	11	8	12	9	5	2	11	8
OPA-14	7	7	7	7	7	7	0	0	5	5	6	6	6	6	1	1
OPC-2	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0
OPC-3	6	4	2	0	6	4	2	0	2	0	5	3	4	2	4	2
OPC-4	6	6	7	7	6	6	6	6	0	0	6	6	6	6	6	6
OPC-16	5	4	7	6	6	5	6	5	3	2	6	5	6	5	7	6
OPC-19	9	9	8	8	8	8	0	0	8	8	9	9	11	11	0	0

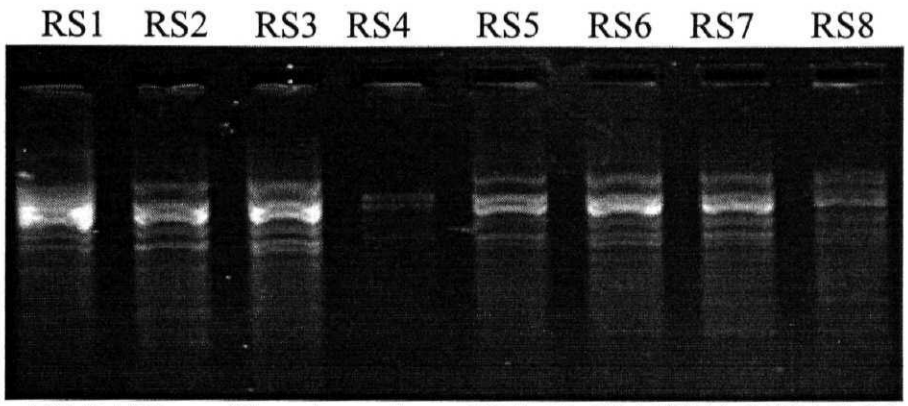


Figure 106:RAPD profile using primer OPA-03 (5'AGTCAGCCAC 3') Monomorphic bands are generated among all the accessions

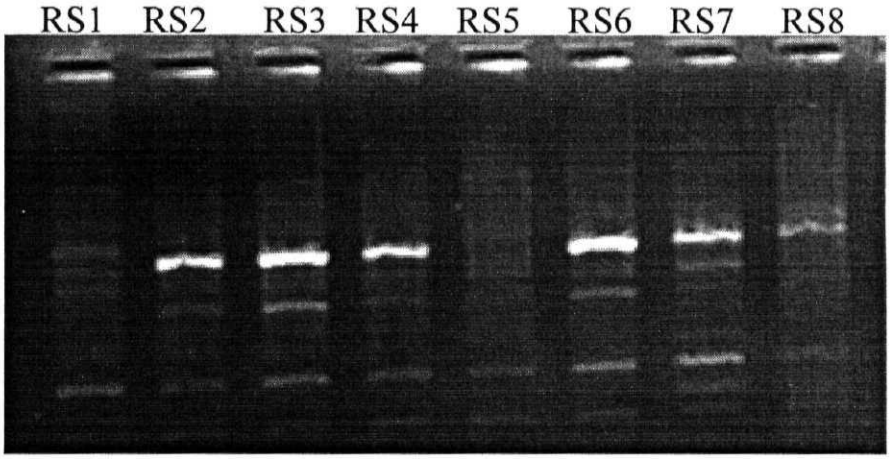


Figure107:RAPD profile using primer OPA-10 (5'GTGATCGCAG3'). Monomorphic bands are generated among all the accessions

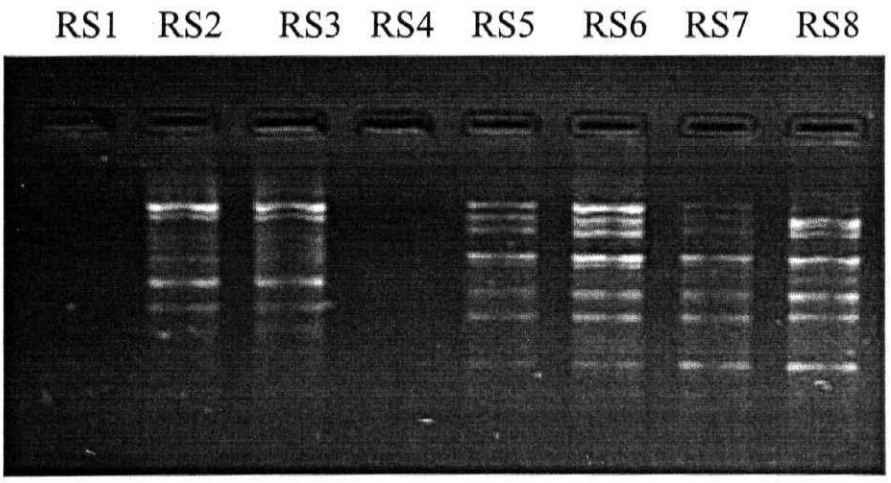


Figure 108:RAPD profile using primer OPA-11(5'CAATCGCCGT 3')Monomorphic bands are generated among all the accessions except in lane RS4 where there is no amplification

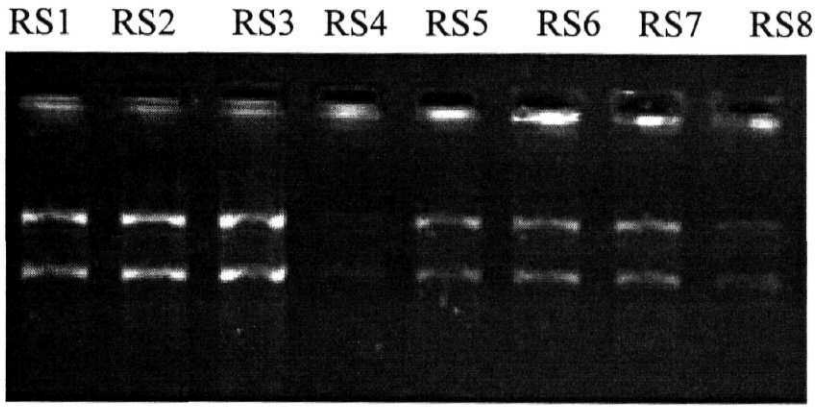


Figure109:RAPD profile using primer OPC-2 (5' GTGAGGCGTC 3').
Monomorphic bands are generated among all the accessions

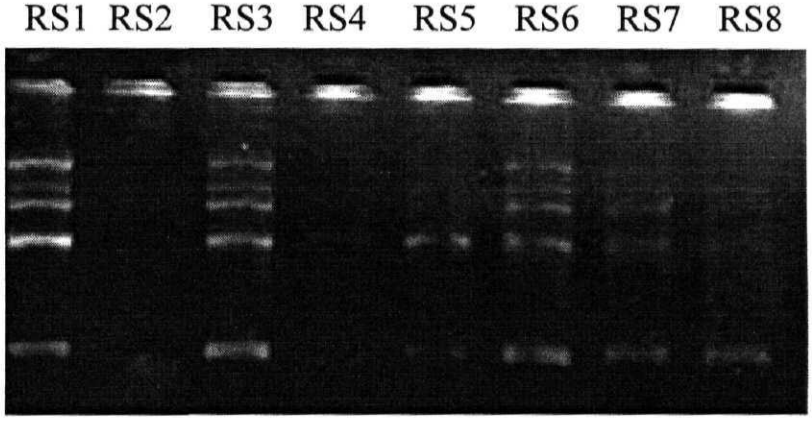


Figure110:RAPD profile using primer OPC-3 (5'GGGGGTCTTT 3').
Monomorphic bands are generated among all the accessions

