

Genetics and Biotechnology of Sandalwood (*S. album* L.)

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Abstract: Genetic improvement of trees, especially forest trees, is an important but neglected and often overlooked research priority, due to several reasons. Modern biotechnology has revolutionized all fields of biology, more so the plant biology, with profound transformational potential to address concerns and to derive opportunities. Judicious application of biotechnological tools can bring about comprehensive changes in the field of genetic improvement of trees. Different methods of molecular biotechnological interventions are needed for the improvement of trees, depending upon tree species, its genetics, its commercial and conservational priorities and response to genetic selection systems. Brief encounters with the myriad of complexities of genetics of heterozygous and heterogeneous perennial trees, w.r. t. sandalwood, are presented. Selected examples of potential genetic and molecular biotechnological interventions in tree improvement are discussed, including molecular genetics of sandalwood oil biosynthesis, rapid multiplication/micropropagation of elite clones, molecular detection and genotyping, tree polymorphism and association mapping, in vitro germplasm conservation, tree "omics" and designer GM trees.

Key words: Sandalwood, micropropagation, Heterozygosity, Heterogeneity, Variability.



INTRODUCTION

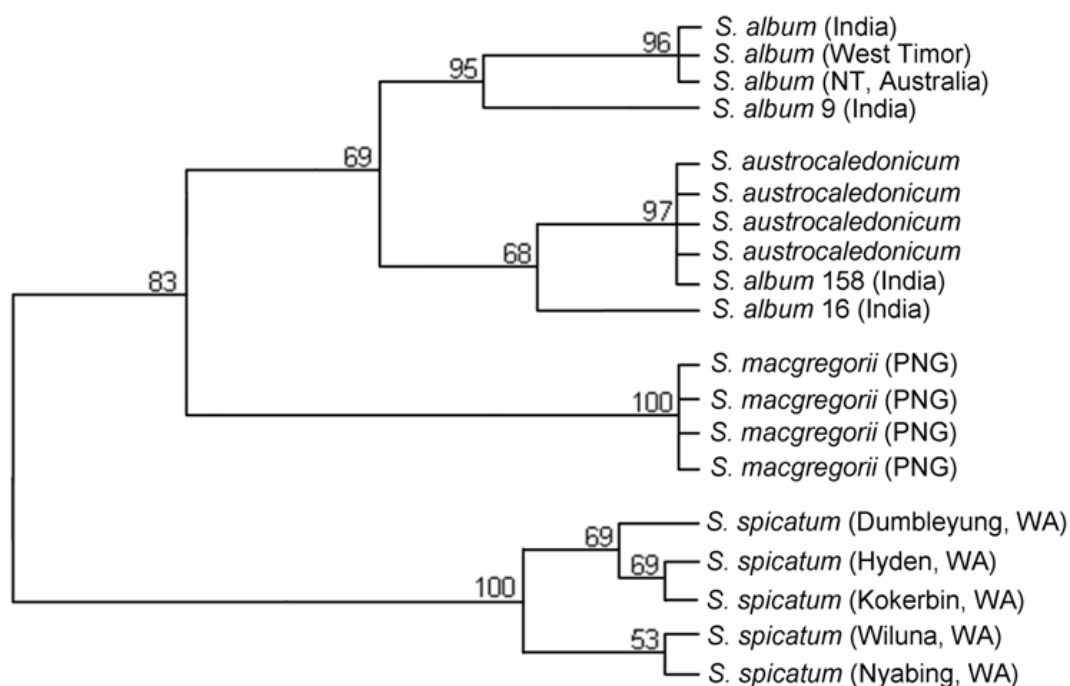
1. Sandalwood genetics

Sandalwood represents a typical less domesticated case of highly heterozygous and highly heterogenous, perennial tree species, often propagated by seeds. Although overall oil content is the trait of commercial interest, heartwood yield, the most important and highly correlated trait influences oil yield, apart from other traits. The major problem in plantation establishment of this species is the supply of genetically improved materials for large scale planting programmes; to overcome shortages both short- and long-term improvement and propagation strategies are proposed. The short-term strategy includes the establishment and management of seed production areas, clonal seed orchards and clone banks for seed and clonal stock production. The mass clonal propagation of selected trees, tissue culture in combination with a plantlet cutting technique, is recommended. Long-term improvement strategies involve the development and management of gene resource populations, breeding populations and propagation populations. Genetic structures and functions of these three populations are discussed. In long-term breeding populations, the formulation, establishment and management of single and multiple breeding populations suitable for breeding programmes are important considerations.

