DISCUSSION
V. DISCUSSION

Although economically very important, the genetic diversity study's on this plant is meager (Venkatesan and Srimathi, 1995). India accounts for nearly 99 percent of sandal oil producer in the world. Sandalwood occupies an important place in the ecological, cultural and spiritual heritage of India. The area under sandalwood is nearly 9000 sq. km. However the rate of production of scented heartwood in natural population is only 450-600gr/hecter/year. However, for successful breeding programme collection of diverse genetic stock is a prerequisite. Information on nature and magnitude of the variability in germplasm collection is very essential for any forest crop improvement. A variety of molecular techniques have been exploited for variability analysis among which RAPD is the most widely used and accepted (Huang et al., 2000).

The result of the present investigation entitled “Assessment of Genetic diversity in Santalum album L. by using Randomly Amplified Polymorphic DNA Analysis and development of specific Primer” have been discussed in this chapter.

5.1 Standardization of the protocol for DNA isolation of high quality DNA

Recently matured leaves from 3rd to 5th node were collected before blooming. Sandalwood leaves collected after the onset of reproductive phase posed difficulty in isolating pure DNA, as it is associated with higher phenol content (Lodhi et al., 1994). Processing of too young leaves resulted in charring of tissue while drying. Similar
results were also reported by Suneetha (2000). Leaves were dried uniformly in hot air oven at 40°C for 24 hrs. Prolonged drying of leaves resulted in poor DNA yield and quality. This can be attributed to the formation of irreversible complexes between polyphenols and DNA as reported by (Wilfinger, 1999).

In earlier protocols for isolation of plant DNA liquid nitrogen was used to assist the grinding of leaf material (Murray and Thompson, 1980; Porebski et al, 1997). Although these protocols yielded good quantities of high quality DNA, the use of liquid nitrogen presented some problems. Once exposed to liquid nitrogen, it is imperative that the tissue should not be allowed to thaw before extraction. Again if large number of samples were to be extracted, then processing of tissue to extractable form and storage of tissue in freezer may lead to problems involving lack of space (Jennifer and Paul, 1990). In addition, plant material that are available from distant sources must be shipped frozen at high cost. Liquid nitrogen is also expensive. To circumvent these problems, it has become a standard practice to lyophilize tissue before extraction. In this freeze dried state, it is not necessary to keep the tissue in a freezer. Shipping of such material is relatively easy and inexpensive but lyophilizers are quite expensive and have only a limited capacity. Besides, lyophilization requires several days to complete and errors in may result in causes degradation of DNA. Therefore, a new alternate method reported by Taiand Tanksley (1990) has been used in the present study, by which plant tissue can be inexpensively dehydrated, efficiently processed and extracted to consistently yield high molecular weight DNA comparable to lyophilized and fresh frozen material. The present attempt to standardize a simple, cheap and yet efficient method of sample
preparation for isolation of genomic DNA from large number of samples yielded favorable results. Further, the dried leaves can be stored for over three months in a scaled polythene cover without degradation in the quality of DNA, while the leaf powder cannot be stored for longer time because of its hygroscopic property.

5.2 DNA extraction and purification

Extraction of high quality DNA from sandalwood leaves is a difficult task due to their rigid cell wall which is composed of complex carbohydrates. Sandalwood leaves are also rich in secondary metabolites, such as polyphenols, tannins and polysaccharides, which pose major problem in DNA purification, as they are difficult to separate from DNA. Many of the initial problems encountered in the extraction of high quality DNA have been attributed to these contaminants (Murray and Thompson, 1980). A modified CTAB method DNA extraction (Probeski et al., 1997) was followed which is relatively quick, inexpensive and consistent for extraction of DNA.

