DALBERGIA AND ALBIZIA : PLANTLET PRODUCTION VIA
TISSUE CULTURE, KARYOLOGICAL EVALUATION,
AND SEED ANATOMY WITH SCANNING
ELECTRON MICROSCOPY

DISSERTATION

Presented to the Graduate Council of the
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By

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A publication by the National Academy of Sciences, USA (1979) outlined some of the research need for a great variety of economically important woody species whose remaining genetic resources need urgently to be collected and conserved. A viable regeneration system was established via tissue and cell suspension culture for *Albizia falcata*ria* and *A. lebbeck*, two important wood yielding leguminous tree species. The culture medium was standardized after several trials to obtain callus from the leaflet explants of these two tree species. The optimum use of casein hydrolysate (w/v) and coconut milk (v/v) in addition to 6-Benzylaminopurine and Indole-3-butyric acid could induce morphogenesis and somatic embryogenesis in the cultured tissue. This reports the first observation on somatic embryogenesis of *A. lebbeck* using leaflets as the explants. Scanning Electron Microscopy and histological studies were done on the different stages plant development following standard techniques. Embryogenesis in suspension culture followed regeneration of plantlets in *A. lebbeck*. In *A. falcata*ria* the regenerative process followed via organogenesis from the shoot buds developed on the leaf explants. After hardening the regenerated plants were transferred to the greenhouse. Some of the trees grew more than 25 feet tall within a few months outside the greenhouse.
Karyotype of the three leguminous trees *Albizia lebbeck*, *A. falcata*, and *Dalbergia sisoo* was analyzed. In *D. sisoo*, various chromosomal anomalies were observed in the cultured tissue. The abnormality indices and ploidy level varied with the age and the frequency of the subculture. In the aged culture the regenerative potential declined but was reinstated to some extent with the addition of two complex growth factors, coconut milk and casein hydrolysate.

Seed anatomy of 26 species of 4 leguminous genera was studied with SEM. The main distinguishing anatomical features observed in the seed sections were uniseriate or multiseriate epidermis, epidermal projections, and number of rows and nature of columns of hypodermal layer, especially the nature of endosperm. Three species of *Dalbergia*, *Acacia* and *Cassia* and two species of *Albizia* are difficult to distinguish externally even with seed coat study under SEM, but this study with cross sections provided enough characteristic features to distinguish one from the other.
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<td>AI</td>
<td>Abnormality Index</td>
</tr>
<tr>
<td>B₃</td>
<td>Gamborg's medium</td>
</tr>
<tr>
<td>BAP</td>
<td>6-Benzyl-amino-purine</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CM</td>
<td>Coconut milk</td>
</tr>
<tr>
<td>CH</td>
<td>Casein hydrolysate</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>Concn.</td>
<td>Concentration</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2, 4-dichloro phenoxy acetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscope</td>
</tr>
<tr>
<td>GI</td>
<td>Growth index</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>Indole-3-butyric acid</td>
</tr>
<tr>
<td>Kn</td>
<td>Kinetin</td>
</tr>
<tr>
<td>Kv</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>M</td>
<td>Molar solution</td>
</tr>
<tr>
<td>μ</td>
<td>Micron</td>
</tr>
<tr>
<td>mg/l</td>
<td>Milligram per liter</td>
</tr>
<tr>
<td>MI</td>
<td>Mitotic index</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog (1962) medium</td>
</tr>
<tr>
<td>N</td>
<td>Normal solution</td>
</tr>
<tr>
<td>NAA</td>
<td>α-Naphthalene acetic acid</td>
</tr>
<tr>
<td>p-DB</td>
<td>Para-dichlorobenzene</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>‘t’-test</td>
<td>Student’s t test, a test of significance (W. S. Gosset, 1908 and R. A. Fisher, 1924)</td>
</tr>
<tr>
<td>v/v</td>
<td>In volume/volume ratio</td>
</tr>
<tr>
<td>w/v</td>
<td>In weight/volume ratio</td>
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CHAPTER 1

INTRODUCTION

A publication by the National Academy of Sciences, USA on tropical legumes (NAS 1979) outlined some of the research needs for a great variety of economically important woody species whose remaining genetic resources need urgently to be collected and conserved. Most of these serious issues remain unaddressed or unsolved. Little work has been done on genetic improvement of the leguminous trees that are the main components of a tropical rain forest. Forest is an important natural resource as well as a most important natural habitat for some wild life. It provides raw materials for many products of daily use. Printing, painting and other industries get turpentine oil, gum, resin and other raw materials from the trees of the forests. It supplies several industries that depend on wood products. In spite of immense scientific achievement the demand of plant products is increasing day by day. Not only is man absolutely dependent on plants for food, but plants are also a major source of clothing, fuel, drugs, and construction materials. The trees and forests play a vital role in protecting the environment by consuming carbon dioxide and releasing oxygen. An acre of forest absorbs 4 tons of carbonic acid gas and recycles 8 tons of oxygen into the environment (Shukla and Chandel 1985).

The presence of forests reduces soil erosion, slows wind speeds, traps sand and moderates the force of rain and slows the run-off of water after heavy rain. In addition,
planting and establishment of trees on degraded lands, industrial waste or sand dunes, can be the first important steps in soil rehabilitation and land reclamation.

It is estimated that the 'second energy crisis' involves loss of forest resources at the rate of eight acres per minute implying that in the next twenty years it would lead to a loss of about one-third of the world's forest cover. If the present rate of deforestation is continued, one can easily predict a disaster on the global scale. There will be exposure and washing away the topsoil, reduction of soil nutrients and decline of agricultural productivity.

Because of the indiscriminate exploitation of forests, large scale destruction of tree habitats, adverse effects of pollution and introduction of alien species, a number of species have already disappeared from the surface of the earth and many others are awaiting a similar fate. All human interference has been responsible for the depletion of plant resources and consequent decline of genetic diversity. Permanent destruction of forest cover by cultivation has eroded many gene resources.

Besides this, ozone depletion, acid rain and global warming are posing further threats to biodiversity. The importance of species diversity conservation is justified by the direct use of species by man, like food sources, fiber, fuel, new chemicals and raw materials.

Two major factors, the energy crisis and environmental pollution steer the thinking for understanding the great and significant roles of plant communities for general welfare of human beings. This necessitates ensuring conservation and protection of the rich varied forest wealth and regeneration of forests.
All aspects of life and human development and practically every human activity are concerned with the utilization of energy in various forms. The increasing world population requires improvement of existing and evolution of newer varieties of plants so that more food and energy are produced. As food and energy are concerned, leguminous plants have made significant contributions in this respect and thereby satisfy a wide range of needs. The family Leguminosae has an estimated 16,000 to 19,000 species in about 750 genera. In economic importance it is second only to the grasses, Gramineae (Poaceae); in size, only to the Orchidaceae and Compositae (Asteraceae). Bentham and Hooker considered Leguminosae as an order with three suborders -- Papilionoideae, Caesalpiniae and Mimosoideae. Engler and Diels considered these three groups as subfamilies while Hutchinson gave those groups the status of family under the order Leguminales. Many species in Mimosae and Caesalpiniae are valuable for their timber, dyes, tannins, resins, gums, insecticides, medicines and for fibers. They are, in addition, the world's handsomest flowering trees, vines and shrubs. Numerous members of the Papilionaceae, moreover are economically important, especially in temperate areas, as edible and highly nutritional crops for human and animal consumption, for forage, fodder, ground cover, green manure, erosion controls and as major honey sources. As pioneer plants in the Arctic regions they form the hub of an efficient nitrogen source for the entire ecosystem (Allen et al. 1981).

The non-renewable sources of energy like coal, gas, oil can not last indefinitely. The fossil fuel will be scarce in the coming century. The present day world is now conscious about the finiteness of hydrocarbon reserves. It is, therefore, desirable to
develop and explore renewable sources of energy that can be developed in a location specific decentralized manner (Report of the firewood study Committee 1982). The leguminous fuelwood trees are selected for their bole wood, branch wood, leaves as well as pods and their percentage contribution in total aerial biomass production. The fuel crisis can be partially solved by raising such multipurpose fuel-cum-fodder-cum-timber trees (Deb Roy 1986b).

In the present investigation, three such important tree species, namely, *Albizia lebbeck* (L.) Benth. and *Albizia falcata* (L.) Fosberg and *Dalbergia sissoo* Roxb. (American Rosewood tree) of the family Leguminosae were taken into consideration for *in vitro* cultural and cytogenetical studies. The application of *in vitro* techniques to trees had a rather late beginning but is now becoming a very active area of research. However, it is still noticeable that only a relatively small number of species have been studied, and only a few technologies applied. Potential technologies are required to stimulate further research into *in vitro* culture of trees (Dodds 1983). Many tree species are propagated vegetatively, that is they are reproduced in ways other than pollination. Some such methods are propagation by cuttings, grafting, layering, etc. These methods produce plants genetically identical to the parent plant, but there are difficulties. Only a small number of plants can be produced in these ways and it takes years to build up enough stock for planting in fields and forests. Sometimes the method proves to be impossible. The long life cycle of trees also makes development of superior varieties very lengthy and tedious. The conventional methods of propagation by seed and vegetative means have many limitations. In cross-pollinated plants there is tremendous heterozygosity.
Seed propagation, therefore, produces plants with different characters. Most of the forest trees fall in this category. Vegetative propagation can take care of this problem to some extent. But these techniques are applicable to very few species and are very slow. Moreover, diseases present in the seeds or the vegetative parts get propagated in the daughter plants. Seeds of a number of plants have long dormancy periods and thus take a long time to germinate.

Tissue culture is a well-known method for mass propagation, multiplication and conservation of plants. It has great potential to improve the traditional method of tree breeding, particularly genotype evolution, with respect to growth rate, hardiness, disease, drought and chemical resistance. In vitro selection has a distinct advantage over other selection systems since it allows significant saving of space, time and money. Micro propagation of tree species is being increasingly recognized as a tool with much potential for application in the field of forestry. It offers a rapid means of afforestation, multiplying woody biomass, and of conserving elite and rare germplasm (Bonga and Durzan 1982; Bajaj 1986).

Modern forest plantations have specific purposes for which use of correct species and germplasm is essential. Every species is a genetic source and needs to be considered. Conservation without having studied the genetic information carried by them will not serve a worthwhile purpose. Sound information should also work to form an index of characters of the species concerned obtained from different fields of study. Forest genetics deals with inherited variation, genetic biology, evolution and collection of data on different species for tree improvement work. Tree improvement is a part of modern
silviculture with the help of which wood and other products can be increased and qualitatively improved upon. Tree genetic improvement gives permanent gain and once achieved this gain requires no further expenditure for its maintenance. A genetic improvement can generate recurring profits, therefore genetic techniques are worth investing in the forestry practice. Genetic techniques usable on common indigenous tree species have received great attention. Improvement programs for gains in vigor, tree form, wood characteristics (color, texture and hardness) and disease resistance has been initiated in several countries. Organized information on many of the tree species is not available so indexing and cataloguing of characteristics of a species is still a requirement for tree improvement. In vitro techniques of regeneration of plants offer an effective and alternative way by which plants can be produced, maintained, multiply and transport disease free propagules or regenerants safely and more economically. The most common commercial use of plant tissue culture is clonal propagation that is also termed as micro propagation. The in vitro technique of clonal propagation requires a quantitative approach that leads to an optimization of the conditions associated with vegetative plant propagation.

The basic advantages of in vitro propagation lie mainly in the rapidity with which plant multiplication can be successfully achieved and the number of plants that can be produced in a relatively short period, with conservation of space and often with a lower cost. Conventional methods of propagation, namely by seeds and standard vegetative means, have limitations. In cross-pollinated plants there is tremendous heterozygosity and offspring may be of poor quality or even unusable. The alternate pathway of regeneration
through tissue culture can overcome this problem. The difference between tissue culture and traditional methods of cloning involve the use of smaller propagules, the provision of an aseptic and artificial environment, and substantially greater plant multiplication (Murashige 1978). Plant tissue culture offers excellent opportunities of mass propagation of plants in test tubes. Indeed the number of plants that can be obtained from the culture of a small segment of a superior tissue is unlimited.

After induction of mutation and genetic manipulation a suitable evaluation system is required for obtaining stable regenerants. In vitro selection has a distinct advantage over other selection systems since it allows significant saving of space, time and money. The process of organogenesis from explants or callus cultured in vitro is a complex piece of plant morphogenesis and serious technical achievement. A number of factors that are known to influence the expression of morphogenesis and proliferation rate of cultured explant or tissue:

i) size, source, physiological status and physiological variation of the explants and donor plants;

ii) cultural environment inside and outside the culture vessel;

iii) composition of the culture media;

iv) genotypes being propagated;

v) various technical problems associated with persistent contamination with bacteria or viruses and with the continued stability of regenerative capacity of cultures.

The initiation of plant tissue cultures, the induction of differentiation and the regeneration of complete plants from cultured cells depend upon being able to manipulate
the process of differentiation. In the present study a suitable technique was devised to culture the selected explant to obtain regenerants. Successful plant propagation through organogenesis by proper manipulation of cultural systems and conditions have been very useful in herbaceous crops and monocots (Murashige 1974; Hussey 1977b, 1978a; 1980a; Vasil and Vasil 1980; Krikorian 1982; Hu and Wang 1983; Hussey 1983, 1985), but much less success has been reported in woody plants.

PLANT GENERA IN THE PRESENT INVESTIGATION

*Albizia lebbeck* Benth.: *Albizia*, a large and complicated pan tropical genus, belongs to the subfamily Mimosaceae, 50 species of which are found in the warmer parts of the world. Two spellings of *Albizia* have been used. Some earlier authors used *Albizzia* but it is written with single in current scientific literature. *Albizias* are well adapted to poor soil and are found up to 1600m. altitude above sea level. They are common in low bush, secondary forests, sandy riverbeds, and in savannas. Most of the species are fast growing. A few of them yield valuable timber. *Albizia lebbeck* (L.) Benth. is known in Europe as East Indian Walnut (Watt 1908). Several *Albizias* are cultivated as avenue trees, and as shade trees in coffee and tree plantations. *A. lebbeck* is a large, erect, deciduous tree with an umbrella shaped crown.

The species is known in Hawaii and the Philippines as women's tongue because the pods remain on the tree for three to five months and make a chattering noise in the wind. In the Andamans it reaches 20-30 m. in height and a girth 1.8-3 m. is common. It occurs scattered in mixed deciduous forests. The tree prefers a moist situation, and is
found to grow on variety of soils.

It can be grown by line sowings on well-loosened soil. The rate of growth is very rapid. The leaves are bipinnate, pinnae 1-5 pairs and leaflets 3-8 pairs. The leaflets are very like those of *A. procera*, a species from which it is distinguished by the rougher and brown bark. The tree flowers in June-July and fruits in November-December. Pods are flat and straw colored. The wood is known in the timber industry as 'siris' or 'kokko'. The wood is hard, heavy and tough with medium coarse textured and has broadly or shallowly interlocked grains. The sapwood is white or yellowish and the heartwood is dark brown, streaked with dark and white shades. The wood is excellent for high-class furniture, agricultural and industrial instruments and furniture, internal decoration and paneling, and is valued for parquet and stripe flooring, and railway carriages. It is also useful for construction, oil extraction machine and cane crushers. The texture of the wood makes it easy to work with and therefore widely usable. It is good fuelwood also. Calorie value of moisture free heartwood is 5166 calories per cubic cm. The leaves of *Albizia lebbeck* are edible for cattle. The genus *Albizia* contains albitocin, hydrocyanic acid and saponin.

*Albizia falcataria* (L.) Fosberg: *Albizia falcataria* (L.) Fosberg, another species of *Albizia* is included in the present investigation. This species is used as the shade tree for different crops. It differs from all indigenous *Albizias* in being evergreen. In young trees there are 14-16 pairs of leaflets and glands at the junction of last seven pairs of pinnae towards the tip. There is another gland on the main stalk near the base. This species has a value in soil improvement and as shade on different crops (Jackson 1910). It is very fast growing and acquires about 3 m. in height and 30 cm. in girth in a year (Willis 1981).
The tree is commonly known as *Albizia moluccana* Miq. in Malaysia and India. For its fast growth rate and excellent biomass production the species has been selected for reforestation programs in different countries. This is one of the quickest growing leguminous trees and offers enormous economic potential as a source of pulpwood (NAS 1979).

*Dalbergia sissoo* Roxb.: The genus *Dalbergia* of the sub-family Papilionaceae (Fabaceae), under the family Leguminosae with some dozen species comprises the rosewoods. *Dalbergia* wood is commonly called rosewood because of its fragrance (Barrett 1981). It is considered as one of the most handsome and valuable woods of the world. *Dalbergia* wood was once a trade article in both the old and the New World (Burkill 1966). The whole tree is utilized. The wood produces highly prized timber, the branches are dried and used as fuel, the leaves are used as fodder, oil extracted from its seeds is used for curing skin diseases, and the saw dust is used as a remedy for leprosy and other skin eruptions (Sinha 1977). The genus *Dalbergia* comprises woody species whose accessible stands are now gone and apart from Java and India there are no plantations or trial plantings (Dodds 1983).

