CHAPTER 2

REVIEW OF LITERATURE
2. REVIEW OF LITERATURE

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

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

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

2.2. POPULATION GENETIC PRINCIPLES

Population genetics is the quantitative study of the amount and distribution of genetic variation/diversity in populations, and the dynamics of the underlying processes. Description of population genetic structure and its dynamics are based on the analysis of allele and genotype frequencies in sampled populations with
simply inherited Mendelian traits (Frankel, 1983). The factors that affect allelic frequencies in a random-mating population are random drift (chance fluctuations), mutation, natural selection and migration. The estimate of allele frequencies at a locus from knowledge of genotype frequencies is forthright under the assumptions of Hardy-Weinberg principle (Hardy, 1908; Weinberg, 1908) which is the foundation for all population genetic investigations.

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

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

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

2.3. TOOLS TO STUDY POPULATION GENETIC DIVERSITY

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

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

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

2.3.1.1. Historical aspects

Coolidge (1939) made the first effort to characterize proteins by electrophoretic mobility through the separation of serum albumin and globulin using a semi-porous medium. Using paper electrophoresis, Durrum (1950) separated five individual protein components of serum. The beginning of isozyme epoch was by the introduction of starch gel as a supporting medium by Smithies (1955). Hunter and Markert (1957) first reported the multiple molecular forms of enzymes in mammals. Markert and Moller (1959) coined and defined the term isozymes as 'structurally different forms of an enzyme with identical or nearly identical function'. In higher plants, peroxidase was initially used to study variation among individuals because of the simplicity of its detection method (Hosoya, 1960; Mc
Cune, 1961). In subsequent investigations, many other enzyme systems were found to consist of multiple molecular forms (Feret and Bergmann, 1976; Gottlieb, 1977).

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

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

The separation of complex mixture of enzymes and other proteins was not satisfactorily performed before the introduction of zone electrophoresis in starch gel as supporting medium (Smithies, 1955). Because of the 'molecular' sieving effect of starch gel, the macromolecules could be separated both by charge as well as by size and shape. With the introduction of PAGE (Ornstein, 1964; Chrampach
and Rodbard, 1971) the resolution of enzyme and protein mixture was improved considerably. Although, PAGE provides maximum resolving power and superior zymograms for the analysis of large number of individuals for many enzymes, because of the complexity of gel preparation and toxic nature of the material used, starch gel electrophoresis is performed still in the field of evolutionary biology and systematics (Bergmann and Hattemer, 1998).

2.3.1.2. Applications in tree species

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

2.3.1.2.1. Genetic fidelity

Adams (1983) used isozymes as an efficient marker to detect labeling errors in Douglas fir seed orchard and to distinguish between trembling aspen and big tooth aspen (Cheliak and Pitel, 1984). Bergmann (1987) reported isozyme markers as an efficient tool to discriminate multi clonal aspen cultivars. Isozyme analysis was carried out to identify clones of Tilia (Maurer and Tabel, 1995). Cabrita et al. (2001) used isozyme marker system to test the fidelity of Ficus carica clones.
Several authors have reported the utility of isozyme phenotypes for identifying cultivars and determining genetic variation in apple (Bournival and Korban, 1987), red raspberry (Cousineau et al., 1993), chestnut (Huang et al., 1994) and pawpaw cultivars (Huang and Layne, 1997).

2.3.1.2.2. Linkage mapping

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

2.3.1.2.3. Mating system

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

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

2.3.1.2.4. Genetic diversity/variation

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

2.3.1.3. Isozyme studies in sandal

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

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

### 2.3.2. Randomly amplified polymorphic DNA

#### 2.3.2.1. Historical aspects

In the 1970's, discovery and use of restriction enzymes and DNA hybridization allowed the analysis of restriction fragment length polymorphism (RFLP). RFLP technique involves the digestion of target DNA by restriction enzymes and the resulting fragments can be size fractionated by electrophoresis and visualised by hybridizing with labeled known base sequences called probes (Botstein *et al.*, 1980). The subsequent advent of modern techniques of molecular biology brought out several methods for the detection of DNA polymorphisms viz. random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), microsatellites (SSRs) (Weber and May, 1989; Morgante and Olivieri, 1993), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), etc. Microsatellite or SSR consists
of segments of DNA containing numerous tandem repeats of a short 'motif' usually of one to six bases (Beckman and Weber, 1992). AFLP technique is intermediate between RFLP and RAPD and combines the power of restriction enzyme digestion and the efficiency of PCR amplification (Vos et al., 1995; Karp et al., 1997). All these DNA markers allow investigation of both coding and non-coding regions of whole genome, independent of growth and developmental stages and are considered as the most powerful tool in the study of population genetics. Deciding which DNA marker to use depends on the financial resources, availability of equipment, skilled personnel and on the study objectives. Because of these unique advantages, molecular markers are gaining enormous popularity day by day (Ahuja, 2001).