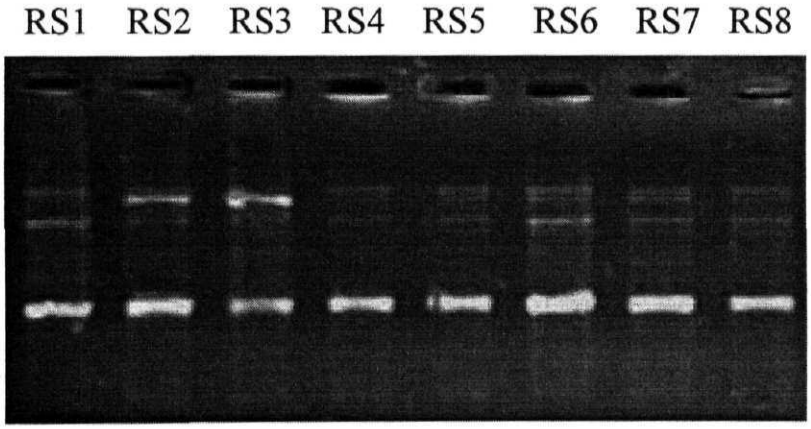


Figure111:RAPD profile using primer OPC-4 (5'CCGCATCTAC3').
Monomorphic bands are generated among all the accessions

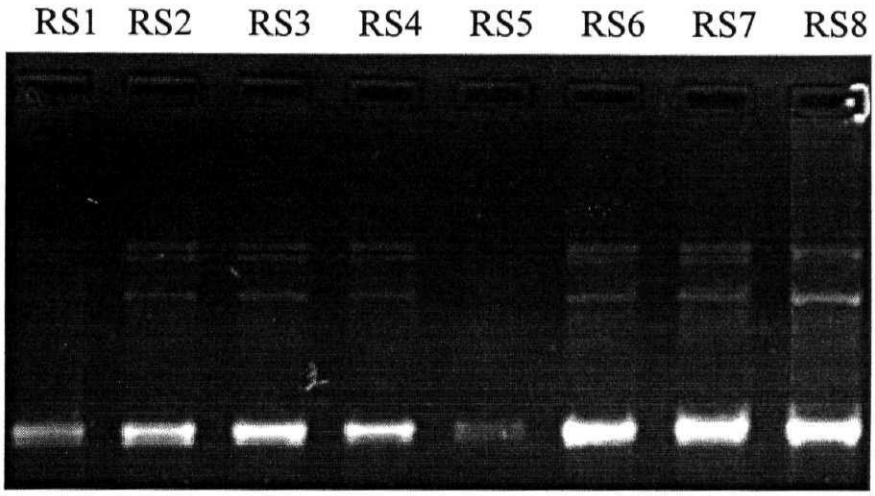


Figure112:RAPD profile using primer OPC-16 (5'CACACTCCAG3'). Monomorphic bands are generated among all the accessions except in lane RS5, one plant specific diagnostic marker was generated.

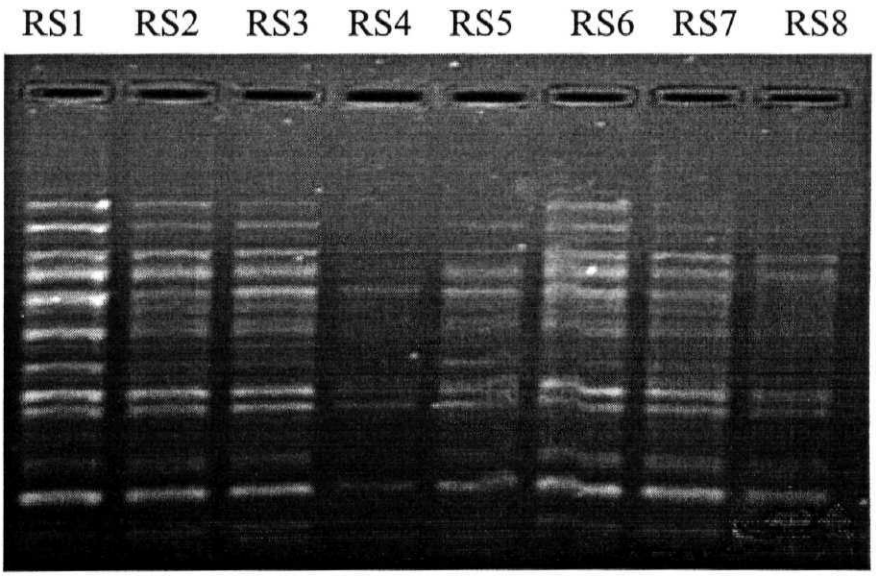


Figure113:RAPD profile using primer OPC-18 (5' TGAGTGGGTG3'). Monomorphic bands are generated among all the accessions.

	1	2	3	4	5	6	7	8
RS1	1							
RS2	0.827	1						
RS3	0.895	0.923	1					
RS4	0.604	0.713	0.673	1				
RS5	0.821	0.810	0.824	0.666	1			
RS6	0.786	0.858	0.858	0.703	0.802	1		
RS7	0.733	0.830	0.831	0.709	0.784	0.873	1	
RS8	0.658	0.781	0.746	0.758	0.717	0.717	0.8082	1

Figure 114: Similarity matrix of *R. serpentina* generated from Dice estimate of similarity based on the number of shared fragments (Dulapally).