Tree improvement strategies involve planning and execution for achieving general objectives, especially of long term breeding, propagation and conservation in the improvement programme. The formulation and development of an improvement strategy requires biological and technological knowledge including: genetic variation and gain, flowering biology, mating system, seed production, clonal propagation, planting techniques etc. Tree populations in an improvement programme consist of genetic resources, breeding, propagation and wood production. The genetic structures of these four populations are the core elements in the breeding programme. Due to the differences in their objectives, genetic structures, variabilities and long- term utilization, these four populations are usually established and maintained separately.

1.1 High levels of heterozygosity and heterogeneity.

Genetic variation depicts a more adaptable genome to changes. Interbreeding populations contain individuals with unique genotypes, identifying a predisposition to yield more heartwood, produce different compounds in the oil and faster growth rate. Based on genetic distances, appropriate breeding methodologies and parents for high heterosis can be selected.



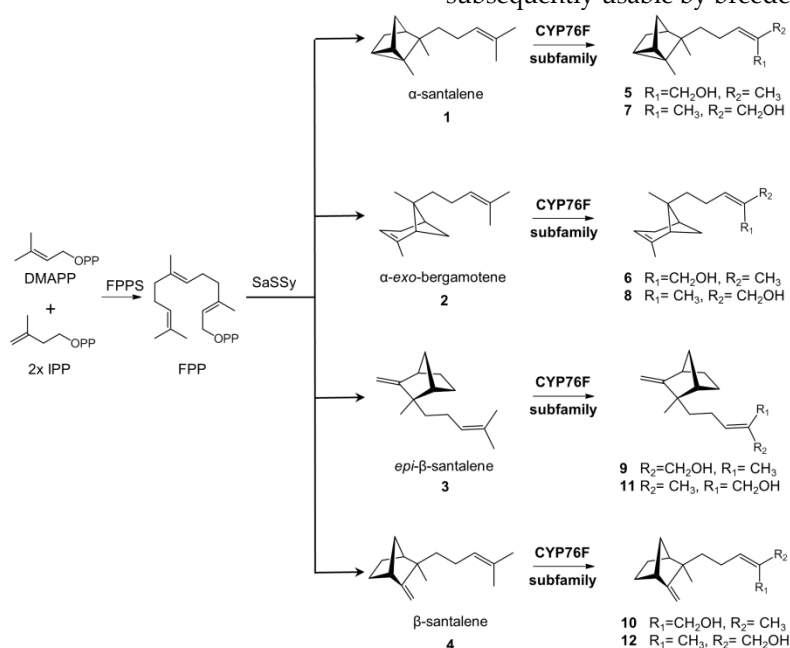
Selection of parents for wide hybridization based on genetic distance (From Jones *et al*⁸)

1.2 Extensive variation in the heartwood developmental biology: Genetics of heartwood development has not been systematically worked out in sandalwood in the absence of appropriate breeding lines. However, extensive genetic variation is available in this trait and should be utilized for tree improvement, although a very

strict correlation between this trait and oil content is lacking.

1.3 Huge variability in the amount of oil produced by individual trees

Oil yield is the final commercial trait of importance in sandalwood. High heterozygosity and heterogeneity in this crop promises the availability of inherently huge variability in the oil content in the sandalwood germplasm and subsequently usable by breeders.



Schematic biosynthetic pathway for santalols and bergamotol in sandalwood.

Compounds identified with numbers are: α -santalene (1), α -*exo*-bergamotene (2), *epi*- β -santalene (3), β -santalene (4), (*Z*)- α -santalol (5), (*E*)- α -santalol (7), (*Z*)- α -*exo*-bergamotol (6), (*E*)- α -*exo*-bergamotol (8), (*Z*)-*epi*- β -santalol (9), (*E*)-*epi*- β -santalol (11), (*Z*)- β -santalol (10), (*E*)- β -santalol (12). Numbers match the numbers. DMADP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; SaSSy, *S. album*santalene synthase.