Young *Santalum album* leaves were sampled and therefore possibilities of high amount of polyphenols, tannins and polysaccharides was excluded. Dealing with such components in mature leaves becomes necessary. Aware of these difficulties, four methods (1 and 2 using CTAB; 3 and 4 using SDS) were compared for isolation of DNA from sandalwood leaves. The protocols involving SDS were uniformly unsuccessful in attempts to extract high quality DNA. The main obstacle was difficulty in re-dissolving isopropanol precipitate DNA in TE buffer. Further, the resulting suspension was sticky and impossible to draw. A brownish
yellow coloured substance, presumably one or more of the polyphe
lols present in sandalwood, often co-purified with the DNA.
Similar observations were also reported in other crops such as cocoa
(Jennifer and Paul, 1990), Bhendi (Kochko and Hamon, 1990) and
cotton (Franklin and Arkesh, 1991) where polyphe
lols were shown
to co-purify with DNA. Based on this problem encountered with
SDS method, the protocols using CTAB (Method 1 and Method 2)
was employed.

According to Barnwell et al., (1998), CTAB is a cationic
detergent that precipitates DNA leaving the neutral polysaccharides
in solution at the DNA precipitation step. The DNA-CTAB pellets
were treated with 1.4 M NaCl (Method 2), so that the sodium
exchanges with the CTAB resulting in clean DNA. The inclusion of PVP
(2%) in the extraction buffer helped to avoid browning of isolated
DNA. The browning occurred chiefly due to binding of tannins to DNA,
which leads to degradation of DNA (John, 1992). Similar observations
were also made by Bortbakur et al (1998), who used 200 mg of PVP in
the extraction buffer for isolation of chloroplast DNA from tea. The PVP
acts as an antioxidant, which binds to the phenolic compounds and co-
precipitates in subsequent centrifugation resulting in DNA free of poly
phenol contamination (Maliyakal, 1992). A concentration of 1% β-
mercaptoethanol was found to be optimal in order to keep the nucleic
acids in a non-oxidative environment and to denature endonuclease
activities (Leofort and Douglas, 1999). The repeated washings with
chloroform-isoamylalcohol (24:1) resulted in clean DNA, consistently
amplifiable by PCR. Centrifugation for 5 min at 6000 rpm, as the
original protocol stated (Method 1), produced a loose mass that impeded
liquid phase separation. Increasing centrifugation time to 20 min at
9000 rpm improved adherence of the solid phase to the tube and allowed better separation of debris and supernatant after cell disruption. Further using higher concentration of PVP (2%), NaCl (1.4M) and repeated washings with chloroform-isoamylalcohol (4 times) removed the interfering contaminants resulting in high quality DNA, as compared to original CTAB method (method 1). The improvement in DNA yield may be explained by the electrostatic interactions between the different chemicals, nucleic acids and proteins. This combination of chemicals seems to efficiently prevent the formation of insoluble complexes of DNA than the classical combination of one detergent, one reductant, and one salt offered by other protocols (Leofort and Douglas, 1999).

In sandalwood it was observed that RNA and protein interfered with PCR amplification and its removal was essential. Though it has been reported that high concentration of RNAses (10 μg/ml) is essential for removing RNA, we were to obtain equivalent result in sandalwood with lower concentration of RNAses (3μg/ml at 37°C Overnight). The proteins were removed by subjecting to phenol-chloroform extraction. Similar results have been reported by Dhanaraj et al., (2002) in cashew.

The method-2 involving the use of high salt concentration (3% CTAB and 1.4% NaCl) to remove polysaccharides, use of PVP (2%) and p-mercaptoethanol (1%) to remove polyphenols and to keep DNA in a non-oxidative state and an extended RNAses treatment followed by phenol-chloroform extraction, consistently gave high amounts of good quality DNA suitable for RAPD analysis.
5.3 Standardization of protocol for DNA amplification

5.3.1 Amplification conditions

Polymerase chain reaction is powerful technology used in many areas of molecular biology which allows in vitro amplification of specific DNA sequence (Innis et al., 1990). PCR is an enzymatic method of making multiple copies of a predicted segment of DNA. It commences with the denaturation step, which ensures the complete denaturation of the DNA followed by primer annealing and extension. Several factors like initial denaturation, primer annealing temperature, final extension and components in the PCR mixture influence the final product. Hence these conditions have to be optimized in order to produce informative and reproducible fingerprints.