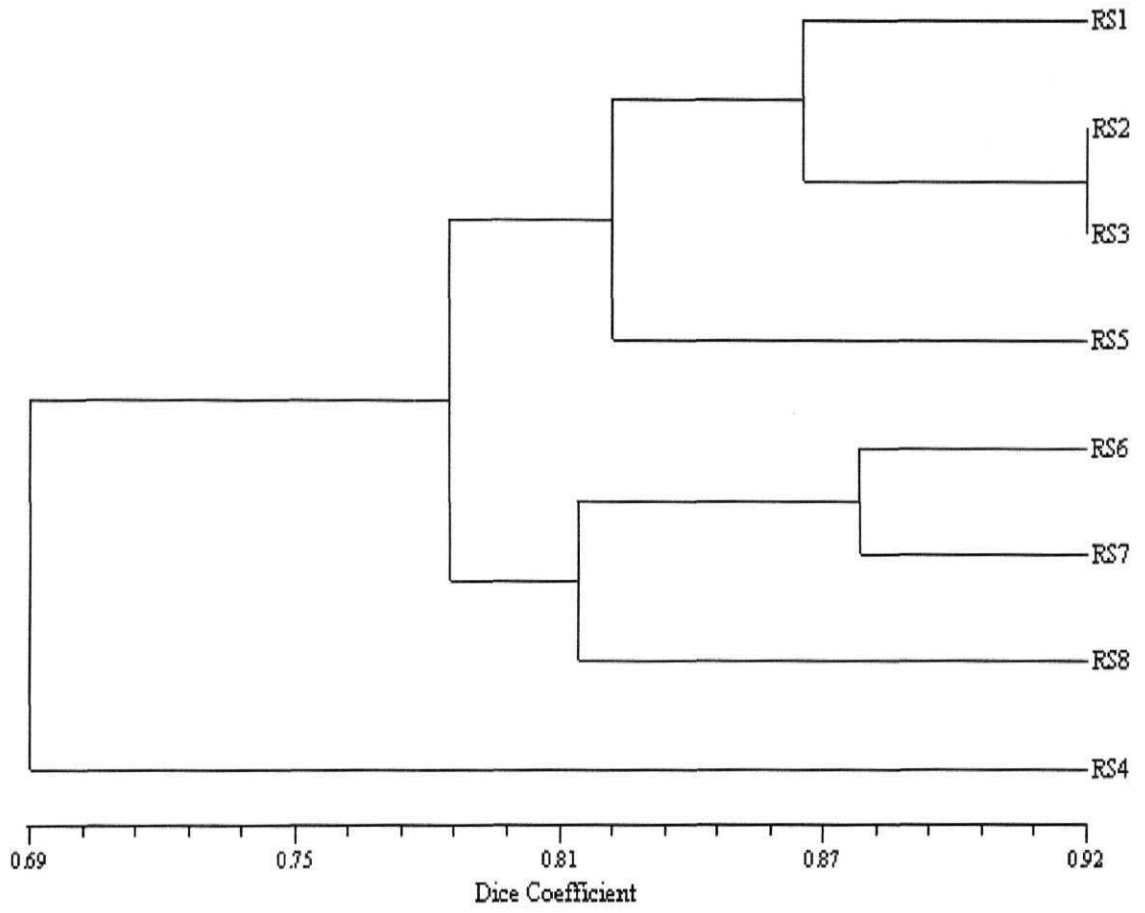


Figure 115: Cluster diagram of eight accessions of *R. serpentina* based on Dice genetic identity (Dulapally)

7. Discussion

Ex *situ* conservation is one of the strategies for conservation of the "red listed" medicinal plants (Figure 1). Assessment of the genetic diversity of the conserved germplasm by RAPDs was considered as one of the easy and simple techniques where the amplification occurs across the total genome as these are random primers (Figure 2). RAPD technology has been applied to a number of medicinal and aromatic plants of conservation concern (Table 1). To date there are many markers investigated which have both advantages and limitations. RAPD technology is the most commonly applied tool, for investigating the molecular diversity of medicinal plants. (Table 2).

7.1 Collection and conservation of chosen species

Pterocarpus santalinus is an endemic and endangered tree species (Figure 6) in Deccan ecoregion (Figure 3). Its distribution is restricted to the Kadapa (Cuddapah), Nellore and Chittoor districts of A.P (Figure 4). Different accessions were collected from 27 different locations during the months of May - September (Table 6). The seeds were air dried and accessions in the form of seedlings and plants (300) were grown in experimental site and gene bank (Figures 8 and 9). *R. serpentina* and *R. tetraphylla* are distributed in different districts of A.P (Figure 5). The seedlings and seeds were collected during the months of January to December from different locations of A.P (Tables 7 and 8) and were conserved in the experimental field and seed bank (Figures. 10 and 11).

Among the various *ex situ* conservation methods, seed storage is the most convenient strategy for long-term conservation as seeds being perennating structures of plants, represent a condition of suspending animation of embryos, which is best suited for storage (Kameshwararao, 2004). According to the Harrington's thumb rule of seed storage, lower the moisture level and temperature of stored seeds, greater is the storage life. Therefore, air dried seeds of all the collected accessions with low moisture level of 4 to 6% were placed in airtight plastic bags and stored in seed bank (Figures. 9, 10 and 11). Their viability was checked periodically every six months.

The importance of morphological features and time of collection of raw material is reported to affect the stored seeds viability (Rawat and Uniyal, 1993). Pod characteristics like length, width and weight, which indicates the amount of seed reserve varied among different accessions of *P. santalinus* (Figures 23 and 24) (Arya *et al.*, 1993). Apart from storage of seeds in seed bank, seedlings are also grown in experimental garden.

Several *in vitro* techniques have been developed for storage of vegetatively propagated and recalcitrant seed producing species. In general, they fall under two categories. Slow growth procedures where germplasm accessions are kept as sterile plant tissues or plantlets on nutrient media. This provides short and medium term storage options. Cryopreservation is another *in vitro* technique where plant material is stored in liquid nitrogen for long-term storage. Experiments conducted revealed that *in vitro* multiplication. in case of *P. santalinus* was not satisfactory. However *in vitro* conservation has some advantages, as the cultures are not subjected to environmental disturbances (Withers and Engelmann, 1997).

In *R. serpentina* and *R. tetraphylla* attempts have been made for micropropagation. Hence in the present study only genetic diversity and similarity in between both the species was investigated.

7.2. Seed germination in *P. santalinus*

Knowledge of seed germination and seedling establishment is a prerequisite for the successful implementation of conservation activities especially for tree species like *P. santalinus* (endangered). Factors affecting seed germination in *P. santalinus* include seed dormancy, temperature, water stress, predation, seed size, light intensity, soil moisture, seasonal variations, different locations of collection etc., According to Kalimuthu and Lakshmanan (1995), *P. santalinus* inherently shows poor seed germination.

Temperature plays a key role in germination of *P. santalinus.* The optimum temperature for seed germination of *P. santalinus* found to be between 20 and 25°C in *P. santalinus* (Teketay and Granstorm, 1997). The rate of germination varied under different light regimes in most of the examined species collected from the dry forests. Differential concentrations of mineral nutrients in embryos and seed coats also influence seedling establishment, irrespective of seed size.

Low viability is usually due to loss of moisture, which is associated with loss of hair from the seed coat. Increased leachate conductivity and decreased fatty acid content due to aging in certain seeds are other reasons for loss of viability and decline in germination percentage (Thapliyal and Connor, 1997).

Competition from annual herbaceous flora is one of the limiting factors for *in vivo* seed germination of *P. santalinus.* Seed size represented a trade off between

seedling establishment and seed dispersal efficiency in wind-dispersed tree species like *P. santalinus.* It influences the dispersal and seed water relations, emergence, establishment, survival and growth of seedlings. Small seeds have a better chance to enter into the soil easily than larger seeds, and thus, facilitate the build up of persistent soil seed banks, crucial for regeneration of plant species. A greater food reserve in larger seeds may enhance its ability to persist by providing metabolic requirements during quiescence period, until the availability of suitable conditions of light or moisture, which stimulates germination, thereby enhancing seedling survival and growth (Milberg and Lamont 1997). The young seedlings from large seeds withdraw nutrients for their successful establishment, survival and early seedling growth. Significant pod variation was noted among the collected accessions (Figure 22).

Dormancy in recalcitrant seeds can be overcome by mechanical or acid scarification or sometimes by transit through animal guts. *P. santalinus* seeds are highly recalcitrant with a dormancy period of one year. In the undisturbed forests of Kadapa (Cuddapah) district of A.P, (Figure 6a), *P. santalinus* is the most dominant species. The seeds are bigger compared to seeds collected from Vishakapatnam (A.P, India) and require shade for survival of the seedlings. Seeds from different locations exhibited differences in germination and seedling growth. Thus, emergence, establishment and growth of seedlings face very heterogeneous situations even in the native territory i.e., in Kadapa district (Cuddapah) and that of Veligonda Hills (Nellore District).

During *in vivo* seed germination many treatments have been used to break the dormancy. Acid, hot water and mechanical scarification have been applied to be found suitable in a majority of forestry species. In *P. santalinus,* mechanical scarification was found to be more effective. Such findings were also reported in *Olea auropaea* and *Podocarpus falacatus* by Teketay and Granstorm (1997). Pods of *P. santalinus* collected from various areas responded only to potassium nitrate (100 ppm) treatment (dormancy breaking agent) but the percentage of germination was very less (2%). Dormant seeds suppress the negative demographic effect of reproductive failure and permit the species to avoid environmental conditions potentially unfavorable for seedling establishment. A hard seed coat prevents entry of moisture during isolated showers in the middle of a long dry season while permitting the same during a sustained rainy season. Dormant seeds generally remain viable for long periods of time as in the case of seeds of *P. santalinus*. The extent of dormancy varies within a species, and as a result, individual seeds become permeable to water at different times, which results in staggered seedling recruitment providing an insurance against spells of unfavorable conditions. Thus the soil seed bank produces seedlings continuously during permeable conditions for several years due to different periods of dormancy.