1.3 Root parasitism means host selection is vital. Also, short, medium and long term hosts means high maintenance silviculture

Secondary selection of host and corresponding parasite (sandalwood) survival traits is important in sandalwood breeding and crop improvement although this involves high main

2. Oil phenotype is susceptible to forces

Simply put, the phenotypic value (P) of an individual is the combined result of its genotype (G) and the effects of the environment (E):

$$P = G + E$$

Genotype refers to the total genetic variation. This includes not only the effects of nuclear genes, but also the effects of mitochondrial genes and the interactions between genes. Genotypic variation can be partitioned into additive and dominance variation:

Additive variation represents the cumulative effect of individual loci, therefore the overall mean is equal to the summed contribution of these loci.

Dominance variation represents interaction between alleles. If a trait is controlled by a dominant allele, then both homozygous and heterozygous individuals will display the same phenotypic value.

Quantitatively varying traits are also affected by the environment (E). This can be further subdivided into pure environmental effect and interaction between genes and the environment. In other words, how different genotypes respond in different environments. Finally, interaction (I) between different genes can modify the observed phenotypes. This is called epistasis, or non-allelic interaction, distinguishing it from dominance. So, the equation above can be better written as:

$$P = A + D + E + I$$

However, since geneticists are interested in studying and identifying variation and its source in a given population, this equation is better described below. The total phenotypic variation (V) of a population is the sum of the variation in

additive (A), dominance (D), gene-interaction (I), environmental (E) and gene-environment interaction (GE) effects:

$$VP = VA + VD + VI + VE + VGE$$

Being able to estimate how the total variance is partitioned between genetic and environmental effects is important to quantitative geneticists trying to improve a given trait. If the proportion of variation is mostly due to genetic effects (heritable), then selecting for individuals that possess the desired genetic value is a worthwhile investment. If however, the genetic variance is low (and therefore the environmental variation has more impact on phenotype), then a more strategic approach would be to optimize environmental conditions.

Heritability estimates how much of the phenotypic variation can be explained by genetic, or genetic-environmental effects. Broad-sense heritability (H^2) refers to the inclusion of all potential sources of genetic variation (additive, dominance, epistatic, maternal and paternal effects):

$$H^2 = VG/VP$$

To only know the ratio of additive genetic variation to the total phenotypic variation observed, VA can be used in the equation instead of VG , and this becomes the narrow-sense heritability (h^2):

$$h^2 = VA/VP$$

Calculating narrow-sense heritability is important for predicting how a trait will respond to selection (response to selection refers to the gain in the mean of the population compared to the mean of the selected parents). However, important to note, is that the estimate of heritability is not an absolute measurement of how genes and environment determine a phenotype, but specific to the population and environment under analysis.

The covariance in phenotype of two traits (A_1 and A_2) can be used to calculate their genetic correlation using the following equation:

$$\text{Genetic correlation} = \frac{\text{Cov}(A_1, A_2)}{\sqrt{V_{A_1} \times V_{A_2}}}$$

In case of quantitative genetic analysis of highly heterozygous and heterogeneous perennial crops like sandalwood, the correlated heritability coefficients are less understood, coupled with small population sizes, absence of breeding lines

and long crop stand durations. However, an effort must be made to initiate such studies.

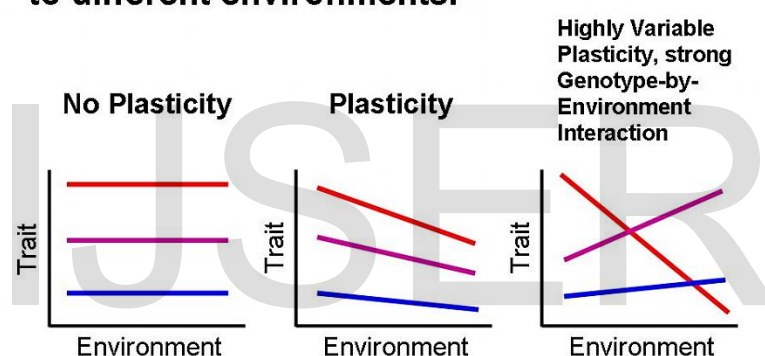
3. "High yielding clone" is deceptive Yield is the most important and complex trait for the genetic improvement of crops. Although much research into the genetic basis of yield and yield-associated traits has been reported, in each such experiment the genetic architecture and determinants of yield have remained ambiguous. One of the most intractable problems is the interaction between genes and the environment. This is partly because of the following reasons:

- 3.1 Yield is a function of many component traits
- 3.2 Absence of replicated trials (multilocation, multiyear and multiparametric)
- 3.3 Oil yield is governed by QTLs
- 3.4 Genetic diversity is unverified and lacking

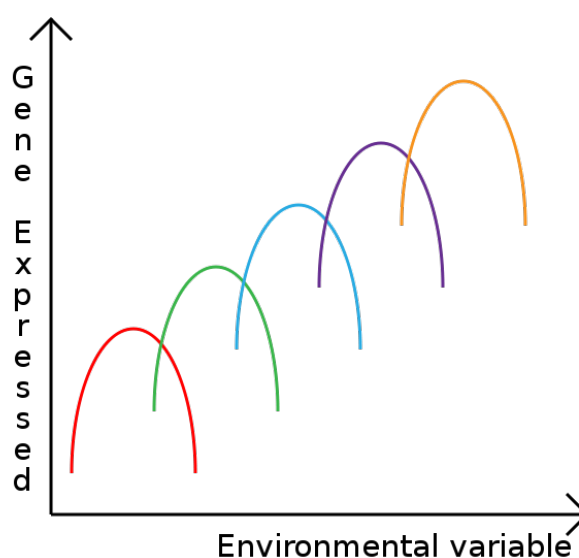
4. Sandalwood phenology is plastic As sessile organisms, plants are unable to seek out environmental conditions optimal for their growth and development but instead must complete their life cycles in the environment in which they are growing. However, plants are remarkably plastic, such that a single genotype is able to give rise to a wide range of phenotypes. Developmental plasticity has profound implications for plant evolution and ecology and can make important contributions to improving yield stability in agriculture and silviculture. Sandalwood is no exception for this reaction norm.

4.1 The ability of one genotype to produce more than one phenotype when exposed to different environments (Reaction Norm):

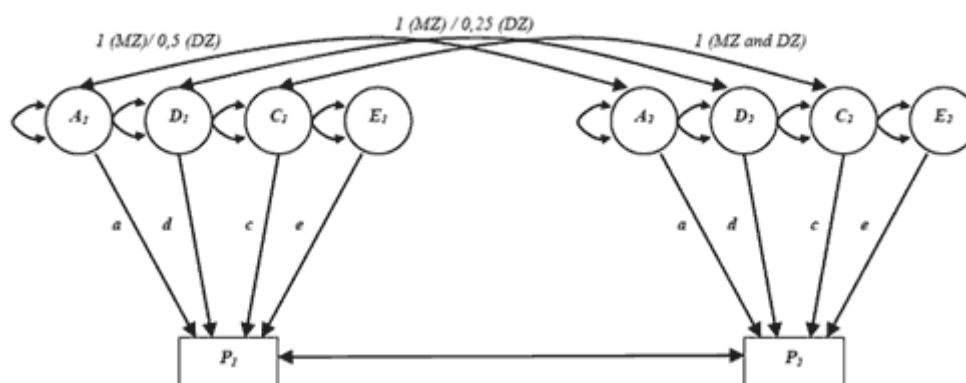
The ability of one genotype to produce more than one phenotype when exposed to different environments.



Each of the colored lines is a "Reaction Norm"

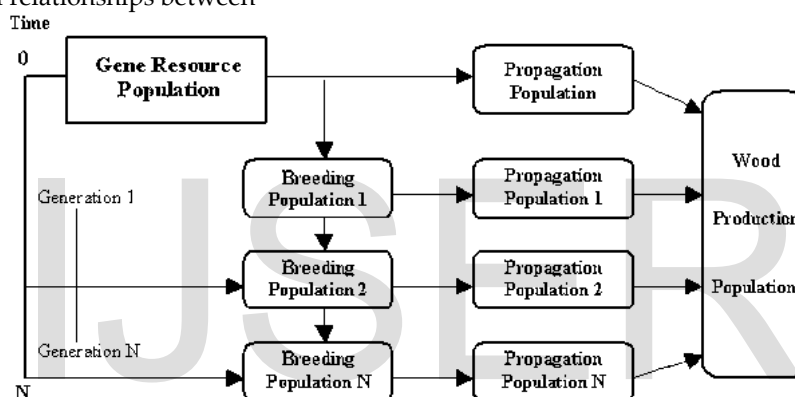


5. The solution to improvement: 3R's
5.1 Reduce environmental variance



Path coefficients diagram for genetic and nongenetic factors. Rectangles represent measurable variables; circles represent latent variables; one-way arrows (or paths) show hypothetical causal relationships between