The results revealed that, one of the most important variables is the concentration of template DNA. Different extraction methods produce DNA of widely different purity. Assays to optimize the template concentration were conducted over the range of 10-50 µg DNA in 25 µl reaction volume. For sandalwood, 25-30 ng DNA per 25 µl reaction volume gave reproducible banding pattern. Too much template (40-50ng) showed smears or lack of clearly defined bands in the gel. Too little DNA (10-15ng) gave irreproducible patterns. Similar findings were reported by Zenglu and Randall, (1993) in soya bean. Both the magnesium ion and dNTPs concentration are known to affect the relative intensity and the number of amplified bands. It was found that a magnesium concentration of 2.0 mM was optimal, since the
lower concentration resulted in less intense bands. Similar findings were reported by Murali (1999) in cashew. The magnesium concentration required for optimum activity of *Taq* polymerase depends on the dNTPs concentration, since dNTPs binds magnesium (Innis and Gelfand, 1990). The results showed a dNTPs concentration of 215 μM for each of the four bases is optimal for generating satisfactory RAPDs. At lower concentrations (< 150 μM) the intensity of stained bands in the gel becomes progressively weaker and at higher concentration (> 225 μM) smearing of the band was evident. Baumforth *et al.*, (1999) reported that higher concentration of dNTPs encourage misincorporation by *Taq* polymerase. Similar findings were also reported by Prakash in Guava (2000).

5.4 DNA fingerprinting and identification of sandalwood using RAPD Markers

Molecular genetic technique have been applied to plant cultivar identification in the past decade by developing molecular markers that detect difference in DNA sequences between cultivars (Thomas *et al.*, 1993). Highly specific marker profiles commonly know as DNA fingerprints, can be developed for each cultivar and used for its identification. Compared to isozymes and flavonoid markers (Nilson *et al.*, 1995). DNA markers have many advantages. They are detectable in all tissues at all ages of the plant. There by enabling early identification. They are virtually insensitive to epistatic pleotropic effect (Williams *et al.*, 1992). They are developmentally stable and are not affected by the environment. Further more, the number of DNA markers available for examination is unlimited because genomes are composed of base pairs.
and many different types of molecular marker systems are available to compare these differences. Trigiano and Caetano-Anolles (1998) described that the DNA fingerprinting is a bar code like DNA fragment pattern generated by multilocus probes after separation of genomic DNA fragments. They were considered to be the ultimate tool for biological individualization. Recently the term DNA fingerprinting profiling is used to describe the combined use of several single locus detection system and is being used as versatile tools for investigating various aspects of the plant genome. These include characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, taxonomy and plant breeding.

The present investigation revealed that the genetic resources and diversity of Santalum album L. India is large. Hence, there is an immediate need to conserve this genetic resource and utilize them for further commercial exploitation. The DNA fingerprints of the sandalwood genotypes developed during the present study would be of immense use in identifying these genotypes individually which would be useful in future patenting and Plant Varietal Rights to safeguard the country's genetic resources. In sandalwood, identification of elite trees still relies on morphological characters of the leaf, flower, fruit etc. However, many of them cannot be readily distinguished by morphological indices, particularly if they are closely related. Furthermore, phenotype identification based on morphological traits is subject to environmental variation (Nielson, 1985). Isoenzymes have been used in distinguishing sandalwood genotypes, but the main limitation is the small number of loci that can be examined (Angadi et al, 1997). This limitation is
overcome by the RAPD method, based on amplification of multiple random segments of the genome using arbitrary primers (Welsh and McClelland, 1990; Williams et al, 1990).

5.4.1 Genetic diversity and relatedness estimated by RAPD

In the conservation of forest genetic resources, priority should be given to both the present rate of extinction as well as the need to ensure the availability of the resources for future use and adaptability to changing environment. Therefore, comparative genetic diversity studies are needed with the objective of saving gene pools by preventing loss of genotypes, genes and gene complexes. Such studies are particularly needed in tropical tree species like sandalwood because of the vicious process of destruction of this particular species has lead to the poor stocking in Southern India by the way of forest fire and by indiscriminate cutting by sandalwood smugglers.

Molecular diversity has great potential for quality control in tree breeding. Assessment of genetic diversity could be of great importance for identification and classification of genotypes, estimating the genetic relationship between them and selection of superior parental combinations useful in hybridization programmes. With the objective of selecting and maintaining elite trees to bring about an overall genetic in improvement in sandalwood for enhanced output of oil and scented heartwood, genotypes have been collected from various regions Tamil Nadu, Kerala, Karnataka and Andhra Pradesh. Which are maintained in the Institute of Wood Science and Technology, Bangalore. However, the evaluation and
characterization of these genotypes have not been conducted hitherto using molecular markers.