In vivo seed germination studies in *P. santalinus* showed maximum seed germination in pods collected from Balpally (Kadapa District, A.P) when compared to other locations (Table 9 and Figures 12 a and b). The larger size of seeds and high germination rate compared to rest of the accessions place them under desirable and elite accessions.

In vitro experiments using Balpally (Kadapa District) seeds on MS (liquid), ½ MS (liquid), 3% sucrose, 1% agar and MS with 0.1% phytagel resulted in 100% seed germination. With 3% sucrose being the most cost effective (Table 10, Figure 13) and successful when cultured with the micropylar end touching the medium facilitating nutrient uptake from the medium. Thus, *in vitro* seed germination proved to be very useful for enhancing seed germination to build up seedling stocks.

7.3. *In vitro* plant regeneration

In vitro propagation of trees using tissue and organ culture has clearly proved to be a useful technique for multiplication of a number of forestry tree species. Direct organogenesis seems to be a desirable method since there is no callus phase involved in the shoots obtained and hence reducing the chance for induced variations. Forest trees in general, and legumes in particular are recalcitrant. Even though there are a few reports on organogenesis and micropropagation of tree legumes like *Acacia sps.* (Aradhana *et al.*, 1989), *Albizia sps.* (Tomar and Gupta, 1988), *Dalbergia sissoo* (Suwai *et al,* 1988) *Sesbania sp.* (Khattar and Mohanram, 1983) etc., regeneration has been mainly from seedling explants which is not desirable from the tree improvement point of view due to chances for variability and lower multiplication rate. Efficient regeneration is a prerequisite for genetic manipulation and transformation studies using different explants.

In nature, *P. santalinus* reproduces via seeds, but the low percentage of germination limits its propagation as it requires a long stratification period of about one year to break the dormancy partly by weathering process or by microorganisms (Arockiasamy *et al.*, 2000). Hence, an alternative method like *in vitro* propagation

was checked to understand whether the plant can be propagated true to its type or not. Among various explants used like nodal segments, shoot tips, leaves, cotyledonary nodal meristem, hypocotyls and seeds for multiple shoot regeneration, only whole seeds showed excellent response (Table.20). The explants used were collected from the plantations of UH campus and also from the in *vitro* regenerated seedlings (avoid problems of contamination). Varied morphogenetic responses were observed with different explants (Table 13).

7.3.1. Role of media, sucrose, agar and orientation

Initially nodal segment explants were cultured on different types of media and no significant difference was found (Table 11). Hence, further experiments were carried out on MS medium which is the most universally accepted standard medium amenable for good and efficient growth of multiple shoots. Different morphogenetic responses were observed when various plant growth regulators at different concentrations in MS medium were used for screening (Table 12). Sucrose at 3% was found to be optimum for multiple shoot production possibly because higher concentrations of sucrose (4 and 5%) may increase the levels of polyphenols, which results in browning of cultures and subsequent growth inhibition.

For different explants used i.e, nodal segments, leaves, seeds, shoot and cotyledonary meristems, of all the different percentages of agar tried, 0.6 % (Table 14) agar was found to be most effective for multiple shoot regeneration due to the semisolid nature of the agar which allows profuse proliferation of shoots when compared to 0.8% agar.

Besides explant type (seeds better compared to others), orientation of the explant in the cultures plays a significant role in induction and proliferation of multiple shoots (Polisetty *et al.*, 1977). It was observed that horizontal orientation of the explant was more effective (Table 15) when compared to vertical orientation because horizontal orientation results in the whole surface of the explant in contact with the semisolid medium. Profuse sprouting was observed from all the exposed surfaces of the explant profusely (Figure 14a) which was later transferred onto medium with similar hormonal composition in vertical orientation (Figure 14 b), for proper growth and maturation of plantlets. Thus as observed in Chickpea (Suhasini *et al.*, 1996), orientation of the explant has a significant effect in *P. santalinus* which was further modified by other factors like age of the explant and season of collection.

A 2-day-old seedling was placed with micropylar end of the seed downward into the medium,did not produce multiple shoots initially. On the other hand, 3-4 days old seedling explants when used in a similar way, numerous multiple shoots were produced. Thus different age and orientation of the explant caused differences in multiple shoot regeneration and can be attributed to increased in time for starch accumulation with BAP treatment resulting in multiple shoots. Heavy and early accumulation of starch in BAP treated explants was related to induction of shoot primordia. Hence, it clearly supports the view that orientation plays a major role in multiple shoots production, which in turn is related to starch accumulation. Breakdown of starch into sugars by alpha amylase had negative influence on the process of differentiation. Thus, treating the explant with BAP resulted in accumulation of starch with a corresponding reduction in solubilization of starch by

alpha amylase, which might be linked with multiple shoot production (Thorpe and Murashige, 1970).

7.3.2. Nodal segments as explants

Induction of adventitious shoot buds in BAP treated explants by suppressing the apical dominance was reported by Polisetty *et al.*, 1997. Successful shoot development or organogenesis was observed with utilization of nodal segment explant with axillary buds (one per node) though multiple shoots formed were very less compared to seed explants. Around 80% of these explants developed actively growing buds, mostly 6-7 shoots per nodal segment after 30 days of culture on MS medium fortified with different cytokinins either singly or in combination. Earlier and higher frequencies of bud break, as well as varying degrees of multiple shoot formation occurred on BAP supplemented medium compared to the control medium without any hormones. In nature, these axillary buds may remain dormant for various periods depending on the growth pattern and environmental conditions, however by culturing the nodal segments on medium containing appropriate concentrations of cytokinins, it is possible to break the dormancy and subsequently enhance development of multiple shoots (Figure 15).

The growth and multiplication of axillary buds was greatly influenced by the season of explants collection. Sprouting was better when they were collected during the months of September to December. During rainy season axillary buds were free from phenolic exudates and resilience is broken and proliferated into multiple shoots. At higher and lower concentrations of cytokinins either singly or in combinations, initiation of callus was greater. Swelling of the dormant axillary bud within a week

followed by differentiation into two to three shoot buds in three weeks was observed along with development of callus in different concentrations of KN and BAP. After 5 weeks maximum number of shoots observed were 6.5, 5.0, 4.8 and 6.0 respectively on MS with 3.0 mgL^{-1} BAP, MS with 3.0 mgL^{-1} KN, MS with 2.0 mgL^{-1} TDZ and MS with 0.1mgL^{-1} BAP + 3.0 mgL^{-1}TDZ (Figure 15).

Superiority of BAP over KN was demonstrated by several workers (Rech and Pires, 1986; Kukreja *et al.*, 1991; Vaneck and Kitto, 1990; Mishra and Bhatnagar, 1995). This may also indicate that these explants (seeds and nodal segments) contain sufficient endogenous level of auxins or are capable of its *de novo* synthesis which can induce shoot formation even in a medium containing cytokinin alone (Julliard *et al.*, 1992).