**EXPERIMENTAL OBJECTIVE**

The ever-increasing pressure of an expanding world population in recent decades has resulted in the exploitation of the forests to disastrous proportions. The increasing demands for biomass for fuelwood, timber and the pulp and paper industry can no longer be met from the existing natural forests alone. Large-scale plantation forestry and
afforestation of degraded forestlands are the solution to ensure future sufficiency in biomass.

Most woody plants are difficult to propagate by vegetative means. Traditional methods of tree breeding and grafting although successful in some instances, still has limitations and can not be applied for all commercially valuable tree species. The practical constraints for improvement and multiplication of trees through conventional techniques are as follows:

Inadequacy of seed germination -- In most of the woody species, especially in case of the hard seed coated legumes the percentage of seed germination is very low. Even if the seed germinates, it can not grow to the seedling stage because of several external and internal factors. Sometimes internal inhibitors in the seed prevent germination. Sometimes seed germination requires enough rainfall to wet the soil thoroughly to bleach out the inhibitors that effect seed germination (Wareing 1963). Tissue culture is a useful tool to bypass these problems.

There is a problem of maintaining elite clones due to genetic segregation through seed propagation. Plant breeding depends conceptually upon the existence of genetic variation, it's recombination and selection of improved genotypes. Sometimes random inbreeding causes the loss of genetic variability as well as the useful characters. Vegetative propagation of trees, offers better maintenance of desirable genetic combinations (Thulin and Faulds 1968; Murashige 1974a).

Traditional breeding methods in trees are limited by their large size and long life cycles. Development of inbred lines to procure predictable genetic traits in offspring
takes far too long. *In vitro* culture and genetic manipulation at the cellular level i.e., clonal forestry is now gaining increasing recognition as a far quicker alternative in tree improvement as for mass propagation of the tree species.

The objective of the present study is to devise reliable, reproducible and rapid methods for mass clonal propagation of the selected tree species. *Albizia lebbeck* Benth., has a moderately poor percentage of seed germination. On examination of several hundreds of pods I found the seeds varied widely in size and weight. The smaller seeds germinated poorly or not at all but a few of the larger seeds successfully germinated.

Leaves from very young seedling were selected as explants to attempt *in vitro* propagation. One reason for selecting young leaves is that these tree species undergoes a shift from juvenile to adult, and explants of mature (adult) parts either do not survive or perform poorly. (de Fossard, Barker and Bourne 1977; Sommer and Brown 1979; Bonga 1980; Rao and Lee 1982; Chevre, Gill, Mouras and Salesses 1983). Another important species of *Albizia*, *A. falcataria* was also studied for *in vitro* culture. For *in vitro* culture the leaflet explant of the *in vitro* germinated seedling was selected for obtaining callus.

The formation of plant organs is an orderly, predictable process. Cells and tissues become determined for specific morphogenetic pathways to the exclusion of others (Lang 1965a; Heslop-Harrison 1967; Meins and Binns 1979; Henshaw et al. 1982). The initiation of plant tissue cultures, the induction of differentiation and the regeneration of complete plants from cultured cells depend on being able to manipulate the process of determination (Yeoman 1987). This manipulation is done by several ways to control differentiation *in vitro*. The main objectives of the present investigation are to find
suitable media to:

i) promote and sustain callus growth from suitable explants, and

ii) induce a high incidence of shoot bud formation or differentiation of the callus.

I attempted by manipulating the *in vitro* cultural conditions and components, to establish tissue culture and regenerate the two tree species under investigation. By changing the combination and concentration of the culture media, components and effected changes in growth rate and morphogenetic potential.

In any conservation program it is essential to record specific data (like cytological, biochemical and EM data) for proper identification and conservation of the germplasm. The second and third part of this work will provide necessary information for firm identification and genomic analysis of the tree species.

**TISSUE CULTURAL PROCEDURES (METHODOLOGY)**

Choice of culture media: In culture, plant cells, tissues and organs are grown *in vitro*, in isolation from the rest of the plant body. In this state they must be supplied with essential inorganic salts, organic factors (vitamins and amino acids), and usually hormones (auxins, cytokinins and gibberellins in various combinations and concentrations), essential for growth. These are provided by a culture medium with suitable nutrients. Different workers have prepared media of varying composition, and these have often been modified to stimulate the growth of a particular plant. There is no single medium capable of supporting growth of all cells, tissues and organs irrespective of their source.
Consequently, the one most suitable for any particular tissue must be determined empirically. Vitamins, e.g., inositol, nicotinic acid, pyridoxine and thiamin, and amino acids, e.g., cysteine, glycine and casein-hydrolysate (an amino acid mixture) may be included to supply organic factors. Most cultures require growth hormones, either an auxin, a cytokinin or both. The auxins (stimulators of cell expansion) commonly used are the naturally occurring and synthetically produced indole acetic acid, together with the synthetic auxins like naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). Cytokinins (stimulators of cell division) includes naturally occurring zeatin, and synthetically produced 6-furfuryl-aminopurine (kinetin), 2-isopentenyl-adenine (2,i-p), and 6-benzylaminopurine (6, BAP). Growth requirements frequently change during culture, requiring alterations in the concentrations of growth regulators in the medium. Hormones are physiologically active in very low concentrations, usually in the range $10^{-5}$ to $10^{-10}$ M. Some cultures will grow only in the presence of complex additions to the medium, such as liquid endosperm e.g., coconut milk, yeast or malt extracts. Such media are chemically undefined, since the exact composition and concentration of growth substances in these additions are unknown. The most important factor governing the growth and morphogenesis of plant tissue in culture is the composition of the medium. Several plant tissue and cell culture media are in common use including formulations devised by Murashige and Skoog (1962), White (1963) and Gamborg et al. (B5 medium, 1968). The most widely used medium is the Murashige and Skoog formulation, which is often used when morphogenesis is required. Generally, sucrose is used as the carbon and energy source in a tissue culture medium. (Table I-1).
Table I-1. Chemical composition of Murashige and Skoog’s basal medium

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (mg/l)</th>
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<tbody>
<tr>
<td>1. Ammonium nitrate</td>
<td>1650</td>
</tr>
<tr>
<td>2. Calcium chloride</td>
<td>440</td>
</tr>
<tr>
<td>3. Magnesium sulfate</td>
<td>370</td>
</tr>
<tr>
<td>4. Potassium dihydrogen phosphate</td>
<td>170</td>
</tr>
<tr>
<td>5. Potassium nitrate</td>
<td>1900</td>
</tr>
<tr>
<td>6. Boric acid</td>
<td>6.2</td>
</tr>
<tr>
<td>7. Cobalt chloride</td>
<td>0.025</td>
</tr>
<tr>
<td>8. Copper sulfate</td>
<td>0.025</td>
</tr>
<tr>
<td>9. Manganese sulfate</td>
<td>22.3</td>
</tr>
<tr>
<td>10. Potassium iodide</td>
<td>0.83</td>
</tr>
<tr>
<td>11. Sodium molybdate</td>
<td>0.25</td>
</tr>
<tr>
<td>12. Zinc sulfate</td>
<td>8.6</td>
</tr>
<tr>
<td>13. Disodium ethylenediamide tetra-acetate(EDTA)</td>
<td>37.3</td>
</tr>
<tr>
<td>14. Ferous sulfate</td>
<td>28.8</td>
</tr>
<tr>
<td>15. Glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>16. Meso-inositol</td>
<td>100</td>
</tr>
<tr>
<td>17. Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>18. Pyridoxine hydrochloride</td>
<td>0.5</td>
</tr>
<tr>
<td>19. Thiamine hydrochloride</td>
<td>0.1</td>
</tr>
<tr>
<td>20. Sucrose</td>
<td>30000</td>
</tr>
</tbody>
</table>

pH 5.8

Agar 0.6% (w/v)
Plant regeneration has been quite difficult among the legumes. Forage legumes, e.g., clovers are more amenable to in vitro plant regeneration than are seed legumes (Phillips and Collins 1983). Twenty-five leguminous species have been regenerated in vitro, but in most cases regeneration is at low frequency or limited by the source of explants. At least eight different media have been used successfully to obtain plant regeneration--MS (Skolmen and Mapes 1976; Ramawat et al. 1977; Raj Bhansali et al. 1978b; Mokhtarzadeh and Constantin 1978; Sen and Gupta 1979a,b; Horvath Beach and Smith 1979; Malmberg 1979; Gharyal and Maheshwari 1981; Meijer and Broughton 1981). B₅ medium (Mukhopadhyay and Bhojwani 1978; Horvath Beach and Smith 1979; Swanson and Tomes 1980; Bharal and Rashid 1979a). White's medium (Shama Rao and Narayanaswamy 1975). Blydes medium (Bingham et al. 1975). 67-V (Crocomo et al. 1976). SH medium (Horvath Beach and Smith 1979). PC-42 (Phillips and Collins 1979), B M (Oswald et al. 1977).

On reviewing the report of plant regeneration in vitro (Evans et al. 1983) of twenty-five legume species it has been noted that MS medium is the medium most often used. Unlike most plant families, in leguminous species few generalizations can be made about the role of plant hormones. A cytokinin, either 6-BAP or kinetin, has been used for callus formation in each of the species except Acacia koa (Skolmen and Mapes 1976) and Trigonella sp. (Sen and Gupta 1979), in which coconut water was used. In most legume species, the auxin: cytokinin ratio is high for callus initiation, but a number of exceptions are evident. Most legume species require higher concentrations of cytokinins than do other plant families. For most species, the frequency of root initiation is quite
high despite the concentrations of auxins and cytokinins. The hormone concentrations successfully used for callus initiation in seed legumes were usually highly specific. In some cases special additives were required.

With this interesting scenario of in vitro culture of leguminous species the most widely used medium i.e., MS medium was the starting point for present investigations. Preparation of media: In all experiments, MS basal medium (Murashige and Skoog 1962) was used, variously supplemented with 6,BAP, 2,4-D, IBA, IAA, NAA. Perhaps the most troublesome problems affecting the initiation of cultures of woody plants is the phenomenon of explant browning and subsequent death. This is usually due to phenolic compounds produced by tissues in response to damage caused by surface sterilization or the dissection process. Several solutions to this problem have been proposed, such as the inclusion in the culture medium of compounds such as cysteine, ascorbic acid, dithiotheritol (DTT), activated charcoal and polyvinyl-pyrrolidone (PVP). Of all the chemicals tried I found 0.5% PVP (w/v) to be most effective for solubilizing and thereby removing phenolic compounds. PVP is an inert chemical with molecular weight about 40,000 that does not alter the pH of the culture medium.

Hormonal supplements, different auxins such as indole-3-acetic acid (IAA), indole-3-butyric-acid (IBA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D) and cytokinin like kinetin, 6-Benzyl aminopurine (6,BAP) etc. were added to the MS basal medium in various concentrations. The pH of all media was adjusted in the range of 5.6 to 5.8 using 0.1 N HCl or 0.1 N NaOH before autoclaving. Sucrose (3%) was used as the carbon and energy source in the media. The media were
gelled with 0.5% agar powder (Sigma, Bacteriological grade). Culture tubes were filled with 15-20 ml of medium and capped.

Sterilization of culture media and instruments: The media, distilled water, working instruments and Petri dishes (glass) were sterilized in an autoclave at 15 pounds per square inch for 25 minutes at 121°C. The minimal sterilization time increases with an increase in liquid volume (Dodds and Roberts 1987). The caps of the culture tubes were loosely fitted so that the medium would not spill during sterilization. After autoclaving the caps were pressed tightly to avoid contamination. During autoclaving, instruments are wrapped in heavy brown paper and culture tubes and vessels are kept in metal containers. Although aluminum foil is commonly used for wrapping, it is impermeable to steam vapors and therefore is not very useful.

After autoclaving, the culture tubes and vessels were kept on a rack in the culture room at a slant angle of 30°-40° until the media gelled. Stabs were made by keeping the culture tubes at a right angle to the surface of the rack. Wrapping of the vessels and instruments prevents contamination during transfer from the preparatory room to the culture room.

Aseptic techniques: The importance of maintaining a sterile environment during the culture of plant tissues can not be over emphasized. By following proper precautions microbial contamination can be avoided. All manipulations were carried out in front of laminar airflow. A spirit lamp is kept in front of the airflow where cultural manipulations are to be performed to ensure the sterility of the air. Scalpels, spatulas and forceps are usually immersed in alcohol until required and sterilized during use by frequent
immersion in alcohol and then flaming. After dipping in ethanol or iso-propanol (90% v/v), instruments are blotted dry on sterile filter paper. Before starting any sterile procedure, the working area was thoroughly scrubbed with a tissue paper soaked in ethanol or iso-propanol (90% v/v). The hands are vigorously scrubbed with soap and hot water for several minutes and finally sprayed with a dilute solution of ethanol to ensure the sterility of the surface of the hands and forearm that extended into the chamber.

Surface sterilization of the seeds and plant parts: The external surface of plants supports a rich micro-flora that must be removed by surface sterilization before aseptic excision of organ or tissue explants. The most suitable surface sterilant, its concentration, and time of sterilization must be determined empirically for the material under investigation. The goal in surface sterilization is to remove all the microorganisms with minimum damage to the plant system to be cultured. In the case of seeds, the use of higher concentrations or longer period of treatment does not appear to improve the decontamination success but it reduced the percentage of germination. For present study the seeds were disinfected with a three-stage process. The seeds were first immersed in a small amount of detergent (5% v/v) Tween-20, then washed thoroughly with distilled water and placed before laminar airflow for inoculation. Just before inoculation the seeds were again immersed in 0.1% sodium hypochloride solution for eight minutes. The optimum period for sterilization for seeds of a particular species was determined after several trials. After immersion in mercuric chloride solution the seeds were thoroughly washed in autoclaved distilled water three times to remove all the traces of the surface sterilant. The seeds were then placed in a petri dish containing filter papers, both of which were autoclaved earlier.
Inoculation of seeds for germination \textit{in vitro}: The seeds were inoculated in the containers containing 1/2 MS or MS medium for germination. The strength of the medium that facilitates seed germination was determined after several trials. Before opening the cap of the container it was placed in front of the flame of the spirit lamp to prevent the chances of contamination. The seeds were placed into the medium using forceps. The forceps were flamed after rinsing in 90% alcohol before each aseptic manipulation. After placing the seeds into the medium the caps of the containers were tightened properly. The containers inoculated with seeds were kept in the dark to facilitate seed germination. Excepting illumination with light the other standard cultural conditions namely, temperature and humidity were maintained in the racks in the usual manner.

Incubation of the cultures: The freshly inoculated cultures were grown under carefully regulated environmental conditions i.e. temperature, light and humidity. The cultures were grown under fluorescent tubes at intensities of 2000-3000 lux for 16 h daily photoperiod, 60% humidity and $25\pm2^\circ$C temperature. The most suitable temperature for callus initiation and growth is usually $25^\circ$C. Some cultures grow best in darkness, and others under light from fluorescent tubes. The temperature and photoperiod were determined after observation on growth behavior of the explants in culture.

Observation: All cultures of an experiment or of a particular treatment were maintained under the same incubation conditions. For each treatment 10 replicates were maintained and each experiment was conducted at least twice. Visual observations of the cultures were taken every week with two-day intervals. The effects of different treatments were quantified on the basis of percentage of cultures showing response and the number of
regenerants per culture. After induction of callus, the growth rate, morphogenetic behavior and response to the treatments were recorded after three weeks. Changes in color and texture were recorded from each culture tube. Photographs were taken regularly for each kind of treatment as well as for different stages of regeneration. Graphs were prepared from the data recorded during observation.

*In vitro* morphogenesis of *Albizia lebbeck*. Benth.

**MATERIALS AND METHODS**

Viｅvable seeds of *Albizia lebbeck* Benth. were collected from a well-known seed supplier M/S SHIDH Seeds Sales Corporation, Dehradun (U.P., India). The seeds were imported with a permit (# 37-77437) from USDA. Dry, mature, healthy seeds with approximately equal size were selected for use in experimental purposes.

**Source of mother explant:** The following plant parts were used as the source of initial explant in *in vitro* culture for callus initiation and growth.

- leaves - with leaflets
- hypocotyl of *in vitro* growing seedlings
- storage tissue i.e. cotyledons

**Sterilization of plant materials:**

Seed sterilization was done with the following steps:-

- **Pre-sterilization**
- **Sterilization**, and
- **Post-sterilization**
During pre-sterilization process, seeds were treated with 5% detergent Tween-20 for 10-15 minutes. Then the seeds were washed in distilled water.

In the sterilization period, the seeds were treated with 0.1% Tween-20 (a detergent) solution for 8-10 minutes followed by thorough washing in sterilized distilled water. This concentration of Tween-20 and duration of treatment was found to be optimum for *Albizia lebbeck* seed sterilization.

In the post-sterilization procedure, washing with sterilized distilled water was continued for several times to remove all the traces of sterilizing chemicals from the surface of the seeds. Proper sterilization is the pre-requisite for *in vitro* germination of the seeds and to initiate aseptic culture using explants of the germinated seedlings. After sterilization the seeds were aseptically inoculated in the culture tubes containing sterile hormone free 1/2 MS medium (Murashige and Skoog 1962) solidified with 0.5% agar agar (Sigma, Bacteriological Grade) at pH 5.7. Use of the 1/2 MS medium or half-strength MS medium was found to be suitable to accelerate seed germination and healthy growth of the seedlings. The culture was maintained in the dark at 22±2°C for 10-15 days. The etiolated hypocotyl, cotyledon and leaves were finally used as the sources of primary explant.