The RAPD method, because of its technical simplicity, has attracted widespread interest in detecting DNA polymorphisms in many species of forests trees (Williams et al., 1990; Deragon and Landry, 1992; Hadrys et al., 1992). RAPDs are generated by applying PCR to genomic DNA samples using randomly constructed oligonucleotides as primers. Each RAPD reaction cycle involves three steps; DNA denaturation (90°C-94°C), primer annealing (30°C-36°C) and primer extension (72°C). The extension step involves the addition of nucleotides using target sequences as template, resulting in the synthesis of a new copy of the target sequence in each cycle. The cycle is repeated generally for 20-45 times and the amplification process follows geometric progression (Welsh and Mc Clelland, 1990). The technique is relatively easy to apply to a wide array of plant and animal
taxa and the number of loci that can be examined is essentially unlimited (Lynch and Milligan, 1994). DNA variation down to the level of a single base substitution can be detected through electrophoretic gels followed by ethidium bromide staining. Because of the dominant nature, RAPD reveals low information content per locus and easily prone to artifacts by contamination of the sample DNA. However, the technique is widely accepted because it offers advantages in speed, technical simplicity, requires only nanogram amounts of DNA and reveals high levels of sequence polymorphisms. There are several applications for RAPD assay, each of these techniques exploits the efficiency of detection of DNA sequence based polymorphisms (Williams et al., 1993).

2.3.2.2. Applications in tree species

2.3.2.2.1. Genetic diversity/variation

Determining nature and extent of genetic diversity in population of forest trees has been the focus of many studies over the past three decades (Wickneswary et al., 1996; Karp et al., 1997). RAPDs have been used as an efficient method to reveal the genetic structure and diversity pattern of trees in natural populations, reserve forests and plantations and the outcome could readily be used to select priority areas for conservation, to design and monitor management strategies in protected areas and to plan conservation strategies for genetic resources (Newbury and Ford-Lloyd, 1993; Young et al., 2000; Uma Shaanker et al., 2001). RAPDs have been successfully used for estimation of genetic diversity/variation and relatedness in \textit{Gliricidia} (Chalmers et al., 1992), \textit{Coffea} (Lashermes et al., 1993; Anthony et al.,
2001), *Malus* (Harada et al., 1993), *Elaeis* (Shah et al., 1994), mahoganies (Chalmers et al., 1994), *Populus* (Yeh et al., 1995; Rajagopal et al., 2000), *Grevellia* (Rossetto et al., 1995), *Eucalyptus* (Nesbitt et al., 1995), *Prunus* (Warburton and Bliss, 1996), Brazil nut (Kanashiro et al., 1997), pistachio (Hormaza et al., 1998), *Quercus* (Kremer and Zenetto, 1997), plums (Shimada et al., 1999), tea (Kaundun et al., 2000; Wachira et al., 2001), *Anacardium* (Mneney et al., 2001), mango (Hemanthkumar et al., 2001) and in *Pinus* (Fazekas and Yeh, 2001).