Nodal explants were more responsive than apical shoot meristems and leaf explants, as multiple shoot production was observed with nodal segments, whereas development of callus was observed with shoot meristems and leaf explants. This differential morphogenetic response could be due to differences between the physiological states of the buds on different regions of the stem when compared to that of other explants (Table 13) (Vieitez *et al.*, 1985). Similar results were also reported in *Syzygium cuminii* and *Morus australis* (Yadav *et al.*, 1990). The less frequent shoot initiation was preceded by callusing in case of apical meristems and leaf explants. Hence, shoot tip cultures and micropropagation using leaves was less desirable when compared to seeds, as the number of regenerated shoots was very few in number. Nodal explants can be used for micropropagation in case of unavailabiltity

of seed explants though the number of regenerated shoots are less compared to seeds (but better than shoot tip and leaf explants).

When cotyledonary nodal meristems were used as explants, multiple shoots (12.0) were obtained on MS medium with 1.0 mgL^{-1} BAP (Figure 14 e) but there was no further elongation of shoots which became stunted. Even when transferred onto the shoot elongation medium like MS with different concentrations of GA_3 (0.1-7mgL^{-1}), no elongation was noticed and may be due to some of the external conditions or the inability of the shoots to further absorb the plant growth regulators from the medium and thus shoots remained inert. Hence, the usage of cotyledonary nodal meristem was not continued further.

All the explants at the initial stage increased in size and became green in colour. In most of the cases callogenesis and adventitious bud initiation took place simultaneously as seen in the case of nodal segments and leaves (callus). The degree of bud induction and callus proliferation varied greatly with the growth regulator composition of the medium. No correlation was observed between the amount of callus and number of shoot buds per culture. The axillary buds at the initial stage appeared as tiny tube like structures that resembled bulbets seen in some of the tuberous crops. The size of the initial explants i.e., nodal segement, hypocotyls and shoot tip is significant for multiple shoot regeneration.

The observed shoot forming ability of all the *in vitro* derived explant types such as nodes, meristem, leaves, cotyledonary nodal meristem, hypocotyls did not influence the rate of proliferation as compared to explants tested from the field grown plants. Repeated subculturing of nodal segments, leaves, and seeds from seed cultures

helped to achieve continuous production of callus free, healthy shoots atleast through five subculture cycles. A similar phenomenon was also observed in *Morus australis* (Pattnaik *et al.*, 1996). From single seed, 80 % of the shoots showed good rooting on MS medium without exogenous hormones. The results suggested that seeds when used as explants, a threshold level of endogenous growth regulators accumulated during culture initiation, which enabled the explants to develop optimum number of multiple shoots initially in the MS basal medium, and at reduced levels of BAP and KN without auxin augmentation. Hence, endogenous hormonal level plays an important role in shoot multiplication.

7.3.3. Seeds as explants

Among all the explants tried seeds, of *P. santalinus* responded most favourably in the presence of BAP and KN for multiple shoot induction (Figures 15-18) as in *Albizzia chinensis,* a tree species (Sinha *et al.*, 2000). Patri *et al.*, (1988) have reported shoots with scaly leaves from cotyledonary node. Arockiasamy *et al* (2000) reported the influence of growth regulators and explant type on *in vitro* shoot propagation, but in all these cases, the number of multiple shoots regenerated were very less when compared to the present study. Seeds from Balpally (Kadapa District) produced 2-3 shoots when cultured on MS basal medium unlike seeds from other locations which showed normal seed germination and were hence used for further comparision with other explants. TDZ, a urea-derived potent cytokinin for woody plant tissue culture was extensively used for the induction of shoot regeneration in several plant species (Huetteman and Preece, 1993; Li *et al*, 2000; Liu *et al.*, 2003). However it had no effect in *P. santalinus.*

During initial subculturing, the mother explant was kept intact with proliferated shoots. Later increasing or decreasing concentration of hormones resulted in decreased rate of shoot regeneration. When the explant was treated with BAP there was a significant increase in phenol content. Thus increasing the concentration of BAP to a higher level leads to decrease in the number of multiple shoots. Hence changes in secondary metabolites like phenols seems to play an important role in determining the BAP induced multiple shoot differentiation. An adverse effect of phenols on differentiation was reported by Bhat and Chandel (1991). At the same time maintenance of auxin and cytokinin, ratio was found to be necessary for differentiation.

When a single node obtained from the multiple shoots was cultured onto MS media with $1mgL^{-1}$ BAP 3-4 multiple shoots were produced (Figure 19c) which were further used for rooting. Thus among all of the explants tried seeds responded most favourably in the presence of BAP and KN in *P. santalinus* . This could be due to many factors prominent among which is that the seeds collected from Balpally (Kadapa District) may be variants and further investigation of the multiple shoots needs to be done to determine their origin (polyembryony or apomixes) (Table 20)

The requirement of embryonic axis along with cotyledons for inducing multiple shoots was very important. Evidently the presence of cotyledons was essential for maximum shoot production potential. The regeneration of multiple shoots from seeds collected from Balpally (Kadapa District) may be due to the phenomenon of either polyembryony; apomixes or due to variations, which has to be further worked out in future (Table 20)

Although several reports on tissue culture of many plants are available, information on plant losses during *in vitro* culture caused by microbial contamination is rather scanty. In *Colocasia esculenta* (Taro) it was found that microorganisms can live within plant tissues for longer periods *in vitro* without being pathogenic and show up in cultures during short environmental changes which may inhibit growth rate and decrease the potential of *in vitro* propagation. This was also a prominent observation in seeds of *P. santalinus*, which were collected from forests of different areas. Contaminants could become pathogenic *in vivo* when the plants are introduced into another climate. Furthermore, metabolites of the contaminants can be toxic to the culture during short climatic changes in the growth room. Leifert *et al* (1991) mentioned that microbial contaminants may lead to heavy loss of plants.

Deleterious pathogenic microorganisms, which exist endogenously, are activated during culture conditions. In case of Taro (*Colocasia esculenta*), high death rate of explants was observed in tissue culture due to the presence of casual endogenous microorganisms (Gunua, 1921). Initially 50% of the seeds of *P. santalinus* were contaminated with bacteria and fungi which were endogenously associated. Contaminated cultures were able to change the colour of the growth medium from colourless to yellow and were able to survive after treatment with an antibiotic (Streptomycin) or (0.1 % Bavastin) fungicide followed by transfer to fresh medium.

7.3.4. Rooting in *P. santalinus*

Rooting was found to be difficult in *P. santalinus.* Auxin in the medium generally promotes rooting, while in the present study, auxins in MS medium were ineffective in rooting of individual shoots. Rooting was observed only when the shoots were not separated and left in clumps. Thus fully grown plantlets with 30-40 expanded leaves and well developed roots in bunches were transferred into magenta boxes successfully (Figure 19 a, b and Figure 20). The survival rate was only 20% when the plantlets in soilrite were transferred to glass house.

The role of activated charcoal in a nutrient medium for tissue culture was discussed by Misson *et al.*, 1982. Charcoal is thought to remove the inhibitory material that may be present in the medium that originate from the explant itself. Anagnostakis, 1974 and Fridborg *et al* 1978 found that compounds excreted from growing cells of *Daucus* and *Allium* could be adsorbed by activated charcoal to allow embryogenesis and root formation that did not occur in cultures lacking activated charcoal. Stenitz and Yahel (1982) found a need for activated charcoal in the medium for the production of bulbets of *Narcissus tazetta.* Activated charcoal incorporated into the media for our study, had no effect. The percentage survival observed in *P. santalinus* was very less (20%).