The present investigation was, therefore, undertaken to document and measure genetic diversity and relatedness in sandalwood using RAPD markers. An advantage of RAPD markers over morphological markers is that they are seldom influenced by the environment, are more polymorphic and can be assessed during any stage of plant development. Assessment of genetic diversity and relatedness in the present study is based on 248 RAPD markers generated by fourteen (10 mer) random primers.


The present investigation revealed that there is a large genetic diversity in Santalum album L. Seventy four (74.1%) per cent polymorphism was detected with 248 RAPD markers indicating a high marker index. RAPD analysis revealed that the genetic diversity of 30 genotypes was high for T3, T24 and T12 which were 45% per cent similar, while T3,T24 and T12 differed by only 1 per cent. Such a wide range in similarity values suggests that the sandalwood germplasm examined represents a genetically diverse population. One of the major contributory factors to the high degree
of polymorphism observed may be on account of its evolutionary status as an predominantly cross pollinating angiosperm (Nelson et al., 1996). Similar observations have been made in oil palm by Perera et al., (1998) where the level of genetic diversity was shown to correlate with the breeding nature of the palms.

A study of the dendrogram showing the genetic diversity/relatedness among the 30 genotypes, indicated that clustering was according to their geographical regions with a very few exceptions. Matching the dendrogram results with their collection sites revealed that the geographical distribution of most genotypes in each of the sub clusters were well defined. The sub cluster Ila and IIc had majority of the genotypes from Karnataka. Likewise Ia and Ib had majority of the genotypes from Tamilnadu. The genotypes in the sub cluster IIe and IIg, eight of the four genotypes in the sub cluster were procured from Tamil Nadu. The genotypes KL and AP4, were the exceptions and there genotypes shared a similarity values ranging from 21-25 per cent. AP4 is the only genotypes examined from Andhra Pradesh and inclusion of AP4 (Hyderabad) in this sub cluster is quite interesting, since it is geographically distant from the rest. It was grouped with KL3 from Kerala exhibiting 13 per cent genetic similarity. It is possible that genotypes from different geographical regions can be genetically similar to genotypes with immediate spatial relationships.

Major cluster II having genotypes from local and distant localities of Karnataka and Tamil Nadu respectively, showed genetic similarity of 41 per cent (excluding exotic genotypes) and this association between genotypes from different regions may be attributed to the broad genetic base of the species, which enable it to
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maintain and exist in different gene combination. Genotypes from Tamil Nadu (T12, Salem and T13, Salem) were grouped in between genotypes from (K16, Chamarajnagar) and (K27, Sagar) regions of Karnataka, which constituted sub cluster IIId sharing genetic similarity of 1-21 per cent. This association between genotypes from neighboring states could be attributed to seed movement and gene flow. It was also observed that T21, Tirunelveli from Tamil Nadu was quite distinct and appeared as a separate entity both in the dendrogram and PCA. It is found to be significantly diverse from the rest exhibiting 19 per cent similarity with T24 from Tamil Nadu. This may be attributed to the unique genetic composition of T21 as compared to other genotypes. Similar observations have been made by Padmesh et al. (1999) in Andrographis puniculata, where isolation of AP29, a native genotype as a separate cluster has been reported. On comparing the generic diversity from the different states, as revealed by the PCA and dendrogram, it was evident that genotypes from Karnataka were less diverse (29%) compared to Tamil Nadu (31%). This could be partly attributed to the duplication of genotypes from Karnataka.

5.4.2 Origin of Santalum album L.

There is some discrepancy in the origin of sandalwood. Gode (1961) believed that sandal is indigenous to India since it has been often referred to in ancient literature. However, Thirawat (1955) reported that sandal is exotic to India since there are many species present in the Malayan archipelago region. Majumdar (1941) had a
different theory for the origin of sandalwood. He proposed its diphyletic origin from India and Timor from the Malayan archipelago. This hypothesis states that sandal could have spontaneously originated in different places about the same time.