Growth substances: For suitable induction and maintenance of callus from different explant sources of *A. lebbeck* MS basal medium supplemented with various combinations and concentrations of auxins such as NAA (α-naphthalene acetic acid) and IBA (Indole-3-butyric acid) were used separately. Cytokinins such as kinetin and 6-BAP (6,benzylamino-purine) were used in combination with above mentioned auxins for obtaining and
maintaining callus in culture.

The sets of media were categorized into four different combinations and they were designated as 'A', 'B', 'C', 'D' according to the nature of hormone used (Table 1). For each experimental set 10 cultures were raised and each experiment was conducted at least twice. The pH of all the sets of media was adjusted to 5.6-5.7 before autoclaving and for gelling the liquid media, 0.55% agar powder was used. Nutrient media, glassware and necessary instruments were sterilized by autoclaving at 15 lb p.s.i. at 121°C for 15 minutes.

Inoculation: The explants were cultured onto 20 ml-agarified culture medium in 15cm X 2.2cm culture tubes. Leaf explants were inoculated into conical flasks (250 ml) containing culture medium. The inoculation process was carried out in aseptic conditions under a laminar airflow. The primary explants such as hypocotyl, cotyledons were cut into pieces of 5-8 mm size whereas the leaves were inoculated directly after excision from the in vitro germinated seedlings. The explants were planted firmly on solidified nutrient agar medium. Observation was taken after each seven-day period on the growth rate, change in color and texture of the calli and morphogenetic responses exhibited by the explants in culture.

Subculturing: Callus cultures are required to be transferred periodically to a fresh nutrient medium. Extensive growth leads to the exhaustion of the nutrients, drying out and concentration of solid media by evaporation of liquids, and the accumulation of tissue materials. The subculture of established callus demands the frequent subdivision and transfer of separated pieces. In this case it is important to transfer small healthy looking
pieces to the surface of fresh agar medium. Very small pieces of tissue tend not to survive the rigor of transfer. Failure to transfer cultures ultimately leads to the death of the callus while a subculture from a degenerative callus tends to grow much less actively than one taken from an actively growing healthy culture. In this situation active growth can often be restored by repeated and sufficiently frequent subculture. Loss in vigor is possibly related to the accumulation of toxic materials that are carried over to the subcultures from the necrotic callus and which are gradually loss during subsequent subculture (Street 1977).

Growth rate studies: To study the growth rate and its pattern, the weight of the inoculum was calculated by noting the initial weight of the culture tube with medium and again re-weighing it after inoculation of the callus tissue (fresh inoculum). For the growth pattern analysis, the growth rate was measured as growth index and this was calculated on the basis of initial fresh weight and fresh weight of callus tissue after the required period of growth as follows:

\[
\text{Growth Index (G.I.)} = \frac{\text{Final weight of the callus} - \text{initial fresh weight}}{\text{Initial fresh weight}}
\]

Callus growth rate study was carried out from 15th to 120th day after the very first culture of the callus tissue. Growth values were recorded with milligrams (mg).

Observation on callus color, texture and morphology from different sets of calli: During observation on cultures the color, texture and morphology of the calli from different sets were studied. From this observation four main types of calli were found.

Green compact type: This type of callus shows very compact, sometimes hard, green in color. The growth rate was slow. In course of subculture the callus grew more compact.
Gelatinous type: This type of callus was obtained from culture on MS medium supplemented with BAP (4.0 mg/l) and IAA (0.25 mg/l). The callus tissues were creamy white in color.

Friable callus: Friable callus was obtained from different sets of culture with high concentrations of IAA. The callus was greenish to yellowish in color. This friable callus was used as the source for the suspension culture.

Nodular type: Leaf explants cultured on BAP (4.0 mg/l), IAA (0.25 mg/l) and coconut milk (10% v/v) produced nodular type. In some sets of this callus type sub orbicular embryoids were noted.

The concentrations, combinations of growth hormones used with MS basal media for induction of callus from leaf explants of *A. lebbeck* is presented in Table 1. The responses of the leaf explants are summarized in Table 2.

Further experimentation was carried out with the sub-globose embryoids. These structures were loosely attached to each other and could be separated easily. The embryonic and non-embryonic calli could be differentiated easily on observation at high magnification. The globular structures directly obtained from the leaf explants were cultured on MS medium supplemented with various concentrations of BAP (0.5-2.0 mg/l) and IAA 0.5-1 mg/l and coconut milk (v/v). The absence of coconut milk was necessary for emergence of shootlets and rootlets. Subculturing of the proembryoids in the medium supplemented with BAP (8 mg/l) and IAA (1.5 mg/l) effected the emergence of shootlets from the subglobose structures (Table 3). Further subculture lead to the roots at the base of the subglobose structures becoming attached to the medium. The regeneration process
was studied under the magnification and photographs were taken at each stage of development.

Proembryoids first developed into multicellular structures from which shootlets emerged. The plantlets developed in this process produced very few roots that were weak. These plantlets were transferred to a liquid MS medium containing different concentrations of NAA and IAA (Table 4). Previous studies on rooting of woody species indicated that liquid medium is much more efficient than the solid medium for rooting. Plantlets regenerated in vitro were transferred to liquid medium containing different concentrations of NAA and IAA to elicit sufficient rhizogenesis for transplantation of the plantlets.

Hardening of the plantlets for transplantation: Transfer of *in vitro* propagated plantlets from culture-vessel to the soil is known to be very critical for tree species. An intermediate process called hardening (Sommere and Caldas 1981) was adopted before transferring the plantlets to the soil. The hardening process was carried out as follows:

i) allowing development of a reasonable balance between root and shoot system.

ii) a gradual transition from a constantly high humidity regime to varying and low humidity.

iii) all traces of agar were removed through a series of subcultures from 1/2 MS solid to 1/2 MS liquid medium- sterile distilled water and thorough washing in tap water.

Transfer of regenerated plants to the soil: The regenerated plantlets, after being maintained in transitional culture for about four weeks, were transferred to the same medium but lacking sucrose, vitamin and hormone. Plantlets were allowed to grow for
20-30 days. During this process a gradual transition of high humidity regime to varying
and low humidity was maintained. Special care was taken to remove all traces of agar
present at the base and regenerated roots of developed plantlets. The plantlets were
washed thoroughly in tap water and transferred to the potted soil. Finally the potted
plants were transferred to the greenhouse after required hardening for three to four weeks.
LEGEND TO THE FIGURES

Morphogenesis in *Albizia lebbeck* Benth.

Fig.-1 Development of White callus from the leaflet explants (X12).

Fig.-2 Green nodular callus (X8).

Fig.-3 Green callus produced shoot buds (X12).

Fig.-4 White and green mixed callus (X20).

Fig.-5 Cultured tissue showing shoot buds after 6-week (X10).

Fig.-6 Shoot buds developing shoots after 9-weeks (X15).

Fig.-7 Shoot primordia (X12).

Fig.-8 A developing shootlet (X5).

Fig.-9 Embryogenic callus (X12).

Fig.-10 Cotyledons developing in embryos (X10).

Fig.-11 Developing shoot buds (X8).

Fig.-12 LS of the embryo (X25).

Fig.-13 Shootlet developing leaves (X8).

Fig.-14 LS of the region of origin of shootlets (X15).

Fig.-15 LS showing development of xylem in the shootlet (X50).

Fig.-16 Multiplication of shootlets in culture.

Fig.-17 Shootlets isolated in culture tubes.

Fig.-18 Shootlets in rooting medium.

Fig. 19 Regenerated plants in the greenhouse.

Fig. 20. Regenerated plants of *A. lebbeck* after 3 months.
Fig. 21. Regenerated plants of *A. lebbeck* after 6 months.

Fig. 22 Regenerated plants of *A. lebbeck* after 12 months.

Fig. 23. Somatic metaphase plate of *A. lebbeck* showing 2n=26 chromosomes.

Fig. 24. Karyotype of *A. lebbeck*.
Table 1. Concentrations and combinations of different growth modifiers used with MS basal medium for induction of callus in various explants of *Albizia lebbeck*

<table>
<thead>
<tr>
<th>Set</th>
<th>Growth regulator</th>
<th>Cytokinin (mg/l)</th>
<th>Auxin (mg/l)</th>
<th>Coconut milk (v/v)</th>
<th>PVP (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>BAP/NAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-1</td>
<td></td>
<td>1.0</td>
<td>-</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>A-2</td>
<td></td>
<td>1.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>A-3</td>
<td></td>
<td>2.0</td>
<td>-</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>A-4</td>
<td></td>
<td>2.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>A-5</td>
<td></td>
<td>4.0</td>
<td>-</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>A-6</td>
<td></td>
<td>4.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>B</td>
<td>BAP/IAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-1</td>
<td></td>
<td>1.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>B-2</td>
<td></td>
<td>2.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>B-3</td>
<td></td>
<td>4.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>B-4</td>
<td></td>
<td>4.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>C</td>
<td>Kn/NAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1</td>
<td></td>
<td>1.0</td>
<td>-</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>C-2</td>
<td></td>
<td>1.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>C-3</td>
<td></td>
<td>2.0</td>
<td>-</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>C-4</td>
<td></td>
<td>2.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>C-5</td>
<td></td>
<td>4.0</td>
<td>-</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>C-6</td>
<td></td>
<td>4.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Set</th>
<th>Growth regulator</th>
<th>Cytokinin (mg/l)</th>
<th>Auxin (mg/l)</th>
<th>Coconut milk (v/v)</th>
<th>PVP (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-1</td>
<td>Kn/IAA</td>
<td>1.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>D-2</td>
<td></td>
<td>2.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>D-3</td>
<td></td>
<td>4.0</td>
<td>0.5</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>D-4</td>
<td></td>
<td>4.0</td>
<td>1.0</td>
<td>10%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Table 2. Response of leaf explants to modified MS medium.

<table>
<thead>
<tr>
<th>Sets</th>
<th>Percentage of leaf explants showing response</th>
<th>Minimum incubation period (in days)</th>
<th>Nature of response</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-1</td>
<td>0-00%</td>
<td>00-05</td>
<td>No response</td>
</tr>
<tr>
<td>A-2</td>
<td>9-12%</td>
<td>30-35</td>
<td>No response</td>
</tr>
<tr>
<td>A-3</td>
<td>20-25%</td>
<td>25-30</td>
<td>Swelling</td>
</tr>
<tr>
<td>A-4</td>
<td>28-33%</td>
<td>25-30</td>
<td>Swelling</td>
</tr>
<tr>
<td>A-5</td>
<td>30-35%</td>
<td>20-25</td>
<td>Poor callusing</td>
</tr>
<tr>
<td>A-6</td>
<td>40-45%</td>
<td>20-25</td>
<td>Slow callusing</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-1</td>
<td>60-65%</td>
<td>25-30</td>
<td>Green friable callus</td>
</tr>
<tr>
<td>B-2</td>
<td>40-45%</td>
<td>20-25</td>
<td>Friable callus</td>
</tr>
<tr>
<td>B-3</td>
<td>50-65%</td>
<td>25-35</td>
<td>No growth</td>
</tr>
<tr>
<td>B-4</td>
<td>68-70%</td>
<td>25-30</td>
<td>Callusing</td>
</tr>
<tr>
<td>Sets</td>
<td>Percentage of leaf explants showing response</td>
<td>Minimum incubation period (in days)</td>
<td>Nature of response</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------------------------</td>
<td>------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1</td>
<td>20-35%</td>
<td>21-22</td>
<td>Twisting of leaflets</td>
</tr>
<tr>
<td>C-2</td>
<td>30-40%</td>
<td>21-24</td>
<td>Poor callusing</td>
</tr>
<tr>
<td>C-3</td>
<td>30-35%</td>
<td>25-30</td>
<td>Friable callus</td>
</tr>
<tr>
<td>C-4</td>
<td>60-70%</td>
<td>28-30</td>
<td>Green granular callus</td>
</tr>
<tr>
<td>C-5</td>
<td>20-25%</td>
<td>25-30</td>
<td>Explants enlarged</td>
</tr>
<tr>
<td>C-6</td>
<td>60-70%</td>
<td>30-35</td>
<td>Light green friable callus</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-1</td>
<td>30-35%</td>
<td>28-30</td>
<td>Callus growth continued</td>
</tr>
<tr>
<td>D-2</td>
<td>40-45%</td>
<td>30-35</td>
<td>Callus growth continued</td>
</tr>
<tr>
<td>D-3</td>
<td>50-55%</td>
<td>30-35</td>
<td>Green compact callus</td>
</tr>
<tr>
<td>D-4</td>
<td>60-70%</td>
<td>28-30</td>
<td>Green granular callus</td>
</tr>
</tbody>
</table>
Table 3. Composition of medium for subculturing the globose callus tissue

<table>
<thead>
<tr>
<th>Set</th>
<th>BAP (mg/l)</th>
<th>IAA (mg/l)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-1</td>
<td>6</td>
<td>1</td>
<td>Callusing</td>
</tr>
<tr>
<td>E-2</td>
<td>6</td>
<td>1.5</td>
<td>Callus growth continued</td>
</tr>
<tr>
<td>E-3</td>
<td>8</td>
<td>2</td>
<td>Shootlets emerged</td>
</tr>
<tr>
<td>E-4</td>
<td>8</td>
<td>2.5</td>
<td>Emergence of shoot let and rootlets</td>
</tr>
</tbody>
</table>

Table 4. Medium for rooting

<table>
<thead>
<tr>
<th>Set</th>
<th>NAA (mg/l)</th>
<th>IAA (mg/l)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-1</td>
<td>0.25</td>
<td>0.25</td>
<td>Slow rooting</td>
</tr>
<tr>
<td>F-2</td>
<td>0.5</td>
<td>0.25</td>
<td>Few roots emerged</td>
</tr>
<tr>
<td>F-3</td>
<td>1.0</td>
<td>0.25</td>
<td>Good rooting</td>
</tr>
<tr>
<td>F-4</td>
<td>2.0</td>
<td>0.25</td>
<td>Fragile deformed roots</td>
</tr>
</tbody>
</table>
Somatic embryogenesis in suspension culture to regenerate *Albizia lebbeck* Benth.: A viable regeneration system was established via suspension culture of *Albizia lebbeck*. The culture medium was standardized after several trials and modifications by adding different growth factors and growth stimulators to obtain somatic embryos in the culture. The cell suspension culture was initiated with modified Gamborg’s B5 medium using green friable callus as the source. I found that the optimum use of casein hydrolysate (w/v) and coconut milk (v/v) in addition to 6-BAP and IBA could induce morphogenesis and somatic embryogenesis in the cultured cells. This reports the first observation of somatic embryogenesis of *A. lebbeck* using leaflets as the explants. I prepared the embryos for SEM study following standard techniques: fixation, dehydration, and critical point drying and gold-coating. On subculturing on fresh media the embryos developed cotyledons. They developed shoots above the surface of the medium and roots inside the medium. I cut sections of the developing structures, dehydrated with ascending grades of alcohol and observed under compound microscope after double staining with safranin and light green. I recorded the different stages of embryogenesis and plant development. After hardening I transferred the regenerated plants to the greenhouse. Some of the trees grew more than 25 feet tall within few months outside the greenhouse.

Source of Callus: Light green friable callus obtained in the previous experiment was used as the source for initiating the suspension culture. I obtained green friable callus from using leaflets as explants (Fig.1). Green friable callus was the most suitable to establish uniform suspension culture. Gamborg’s B5 medium (Gamborg et al. 1976) was used to establish suspension culture. The friable callus was centrifuged at 3000xg for 15 minutes.
The pellet was suspended in the B₅ medium in 500ml conical flasks. The flasks were kept on a shaker in the culture room following 50 resolutions per minute (Fig. 4). The suspension cultures were maintained at 25±2°C under a photoperiod of 15h. Subculturing to the fresh medium was done with 21-day intervals.

Methodology for suspension culture: Of the three types of callus tissues (green compact, green and white mixed, light green friable: Figs. 1, 2, 3) obtained from leaflet explant, light green friable callus was the best for suspension culture. This tissue was soft and fragile, easily separable in the medium on centrifugation at a low speed (3000-rpm). Cell suspension cultures are generally initiated from compact or friable callus, defined by Green (1982) as type I and type II, respectively, of which the latter type is generally considered the most suitable. After one week of culture I observed the single cells (Fig. 5). On addition of adenine-sulfate (50-150 mg/l) the single cell aggregates formed multi-cellular structures (Fig. 6). The photographs were taken after staining the cells in light safranin dissolved in 30% alcohol. After two weeks of culture, some of the cells became elongated and fibrillar (Fig.7). In the third week multi-cellular structures developed with various forms, fibrillar (Fig. 8), ovoid (Fig. 9), heart-shaped (Fig. 10). The heart shaped structures formed embryos. The embryos were observed at different magnifications with dissecting scope (Fig. 13), compound microscope (Fig.14) and Scanning Electron Microscope (Fig.12). For SEM, the embryos were fixed in 4% gluteraldehyde in 0.1M phosphate buffer, post fixed in osmium-tetroxide (OsO₄) and dehydrated in ascending concentrations of ethanol and finally in isoamyl acetate. The tissues from suspension
culture were dried following critical point drying (CPD), coated with gold, and scanned with SEM.