Hence, micropropagation has been advocated as one of the most viable biotechnological tools for *ex situ* conservation of germplasm. Similar reports are plenty and many of the groups have been successful in the micropropagation of number of plants maintaining genetic stability of the tissue cultured clones. (Gangopadhyay *et al.*, 2003;. Ramalakshmidutta *et al.,* 2003) but whereas in case of

P. santalinus micropropagation technique was not so effective in terms of conservation management purposes since the percentage survival of plants in field conditions after transfer was very less due to many factors one of them being the highly recalcitrant nature of the species (20%) during hardening. Further studies need to be planned to emphasize on identification, description, documentation, and to find out the relationship of these microorganisms with *P. santalinus*. Hence both *in vivo* and *in vitro* seed germination would be the most feasible .

7.4. Morphological variations in *P. santalinus*

The morphological variations are not significantly correlated with the geographical distances. Morphological data indicated considerable phenotypic variations among various accessions of *P. santalinus* (Figure 22).

The study of natural variations has proved to be useful for analyzing the genetic basis of some developmental processes in the model system *Arabidopsis thaliana* (Perez-Perez *et al.*, 2002). The large phenotypic variability obtained for the quantitative traits facilitated a clear distinction among the sixteen accessions collected from different geographical locations indicating the existence of region specific adaptations due to the influence of environmental and edaphic factors among all the locations. The accessions of *P. santalinus* showed large differences same could be true here for the large differences observed in pod weight between Araku (Vishakapatnam District) and Papavinasanam (Chittoor District) pods, pod length between pods of Balpally (Kadapa (Cuddapah) District) and Vishakapatnam, pod width between Papavinasanam (Chittoor District) and Rapur (Nellore District) pods, number of shoots between Rapur and Sanipaya (Kadapa (Cuddapah) District) plants,

number of nodes between Rajamundry (E. Godavari District) and Sorakaipalem (Chittoor District) plants and leaf length and width between plants from Sorakaipalem (Chittoor District) and Papireddypally (Kadapa (Cuddapah) District) (Figures 23-27). The conventional morphological markers used for characterization of genotypes cannot be relied upon as their expression is influenced by environmental factors and developmental changes.

Plant populations under different environmental selection pressures generally show phenotypic differences. Such phenotypic differences are due to genetic diversity. The high levels of genetic diversity as observed in case of accessions of *P. santalinus.* also accounts for the high levels of allelic diversity.

Generally qualitative traits reveal less genetic diversity than quantitative traits. The available moisture of the growing environment is very important for plant growth. Phenotypic traits are controlled by genes and affected by environment. In *P. santalinus* the phenotypic data also revealed polymorphism which indicate genetic variation. Therefore, phenotypic traits further strengthen the occurrence of molecular divbersity (Perry and McIntosh, 1991).

7.5. RAPD analysis in *P. santalinus, R. serpentina* and *R. tetraphylla*

RAPD markers have been employed as an alternative for morphological and biochemical markers. (Dawson *et al.,* 1993; Pei *et al.*, 1995; Su *et al.,* 1999; Wolfe and Liston; 1998, Yoon and Glawe, 1993; Esselman *et al.*, 2000).

The data from RAPDs do not depend on strict dominant and recessive allelic frequency. Estimating the genetic differentiation coefficient among populations using RAPDs has been problematic due to their dominance, and analytical methods usually

rely on knowledge of the selfing rate or assume Hardy-Weinberg equilibrium (Lynch and Milligan, 1994). This assumption does not hold when populations exhibit fixed heterozygosity, hence in our studies an alternative method i.e., Dice coefficient was used to partition the genetic diversity which is supposed to be in accordance with the RAPD data and it was found consistent in showing the variation within accessions. DNA based polymorphism contributes towards assessing phylogenetic relationships among different species and genera. The tissue age, pathogen infestation, intra population contamination and PCR conditions are reported to introduce some levels of error in RAPD analysis. Therefore DNA from young uninfected plant tissue, is the best suited material to achieve consistent results (Staub *et al.*, 1996

. One of the objective of the study is to find out the genetic distance between different accessions of *P. santalinus*, *R. serpentina* and *R. tetraphylla* individually and genetic similarity in between *R. serpentina* and *R. tetraphylla. P. santalinus* is a woody plant species where the leaves are exceptionally rich in of polysaccharides, polyphenols, tannins, hydrocolloids (sugars and carragenans), and other secondary metabolites such as alkaloids, flavanoids, phenols, terpenes and quinines which have interfered with the DNA isolation and further experiments in molecular technology. Polysaccharides interfere with the PCR by inhibiting *Taq* polymerase activity (Fang *et al.*, 1992) which inturn can inhibit RAPD reactions. Polysaccharides like contaminants, which are undetectable by most criteria, can cause anomalous reassociation kinetics but polysaccharide co-precipitation is avoided by adding a selective precipitant of nucleic acids, i.e., CTAB to keep polysaccharides in solution.

The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of DNA by binding covalently making it useless for most research applications (Katterman and Shattuck 1983; Peterson *et al.*, 1997; Loomis, 1974). Additionally tannins, terpenes and resins are difficult to separate from DNA (Doyle and Doyle, 1987, Ziegenhagen and Scholz, 1998).

Therefore the problems encountered in the isolation and purification of DNA specially from medicinal and aromatic plants include degradation of DNA due to endonucleases, coisolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions and moreover the contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Pikkart and Villeponteau, 1993), interference with DNA amplification involving random primers, e.g. RAPD analysis and improper priming of DNA templates during thermal cycle sequencing (Mejjad *et al.*, 1994; Yoon and Glawe, 1993) .

These factors do not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weishing *et al.*, 1995). Hence *P. santalinus* DNA was isolated by using Plant DNA Zol isolation Kit where a good quality and quantity of DNA was obtained which was used for RAPD reactions (Figure 28). In case of *R. serpentina* and *R. tetraphylla* DNA isolation was comparatively easier with reference to that of *P. santalinus* and

pure DNA was obtained following the established CTAB method with few modifications (Figures 55 and 56).

The differences among accessions of *P. santalinus*, *R. serpentina* and *R. tetraphylla,* collected from different locations, could partly be explained as a result of both abiotic (geographical, e.g., hydrographic connections, or climactic differentiation. e.g., annual rainfall differences) and biotic (pollination between populations and seed dispersal etc) factors. It is expected that obligate outcrossing species show more genetic variation at the population level (Apostol *et al.*, 1996; Cardoso *et al.*, 1998) as observed in *P. santalinus, R. serpentina* and *R. tetraphylla.*

For a species with limited gene flow and over 50% variation among populations, it is necessary to collect samples from at least six locations (based on which accession no. is given) in order to conserve 95% of the genetic diversity of the species. Hence, our minimum size of the accessions was atleast six to minimize the external effects. For a species with only 20% variation among populations, samples taken from two populations are enough to get the same results as above. (Pei *et al.,* 1995).

With all the tested 40 primers genetic polymorphism was 100% in *P. santalinus*, whereas in *R. serpentina* and *R. tetraphylla* it was 70% and 50% respectively. Genetic similarity between *R. serpentina* and *R. tetraphylla* was found to be 85%. exhibiting a higher genetic diversity among the collected accessions (Tables 23,24, 29, 32, 34 and 35, Figures 30-53 and Figures. 58-97).

Among the forty primers tested, *P. santalinus* showed 100% polymorphism with 26 primers (Table 22) indicating higher genetic diversity within populations of

P. santalinus. Distinct polymorphic bands have been observed on 2% agarose gels (Figures 29-52) (Hamrick *et al.*, 1992) *R. serpentina* showed 100% polymorphism with 16 primers and in *R. tetraphylla* only with 3 primers (Figures 57-96, Tables 28, 31 and 33). In case of accessions of *R. serpentina* the genetic variation is more when compared to that of *R. tetraphylla* but both the species show a genetic similarity of around 85%. RAPD data suggests that in woody legumes most of the variation is maintained within the populations (Schierenbeck *et al.*, 1997). Similar results are reported in tropical in similar to the genetic variation of a tropical tree legume, *Gliricidia sepium* which showed more than 60% genetic variation (Chalmers *et al.*, 1992).