variables; two-way arrows show covariances and dispersions. Selection within and among geographically close trees.



Over time, genetic material is progressed through the series of populations by a process of selection and mating.

Operations and management in an improvement programme are mostly concerned with the availability of human resources, financial resources, infrastructure and organization, knowledge of the genetic parameters and reproductive biology of the species, information, technologies, etc. Research and development in an improvement programme is essential to solve certain key problems. Moreover, the development of appropriate technologies will assist and facilitate the breeding activities (e.g. pollen extraction and storage, flowering induction, etc.) and propagation operations (e.g. seed production, cuttings, tissue culture, etc.). Provenance variations in wood quality, growth rate, stem form, seed morphology and germination and other characters are substantially reported. Heritability values of

certain characters at provenance level have been estimated to understand tree genetic parameters. Although several breeding methods have been developed, i.e. to maximize gain and to maintain genetic variability of the breeding population, few methods are being used in the tree programme, e.g. simple recurrent selection (SRS), simple mass selection (SMS), simple recurrent selection for general combining ability (SRS-GCA) and selection and mass vegetative propagation (MVP). These methods may be operated for a short term seed production or propagation programme or for a long term breeding and propagation programme. In a long term programme, it may be operated as a basic single population programme or as a multiple populations programme. However, these tree breeding systems are managed and operated based on the assumption that: a) there is no controlled pollination; b) an open pollinated progeny test at the initial stage is not feasible;

and c) mass clonal propagation, i.e. through tissue culture and seedling cutting is applicable. It is well accepted that controlled pollination is the most difficult part in the breeding programme. As for sandalwood, the pollination technique, at this stage, is still not feasible for a full-sib progeny testing programme.

Simple Recurrent Selection (SRS). A SRS, which is the simplest and cheapest breeding method, is widely advisable in a short term seed production programme. In this method, clonal and progeny tests are not essential. This method is most suitable for short term seed production programme. Recurrent selection for general combining ability is a modified SRS. The main purpose of a long term breeding programme is to generate cumulatively improved plant material as well as to create genetic diversity of the breeding population for as many generations as possible. Multiple breeding populations, designed to manage larger breeding populations, i.e. for larger genetic diversity and gain, have been widely developed. Exploit seedling

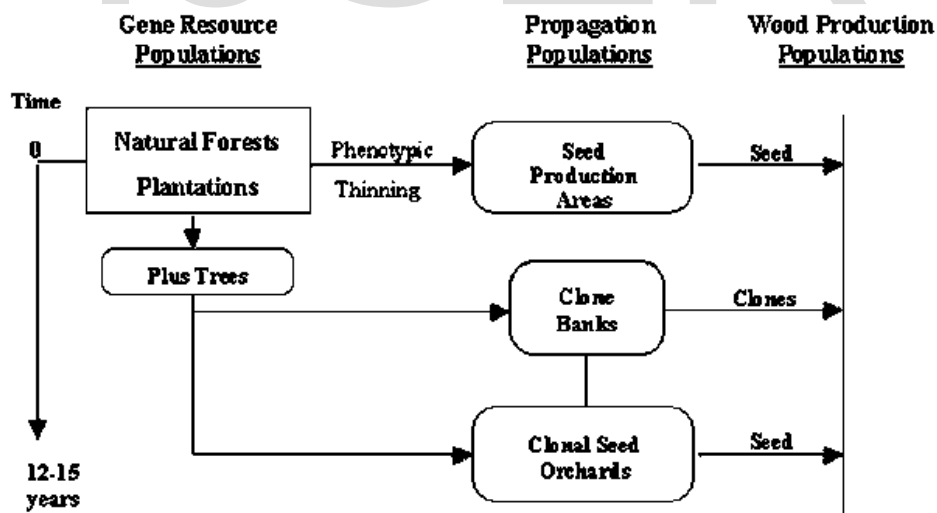
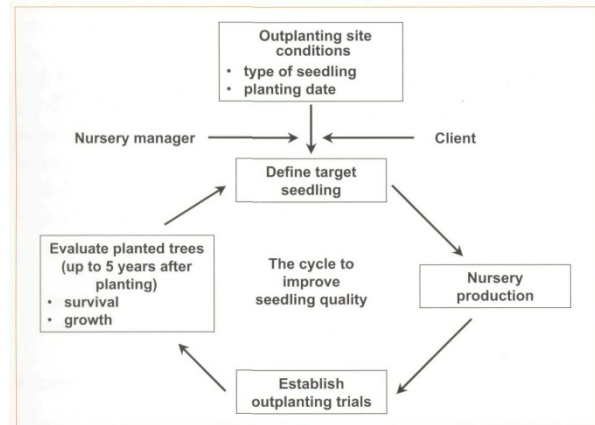
5.2 Replicate best clones