The developing embryos were transferred to fresh liquid Gamborg’s medium supplied with adenine sulfate (120 mg/l) and NAA (2 mg/l). Increased concentration of IAA induced profuse rooting. The plantlets were kept in the liquid medium to obtain enough roots to support the shoot system. Hardening critical in in vitro propagation of any plant species. The standard procedure for hardening is to place the plantlets in sterile distilled water. In my investigation I found that a pre-exposure of the rooted plantlets in sterile Knop’s medium (10%)(Salisbury and Ross 1985) for 5-6 days yielded a better survival rate in the greenhouse than is the case with sterile distilled water. The plantlets were kept in Knop’s medium and covered with an inverted beaker to maintain high humidity in the laboratory condition. In the next step the plantlets were transferred to sterile potted soil. These measures prevent sudden shock or environmental stress to the plantlets. After 5-6 weeks when the plants were about 5-6 cm long they were transferred to larger pots in the green house (Fig. 17). Following the transfer from culture room to pots, 55-60% plants survived. The survival rate was less (30-40%) when plants were not hardened in Knop’s medium.
LEGEND TO THE FIGURES

Somatic embryogenesis in suspension culture to regenerate of *Albizia lebbeck* Benth.

Fig.-1 Development of callus from the leaflet explants (X12).

Fig.-2 Green and white friable callus (X20).

Fig.-3 Light green friable callus (Source for suspension culture) (X12).

Fig.-4 Flasks in shaker containing suspension culture.

Fig.-5 Cultured cells after one-week (X100).

Fig.-6 Multi-cellular structure developed after one two weeks (X125).

Fig.-7 Fibrillar cell developed after two weeks (X125).

Fig.-8 Multi-cellular structure developed after two weeks (X75).

Fig.-9 Ovoid multi-cellular structure developed after three weeks (X50).

Fig.-10 Cordate multi-cellular structure developed after three weeks (X10).

Fig.-11 LS through a developing embryo (X8).

Fig.-12 SEM of an embryo (X75).

Fig.-13 An embryo under dissecting scope (X10).

Fig.-14 Embryos developing shoots and roots in culture (X15).

Fig.-15 Profuse rooting in liquid medium with α-NAA (2mg/l).

Fig.-16. Hardening of the plantlets in liquid medium.

Fig.-17 Regenerated plants in the greenhouse.
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Leaflet to plantlet: rapid in vitro propagation of

*Albizia falcataria* (L.) Fosberg

using leaf explants

This part of research work was aimed at successful regeneration of *Albizia* falcataria, a leguminous tree species using leaf explants in vitro. I cultured leaf explants that showed varied responses in culture with different plant growth regulators and supplements. The leaf explants produced adventitious shoot buds directly on culturing on Murashige and Skoog's medium supplemented with 6-benzylaminopurine (6-BAP), indole-3-butyric acid (IBA) (4.0/0.05 mg/l), 8%-10% coconut milk (v/v) and casein hydrolysate (70-80 mg/l). Simultaneous use of both growth factors (CM and CH) showed a stimulatory effect that was also concentration dependent. The concentrations of CM and CH that produced the maximum number of shoot buds (69±SD 3.6) were CM 8% (v/v) and CH 80 mg/l (w/v). The regenerated shoots produced roots after transfer to a liquid MS medium modified with NAA (2 mg/l) and IBA (0.25 mg/l). After proper hardening I transferred the plants to soil. I found that a pre-exposure to 5% Knop's solution for 6-7 days increased the percentage of survival among the regenerants. The transferred plants showed 65% survival in the greenhouse, providing a new technique of mass propagation of this important leguminous tree species.

*Albizia falcataria* (L.) Fosberg is one of the fastest growing leguminous trees and offers enormous potential as a source of pulpwood (NAS 1979). Burkill (1966) described the species as one of the fastest-growing trees of Malaysia. *A. falcataria* trees reach a height of 16 m in less than 3 yr., 33 m in 9 yr. and 45 m in 17 year (Burkill 1966). It is
used as a shade tree on tea and coffee plantations (Dassanayake and Fosberg 1980) and can also be utilized for biomass production in social forestry programs (Sinha and Mallick 1993).

*In vitro* culture studies on different species of *Albizia* have been reported including *A. julibrissin* (Sankhla et al. 1993); *A. lebbeck* (Gharyal and Maheswari 1981, 1983; Upadhyya and Chandra 1983; Bhargava and Chandra 1987; Varghese and Kaur 1988, Tomar and Gupta 1988b); *A. procera* (Ghosh and Chatterjee 1992); *A. richardiana* (Tomar and Gupta 1988a). Sinha and Mallick (1993) first reported in vitro culture of *A. falcataria*. The effect of Gamma-radiation on *in vitro* culture of this species was also reported (Ghosh et al. 1993 1995). In these studies cotyledon explants were used to establish the cultures. The present communication reports on successful *in vitro* regeneration of plantlets from shoot buds directly produced on leaflet explants, an accomplishment not previously reported for *A. falcataria*.

The potential application of *in vitro* techniques for the genetic improvement and propagation of trees of various economic uses has been described (Venkateswaran and Gandhi 1982). These techniques offer an effective way by which large number of genetically stable plants can be produced, maintained, multiplied and transported as disease free propagules or regenerants, safely and economically.

Callus cultures of forest trees, similar to callus cultures of herbaceous plants, display genetic instabilities: polyploidy, aneuploidy, etc. (Bonga 1977; D’Amato 1978). As it is evident that polyploidy and other chromosomal instabilities occur in tree callus cultures, it seems reasonable to expect that the apparently strong tendency away from
polyplody and homozygosity displayed in forest trees should ensure that regenerated plants will be normal diploids (Conger 1981).

A member of Mimosaceae, *Albizia falcatoria* has bipinately compound leaves. In the present investigation, I achieved the regeneration of this tree species using young leaflets as the explants without callus intervention. This opens a new way of *in vitro* culture of this important tree species that could be useful for mass propagation and genetic improvement of this woody species.

**MATERIALS AND METHODS**

I collected viable seeds of *Albizia falcatoria* (L.) Fosberg from SHIDH Sales Corporation of Dehra Dun (UP), India and imported them to the USA (permit no. 37-77437). Dry, mature, apparently healthy seeds were selected for our experiments. As seed size often influences the germination rate (Foster and Janson 1985), only uniform large seeds were selected. I washed the seeds thoroughly in tap water and submerged them in 5% Tween-20 for 15-20 min. The detergent was decanted and the seeds were washed with sterile distilled water several times and held in sterile distilled water for 2-3 h. Finally, the seeds were washed thoroughly in sterile distilled water several times to remove the traces of detergent from the seed surface.

Culture media: Half strength hormone and vitamin-free MS medium (Murashige and Skoog 1962) with 0.6% agar (w/v) (Fisher Scientific, A360-500) was used in vessels (77x77x97 mm, cat no. V8505, Sigma) for seed germination. After 4-5 weeks when the leaves expanded the young leaflets were excised and inoculated aseptically on the
modified MS medium.

I cultured the explants on MS medium modified with different concentrations of the following growth regulators: 6-BAP, kinetin, IBA, NAA and growth supplements: coconut milk (v/v) and casein hydrolysate (w/v) (Table 1). Sucrose 3% (w/v) was used as the carbon source in the medium and it was gelled with 0.5% (w/v) agar powder.

The pH of the media was adjusted to 5.8 before autoclaving. I poured 15-20 ml of medium in each of the culture tubes and 80-100 ml medium in each vessel. I autoclaved the media, petri dishes and equipment at 1.05 kg/cm² and 121° C for 16 minutes. I carried out all aseptic manipulations in a laminar airflow cabinet. I inoculated 100-110 mg leaf explants per culture tube and I used ten tubes as replica for each treatment (Table 1). After inoculation the culture tubes (25x150 mm cat no. C5916 with closures, cat no. C2048) and vessels (77mmx77mmx97mm, cat no. V8505) were maintained in a rack in the culture room at 25±1°C with 60% relative humidity under a 16-h photoperiod with 36 μE/cm²/sec from cool white fluorescent light.

Multiplication of shootbuds: Of the different sets of culture, the leaf explants produced adventitious shoot buds directly on culturing on Murashige and Skoog’s medium supplemented with 6-Benzylaminopurine (6-BAP), Indole-3-butyric acid (IBA) (4.0/0.05 mg/l) and 10% coconut milk (v/v)(Fig. 1&2). There was an increase in the number of shoot buds produced per 100 mg of leaflet explant in the casein hydrolysate and coconut milk supplemented sets (Graph 2).

Rooting and transfer to the field: I excised the regenerated microshoots (Fig. 3) of 2-2.5 cm length to transfer them to the two sets of rooting media, one with agar and the other
without agar (liquid) supplemented with IBA and NAA of different concentrations (Table 2). I transferred the rooted microshoots to Knop's medium. After 6-7 days exposure to the Knop’s medium I transferred the plantlets to pots in the green house (Fig. 7).

Cytology of the regenerated plants: For cytological observations, I excised root tips from the regenerated plantlets, pretreated with a mixture of saturated solutions of pDB (para-Dichlorobenzene) : 0.0002 M oxiquinolene (1:1) for 3 h, fixed in Carnoy’s fluid for 24 h, hydrolyzed with 2N HCl at 60°C for 8 min and stained with 2% aceto-orcein for 45 min. After squash preparation (Sharma and Sharma 1980), I observed the well-scattered metaphase plates under the high power and oil immersion lenses. Photomicrographs were taken with necessary attachments.

RESULTS AND DISCUSSION

On reviewing the report of plant regeneration in vitro (Evans et al. 1983) of 25 legume species I noted that MS medium is the one most often used for the regeneration of legumes. In vitro culture requires a cytokinin for growth. I tried 6-BAP and Kinetin of which 6-BAP showed better morphogenetic response in culture. Sometimes the addition of coconut milk improved morphogenetic response and the leaf explants responded differently to combinations and concentrations of growth factors added to the basal MS medium (Table 1). I achieved direct regeneration of shoot buds using MS medium supplemented with 6-BAP, IBA (4.0/0.05 mg/l) and coconut milk (CM) 10% (v/v) (Fig. 1). The addition of 2-12 mg/l casein hydrolysate (CH) with coconut milk (50-100mg/l) increased the number of shoot buds per propagule (leaflet) (Graph 1).
In preliminary observations, I noticed moderate to heavy browning of the culture medium in 90%-95% of the culture sets. Both the culture medium and the explants turned brown. Browning is generally considered to result from the oxidation of phenolic compounds released from the cut ends of the explants by polyphenoloxidases (Mayer and Harel 1979), peroxidases (Loomis and Battaile 1966; Vaughn and Duke 1984) or air (Robinson 1983). The oxidized products, quinones, are known to be highly reactive, inhibit enzyme activity and lead to the death of explants (Hu and Wang 1983). The inclusion of an antioxidant, polyvinylpyrrolidone (PVP) inhibited browning and subsequent death of the leaf explants. I found 0.5% PVP (w/v) prevented browning. PVP and PVPP were added to success in anther culture experiments resulting in increased pollen embryogenesis probably due to adsorption of the phenol complexes or other inhibiting compounds (Tyagi et al. 1981; Babbar and Gupta 1982).

The number of shoots produced per explant varied with the concentrations of CM and CH added to the media. Simultaneous use of both growth factors showed a stimulatory effect that was concentration dependent. The concentrations of CM and CH that produced the maximum number of shoot buds (69 ± SD 3.6) per 100 mg explants were CM 8 % (v/v), CH 80 mg/l (w/v). Increased concentration beyond this optimum of either CM or CH separately or together reduced the production of shoot buds (Graph 1).
Table 1: Response of leaf explants of *A. falcataria* in MS medium modified with growth hormones and coconut milk. (The data represented from the observations taken from three replicates of each sets of culture.)

<table>
<thead>
<tr>
<th>6-BAP mg/l</th>
<th>Kinetin mg/l</th>
<th>IBA In mg/l</th>
<th>CM % (v/v)</th>
<th>Response of leaflet explants (after 21 days)</th>
<th>% of explants responded</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.005</td>
<td>10</td>
<td>No response</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.01</td>
<td>10</td>
<td>White friable callus non-regenerative</td>
<td>15-20</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.02</td>
<td>10</td>
<td>Light green friable callus</td>
<td>15-20</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.03</td>
<td>10</td>
<td>Green compact callus</td>
<td>25-30</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.04</td>
<td>10</td>
<td>Green friable callus</td>
<td>45-50</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>0.05</td>
<td>10</td>
<td>Hard compact green callus</td>
<td>60-70</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>0.1</td>
<td>10</td>
<td>Yellowish green friable callus</td>
<td>45-50</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.005</td>
<td>10</td>
<td>Leaflets enlarged</td>
<td>20-25</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.01</td>
<td>10</td>
<td>A few rows of cells emerged from the leaflets</td>
<td>20-25</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.02</td>
<td>10</td>
<td>The leaflets became twisted, but no callusing</td>
<td>15-20</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.03</td>
<td>10</td>
<td>Light green callus emerged from the cut end of the leaflets</td>
<td>40-45</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.04</td>
<td>10</td>
<td>Dark compact callus, non-regenerative</td>
<td>65-70</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>0.05</td>
<td>10</td>
<td>Green globose shoot buds from the leaflets</td>
<td>65-70</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>0.1</td>
<td>10</td>
<td>Green granular callus</td>
<td>55-60</td>
<td></td>
</tr>
</tbody>
</table>
Graph 1: Effect of coconut milk and casein hydrolysate on shoot bud regeneration in *in vitro* culture of *A. falcata*aria. (Number of shoot buds ± SD counted per 100mg leaflet (propagule.))

<table>
<thead>
<tr>
<th>Media</th>
<th>Concentration of CH in mg/l; and of CM in % (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>MS+ CH</td>
<td>14±1.14</td>
</tr>
<tr>
<td>MS+ CM</td>
<td>25±1.70</td>
</tr>
<tr>
<td>MS + CM + CH</td>
<td>28±0.95</td>
</tr>
</tbody>
</table>

Set A = CH 50 mg/l, CM 2% (v/v)
Set B = CH 60 mg/l, CM 4% (v/v)
Set C = CH 70 mg/l, CM 6% (v/v)
Set D = CH 80 mg/l, CM 8% (v/v)
Set E = CH 90 mg/l, CM 10% (v/v)
Set F = CH 100 mg/l, CM 12% (v/v)
In irradiated callus culture of *Dalbergia sissoo* Roxb. I found that addition of casein hydrolysate had a radio-protective effect on the callus tissue. Its use sharply decreased the abnormality indices of the irradiated tissue in comparison to the culture that was not supplemented (Ghosh 1993).

According to Christianson (1987) the formation of organs from explants or callus cultured *in vitro* is a complex piece of plant morphogenesis that is usually influenced by hormonal constitution and growth factor concentration of the culture medium (Yeoman 1987) interacting factors complex enough to be very difficult to achieve in the laboratory. Independent of BAP concentration, elevated concentration of IBA yielded green shoot buds with white patches, which failed to produce shootlets. At higher concentrations of CM and CH the shoot buds were deformed in shape and failed to produce any shootlet. After a subculture it took 2-3 weeks for full emergence of the shootlets from shoot buds. Shoot buds that formed above the agar medium produced shootlets while those submerged in the agar medium remained recalcitrant and did not produce any shoot until they were subcultured to the surface of the medium.

An exponential increase of the production of shoot buds was noticed on repeated subculturing on fresh medium for a 4-5 month period after which there was a sharp decline in regeneration efficiency. The number of shoot buds produced varied with frequency of subculture (Graph 2). In seven months of culture the regenerative potential declined by a significant level, similar to the findings of Franclet et al. (1989). I established this to an accumulation of genetic errors in cultured tissue as I noticed in long term culture of another leguminous tree *Dalbergia sissoo* Roxb. (Ghosh et al. 1998).
Microshoots of 3-4 cm size were transferred to two different sets of medium. One set was agarified 1/2 MS media the other was liquid 1/2 MS media. I placed filter paper bridges in culture vessels with liquid 1/2 MS medium. Upon which shootlets were placed to achieve rooting. The addition of NAA and IBA at concentrations higher than the optimum concentrations (2 mg/l, 0.25 mg/l) resulted in the production of deformed or feeble roots which atrophied on subsequent subculture of the plantlets. Liquid 1/2 MS media modified with NAA (2 mg/l) and IBA (0.25 mg/l) yielded the best results for rhizogenesis from the transferred microshoots (Table 2) (Fig. 6). The transfer from the liquid medium to the hardening solution was easier in comparison to the solid medium where there is a higher incidence of damage to the fragile root system.

Table 2: Liquid MS medium (half concentration) for rooting in the microshoots. (The data represented from the observations taken from three replicates of each sets of culture.)