The high levels of variation found within different accessions of chosen species suggests that sampling from a few localities for either breeding or conservation could capture a large proportion of the variation within the species. The genetic diversity can be explained by the aid of calculation of polymorphism levels and cluster diagram.

The mean level of genetic diversity within 15 accessions of *P. santalinus* is 3.168. The range of genetic diversity calculated in terms of genetic distance is 0.14 - 0.76 (Figure 53). Similarly the mean level of genetic diversity among accessions of *R. serpentina* is 3.168 and that of *R. tetraphylla* is 2.733 and when both of the species are analyzed it is 3.940. The range of genetic diversity calculated in terms of genetic distance for *R. serpentina* is 0.596-0.928 (Figure 97). *R. tetraphylla* it is 0.816-0.932 (Figure 99) and for both of them it is 0.407-0.955 (Figure 101). From this it is evident that the accessions from different geographical locations exhibited a wide range of

genetic distance, which did not show any correlation with geographical distances between the collection sites, negating a simple isolation by physical distance. .

In *P .santalinus*, cluster analysis based on Dice coefficient showed two major groups (Figure 54) indicating that in cross pollinated plants, high levels of differentiation among populations and relatively less within-population genetic variation exists. The dendrogram obtained by the aid of similarity matrix, revealed that there is a similarity of 76% between the accessions collected from Kerala, India and Raichoti (Kadapa District) which clearly depicts that genetically they are similar which was confirmed from the owner of the nursery that they were collected from A.P and were grown in Kerala as plantations. There is also a close similarity of 76% observed between the accessions collected from Talakona (Chittoor District, A.P) and Gadela (Kadapa District, A.P) though geographically they are distantly placed in contrary. The accessions collected from Tirupathi (Chittoor District,A.P) and Narsingapuram (Chittoor district, A.P) though closely placed geographically, their a genetic similarity of only 32%, which clearly indicates that there is no correlation between genetic make up and geographical distances.

The pattern of genetic diversity in *P. santalinus* may be maintained due to effective gene flow within populations. Animal drops which aid in seed dispersal may also contribute for inducing variations indirectly within the populations thus accounting for the high levels of genetic variation (Loveless and Hamrick, 1984; Hamrick and Godt, 1989). Distribution range and population size have been identified as the major correlates within population genetic variation in tropical tree species with restricted populations showing significantly less variation than those with

broader distribution (Loveless, 1992; Travis *et al.*, 1996). In case of *P. santalinus*, inspite of its smaller population size and being endemic in certain districts of A.P i.e., Kadapa (Cuddapah), Chittoor, Nellore and Kurnool, high genetic variation is observed, which might be due to highly cross pollinated nature of the plant. It was reported that outcrossed wind pollinated species exhibit vast variation within populations (Loveless, 1992; Loveless and Hamrick, 1984)

In case of *R. serpentina* and *R. tetraphylla*, when the RAPD data was analysed comparitively two distinct groups were observed (Figure 102). In the dendrogram of *R. serpentina* the accessions collected from forests in Sukumamidi (East Godavari,A.P) showed 62% genetic similarity with other accessions collected from various locations and falls in a separate cluster. Whereas accessions from Araku (Vishakapatnam, A.P) and Hyderabad (A.P) show a similarity of 95% though they are very distant geographically. Similarly the accessions of *R. tetraphylla* collected from Dulapally (Medak District, A.P) and Vijayawada (A.P) show a genetic similarity of 93% though they are quite distant geographically. This situation arises only in the case of natural populations where there is a free/random pollen flow and fertilization, as is the case of the cross-pollinated species.

The grouping of these populations is independent of the geographical distance. Study of interspecific variations and assessment of the genetic similarity among populations of *R. serpentina* and *R. tetraphylla* showed that the 13 populations were divided into two distinct groups based on the difference at species level as evidenced by the dendrogram. Polymorphism of 90 % was observed among populations of *R. serpentina* and *R. tetraphylla* in interspecific diversity analysis.

There is 85% genetic similarity between *R. serpentina* and *R. tetraphylla* by cluster analysis in Unweighted Pairwise Group Matrix for Arithmetic Average (UPGMA).

R. tetraphylla which is a common plant, can be useful for genetic improvement of *R. serpentina* .

The analysis of genetic similarity and dissimilarity in terms of similarity matrix and cluster analysis i.e., genetic diversiy of *R. serpentina* and *R. tetraphylla* species by using limited set of primers proved to be promising for further investigation. It proved that the accessions collected from different locations showed similar morphologies (leaf morphology, leaf length, colour of the petiole, colour of the midrib, flower morphology, fruit morphology), their RAPD fingerprinting differed markedly. On the other hand morphological differences were observed between species in leaf, flower and fruits, hence both genetic similarity and diversity between both the species of *Rauvolfia* was noticed.

Therefore, analysis of RAPD data could be useful to detect genetic differentiation as well as similarity between accessions of *R. serpentina* as well as *R. tetraphylla*. A close phylogenetic proximity between *R. serpentina* and *R. tetraphylla* was shown as per the dendrogram. RAPD marker provides equivalent levels of resolutions for determining genetic relationships (Santo *et al.*, 1994). Reliability of RAPDs among closely related taxa and the limitation of RAPD data for producing expected associations among more divergent taxa was observed in *Pisum* species (Hoey *et al.*, 1996). Similarity between *R. serpentina* and *R. tetraphylla,* may be in terms of some of the morphological or genetic traits and same is the case with

diversity. To analyze in detail further work has to be done so that the appropriate reasons for both the similarity and diversity between both the species can be unraveled.

The significant variations in the accessions of *P. santalinus* collected from Tirupathi (Chittoor District, A.P) when compared to other accessions from various locations need to be investigated further. Similar is the case with one of the accession of *R. serpentina* collected from Sukumamidi (East Godavari, A.P). Such observations have been reported previously in *Hordeum spontaneum* populations by Dawson *et al.*, (1993). It can be inferred that in the accessions, which are clustered in similar groups, there is an effective gene flow in those areas but whereas with the accession of *P. santalinus* collected from Tirupathi and *R. serpentina* collected from Sukumamidi, the gene flow is less hence, they are highly divergent when compared to other accessions (Figures 56 and 99). This may be due to highly cross-pollinated nature of the plant and due to the occurrence of some mutations and rearrangements in the genome, resulting in variation. In *P. santalinus* some of the morphological parameters like pod weight, pod length and leaf length were in accordance with that of the molecular data but it may not represent the exact trait as there may be many other phenotypic traits which may exhibit variation.

Genetic variation decreases with decrease in population size (Mosseler *et al.*, 1992; Baskauf *et al.*, 1994; Gray, 1995; Kappe *et al.*, 1995; Frankham, 1997; Palacious and Gonzalez-Candelas, 1997). One would therefore expect rare and endemic species of small population size, often associated with increased inbreeding and genetic drift, processes that lead to loss of genetic variation (Ellstrand and Elam,

1993; Gaston and Kunin, 1997a) . Inbreeding is avoided in all the accessions of *P. santalinus* because the plants are dioecious, although within-population gene exchange is unavoidable. This situation may arise in natural populations where there is a possibility of free/random pollen flow and fertilization as in the case of most of the cross pollinated species or may be attributed to the formation of hybrids due to introgressive hybridization. Mutations may also play an important role in causing variations. In *P. santalinus* and in possibly other species which are rare and endemic high levels of genetic variation is maintained the reason for which it is not clear which is not yet clear. If a population has always occurred in small numbers, it means that it is adapted to that local conditions (Milligan *et al.*, 1994).