Single hotspot and Multilocational trials

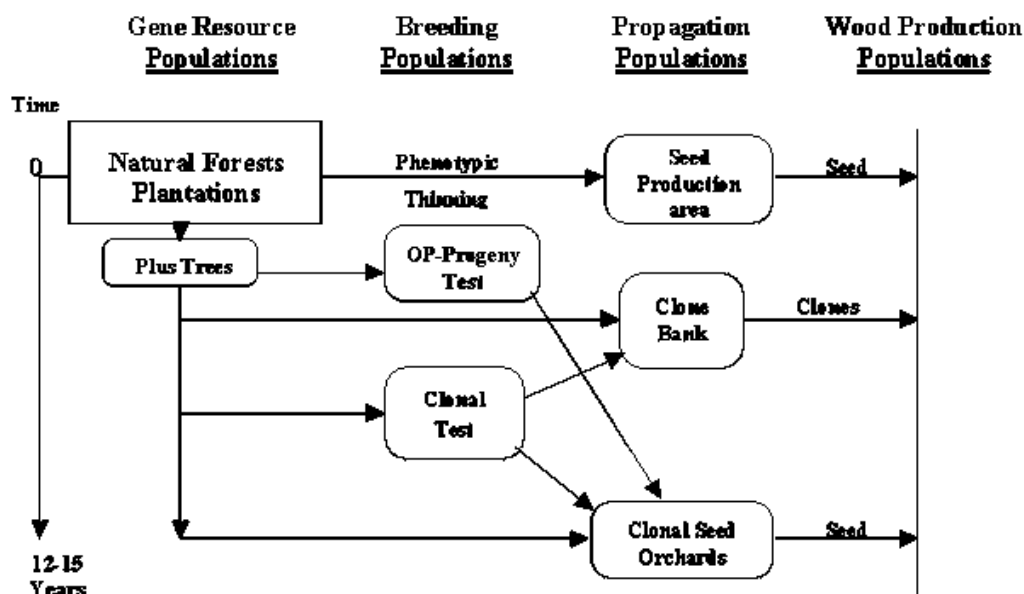
5.3 Revisit recombinants

Breeding among elite clones and select rare recombinants

variability The most straightforward and comprehensive methodology of perennial tree breeding is the exploitation of inherent heterozygosity/heterogeneity and variation through selection from the seedling variability as depicted below:



Simple recurrent selection in a tree improvement programme



Recurrent selection for general combining ability in the tree breeding programme

6. Non-GM options Clonal selection. Although deceptive as discussed earlier, clonal selection needs to be scientifically followed through sound genetic principles.

6.1 Seedling variability. Needs to be exploited in sandalwood improvement religiously for a straightforward genetic gain.

Micropropagation Fruit is produced after three years and viable seeds are mostly available after five years. Like most forest tree species, seed propagation is common to *Santalum*, however, seedlings are extremely heterozygous due to out-crossing. Vegetative propagation via grafting, air layering and root suckers can be used but the production of clones is inefficient and time consuming.