<table>
<thead>
<tr>
<th>Set</th>
<th>NAA Conc. in mg/l</th>
<th>IBA Conc. in mg/l</th>
<th>Response of microshoots (after 14 days)</th>
<th>% of microshoots responded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.1</td>
<td>0.005</td>
<td>No response</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>0.2</td>
<td>0.01</td>
<td>No response</td>
<td>0</td>
</tr>
<tr>
<td>3.</td>
<td>0.5</td>
<td>0.02</td>
<td>White friable callus from the base of the microshoot</td>
<td>18-20</td>
</tr>
<tr>
<td>4.</td>
<td>1.0</td>
<td>0.20</td>
<td>Rooting, roots are fragile</td>
<td>35-40</td>
</tr>
<tr>
<td>5.</td>
<td>2.0</td>
<td>0.25</td>
<td>Profuse rooting from the microshoots</td>
<td>55-50</td>
</tr>
<tr>
<td>6.</td>
<td>4.0</td>
<td>0.50</td>
<td>Few roots developed</td>
<td>25-30</td>
</tr>
<tr>
<td>7.</td>
<td>6.0</td>
<td>0.1</td>
<td>Roots are fragile</td>
<td>10-12</td>
</tr>
</tbody>
</table>
Graph 2: Regeneration of shoot buds via *in vitro* culture of leaf explants of *A. falcataria* varied with the age and frequency of subculture.

<table>
<thead>
<tr>
<th>Frequency of subculture</th>
<th>3 months</th>
<th>5 months</th>
<th>7 months</th>
<th>9 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 times</td>
<td>16±1.14</td>
<td>19±1.94</td>
<td>12±2.97</td>
<td>5±1.14</td>
</tr>
<tr>
<td>2 times</td>
<td>11±0.70</td>
<td>12±0.66</td>
<td>6±2.69</td>
<td>3±1.24</td>
</tr>
<tr>
<td>1 time</td>
<td>9±3.07</td>
<td>6±3.29</td>
<td>4±3.42</td>
<td>1±1.81</td>
</tr>
</tbody>
</table>
It was difficult to induce roots in the microshoots in the agarified media, few sets of culture produced white friable callus at the base of the microshoot and failed to accomplish any rhizogenesis. Plantlets with a healthy and well formed root system were transferred to fresh liquid MS medium without the addition of any kind of growth factor for hardening.

Hardening is a critical step in *in vitro* propagation of any plant species. The standard procedure for hardening is to place the plantlets in sterile distilled water. In our investigation I found that a pre-exposure of the rooted plantlets in sterile Knop’s medium (Salisbury and Ross 1985) for 5-6 days yielded a better survival rate in the greenhouse than is the case with sterile distilled water.

In the next step the plantlets were transferred to sterile potted soil (Fig.7) and covered with an inverted beaker to maintain high humidity. These measures were taken to prevent sudden shock or environmental stress to the plantlets. After 5-6 weeks when the plants were about 5-6 cm long they were transferred to larger pots in the green house. Following the transfer from culture room to pots, 55-60% plants survived. The survival rate was less (30-40%) when plants were not hardened in Knop’s medium.

**DISCUSSION**

Callus-mediated clones are usually genetically unstable. Plants regenerated from long term callus culture often lack morphological uniformity and this is not desirable except in some special cytogenetic improvement program when extensive screening for varied characters is needed. Cloning in vivo and *in vitro* of adult or mature woody plants
is adversely affected by characteristics accompanying maturation such as reduced growth rate, reduced or total lack of rooting ability or sometimes the unpleasant phenomenon of plagiotropy (Thompson et al. 1984; Hutchinson et al. 1988). Culture initiation also depends on the genotype of the plant and the developmental state of the tissue (Murashige 1974; Thomas et al. 1979; Green and Rhodes 1982; Harms 1982). That is why juvenile leaflets were selected as the initiating propagule for the establishment of *in vitro* culture. In this investigation the leaflet explants showed a distinct morphogenetic response to particular concentrations of 6-BAP and IBA. The morphogenetic response could be increased by the addition of two complex growth factors, CM and CH. From the leaflet explants a large number of plantlets can be produced and that can be increased exponentially by subsequent subcultures into fresh nutrient medium at least for a period of time. This avoids the anomalies associated with plants regenerated from callus, cell suspension and protoplast culture (Karp 1989; Larkin and Scowcroft 1981). I obtained culture clones from leaf explants in a very short period and thus harmful interference of long-term callus culture was avoided. The regenerated plants were morphologically identical and were uniform in genetic constitution. Somatic metaphase plate from the root tips of plantlets regenerated *in vitro* showed a constant 2n=26 chromosome number in a standard karyotype of *Albizia falcata*aria (Fig. 8). This corresponds to karyotypes from root tips of *Albizia falcata*aria grown from seed (Ghosh 1993). The potential technology of clonal multiplication from leaflet explants without callus intervention will stimulate further research into *in vitro* culture of tree species, an area of research that is still in its infancy.
LEGEND TO THE FIGURES

Leaflet to Plantlet: Rapid in vitro propagation of *Albizia falcata*ria* (L.) Fosberg using leaf explants

Fig.-1 Development of shoot buds and shootlets from the leaflet explants. 
(S = Shootlet, B = Shoot bud).

Fig.-2 Emergence of shootlets from growing shoot buds. 
(GB = Growing shoot bud, S = Developing shoot).

Fig.-3 Shoots (3-4 cm) developed from shoot buds in culture.

Fig.-4 Proliferation of shootlets in culture.

Fig.-5 Subculture of a number of separated shootlets in a larger vessel.

Fig.-6 Rhizogenesis in liquid medium.

Fig.-7 Regenerated plant in potted soil.

Fig.-8 Somatic metaphase plate from the regenerated plant shows diploid set 2n=26 chromosomes.
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Somaclonal variation in *in vitro* culture of *Dalbergia sissoo* Roxb.

Plant cell culture is an important source for obtaining genetic variability. Callus culture of *Dalbergia sisso* was initiated on a modified MS medium (6-BAP 4.5 mg/l, IBA 0.02 mg/l, PVP 0.5 % w/v) using hypocotyl explants. I noticed a number of chromosomal abnormalities during the mitotic division of the cultured tissue. The abnormality indices and ploidy level varied with the age and the frequency of the subculture. In the aged culture the regenerative potential declined but was reinstated to some extent with the addition of two complex growth factors, coconut milk and casein hydrolysate. The addition of these growth factors showed some anti-aging effects by rejuvenating the cultured tissue and thereby reducing the frequency of chromosomal anomalies in comparison to the control sets (-CH and -CM). SEM study was done to differentiate the regenerative and non-regenerative calli.

The genus *Dalbergia* of the sub-family Papilionaceae (Fabaceae), under the family Leguminosae with some dozen species comprises the rosewoods. *Dalbergia* wood is commonly called rosewood because of its fragrance (Barrett 1981). It is considered as one of the most handsome and valuable woods of the world. *Dalbergia* wood was once a trade article in both the old and the New World (Burkill 1966). The whole tree is utilized. The wood produces highly prized timber, the branches are dried and used as fuel, the leaves are used as fodder, oil extracted from its seeds is used for curing skin diseases, and the saw dust is used as a remedy for leprosy and other skin eruptions (Sinha 1977). A 1979 publication by the National Academy of Sciences on tropical legumes (NAS 1979)
outlined some of the research needs for a great variety of economically important woody species whose remaining genetic resources need urgently be collected and conserved. The genus *Dalbergia* comprises such woody species whose accessible stands are now gone and apart from Java and India there are no plantations or trial plantings (Dodds 1983).

High polyphenol exudation from the seed coat and the cotyledon during germination causes the browning of the seedling, leading to death, and conservation by cuttings is virtually impossible since there is no viable method of vegetative propagation. There is therefore an urgent need to develop reliable methods of *in vitro* propagation for conservation and restoration of the species. A new method of mass propagation is vital to the survival of this important tree species. In my investigations I found that modification of the culture medium by the addition of PVP (Polyvinyl- pyrrolidone) can solubilize the phenolic compound secreted from the cut end of the explant within the culture vessel and increase considerable survivability (Ghosh and Chatterjee 1991, 1992; Ghosh et al. 1995, 1997).

Usually, regeneration of plant from callus culture is not preferable because of genetic instability, but there are occasions when a callus stage can be used to diversify the genetic base of a crop (Dodds 1983). I established the callus culture of *Dalbergia sissoo* on MS medium modified with growth regulators and observed chromosome instability in the callus, particularly, in the aged cultures (Ghosh et al. 1995). The aged cultures had also lost their ability of regeneration and rejuvenation of the aged callus was possible by adding two growth supplements casein-hydrolysate (CH) and coconut milk (CM). The abnormality index was reduced in the CH and CM added sets of culture in comparison to
the controls. We also observed different sets of calli under Scanning Electron Microscope (SEM) after suitable processing, alcohol dehydration, critical point drying (CPD) and gold coating. Since, SEM gives direct visualization of tissues in three dimensions, my specific objectives in the current study were to determine:

(i) how and from which tissues, shoot and root primordia form and (ii) to establish the structural relationship between the distinct developmental structures observed on the callus surfaces and morphological characterizations leading to plantlet regeneration.

Looking at their micro-morphology under SEM I could differentiate the regenerative and non-regenerative calli of *D. sissoo* and other tree species like *Albizia* (Ghosh et al. 1997).

I also performed ‘t-test’ along with the determination of mitotic indices and abnormality indices of the four different sets of callus tissues. The tests revealed significant differences between the four different aged tissues in terms of mitotic indices and abnormality indices. The most aged callus tissue exhibited highest degree of instability in the genomic constitution. I found higher frequency of polyploidy in the aged callus tissues in comparison to the younger ones.

**MATERIALS AND METHODS**

Viable seeds of *Dalbergia sissoo* Roxb. were collected from a seed supplier (SHIDH Sales Corporation of Dehradun, UP, India) and were imported to the USA by proper permission from USDA (permit no. 37-77437). Dry, mature, healthy seeds were selected for the experiment.
As the seed size often interferes with the germination rate, the seeds with uniform morphology were selected. The seeds were washed thoroughly in tap water and submerged in 5% Tween-20 for 15-20 min. The detergent was decanted and the seeds were washed with sterile distilled water for 2-3 h. The seeds were germinated in vessels containing half strength MS medium (Murashige and Skoog 1962). It took 3-4 weeks to develop the seedlings. I used different parts of seedlings for induction of callus of which cotyledon explants showed the highest degree of callusing. I used different types of growth regulators, viz., auxins like indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), \( \alpha \)-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy-acetic acid (2,4-D) and cytokinin like kinetin, 6-benzylaminopurine (BAP) etc. in different concentrations to modify the MS medium. Some undefined complex media, for example, coconut milk and casein hydrolysate, were also added to the MS medium to modify it. I used 5% (w/v) polyvinyl-pyrrolidone (PVP) to dissolve the phenolic compounds exuded from the cut end of the explant. Before autoclaving the medium, I adjusted the pH between 5.6-5.8. I used 0.6% (w/v) agar to gel the medium and used culture tubes (25mmx150mm) and vessels (77mmx77mmx97mm) for culturing; and autoclaving the vessels, tubes, petri dishes, instruments and distilled water at 1.05 kg/cm\(^2\) at 121°C for 16 min. All tissue cultural manipulations were carried out in a laminar airflow cabinet. The culture vessels were maintained in a rack in the culture room at 25±1°C under 16-h photoperiod with 35 \( \mu \)E/cm\(^2\)/sec from cool fluorescent light. I maintained ten replicates for each culture set. Observations were taken with periodic intervals on the color, morphology, texture and
growth rate of the calli (3-4 days). I took photographs of all morphogenetic responses. The nature and degree of callusing was recorded in respect to explant sizes (Table 1).

The effect of different concentrations of coconut milk (v/v) and casein hydrolysate added to modified MS medium (w/v) is shown in Table 2. The browning of the explant could be prevented by the use of different percentages (w/v) of PVP. It was found that excess amount of PVP inhibited the callus growth while the lower concentrations were inefficient in preventing browning. The optimum concentration (w/v) was determined after several trials. The nature of response of the explants to the different concentrations of PVP is presented in Table 1A. The growth indices of the calli derived from different explants were calculated by determining the initial and final fresh after a certain period of culture.

For cytological observations the calli were cut into small pieces, pretreated with para-dichlorobenzene and aesculin (1:1) mixture for 3 h at 8-12°C and fixed in Carnoy’s fixative (1:3:6-acetic acid : chloroform : ethanol) for 3 days. After a brief fixation with 70% ethanol we hydrolyzed the callus tissue at 5N HCl for 1 hour at 24-28°C (Fox, 1969). Staining was done with 1% aceto-orcein solution for 45 minutes to 6 h. HCl was not used during staining because I already hydrolyzed the tissue with 5N HCl. After squash preparation (Sharma and Sharma 1980) well-scattered metaphase plates were observed under high and oil immersion lenses of the compound microscope with necessary attachments (PM-10AD) and photomicrographs were taken.
For SEM, calli were cut into pieces, fixed in 4% gluteraldehyde in 0.1M phosphate buffer, post fixed in osmium-tetroxide (OsO₄) and dehydrated in ascending concentrations of ethanol and finally in isoamyl acetate (Table 3). The calli samples were subjected to critical point drying (CPD), coated with gold, and scanned with SEM.

RESULTS AND DISCUSSION

Somaclonal variation seems not to be species or organ specific and variation among somaclones has been observed for a wide array of characters. Doubtless, the genetic mechanisms that give rise to such variation will prove a productive field of investigation (Larkin and Scowcroft 1981). In the present investigation, with varied combinations and concentrations of growth factors the morphology of the calli also varied. I used different parts of the germinated seedlings for induction of callus. Of these, the hypocotyl explants were found to be the most efficient for callusing.

During observation on callus cultures, the color, texture, and morphology of the different sets of calli were studied. From these observations, four main types of calli were found:

1. Compact dark green type: The callus was very compact, sometimes hard, green in color with a slow growth rate. In the aged sub-cultures the callus grew more compact. Some of the callus tissues developed well-differentiated stomata on their surface (Fig. 1).

2. Gelatinous type: This type of callus was obtained by using MS as a basal medium with hormonal supplements (BAP 4 mg/l, IAA 1 mg/l, coconut milk 10% v/v). The callus was gelatinous, creamy-white in color.
3. Friable yellowish callus: Friable callus was obtained from different sets of culture supplemented with growth factors (casein hydrolysate 70 mg/l, w/v). The callus was yellowish, loose, friable, and could be separated easily.

4. Nodular type: Nodular type of callus was obtained from the leaf explants by using MS basal medium supplemented with BAP (6 mg/l), IAA (0.5-1 mg/l) and coconut milk (10% v/v). The resulting callus was found to be regenerative. This type of callus, ultimately, gave rise to sub-orbicular embryoid like structures.

Further experimentation was carried out with the sub-globose embryoid like structures. The structures were loosely attached to each other and could be separated out easily with a scalpel or a needle. Light microscopic studies could not easily differentiate the embryogenic and non-embryogenic calli. Besides, with the different treatment with growth regulators and modifiers, the texture and the color of the callus tissues also varied with the age of the callus (Table 4). SEM studies revealed the specialized surface structures (Fig. 5) by which the regenerative (Fig. 6) and non-regenerative calli (Fig. 3,4) could be differentiated (Fig. 3-6). Different types of surface patterns were observed from different sets of culture tissues. Some of the callus tissues exhibited uniform reticular (Fig. 3), fibrillar (Fig. 4) rugose, colliculate, tuberculate (Fig. 5) patterns. SEM study of the developmental sequence of the callus tissue showed the presence of small, knob-like, often smooth surfaced, globular nodules with characteristic cellular orientation on the surfaces, which were mostly the regenerative ones (Fig. 5-6). It was found that the exclusion of coconut milk from the medium was necessary for the emergence of the
shootlets. The sub-culturing of the pro-embryoids in a medium supplemented with BAP (8 mg/l) and IAA (1.5 mg/l) resulted in the emergence of the shootlets from the sub-globose structures.

In the next stage of morphogenesis, two weeks following their transfer to a plant regeneration medium (BAP 4.0 mg/l, Kinetin 0.25 mg/l, NAA 0.05 mg/l) the development of leafy shootlets and shoot primordia was observed. In later stages of development, elongated shootlets emerged from the regenerative callus (Fig. 1). The root primordia developed into rootlets with root hairs (Fig. 2). The other set of culture (BAP 8 mg/l, NAA 0.5) produced pro-embryoids, which later developed into fully expanded embryos (Fig. 8). The embryogenic culture when supplied with BAP (4 mg/l), Kinetin(0.25 mg/l), and NAA (0.05 mg/l) developed plantlets with shoots above and roots piercing the medium below. After two weeks of sub-culture, the plantlets were transferred to the liquid half strength MS medium supplemented with different concentrations of NAA. Pre-exposure of the plantlets to Knop’s medium (Evans and Nason 1953) increased the rate of survival in the greenhouse, a result not previously reported.

The addition of coconut milk altered the growth indices in different sets of calli. From the growth indices calculated after 21, 42, 63 and 84 days it is evident that the addition of 12% and 15% coconut milk could increase the growth indices almost to double that of 5% coconut milk (v/v). In the 5% coconut milk supplemented set the growth indices were 1.394±0.338 and 1.666±0.272 after 63 and 84 days. In the 12% coconut milk supplemented set the growth indices were 1.394±0.3388 and 2.34±0.320
and with 15% coconut milk the growth indices increased to $2.222 \pm 0.2387$ and $3.46 \pm 0.3209$ (Table 2).

I analyzed the karyotype from a well spread metaphase plate (Fig. 12A) from the squash preparation of the root tips. The cytological analysis of the tree species *Dalbergia sissoo* Roxb. Revealed a common diploid chromosome number $2n=20$. The chromosome complements represented more or less symmetry in their karyotype, though they varied in size. The detail of the karyotype of *D. sissoo* (Fig. 12b) is listed in Table 5. The length and position of the centromere were the main criteria for this classification.