Sources of polymorphisms in RAPD assay may include base change within priming site sequence, deletions of priming site, insertions that render priming sites too distant to support amplification, and deletions or insertions that change the size of a DNA fragment without preventing its amplification (Williams *et al.*, 1990). In addition the polymorphisms of RAPD markers were observed as different sized DNA fragments from amplification. In *P. santalinus* the strict out crossing results in higher levels of heterozygosity (Wolff *et al.*, 1994).

The differences found among the dendrograms generated by RAPDs could be partially explained by different number of PCR products analyzed reinforcing the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among the accessions of *P. santalinus*; *R. serpentina* and *R. tetraphylla* (Figures 54, 98, 100 and 102).

Another explanation could be low reproducibility of RAPDs (Karp *et al.*, 1997). The putatively similar bands originating for RAPDs in different accessions are not necessarily homologous although they share the same size in base pairs. This situation may lead to erroneous results when calculating genetic relationships. Problems of the reliability and repeatability of RAPD markers are well known (Ellsworth *et al.*, 1993). However in our experiments, high reproducibility with PCR products for RAPDs was observed.

The gene flow in higher plants is accomplished by dispersal of seeds and pollen as well as by vegetative mobility (Handel, 1985; Parker and Hamrick, 1992). Gene flow by pollen dispersal is often low in herbaceous plants (Widen and Swenson, 1992). In *P. santalinus, R. serpentina and R. tetraphylla* none of the accessions collected for our study have less than 15 Km distance to each other. Hence, the genetic structure of any of these accessions is stable and free from any gene flow into them. Hence there is a wide range of genetic differentiation. The genetic variation is related to the distances of pollen and seed dispersals. The seeds of *P. santalinus* are winged which favours the seed dispersal over long distances. *P. santalinus* species is bee pollinated and hence there are more chances of pollen dispersal resulting in a broad range of variations. A detailed on all the aspects related to variations is warranted.

Genetic drift over thousands of generations would lead to significant divergence. This trend may be reinforced for adaptive traits by selection of important ecological differences existing among the areas from where the accessions were sampled. Results from RAPD analysis indicates that genetic drift might have

occurred among the studied accessions of *P. santalinus, R. serpentina* and *R. tetraphylla* thereby producing population differentiation. The main reason being an overexploitation leading to shrinkage of their habitat. With a larger area of population, the probability of crossing among the individuals increases, which results in the retention of genetic variation. Though many individuals of these species were reported earlier in due course of time they have disappeared gradually along with environmental changes in their habitat. For decades much attention has focused on the genetic risks associated with small population size, not only from inbreeding and genetic drift, but also from gene flow. Until now, a precise empirical assessment of how well diversity has been characterized is unavailable (Ellstrand and Elam, 1993).

The wide range of variation observed among selected species may also be due to two evolutionary forces like pollen flow and local selection pressures. Pollen can be dispersed over large distances; this long-term reciprocal movement of pollen must also have contributed to the variation. Recent experiments using pollen traps have shown that oak pollen can migrate at several kilometers (Lahtinen *et al.*, 1996).

The local selection pressures may be due to the effects of environmental factors and due to struggle for existence in nature. The wide spread occurrence of the wind pollination and breeding systems that promotes outcrossing may lead to higher genetic diversity. Palynological and anthropogenic influences may also be attributed to high levels of genetic variation.

7.6. Intrapopulation variation in *R. serpentina* collected from Dulapally

Intrapopulation diversity in *R. serpentina* among eight plants collected from a nursery maintained by Forest Department of A.P, in Dulapally (Medak District) (Figure. 103) showed that one of the plant was highly variant and was falling into an entirely different group, it may be due to the highly crosspollinated nature of the particular plant and also some internal rearrangements occurring in the genome. The monomorphism exhibited by different plants collected from the same location indicates the occurrence of self-pollination in all the plants where homogeneity is being maintained. When intrapopulation variation analysis was carried out in one of the accessions of *R. serpentina* collected from Dulapally it was found that a very low level of genetic variation was found, presumably as a consequence of the techniques applied to seed production, responsible for genetic drift (Figures 104-115) (Tables 36 and 37). This stresses the need to address breeders to apply appropriate techniques for seed sampling. It also underlines the need for further monitoring of the genetic and demographic status of populations, if they decrease too much in size, they will become critically stochastic events (Lanteri *et al.*, 2003)

8. Conclusions

During 2001-2004 field seasons, a total of 300 plants of *P. santalinus* and 30 plants each of *R. serpentina* and *R . tetraphylla* were collected from various locations of Andhra Pradesh and are being grown in the experimental field, field gene bank and seeds were stored in the seed bank. Seeds of *P. santalinus* collected from Papireddypally and Balpally (Kadapa District) were found to be of superior quality, in comparision to seeds from other six locations based on *in vivo* and *in vitro* germination studies.

In vitro regeneration studies of *P.santalinus* revealed that seeds collected from Balpally showed maximum number of multiple shoots. Since the percentage survival was very low this protocol cannot be recommended for *ex situ* conservation. This protocol is genotype and location dependent (Balpally) unlike the seeds collected from any other locations. Seed germination and *in vitro* regeneration studies indicated that Balpally area in Kadapa District could be considered as the reservoir of elite germplasm.

Distinct morphological variations were observed in *P. santalinus* pod characteristics (weight, width, and length), leaf characteristics (length, width), shoot length and number of nodes in five months old germinated seedlings collected from 16 different locations, which could be due to environmental and edaphic factors.

The wide variation in genetic distance among the accessions of *P. santalinus; R. serpentina and R. tetraphylla* revealed by RAPD markers reflected a high level of DNA polymorphism due to outcrossing.

P. santalinus is endemic to Kadapa (Cuddapah), Nellore, Chittoor and Prakasam Districts of A.P. Endemics are generally reported to have low levels of genetic variation. However in the presently investigated accessions, 100% polymorphism was observed. Therefore, possibly the Kadapa (Cuddapah) district of A.P might be the center of diversity for *P. santalinus.*

The presence of many unique markers in *P. santalinus; R. serpentina* and a few in *R. tetraphylla* may be due to the relatively high rate of mutations in RAPD loci. Such markers are important, as they may be diagnostic for particular regions of the genome and are accession specific.

P. santalinus and *R. serpentina* exhibited high genetic polymorphism . Based on the observations of this study and cluster analysis it can be suggested that for *P. santalinus* the *in situ* conservation measures should include Balpally (Kadapa District) and Tirupathi (Chittoor District) and for *R. serpentina* Sukumamidi (Khammam District) and Araku (Vishakapatnam District). Seeds of these sites need to be conserved (*ex situ*). Further sampling covering wider geographical areas, covering larger population and using more number of RAPD primers would be useful for diversity analysis.

The presence of similar markers between *R. serpentina* and *R. tetraphylla* indicates the possibile intragenomic recombinations or translocations. These observations are important to explore the possibility of transferring genes of agronomic and commercial importance from *R. serpentina* (for eg. reserpine) to *R. tetraphylla* which produces abundant biomass and has wider ecological amplitude.

Considerable genetic variation was observed in Sukumamidi (Khammam District) accessions of *R. serpentina* important for conservation and further progeny trials.However accessions from Dulapally (Medak District) exhibited monomorphism (genetically homogenous).

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