2.3.2.2. Linkage mapping

Genetic linkage maps of molecular markers offer a powerful tool to investigate the genetic architecture of quantitatively inherited traits and assist in breeding through identification and manipulation of the individual genetic factors controlling such traits. One of the practical uses of RAPD is in the creation of high-density genetic maps, since RAPD markers can be shown to segregate in Mendelian fashion. Genetic maps allow extensive coverage of genomes for detailed genetic analysis, the decomposition of complex traits into its discrete Mendelian components and the canalization of linkage information between markers and traits of interest into breeding strategies (Neale and Williams, 1991). Genetic maps also enable marker assisted breeding; since forest trees have particularly long breeding cycles, a tool that helps shorten the breeding cycle could be quite valuable. Many desirable attributes require several years to develop; if individuals can be selected for controlled crossing based on RAPD markers linked to the desired trait, then
breeding cycle could be shortened appreciably (Neale and Harry, 1994). RAPDs have been successfully used in the construction of linkage maps in *Populus* (Bradshaw and Stettler, 1994), *Eucalyptus* (Byrne et al., 1995; Grattapaglia et al., 1995; Vaillancourt et al., 1995; Verhaegen and Plomion, 1996; Grattapaglia et al., 1996; Byrne et al., 1997b), *Pinus* (Plomion et al., 1995; Yazdani et al., 1995) and larch (Arcade et al., 2000).

2.3.2.2. Genetic fingerprinting

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

2.3.2.2.4. Clonal fidelity

The reproducibility of RAPD markers and their ability to differentiate between individual genotypes have been utilized in verification, identification and characterization of clones of forest trees. Such type of clonal identification was performed in *Eucalyptus* (Lange et al., 1993; De Lala et al., 2000), *Populus* (Lin et
al., 1994; Sigurdsson et al., 1995); Salix (Auriol et al., 1994), Hevea (Varghese et al., 1997) and in Pinus (Scheepers et al., 1997).

2.3.2.2.5. Resistance breeding

Breeding for resistance to both abiotic and biotic stresses requires the development of suitable and reliable screening techniques and identification of heritable resistance characters. Molecular markers are considered to be an important screening tool in disease resistance breeding programmes. Identification and cloning of resistance genes could find application in evolving transgenic plants (Licy et al., 2000). An efficient and relatively simple way to generate molecular markers for disease and pest resistance is to search for randomly amplified polymorphic DNAs (RAPD) and this strategy has already proven valuable along with restriction fragment length polymorphism (RFLPs) (Lefebvre et al., 1997). In association with the appropriate planting material such as bulks of F₂, doubled-haploid individuals, near-isogenic lines etc., RAPD markers have been efficiently used to unveil the genomic regions near specific loci (Paran and Michelmore, 1993). RAPDs have been used to identify markers linked to nematode resistance gene in tomato (Klein-Lankhorst et al., 1991), downy mildew resistance gene (Dm5/8) in lettuce (Michelmore et al., 1991; Paran and Michelmore, 1993), mildew resistance gene in Hevea (Chen et al., 1994; Chen et al., 1999), tomato spotted wilt virus in Capsicum (Black et al., 1996), club root-resistance gene in Brassica (Kuginuki et al., 1997) and potato Y potyvirus (PVY) resistance gene (Ry stds) in Solanum (Kasai et al., 2000). Marker assisted selection with RAPD is not always
possible because RAPDs designed for one population are not always polymorphic for another. To overcome this problem, it is possible to convert a RAPD fragment to a RFLP probe or sequence characterized amplified region (SCAR) (Paran and Michelmore, 1993). To retrieve the monomorphic amplifications of SCAR, CAPS (cleaved amplified polymorphic sequence) has been developed from SCAR in Arabidopsis (Konieczny and Ausubel, 1993).

2.3.2.2.6. Genetic fidelity in tissue culture

RAPDs have been used to test the genetic fidelity of tissue culture raised plants. Various species of plants regenerated by callus formation may show genetic variability known as somaclonal variation (Brown, 1989; Karp, 1991; Godwin et al., 1997; Hashmi et al., 1997). Somaclonal variation, which occurs during the period of cell differentiation in the regeneration process, can be caused by changes in chromosome number or structure (deletions and rearrangements), DNA amplification, DNA modifications and activation of transposable elements. Some changes in the genome can not be observed at morphological or physiological levels because different gene structures may not alter the biological activity enough to modify the phenotype (Sabir et al., 1992). Molecular markers can be used in the juvenile phase of the plant before mature characteristics appear, allowing for early selection of true-to-type plants. RAPD has been used in the rapid screening of somaclonal variants in rice (Muller et al., 1990), Triticum (Morere-Le Paven et al., 1992; Brown et al., 1993), Picea (Isabel et al., 1993), Populus (Rani et al.,
1995), Piper (Parani et al., 1997), Andrographis (Padmesh et al., 1999) and sugarbeet (Jazdzewska et al., 2000).