The chromosome size in this tree species is very small which also varied with different treatments and the average size was taken from observation on 5-10 metaphase plates. Several workers (Patil 1958; Nanda 1962; Mehra and Hans 1971, 1972; Mehra and Sareen 1973) have reported the diploid chromosome number for *D. sissoo* as $2n=20$. By comparing the normal karyotype with the karyotype of the callus tissues of different ages it was possible to detect the changes in the ploidy level and other chromosomal anomalies.

In the present investigation, the frequency of sub-culturing correlated positively with the degree of stability in the callus tissue (Table 6). An increase in frequency of sub-culturing decreased the abnormality index to some extent in all the four sets of different aged cultures. In 42 days, normally two sub-culturing were done (i.e., sub-culturing in a 21 day interval). With a single sub-culture in 42 days, a significant increase in the abnormality indices and decrease in the divisional frequency of the cells (mitotic indices) was noticed. A further increase in the frequency of subculturing (i.e., three in 42 days) reduced the abnormality indices even more. Evans and Gamborg (1982) suggested
that the frequency of the sub-culture can increase chromosomal stability of the cell cultures. With frequent subculturing, aneuploid cells did not accumulate in the cell culture. When cells were sub-cultured in the late exponential growth they never entered into a stationary phase of growth. Therefore, cells do not lag when subcultured (Evans et al. 1983).

The different types of chromosomal anomalies observed were laggard (Fig. 9), sticky bridges (Fig. 10), early, unequal and late separations (Fig. 11) which were mostly associated with 5 and 7 month old cultured tissue. The highest percentage of sticky bridges, aneuploid and polyploid cells were observed from cytological preparations from late 7 and 9-month old callus tissue. Mitotic indices and the distribution of abnormalities in different aged callus tissues are presented in Table 7 and Fig. 13.

Henshaw (1982) suggested that more effort be directed towards establishing causal relationships between storage/culture conditions and the generation of instability in vitro cultures. From the Table 8 it is very clear that with the increase in age of the callus the mitotic indices gradually decreased and abnormality indices gradually increased. A significant change in the ploidy level occurred in callus tissue with age (Table 6, Fig. 14).

The ‘t-test’ of mitotic indices and abnormality indices of the four different aged callus indicated a significant difference between the four different calli ages (Table 9). The apparent difference in the mitotic indices among the 3-month and 5-month old calli was insignificant. I conclude that the calli from 9 months old culture showed highest degree of instability in terms of the genomic constitution and the frequency of polyploidy. In general terms, this genetic instability is undesirable, but there are
occasions when a callus stage can be purposely utilized to diversify the genetic basis of the plant. By appropriate manipulation of the culture medium it is normally possible to induce the formation of roots or shoots (Skoog and Miller 1957). Krishnamurthi and Tlaskal (1974) suggested that some commercially valued form of the economically important plant species can be obtained by stabilizing the polyploidized plants regenerated from callus tissue. I obtained such callus raised trees that showed very fast growth rate in and outside the greenhouse.
LEGEND TO THE FIGURES

1. SEM on the surface of a regenerative callus tissue showing stoma (S) and shoot primordium (SP).

2. Root initial showing root hairs on the surface (SEM).

3. SEM on a non-regenerative callus tissue showing reticulate surface pattern.

4. SEM on a non-regenerative callus tissue showing fibrillar surface pattern.

5. SEM on a regenerative callus tissue showing tubeculate surface pattern with protruding structures.

6. Regenerative callus tissue under SEM showing leaf buds (LB) and leafy shoots (S).

7. Green compact callus under light microscope (X10).

8. Regenerative callus showing development of embryoids (X12).

9. Somatic anaphase of *D. sissoo* showing a laggard or lag chromosome.

10. Somatic anaphase of *D. sissoo* showing a sticky bridge.

11. Unequal separation during mitotic division of *D. sissoo*.

12a. Karyotype of *D. sisoo* Roxb.

12b. Somatic metaphase plate of *D. sisoo* showing 2n=20 chromosomes.

LEGEND TO Photographs from Cytology:

Fig. 1 Sticky anaphase from 5 months old *D. sissoo* callus (X160).

Fig. 2 Laggards from 5 months old *D. sissoo* callus with one subculture (X900).

Fig. 3 Polyploid cell from 7 months old *D. sissoo* callus with one subculture (X850).

Fig. 4 Polyploid cell from 7 months old *D. sissoo* callus with one subculture (X750).

Fig. 5 Polyploid cell from 9 months old *D. sissoo* callus with one subculture (X900).

Fig. 6 Polyploid cell from 9 months old *D. sissoo* callus with one subculture (X900).
Fig. 13. Graphical representation of distribution of abnormalities in different aged callus tissue.

Fig. 14. Graphical representation of percentage of ploidy in different aged callus of D. sissoo.
<table>
<thead>
<tr>
<th>Name of the explant</th>
<th>Size</th>
<th>Percentage of culture showed response</th>
<th>Growth Index (after 6 weeks)</th>
<th>Degree of callus</th>
<th>Nature of callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledon</td>
<td>Complete cotyledon</td>
<td>90%</td>
<td>0.58 ± 0.0132</td>
<td>++</td>
<td>Loose green callus</td>
</tr>
<tr>
<td></td>
<td>Longitudinal half</td>
<td>25%</td>
<td>0.068 ± 0.012</td>
<td>+</td>
<td>Green callusing</td>
</tr>
<tr>
<td></td>
<td>Transverse proximal half</td>
<td>80%</td>
<td>0.254 ± 0.061</td>
<td>+</td>
<td>Loose green callus</td>
</tr>
<tr>
<td></td>
<td>Transverse distal</td>
<td>15%</td>
<td>0.065 ± 0.098</td>
<td>++</td>
<td>Callus became brown</td>
</tr>
<tr>
<td></td>
<td>Cotyledon segment</td>
<td>70%</td>
<td>0.538 ± 0.141</td>
<td>+++</td>
<td>Green compact callus</td>
</tr>
<tr>
<td></td>
<td>Complete hypocotyl</td>
<td>100%</td>
<td>1.6 ± 0.083</td>
<td>++++</td>
<td>Green compact callus exuberant growth</td>
</tr>
<tr>
<td></td>
<td>Complete epicotyl</td>
<td>50%</td>
<td>0.512 ± 0.083</td>
<td>++</td>
<td>Loose green callusing</td>
</tr>
</tbody>
</table>
Table 2: Effect of different concentrations of coconut milk (v/v) and casein hydrolysate (w/v) on growth of callus

<table>
<thead>
<tr>
<th>Coconut milk (in %) (v/v) (CM) added to MS + BAP (0.4 mg/l) + IBA (0.5 mg/l)</th>
<th>Growth Index ± SD after 21 days</th>
<th>Growth Index ± SD after 42 days</th>
<th>Growth Index ± SD after 63 days</th>
<th>Growth Index ± SD after 84 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without coconut milk or casein hydrolysate 5%</td>
<td>0.0252±1.9235</td>
<td>0.56±0.2509</td>
<td>1.16±0.1140</td>
<td>1.26±0.1516</td>
</tr>
<tr>
<td>8%</td>
<td>0.028±0.0130</td>
<td>0.54±0.0894</td>
<td>1.394±0.3388</td>
<td>1.666±0.272</td>
</tr>
<tr>
<td>12%</td>
<td>0.44±0.2533</td>
<td>0.722±0.1198</td>
<td>1.7±0.1581</td>
<td>2.04±0.6877</td>
</tr>
<tr>
<td>15%</td>
<td>0.58±0.2167</td>
<td>1.061±0.3896</td>
<td>1.86±0.1516</td>
<td>2.34±0.320</td>
</tr>
<tr>
<td>18%</td>
<td>0.68±0.1643</td>
<td>1.32±0.4836</td>
<td>2.22±0.2387</td>
<td>3.46±0.3209</td>
</tr>
<tr>
<td>20%</td>
<td>0.308±0.2081</td>
<td>0.72±0.2683</td>
<td>1.5±0.1224</td>
<td>2.22±0.2387</td>
</tr>
</tbody>
</table>

(SD= Standard deviation of 5 replicates)
<table>
<thead>
<tr>
<th>Concentration of Casein Hydrolysate (w/v) added to the medium (mg/l)</th>
<th>Growth Index ± SD after 21 days (SD= Standard deviation of 5 replicates)</th>
<th>Growth Index ± SD after 42 days</th>
<th>Growth Index ± SD after 63 days</th>
<th>Growth Index ± SD after 84 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.04±0.0158</td>
<td>0.484±0.1314</td>
<td>1.22±0.1483</td>
<td>1.6±0.2738</td>
</tr>
<tr>
<td>100</td>
<td>0.065±0.0151</td>
<td>0.58±0.2167</td>
<td>1.28±0.1923</td>
<td>2.28±1.728</td>
</tr>
<tr>
<td>200</td>
<td>1.056±0.2523</td>
<td>1.68±0.1643</td>
<td>1.820±0.1923</td>
<td>2.66±0.1816</td>
</tr>
<tr>
<td>300</td>
<td>1.32±0.3420</td>
<td>1.56±0.2190</td>
<td>2.4±0.2</td>
<td>3.08±0.3492</td>
</tr>
<tr>
<td>500</td>
<td>0.77±0.28867</td>
<td>1.38±0.3346</td>
<td>1.98±0.03271</td>
<td>2.2±0.2387</td>
</tr>
</tbody>
</table>
Table 3: Schedule for fixation and dehydration of callus tissue for SEM studies.

<table>
<thead>
<tr>
<th>Solutions: Millonig's phosphate buffer (pH - 7.2 to 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solutions:</td>
</tr>
<tr>
<td>a. NaH₂PO₄·H₂O</td>
</tr>
<tr>
<td>b. NaOH</td>
</tr>
<tr>
<td>c. Glucose</td>
</tr>
<tr>
<td>d. Calcium chloride</td>
</tr>
<tr>
<td>Buffer composition:</td>
</tr>
<tr>
<td>a. 2.26% phosphate</td>
</tr>
<tr>
<td>b. 2.52% NaOH</td>
</tr>
<tr>
<td>c. 5.4% glucose</td>
</tr>
<tr>
<td>d. 1.0% calcium chloride</td>
</tr>
</tbody>
</table>

Prefix: 2% glutaraldehyde in buffer - 1.5 to 2.0 hours.  
( Mix 10 ml of 8% GA with 30 ml buffer. NOTE: GA is toxic).  
Wash: 3X in phosphate buffer.  
Post-fix: 2% osmium tetroxide in phosphate buffer - 1.0 hour.  
( Mix 2 ml of 4% osmium tetroxide with 2 ml of buffer).  
NOTE: Osmium tetroxide is very toxic. Use in hood with gloves).  

Wash: 2X with water.  
Dehydration in ethyl alcohol (10 - 15 minutes per step).  
15%  
30%  
Saturated uranyl acetate in 50% EtOH.  
70%  
95%  
100% 3X - last time for 1 hour.  

*After dehydration with 100% ethyl alcohol the tissue was subjected to Critical Point Drying (CPD).
Table 4: Variation of texture and color of callus tissue with age

<table>
<thead>
<tr>
<th>Age of the callus (in month)</th>
<th>Weight of callus</th>
<th>Texture and color of callus tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>146 ± 20 mg</td>
<td>Compact light green callus</td>
</tr>
<tr>
<td>5</td>
<td>260 ± 15 mg</td>
<td>Compact dark green callus</td>
</tr>
<tr>
<td>7</td>
<td>370 ± 15 mg</td>
<td>Friable yellowish-green callus</td>
</tr>
<tr>
<td>9</td>
<td>460 ± 15 mg</td>
<td>Friable yellowish callus</td>
</tr>
</tbody>
</table>

* Mean final weight in mg. - (Average value of 20 individual hypocotyl explants)

Table 5: Details of karyotype of *Dalbergia sissoo* Roxb.

<table>
<thead>
<tr>
<th>Karyotype Diploid number</th>
<th>Formula</th>
<th>Average length (in μ)</th>
<th>Mean length chromosome (in μ)</th>
<th>F%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>12 A₁, 4B₁, 4B₂</td>
<td>77.6</td>
<td>3.88</td>
<td>40.65</td>
</tr>
</tbody>
</table>
Table 6: Percentage of ploidy in different aged callus.

<table>
<thead>
<tr>
<th>Age of the callus (in months with 21 days sub-culturing)</th>
<th>MITOTIC INDICES</th>
<th>DIPLOID</th>
<th>ANEUPLOID</th>
<th>POLYPLOID</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10.2</td>
<td>2%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>6.4</td>
<td>1.1%</td>
<td>0.5%</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>4.5</td>
<td>0.9%</td>
<td>0.3%</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>3.4</td>
<td>0.6%</td>
<td>0.2%</td>
<td>1.2%</td>
</tr>
</tbody>
</table>

The percentage was calculated on observing 1000 ± 20 cells from at least 3 slides and the abnormal cells are not included in this counting.
Table 7: Distribution of abnormalities in different aged calli tissue.

<table>
<thead>
<tr>
<th>Age of callus (in months)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10.2</td>
<td>96</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>6.4</td>
<td>61.0</td>
<td>9.5</td>
<td>19.3</td>
<td>10.2</td>
</tr>
<tr>
<td>7</td>
<td>4.5</td>
<td>73.0</td>
<td>20.5</td>
<td>6.5</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>3.4</td>
<td>65.8</td>
<td>16.2</td>
<td>8.5</td>
<td>9.5</td>
</tr>
</tbody>
</table>

A = mitotic index,
B to E percentages of different types of chromosomal abnormalities
B = clumping and stickiness
C = early and unequal separations
D = laggard chromosome
E = anaphase bridges
Table 8: Variation of M.I. and A.I. with the frequency of subculturing of callus of *D. sisso* Roxb.

<table>
<thead>
<tr>
<th>Age of the callus (in months)</th>
<th>Frequency of subculturing per 42-day period (6 weeks)</th>
<th><em>M. I. ± SD</em></th>
<th><strong>A. I. ± SD</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>12.5 ± 2.620</td>
<td>0.3 ± 0.1643</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10.2 ± 1.505</td>
<td>0.6 ± 0.1870</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.8 ± 1.209</td>
<td>1.2 ± 0.4722</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>7.6 ± 1.246</td>
<td>0.5 ± 0.1949</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.4 ± 0.1516</td>
<td>1.5 ± 0.2236</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.3 ± 0.9757</td>
<td>1.7 ± 0.2588</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>5.3 ± 1.4805</td>
<td>1.9 ± 0.1516</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.5 ± 0.1414</td>
<td>2.6 ± 0.2701</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.3 ± 0.1144</td>
<td>3.4 ± 0.5449</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>3.9 ± 0.7436</td>
<td>2.9 ± 1.0025</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.4 ± 3.001</td>
<td>3.8 ± 0.3286</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.2 ± 0.2069</td>
<td>4.6 ± 0.2880</td>
</tr>
</tbody>
</table>

*M. I.* = Mitotic Index  
**A. I.** = Abnormality Index  
SD = Standard deviation of 5 sets of replicate
Table 9: ‘t-test’ with the abnormality indices of 3, 5, 7 and 9 months old callus of *Dalbergia sisso* Roxb.

<table>
<thead>
<tr>
<th>Age of the callus (in months)</th>
<th>Abnormality Index Mean (x)</th>
<th>S. E. (Sx_A)</th>
<th>Age of the callus (in months)</th>
<th>Abnormality Index Mean (x)</th>
<th>S. E. (Sx_B)</th>
<th>Interaction between passages</th>
<th>‘t-value’</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.6</td>
<td>0.2653</td>
<td>5</td>
<td>1.5</td>
<td>0.2135</td>
<td>3 months vs. 5 months</td>
<td>2.643*</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>0.2135</td>
<td>7</td>
<td>2.6</td>
<td>0.1936</td>
<td>5 months vs. 7 months</td>
<td>3.8194*</td>
</tr>
<tr>
<td>7</td>
<td>2.6</td>
<td>0.1936</td>
<td>9</td>
<td>3.8</td>
<td>0.0416</td>
<td>7 months vs. 9 months</td>
<td>6.060**</td>
</tr>
<tr>
<td>9</td>
<td>3.8</td>
<td>0.0416</td>
<td>5</td>
<td>0.6</td>
<td>0.2135</td>
<td>5 months vs. 9 months</td>
<td>10.58***</td>
</tr>
</tbody>
</table>
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CHAPTER 2

Cytology in vivo and in vitro

Chromosomes provide the genetic basis of inherited variation and evolution of tree races and species. The contrasting patterns of cytogenetic variation show the need for further research on the cytology of woody groups. Chromosomal variation of indigenous species with control of germplasm must be investigated on a much larger scale than before for use in the tree improvement work. In contrast to microbial cells, plant cells maintained in culture and plants regenerated from such cells display considerable phenotypic variability (Heinz et al. 1977; Shepard et al. 1980). Cytological studies offer direct evidence that at least some of this variability is due to the occurrence in vitro true genetic changes (Bayliss 1973, 1975; Sunderland, 1977; Orton 1980). Cell lines cloned and plants regenerated from plant cell cultures exhibit a wide range of variability. Variation has been reported in pigmentation and nutritional requirements of cultured plant cells, as well as in leaf shape, color and growth habit of regenerated plants (Skirvin 1978). In addition frequent karyotypic changes have been documented by cytological analyses (Sunderland 1977), suggesting that at least some of these variant phenotypes have a genetic basis. In the last few years technical advances in cell culture and chromosome identification have resulted in an explosive increase in our knowledge of plant cytogenetics. It is quite clear that within a relatively short time the reservoir of information in this field of biology will be greater for plants and will be remarkable
useful in plant improvement, in betterment of crop varieties, enhancement of biomass production and in increasing per area production of food and fodder.