For centuries, sandalwood has been an important commercial industry in the Pacific. Sandalwood species are grown in island countries like: *Santalum macgregorii* (in PNG Papua New Guinea), *Santalumaustroledonicum* (New Caledonia and Vanuatu) and *Santalum yasi* (Fiji and Tonga). *Santalum album*, however, is also planted and grows well in most Pacific countries from New Caledonia to French Polynesia and the Cook Islands.

The long-term exploitation of sandalwood has had a serious impact on the industry and there is now an urgent need to undertake some extensive planting. To achieve the level of planting required using conventional propagation methods would be extremely inefficient and time-consuming. An effective micro propagation protocol would greatly benefit the development of the sandalwood "industry" and increase

productivity. As stated by Beck and Dunlop (2001) plant tissue culture based biotechnology has been employed to generate quality planting material with many forest tree species.

The sandalwood research in the CePaCT (Centre for Pacific Crops and Trees) aims to establish a micro propagation protocol that would address initiation, multiplication and rooting of the *Santalum* sp. Multiple shoots of *Santalum yasi*, *Santalum album* and hybrid have been induced from nodal shoot segments derived from adult trees at Colo-i-Suva (Forest park in Suva Fiji) and juvenile plants excised from seed-derived plants in the CePaCT nursery. Both explants taken from the adult trees and the nursery plants were cultured on MS (Murashige & Skoog, 1962) medium supplemented with different concentrations of α -naphthalene-acetic acid (NAA) and 6-benzylaminopurine (BA). Three different treatments are currently being investigated for root establishment, namely: pulse treatment of explants followed by soil planting, culturing explants on MS medium supplemented with host plant extracts; culturing explants on MS medium.

6.2 Genome-wide Association mapping and genomics

The genome-wide association approach (GWAS) overcomes several limitations of traditional gene mapping by (i) providing higher resolution, often to the gene level, and (ii) using samples from previously well-studied populations in which commonly occurring genetic variations can be associated with phenotypic variation. The advent of high-density single-nucleotide polymorphism (SNP) typing allowed whole-genome scans to identify often small haplotype

blocks that are significantly correlated with quantitative trait variation. These approaches have enabled both large studies of human disease, which have identified important loci, and recent plant studies that have been successful in identifying loci that explain large portions of phenotypic variation. Sandalwood offers an excellent candidature in GWAS.

Somatic cell breeding. The study of mechanisms of inheritance in animals and plants by using cells in culture. In such cells, chromosomes and genes can be reshuffled by parasexual methods, rather than having to depend upon the chromosome segregation and genetic recombination that occur during the meiotic cell divisions preceding gamete formation and sexual reproduction. Genetic analysis is concerned with the role of genes and chromosomes in the development and function of individuals and the evolution of species. Genetic analysis of complex multicellular organisms classically required multiple-generation families, and fairly large numbers of progeny of defined matings had to be scored. As a result, analysis of animals and plants with long generation times, small families, or lack of controlled matings was difficult and slow. Somatic cell genetics circumvents many of these limitations. It has enhanced the scope and speed of genetic analysis in higher plants and animals, especially when combined with the powerful techniques of molecular biology and the ability to generate fertile plants and animals from single cultured cells. With these methods, every gene in any species of interest can be identified and mapped to its position on a particular chromosome, its functions determined, and its evolutionary relationships to genes in other species revealed. Cross-species comparisons have provided essential insights into such poorly understood areas as embryonic differentiation and the development of complex nervous systems. Sandalwood in vitro genetics is an entirely new field and offers opportunities for innovations.

6.2 Look beyond *S. album*

Evolutionarily related *Santalum* species which are not so intractable like *S. album* need to be explored. Besides, other trees that provide biochemical raw precursor molecules for essential oil biosynthesis equivalent to *S. album* need to be explored, reducing commercial pressure on sandalwood while providing alternate routes to synthetic biochemistry.

7. GM options

Although a moratorium has been in place for all GM crops except Bt cotton in India currently, GM options such as the following should be kept open for R&D and when commercialization opportunities are opened up, GM technologies can usher revolutionary progress in *S. album* biotechnology:

Engineering rate limiting enzymes and pathways
Engineering climate resilient sandalwood.

Express oil components in whole trees and seedlings.
Bioreactor production of tissues, planting materials and oil.

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