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

2.3.3. Tissue culture

2.3.3.1. Historical aspects

Plant tissue culture plays an important role in crop improvement by which a plant breeder or grower is no longer constrained by the season for developing large number of plants with desirable characters within a short span of time and space (Gupta et al., 1993). Plant tissue culture is a collection of techniques by which isolated cells or tissues from either root, stem, leaf or any other part of plant body, if provided with a suitable condition, would develop into a plantlet in a test tube (Bajaj, 1995).
The science of plant tissue culture began in 1902 with the attempt to culture plant cells by Haberlandt (1902). The possibility to cultivate plant tissue for unlimited periods of time was announced independently and simultaneously by White (1939), Nobecourt (1939) and Gautheret (1939). Skoog and Miller (1957) discovered the interaction between auxin and cytokinin and regeneration of entire plant was achieved by Steward (1958) from callus tissue of carrot. Vasil and Hildebrandt (1966) have demonstrated for the first time, cellular totipotency in the single isolated cell of a tobacco hybrid. In 1960, Cocking isolated protoplasts by enzymatic digestion of cell wall. Later, in 1971, Takebe et al. (1971) achieved division and regeneration of protoplasts. This was followed by first successful fusion of protoplast and recovery of hybrid plant (Carlson et al., 1972).

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

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

2.3.3.2.1. Direct regeneration

Direct regeneration of plantlets or the formation of somatic embryos from the explants such as buds, meristems, cuttings, etc. ensures the cloning of genetic stocks. For producing true-to-type plantlets, success of direct regeneration is reported in a number of tree species such as Alnus (Brown and Sommer, 1982), Tectona (Gupta et al., 1980; Bonal and Monteuis, 1997), Castanea (Vieitez et al., 1983), Santalum (Rao and Bapat, 1978; Bapat and Rao, 1984; Lakshmi Sita, 1986; Rao et al., 1984; Das et al., 1998; Sanjaya et al., 1998; Pradhan and Saiju, 1999), apple (Karhu, 1997), Dendrocalamus (Ravikumar et al., 1998), Albizia (Kumar et
al., 1998; Sinha et al., 2000), chestnut (Gonialves et al., 1998), almond (Ainsley et al., 2000), Aegle (Islam et al., 2000), Dalbergia (Pattnaik et al., 2000), etc.

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

2.3.3.2. Indirect regeneration

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

In contrast to the direct method, which ensures clonal stability, the indirect regeneration of plants from callus may result in genetic variability. The callus cultures on periodic subculturing undergo genetic changes such as mutations, changes in ploidy, etc. The advantage of callus cultures is the large number of plants that can be produced from a single culture, as every cell is a potential plant (Gupta, 1991). Callus regeneration is efficiently exploited in many tree species such as Citrus (Chaturvedi and Mitra, 1974), Prunus (Druart, 1980), Sapium
(Venkateswaran and Gandhi, 1982), pine (Tang et al., 1998), apple (Rugini and Mugano, 1998) and pear (Zhu and Welander, 2000). In view of the enormous number of propagules, this system of regeneration can be used in reforestation programmes where the quantity and not necessarily the quality of the biomass is of importance (Zhu and Welander, 2000).

2.3.3.2.3. Somatic embryogenesis


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

In spite of all this excitement in the area of somatic embryogenesis, some basic problems needed further attention. Plants regenerated through somatic embryogenesis exhibit phenotypic variation, which may have a genetic, or non-genetic basis. Genetic variation has been termed somaclonal variation (Scowcroft and Larkin, 1982). For obtaining true-to-type propagules from a selected genotype, somaclonal variation is undesirable. On the other hand, somaclonal variation offers prospects for the recovery of beneficial variant and creates variability useful for plant improvement (Larkin and Scowcroft, 1981; Svobodova et al., 1999). Difficulties for induction of somatic embryos in some species/genotypes have been
reported (Bonga and Durzan, 1982; Tang, 2001). Problems with maturation and conversion of somatic embryos and poor development of viable somatic seedlings are also reported in a number of forestry species (Faure et al., 1996; Tang, 2001; Ilah et al., 2002). The anatomical, biochemical and molecular basis for such abnormalities have to be elucidated for further normalization, improvement and proper utilization of the technique.

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