It is well documented that a consequence of growth *in vitro* is the appearance of dividing cells with chromosome numbers and karyotypes not usually found within the intact plant (Partanen 1963a; Sheridan 1975). These changes are similar to the changes found in animal cells cultured for prolonged periods (Hsu 1961; Harris 1964) as well as in animal and plant tumors (De Torok and White 1960) clearly the changes in cell genotype have occurred during culture growth.

Chromosomal instability *in vitro* is a ubiquitous phenomenon associated with tissue and cell culture. Chromosomal variation in cultures of at least 55 plant specimens has now been published (Bayliss 1980). Though certain plants are reported to be stable *in vitro* (Sheridan 1975) but a majority of them show chromosomal instability in culture. It is evident from the reports reviewed that the extent of chromosomal instability ranges from slight to substantial regardless of chromosomal condition of the source plant. It is also evident that so many factors are involved in the phenomenon that characteristic levels of chromosome instability for a species can not be cited. The most common chromosomal changes include the changes in number or ploidy level, though structural changes also are sometimes observed. It may appear as early as in first subculture (Guo 1972) but is more frequently associated with long term culture (Sheridan 1974). However, it is now well established that chromosome number variations in cell culture do not prevent morphogenesis and that with wide range of polyploidy, aneuploidy and chromosome mosaic plants can be regenerated (D'Amato 1978).
Although a wide range of plant species have been regenerated from tissue and cell culture, only a limited number have been studied from the point of view of chromosome constitution both of cultures and regenerated plants. Chromosome instability can be an asset, liability or innocuous, depending on the specific research objective. Many forms of chromosomal changes may arise because of growth in culture. Final chromosomal constitution of a culture seems to be much more dependent on the relative competitive ability of different cell genotypes of the explant rather than on their frequency in the original explant. Suitable selective conditions then allow retention of desired chromosome complement. In a majority of cells the chromosomal variability in culture may also appear as a result of increased mitotic abnormalities, a function of growth regulators (Bayliss 1980).

Finding chromosomes----a matter of method: Techniques for finding chromosomes are designated to fix, stain and preserve the chromosomes so they can be profitably studied. Fixation is an attempt to kill the material rapidly in such a way that the internal structures are preserved in a life like form. Because fixation involves such processes as denaturation of proteins, some alteration of structure is always induced and care has to be taken in interpretation to recognize artifacts (those features that are induced by the treatment and not present in the living cell). Good fixation is a rather subjective assessment indicating that the appearances after treatment suggest a structure similar to that deduced from phase contrast images of living cells and from cells fixed by other techniques. However, an additional step, often referred to as pre-treatment, can be inserted into the preparative technique to facilitate the subsequent interpretation of many features of the
chromosomal complements in the finished slide. Pre-treatment involves the exposure of living material before fixation to the effects of one of a number of chemicals or physical treatments that inhibit the activity of the spindle during division, resulting in the accumulation of cells with chromosomes scattered through the cytoplasm instead of aggregated on the spindle equator and shorter and straighter than normal due to over-contraction. For the study of chromosomes either in plant or in animal tissue several chemicals are used as the pretreating chemicals. Pre-treatment is carried out for:

i) clearing of the cytoplasm,

ii) separation of the middle lamella causing softening of the tissue,

iii) bringing about scattering of chromosomes with clarification of constriction regions,

iv) rapid penetration of the fixative by removing undesirable deposits on the tissue for the study of spiral nature of chromosomes.

The first two applications involved removal of extra nuclear membrane; whereas the third and most important one, exerts a direct effect on chromosomes. The underlying principle of these important aspects of pre-treatment is the viscosity change in the cytoplasm. As spindle formation is dependent on the viscosity balance between cytoplasm and spindle constituent, a change in cytoplasmic viscosity brings about destruction of the spindle mechanism, with the chromosome remaining free, or more precisely not attached to any binding force within the cell. The chromosome undergoes differential hydration in its segments, and due to this differential effect constriction regions in chromosomes appear beautifully clarified.
After pre-treatment and fixation the material can be stored until required, usually in alcohol (70%), or immediately stained for observation. Storage rarely, if ever, improves the final preparation, frequently accentuating the artifact and sometime reducing the staining. A disadvantage of orcein is that staining is sometimes less effective after storage in alcohol. Temporary orcein preparations made by the techniques will keep satisfactorily for days, and sometimes for weeks or even months, while permanent preparations show little or no change after fifteen years.

Staining is necessary because without it the colorless chromosomes are difficult to distinguish from the equally colorless cytoplasm. A number of dyes have been used to give preferential staining of chromosomes by absorption, taking advantage of their characteristic surface properties. Several naturally occurring dyes like carmine and orcein, dissolved in simple organic acids have been used successfully although they induce some swelling of chromosomes.

Orcein in propionic acid gives better differential staining than other solutions because, while the chromosomes are deep purple, the cytoplasm shows little or no staining. Counter staining of the rest of the cell using a second dye with appropriately different adsorption properties is possible, but rarely beneficial for chromosome studies as it inevitably reduces the contrast between the chromosomes and their background. In this study, cytological analysis was done from different sources of plant materials, namely, normal root tips, calli of different ages and regenerated plantlets raised from in vitro culture. Karyotypic analysis was done from the well-scattered metaphase plate obtained from normal root tip cells.
This part of the investigation is to analyze the cytological changes occur during differentiation in vitro and to determine the cytological stability of the regenerated plants.

MATERIALS AND METHODS

Seeds of *Albizia falcatoria* (L.) Fosberg., *A. lebbeck* Benth. and *Dalbergia sissoo* Rroxb. were used for this study. The seeds of these tree species were imported from SHIDH Sales Corporation (Dehradun, India) with necessary permission from USDA. The seeds were soaked in tap water overnight after removal of the covering of the pods. Then the seeds were washed thoroughly in distilled water and kept on moist filter paper in petri dishes. The petri dishes were kept in the dark to facilitate seed germination. Water in the petri dishes was changed regularly to avoid infection and to wash out the exudates secreted into water by the seeds. After 4-5 days small root tips emerged. For cytological studies root tips were excised. The time for maximum meristematic activity was determined following several trials and the optimum cell division activity was found to be from 10.30 A.M. to 12.00 P.M.. During this period, fresh healthy root tips were harvested and cut into 3-4 mm. segments. Before the pre-treatment, the root tips were washed thoroughly in water to remove any dirt or unwanted particles adhered to root meristematic tissue. A pre-treatment in saturated solution of para-dichlorobenzene for three and half-hours at 12°-16°C was found to be most suitable to study the morphology of the chromosomes and karyotypic analysis of the plant material. Pre-treatment was followed by proper fixation of dividing cells of the meristematic tissue. A number of fixative chemicals including acetic acid and ethanol (1:2, 1:3), propionic acid and ethanol
(1:2, 1:3) mixtures were employed at varying periods. Overnight fixation in acetic acid and ethanol mixture (1:3) at 16 °C was found to be suitable for good fixation and scattering of chromosomes in the divisional cell plates. After proper fixation, root tips were treated with 45% acetic acid for 2-5 minutes and stained in 2% aceto-orcein solution for 1-2 h. The very tip of the root was squashed in 45% acetic acid, excess fluid was blotted off and a uniform pressure was given for better scattering of the meristematic cells of the root tip. The slide was sealed with molten paraffin and observed under the low, high and oil immersion lenses of the compound microscope.

**OBSERVATION**

Cytological analysis of three different species of *Albizia* revealed a common diploid chromosome number 2n=26. The chromosome complement represented reasonable symmetry in their karyotypes, though the size of the chromosome differed among the species. The length and the position of the centromere were the main criteria for this classification (Table C-1). The centromeric index (F%) was calculated from the following formula:

\[
\text{Centromeric Index (F%) = } \frac{\text{Length of the short arm}}{\text{Whole length of the chromosome}} \times 10
\]

The four different types of chromosomes observed in the three species of *Albizia* are:

Type A₁: Short chromosomes within 1.8 - 3.6 μm in length with nearly median primary constriction; F% between 49.00 - 37.51.
Type B₁: Short chromosomes within 1.6-3.3 μm in length with nearly submedian primary constriction. F% between 37.50 -25.01.

Type B₂: Short chromosomes with submedian primary constriction. Length varies from 1.6-3.2 μm. F% is 25.

Type C₁: Short chromosomes with nearly median secondary constriction. Length varies from 1.6-3 μm. F% lies between 18.74-12.51.

Type D₁: Short chromosomes. Only one pair was found in A. odoratissima. length is 2μm with nearly subterminal centromere. F% lies between 12.40-6.25.

Type D₂: Short chromosomes; length varies from 1.6-3μm, with subterminal centromere, F% is 12.5.
Table C-1: Centromeric Index (F%)

<table>
<thead>
<tr>
<th>Centromeric Index (F%)</th>
<th>Nature of constriction</th>
<th>Centromeric Index</th>
<th>F%</th>
<th>Nature of constriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.00</td>
<td>Median</td>
<td>18.74 - 12.51</td>
<td>Nearly subterminal</td>
<td></td>
</tr>
<tr>
<td>49.99 - 37.51</td>
<td>Nearly Median</td>
<td>12.50</td>
<td>Subterminal</td>
<td></td>
</tr>
<tr>
<td>37.50 - 25.01</td>
<td>Nearly Submedian</td>
<td>12.49 - 6.25</td>
<td>Nearly subterminal</td>
<td></td>
</tr>
<tr>
<td>25.00</td>
<td>Submedian</td>
<td>6.24 - 1.00</td>
<td>Nearly terminal</td>
<td></td>
</tr>
<tr>
<td>24.99 - 18.75</td>
<td>Nearly submedian</td>
<td>1.00</td>
<td>Terminal</td>
<td></td>
</tr>
</tbody>
</table>

Table C-2: Details of karyotype of three species of *Albizia*:

<table>
<thead>
<tr>
<th>Name of the sp.</th>
<th>Somatic Chromosome No. (2n)</th>
<th>Karyotype formula</th>
<th>Total length (μ)</th>
<th>Average length (μ)</th>
<th>Mean F%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Albizia falcataria</em></td>
<td>26</td>
<td>16A₁6B₁4C₁</td>
<td>66.8</td>
<td>2.56</td>
<td>34.94</td>
</tr>
<tr>
<td><em>Albizia lebbeck</em></td>
<td>26</td>
<td>14A₁4B₁4C₁4D₂</td>
<td>69.6</td>
<td>2.67</td>
<td>32.49</td>
</tr>
<tr>
<td><em>Dalbergia Sissoo</em></td>
<td>20</td>
<td>12A₁4B₁4B₂</td>
<td>77.6</td>
<td>3.88</td>
<td>40.65</td>
</tr>
</tbody>
</table>
In the three species of *Albizia* the diploid chromosome number found was 2n=26. The average chromosome size is very small in tree species. In *Albizia falcatoria* the total chromosomal length was found to be 66.8μ, the average chromosome length being 2.56μ. In *Albizia lebbeck* the total chromosomal length was calculated to be 69.6μ, the average chromosome length being 2.67μ. In *D. sissoo* the diploid number was 2n=20, the total chromosome length was 77.6μ and the average chromosome length was 3.88μ. The F% of the two species of *Albizia* was found to be 34.94, 32.49 indicating thereby the nearly submedian nature of the most of the chromosomes (Table C-2). Study of the normal karyotype of a plant species is essential for its improvement program that may help in detection of any change occurred in the genome through *in vitro* culture. By comparing the normal karyotype with the karyotype of the cultured tissue the particular change in the chromosomal set or ploidy level can be easily detected which is the first step towards the genetic improvement of a plant species.
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CHAPTER 3

Comparative micro morphological studies on the seed coat (spermoderm) of seven species of *Albizia*

The next part of my investigation was to find out the taxonomic interrelationship of the tree species under investigation and to standardize a procedure for identifying the seeds through Scanning Electron Microscopy (SEM) on the spermoderm or seed surface. With the advent of modern techniques and refinement of methodologies, different instruments have come into use in the field of Biological sciences. For a long period SEM has been used as an important tool to resolve finer structures of biological specimens for their specific identification and characterization. The use of the electron microscope as a tool to provide new data in plant systematics and the first attempts of their application to angiosperm classification date back to the late fifties but until more recently have remained without influence upon the shaping of angiosperm systems. Over a long period, this disregard of ultra structural observation was due to relatively little comparative data available. As early as 1969, during the XI International Botanical Congress at Seattle, a first attempt on broader concepts has been made in a congress symposium, 'Relation of ultra structural studies to systematics', next EM-characters used in plant classification were compiled by Cole and Behnke (1975). Now, EM-data are increasingly included and discussed in revisions and circumscription of angiosperm families and orders (e.g., Heywood 1971; Mabry and Behnke 1976, Vaughan et al. 1976, Heywood et al. 1977;
Hawkes et al. 1979, Polhill and Raven 1981). However, not before the ICSEB-II at Vancouver, 1980, an overall view of the applicability and application of EM-data to the entire group of seed plants had been achieved (Behnke 1981a). The seed as a very characteristic organ of angiosperms receives due attention in all kinds of botanical investigations, including taxonomic ones. The seed coat studies have already become invaluable for certain classification purposes. Within the micro morphological field (SEM) the surprisingly high structural diversity of plant epidermis (the natural primary surface that covers roots, stems, leaves, flowers, fruits and seed) has increasingly provided valuable new information for the classification of the angiosperms.

Seeds are matured ovules that usually contain one embryo. The embryo is a miniature plant consisting of seed leaves (cotyledons) attached to the rudimentary stem (hypocotyl) with a growing tip (plumule) and a root tip (radicle) at opposite ends. Seeds of a particular species are less variable than other organs of it. Seeds and small fruits exhibit a complex and high morphological and micro-morphological diversity, providing valuable taxonomic information. Their overall shape, color, size and in particular, their internal structure (including ultra structural characters) can be of high systematic significance. In practice, most data have been provided by SEM examination of the epidermal surfaces. These surface features may be grouped into four categories: 1) Cellular arrangement or cellular pattern, 2) Shape of cells (the "primary sculpture" of a surface), 3) Relief of outer cell walls (the "secondary sculpture" superimposed on the primary sculpture), caused mainly by cuticular striations and superficially visible cuticular wall thickening, 4) Epicuticular secretions (the "tertiary sculpture"
superimposed on the secondary sculpture), i.e., mainly waxes and related substances. A recent SEM study on seed surface study of different plants has emphasized the importance of this pattern as taxonomic criteria. The application of seed data is rapidly increasing. There is evidence that this source of taxonomic information will play a role as important as pollen morphology has over recent decades (Barthlott 1984). The application of SEM during the last two decades has greatly increased our knowledge of plant surfaces that have a greater importance in systematics and radiation genetics (Stace 1965; Heywood 1973; Brisson and Peterson 1976; Barthlott 1980, Behnke and Barthlott 1983). The major application of SEM has been worked out probably to seed surfaces (Skvortsov and Rusanovitch 1974) as Brisson and Peterson listed 273 references on seeds alone up to 1976 rather than leaf surfaces or cuticular patterns (Heywood 1973; Ehler and Barthlott 1978; Trivedi et al. 1978; Barthlott and Voit 1979).

In his classical book of seed structure of angiosperms, Netolitzky (1926) has admirably summarized our knowledge up to the end of 1923. After a long gap of fifty years Corner published his two volumes on the seeds of the dicotyledons in 1976. These works, complemented by the seed bibliography of Barton (1967) with some 20,000 entries, remain the basis for research in seed structures. Many data on monocotyledonous seeds are provided by Dahlgren and Clifford (1982), two of the few authors who actually integrated seed data in their classification.

During the last decade the use of SEM has greatly increased our knowledge of the microstructure of seeds. Mature seed coats are rather thick walled and stable in a vacuum: this allows quick preparation for SEM examination, without the need of complicated
dehydration techniques. The low level of technical expenditure required, in combination with the high structural diversity exhibited and the intuitive ability to understand the "three dimensional", often aesthetically appealing micro-structures visualized, has turned seed-coat studies into a favorite tool of many taxonomists. The most helpful bibliography of the many papers on the SEM of seeds covering the period from 1967 upto 1976 has been published by Brisson and Peterson (1976); more recent papers have been reviewed by Barthlott and Schill (1981). In earlier literature micro-structural features of seed (and epidermal) surfaces have been reported to be of only restricted diagnostic value. Surface features of seed coats are surprisingly little affected by the environmental condition in which a plant grows. Differences in seed coat characters seem always to reflect genetic phylogenetic differences in the plants concerned. Therefore, it is no surprise that, for example, Denford (1980) could indicate a correlation between the flavonol glycoside profiles in leaves and the micro morphology of seed coats in the genus *Epibolium* (Onagraceae). The seed surface or spermoderm ornamentation are species specific. In the recent SEM studies on the testa pattern of Solanaceae (Axelius 1992) this fact is reflected. There are also reports on infra-specific variations of seed surface ornamentation in Pyroloidae (Ericaceae)(Takahashi 1993).