In order to confirm any of these hypotheses the Indian genotypes used in the present investigation were grouped into three classes based on their distance from (Cape Comorin) Kanyakumari and the diversity was estimated from each of this group. The first group consisted of thirteen genotypes from 8.44 °N to 11.56 °N, second had eleven genotypes from 12.08°N to 12.58°N and the third group consisted of six genotypes from 13.04°N to 17.20°N, the diversity of the genotypes from the first group (southern most of the Indian peninsular) was the maximum with K16 and T19 being most similar (31 percent). The diversity of the other two regions were relatively higher than that of the first region. In the second group, T24 and K10 were similar by 32 percent. In the third group T15 and AP4 were similar by 35 percent. Since the diversity of the genotypes from the southern tip of peninsular region was maximum when compared to the interior regions, it could possibly indicate that sandal came to India via (Cape Comorin) Kanyakumari from the Malayan archipelago. In cashew it has been reported that it was first introduced to Malabar Coast of India from where it spread to other interior parts of the country over the years. Hence, the diversity of the accession for the Malabar Coast was more when compared to other regions of the country (Dhanaraj, 2002). In sandalwood the diversity of genotypes from other regions of south east Asia should be estimated and compared with that of the Indian genotypes in order to strengthen the earlier reports regarding its Origin(Dhanaraj, 2002). If the diversity of the genotypes from other regions is lesser than that of the Indian
once, then it could be confirmed that India is center of the origin of sandalwood, and as revealed by the study the peninsular tip being a hot spot for sandalwood with the most genetically diverse genotypes (Fig. 19a).

5.5 Correlation of the RAPD and morphological data

Harrison et al., (1997) correlated the morphological and RAPD analysis in strawberry. In Santalum album when we compared the data of morphological markers data it could not discriminate the genotypes studied even though there was a positive correlation between the similarity matrices generated by RAPD and morphological data (r = 0.30217). Morphological characters can be used for the evaluation of diversity. But most often, the desired phenotypic characters are phylogenetically inherited and are highly influenced by environmental conditions. Hence, the data obtained by such evaluations are not easily understood at genetic level, often resulting in taxonomic confusion and in maintenance of duplicate accessions. Moreover, in perennials it is required to grow for many years to observe growth and reproductive parameters, which is economically not feasible. In this regard, the advent of molecular markers has revolutionized the entire scenario of plant sciences (Hemmat, 1998). Ever since their development, they are constantly being modified to enhance their utility in the process of genome analysis (Swati et al., 1999). DNA based markers viz., RAPD, RFLP, AFLP and SSRs provide excellent tools for: studying diversity at the DNA level, which assist in elimination of duplicates in germplasm; discriminating cultivars and elucidating misnaming, genetic purity analysis; development of marker based gene tags; map based cloning of agronomically important

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5.6 Gene cloning and Primer designing

After DNA fingerprinting of 30 *Santalum album* L. genotypes seven unique bands were identified using fourteen random primers. Such unique bands can be converted into genotype specific RAPD markers, which may be used for the identification of genotypes. Out of the seven bands the two bands which were unique for the superior genotypes AP4 and K6 were selected, cloned and sequenced. The sequence data obtained was fed to Primer3 software and design 24-mer oligonucleotide primers for each end. These primers can be used for the discrimination of the superior genotypes from the rest of the population. Such have been carried out in lettuce by Paran *et al.*, (1992) who Sequence characterized amplified regions (SCARs) that were derived from eight random amplified polymorphic DNA (RAPD) markers linked to disease resistance genes.

Dean *et al* (2001) developed primer for objectively determining the genotype of cucurbit plants, particularly species of melon, with respect to resistance or susceptibility to Fusarium wilt infection. They used a polymerase chain reaction to amplify sample DNA using either...
an AM or FM oligonucleotide primer pair. The PCR product which results from either primer pair differed in their size, depending upon whether the template DNA was obtained from a plant susceptible or resistant to Fusarium wilt, permitting easy and rapid identification of plant genotype. And Robin Beat Gasser et al. (2002) developed a PCR-based method for the identification of species of the genus Eimeria, (commonly known as coccidia).