Like all taxonomic criteria, seed-coat microstructures must be interpreted with great circumscription. There is a remarkable contrast between the generally accepted opinion that morphology and micro morphology of seeds are a source of important information for the classification of angiosperm taxa and the way these data are ignored in the existing classifications. Only over the last few years have seed data been
increasingly integrated in taxonomic and systematic treatments (e.g., by Dahlgren and Clifford 1982). The Leguminous seed is generally of medium or large size, more or less compressed and exalbuminous, with large embryo and hard, dry smooth testa. Distinctly albuminous seeds occur in all three subfamilies of Leguminosae.

Objective and summary of the work: This investigation was undertaken to find out the taxonomic interrelationship of seven species of Albizia (Mimosoidae, Leguminosae) and to standardize a procedure for identifying the seeds utilizing the Scanning Electron Microscopy (SEM) on the seed coat of these species. Seeds of a particular species are often less variable than other organs. The simple microscopic and stereomicroscopic examinations revealed the color and the nature of the seed surfaces including pleurograms, fissures and hilum at lower magnification.

SEM studies revealed the specific ornamentation pattern of the seeds of each species. The ornamentation including undulations, reticulations and rugae could be observed under SEM at various magnifications. Characteristic cracking was evident in Albizia falcataaria, A. lebbeck and A. odoratissima. Cracking may be connected with the seed pleurogram and respond to humidity inside and outside the seed by stretching and shrinking. Dehydration of the seeds at maturation and absorption of water during germination probably also takes place through these cracks (Trivedi et al. 1980). The cracking pattern of these three species are similar, all of them possess tile shaped cracking on the seedcoat, but, each species exhibited different size and shaped plates. The characteristic size and shape of the plates outlined by the cracks could easily identify each species.
MATERIALS AND METHODS

Dry mature seeds of seven species of *Albizia* were collected from the eminent seed suppliers of Dehradun (U.P., India). The species of *Albizia* selected for this study was: *Albizia falcataria* (L.) Fosberg (Cultivated as *A. moluccana* Miq. in India) *A. lebbeck* Benth., *A. lucida* Benth., *A. odoratissima* Benth., *A. procera* Benth. in Hook., *A. richardiana* King and Prain, *A. stipulata* Boivin. (*A. marginata* Merr. of Cowan’s list). Fifteen seeds were studied for each species. I washed the seeds by sonicating in dehydrated alcohol before use. The extraneous materials were removed from the seeds by sonication for 3-5 minutes and they were mounted on stubs (aluminum holder) with double stick tape and coated with 100-200 A thick layer of gold in a rotating and tilting vacuum coating apparatus. At the base of the seeds silver paint was applied to prevent charging of the surface during scanning. The coated seeds were scanned under a constant tilt angle (0°) and an acceleration potential of 15 Kv. 10-12 photographs were taken for each sample at various magnifications.

OBSERVATION

First the seeds were observed under the dissecting scope with different magnification (X4-X20). The seed color, texture, dimension and specific identifying characters were recorded. The average dimension was taken from the measurement of twenty mature and ripe seeds. The dimension (maximum axial length x maximum breadth) was measured by placing the seeds on a glass slide attached with a graph paper on the lower surface. The mean dimension of twenty seeds was considered as standard.
The observations were also taken with a stereomicroscope and SEM. The photographs were taken with attached cameras. The images were selected on viewing the seed surface on the SEM screen. During the light-microscopic studies special attention was given to the pleurogram, color of the seed coat, shape size and position of the hilum.

**OBSERVATION**

*Albizia falcataria: A. falcataria* seeds were green to greenish brown in color. The unripe seeds were green while the mature seeds develop a greenish brown color. The seeds were ellipsoid in shape, elongated with hard and rough seed coat. I noticed distinct horseshoe shaped elongated groove of pleurogram running parallel with the margin of the seed that divides the seed into outer and inner regions. The outer zone to the groove was slightly uplifted compared to the inner one. The funicle was attached to the hilar end, if not broken during collection and handling the seeds.

The dimension of the seed with a maximum axial length and breadth of 7 mm x 4 mm. I observed the presence of characteristic cracking on the seed surface this species at different magnifications (X200, X400), X800). The plates formed by the cracking were more or less tile-shaped. The outlines of the tiles are straight. At the higher magnification (X2000) the seed surface revealed characteristic pattern of elongated rugae connected to each other. The seed samples collected from different agro-climatic zones revealed the same characteristic cracking on the seed surface at various magnifications under the SEM. The tile formed by the cracking had straight outlines with triangular or polygonal shapes.
Albizia lebbeck: *A. lebbeck* seeds were light brown to chocolate brown in color. The unripe seeds were mostly light brown in color that develops to a chocolate to darker brown color on maturity. The seed is ovoid in shape, mostly narrowed towards the hilar end. The seed has a centrally placed, flat, circular to horseshoe shaped pleurogram. The seed coat is very hard and smooth. Dimension of the seed varies from 12-14 mm x 6-8 mm. Light-microscopic examinations at the higher magnifications showed minor cracking that is distributed throughout the seed surface. The SEM study on seed surface of the tree species showed characteristic fissures and cracking at various magnifications (X400, X800). At higher magnification (X3500) a distinct outline of palisade as represented by protuberance could be seen on the seed surface. The rugae were larger and thicker forming round bulges. These are closely packed, sometimes irregular furrows are present on the surface.
*Albizia lucida:* The seeds of *A. lucida* were yellowish brown in color; completely circular in margin, flat on both the surfaces, somewhat disc-like structure. The seed coat was thin and not very hard, smooth and shiny. From the flat, circular disc-like appearance the seeds could be easily distinguished from most other seeds of *Albizia*. No centrally placed furrow or pleurogram is revealed on examination at the higher magnifications. Only some depressions are found to develop on the seed surface due to dehydration during maturity. I noticed a circular pleurogram present on the seed surface. The diameter of the seed was 10-12 mm. On SEM photos the seed surface shows prominent rugae at the higher magnifications (X1600, X3200). These were aggregated to form somewhat raised, mound-like areas at the regular intervals. The rugae were surrounded by a network of lines that gave rise to somewhat netted appearance of the seed coat with a special design.

*Albizia odoratissima:* The seeds of this tree species were light brown to chocolate brown in color and oval in shape. The seed became narrow towards the micropylar end. Seed surface was smooth, shiny, often wrinkled or striated finely. A horseshoe shaped pleurogram was evident that is dark green in color and present just inside the margin of the seed. The dimension of the seeds in average maximum axial length × breadth was: 6-7 mm × 4-5 mm. On SEM study seed surface showed irregular cracking distributed throughout the seed surface (X200). At higher magnification (X1500) the seed surface appeared to be rugose. The rugae were not very prominent and form hump-like elevations from place to place. Irregular lines of cracking were evident on the seed surface that is totally different from those of *A. falcatoria* and *A. lebbeck*. 
*Albizia procera*: The seeds of *A. procera* were ovoid, dark brown in color. Horseshoe shaped ridges or pleurograms were present on both surfaces running parallel to the margin of the seed. The seed coat is smooth and shiny with fine striations evident on Light microscopic examination. The seed dimension varied: 5-6 X 4-5mm. The SEM revealed wrinkled cracking at lower magnification (X400). At higher magnification (X3200) the rugae were very prominent. Undulations were irregular at higher magnification that on closer examination revealed specific seed surface ornamentation.

*Albizia richardiana*: *A. richardiana* seeds were the largest among the seven species of *Albizia* investigated. The seeds were ellipsoid with outer chocolate brown zone and inner dark brown zone on both the surfaces. The two zones were separated with a yellowish ring-like striation. The seed coat was very hard, smooth and shiny. The dimension varied: 22-25 mm X 9-10 mm. The SEM showed ridges with distinct grooves distributed throughout the seed surface. The ridges are irregular, branched or unbranched. The characteristic feature of the seed coat is deep grooves that are regular in margin and very regular both in size and depth.

*Albizia stipulata*: The seed of this species was ovoid, flat on both the surfaces, greenish brown in color with a dull and hard seed coat. The seed was narrow towards the hilar end. The coloration of the seed coat is a characteristic feature by which it could be easily distinguished from the other species. The one-third of the seed towards the hilar end showed distinct yellowish green color and rest was dark brown. A distinct horseshoe-shaped ridge or pleurogram was present on the hilar end. The pleurogram covered the one-third yellowish-green zone that was very small in comparison to that of
the other species. No other mark or color was observed in the dark brown zone that is uniform in color. The dimension of the seed was: 5-6 mm X 4-5 mm. SEM photos of the seed surface revealed a typical arrangement of wrinkled ridges that were present in a regular manner. The rugae were very prominent and were of even thickness. These aggregate to form somewhat uplifted undulations.

DISCUSSION

The Scanning Electron Microscope has been used as a very powerful tool for characterizing the seed surface. Epiderm, the natural primary surface of all plant organs presents an array of structural diversities and provides valuable information for application to systematics and evolution (Lersten and Gunn 1979; Trivedi et al. 1980; Lersten 1981; Rugenstein and Lersten 1981; Behnke and Barthlott 1983; Bragg et al. 1984; Haridasan and Mukherjee 1988; Takahashi 1993). Trivedi et al. (1979) observing seed surface of six different species of Bauhinia with SEM, concluded that the seed surface revealed ornamentation that differed considerably from species to species. According to Barthlott (1981) seed surface features are little affected by the environmental conditions and seem to reflect genetic-phylogenetic differences in the plants concerned. Bragg et al. (1984) provided valuable information on testa pattern of selected genera of Caesalpinoideae, which may prove useful in the taxonomy of these genera. Haridasan and Mukherjee (1988) from their study on 28 Indian species of the family Campanulaceae concluded that the SEM ornamentation are species specific can not be used as generic marker. In the context of this study attempts have been made to
characterize the seeds of the seven species of *Albizia* for taxonomic delimitation as well as for proper species identification.

Most of the species of *Albizia* in my study possess very hard to moderately hard seed coat. Interestingly, the seed surface of papilionaceous seeds shows heavy to scanty wax deposition (Trivedi 1978b; Wolf et al. 1981). But, the wax deposition in the mimosoid seeds occurs only rarely. SEM observations of the seed surface pattern show that the three species of *Albizia, falcata*, *lebeck* and *odoratissima* out of the seven examined showed similar seed surface nature indicating their close relationship. Clearly some of the species of *Albizia* are more closely related than the other species. Evidently that the genus is an assemblage of related and unrelated taxa. The cracking exhibited by the seed surface of the three species has typical characteristic patterns. The plates formed by the cracking of *A. falcata* are tile shaped with rectangular, polygonal or irregular outline. The cracking of *lebeck* and *odoratissima* are mostly reticulate type. Though the presence of cracking on the seed surface is a common character of the three species, each of them can be easily distinguished by the pattern of cracking. Presence of such cracks has been reported by Trivedi et al. (1979) in *Prosopis stephaniana* and *Acacia catechu*. The prominent cracks present in three species of *Albizia* are connected the seed pleurogram and respond to humidity inside and outside the seed by stretching and shrinking. Dehydration of seeds at maturation and absorption of water during germination probably also takes place through these cracks (Trivedi 1980).
It seems reasonable to conclude that the seed surface ornamentation is a species-specific character. My study corroborated earlier findings by the other workers (Haridasan and Mukherjee 1988; Takahashi 1993), so, it may be concluded that this is a genetically controlled trait. However, further studies will be required on its inheritance patterns in inter-specific hybrids and segregation in subsequent generations to find out the exact nature of gene control of this trait.
LEGEND TO THE FIGURES

Comparative micro morphological studies on the seed coat (Spermoderm) of seven species of *Albizia*:

Fig.-1 A seed of *Albizia stipulata* after gold coating (X15).

Fig.-2 Seed surface of *Albizia falcataaria* at low magnification (X35).

Fig.-3 Seed surface of *Albizia falcataaria* at high magnification (X2000).

Fig.-4 Seed surface of *Albizia lebbeck* at high magnification (X3500).

Fig.-5 Seed surface of *Albizia lucida* at low magnification (X750).

Fig.-6 Seed surface of *Albizia lucida* at high magnification (X2000).

Fig.-7 Seed surface of *Albizia procera* at low magnification (X500).

Fig.-8 Seed surface of *Albizia procera* at high magnification (X1000).

Fig.-9 Seed surface of *Albizia richardiana* at high magnification (X750).

Fig.-10 Seed surface of *Acacia arabica* at high magnification (X4000).

Fig.-11 Seed surface of *Cassia glauca* at low magnification (X500).

Fig.-12 Seed surface of *Cassia glauca* at high magnification (X1000).

Fig.-13 Seed surface of *Cassia marginata* at low magnification (X750).

Fig.-14 Seed surface of *Cassia spectabilis* at low magnification (X1500).

Fig.-15 Seed surface of *Cassia grandis* at low magnification (X1500).
SEM studies on the seed anatomy of 26 Leguminous species

Seed data is increasingly included and discussed in revisions and circumscription of angiosperm families and orders. In practice, most data have been provided by SEM examination of the epidermal surfaces. Sometimes data only from seed surface are insufficient for proper identification of the species. In this investigation I cut sections of twenty-six species of four leguminous genera to study the seed anatomy under the dissecting microscope and the SEM. Under the dissecting scope the color of the different layers of tissue could be seen in some cases, but detailed cellular pattern was evident only under the SEM. The cellular organization of the epidermis, endosperm and internal structural details of the seeds were clearly observable with the SEM. I found distinctive anatomical features in the cross sections of seeds of the different species of the same genus. The main distinguishing characters observed were uniseriate or multiseriate epidermis, epidermal projections, the number of rows and nature of columns of the hypodermal layer, and the nature of endosperm. Three different species of Dalbergia (assamica, latifolia and sissoo) and two species of Albizia (odoratissima and procera) are difficult to distinguish externally even with a seed surface study by the SEM, but SEM photos of cross sections provided enough characteristic features to distinguish from the other. Two species of Acaia (arabica and catechu) and three species of Cassia (glauca, siamia and spectabilis) are difficult to distinguish by standard morphological observation while these can be distinguished easily from anatomical studies with the SEM. I also noted anatomical features of the epidermal and endosperm tissues of seeds that will
be helpful in distinguishing them for characterization and taxonomic interpretation.

MATERIALS AND METHODS

I used 26 species of four leguminous genera.

*Acacia*

* A. arabica
* A. auriculiformis
* A. catechu
* A. centiales
* A. farnesiana
* A. holrisia
* A. melanoxyton
* A. tortillia

*Albizia*

* Albizia falcata*aria
* A. lebbeck
* A. lucida
* A. odoratissima
* A. procera
* A. richardiana
* A. stipulata
**Cassia**

*Cassia glauca*
*C. grandis*
*C. javanica*
*C. marginata*
*C. multijunga*
*C. nodosa*
*C. siamea*
*C. spectabilis*

**Dalbergia**

*Dalbergia assamica*
*D. latifolia*
*D. sissoo*

Dry, mature seeds selected from the above twenty-six leguminous species were collected from a seed supplier of Dehradun (U.P., India). Extraneous materials were removed from the seeds by sonication in dehydrated alcohol for 3-5 minutes. Transverse sections through the midplane of the seeds were mounted on stubs using double stick carpet tape and sputter-coated to approximately 30 nm thickness with a gold target in a vacuum coating apparatus. Silver paint applied to the base of the seed sections prevented charging from the seed surface. At least 8-10 seeds were cut near the midseed and the hilum and examined with SEM at an accelerating voltage of 15
RESULTS AND DISCUSSION

Length and width and average weight of the seeds of each species were measured. I took the average weight of 20 seeds of each species. Seeds were immersed in deionized water for 24h. and weighed to determine the imbibition rate of each species. The available data are summarized in the Table 1 at the end of this chapter.

I scanned the seed sections with SEM and observed the distinctive anatomical features of each species by which they could be differentiated from the other species. Many previous workers including myself have worked on the topography and the structure of the seed coats, but the anatomical features and characteristic epidermal organization of seeds were previously unaddressed. In this study I used 26 species of 4 genera of Leguminosae, viz., Acacia, Albizia, Cassia and Dalbergia. The detailed cellular pattern of the epidermis in the seed sections was not very clear under the dissecting scope but was visible with SEM. The size, shape, and number of tiers of the epidermal and hypodermal layer, and the outer layer of the endosperm of each species differed from the others. The cells were round, columnar, cuboidal or sometimes elliptical. The characteristic presence of “hour glass cells” was noted in the hypodermis of some species of Caesalpinoidae. In two species of Cassia (siamia and spectabilis) contained hourglass cells although the number and orientation of
those cells varied from each other. In *Albizia* the epidermal layer was composed of elongated cells that were visible at X400 magnification (Fig. 10-18). In *A. falcata ria* a green storage tissue was noted under the dissecting scope. This zone possessed stoma everywhere that were visible with higher magnification with SEM (X800). In all three species of *Dalbergia* the epidermal layer was uniseriate but the hypodermal layer was broad having a pattern characteristic to each species. In *D. sissoo* I found distinct stomata evenly distributed in the storage tissue.

In this study, I observed the multiseriate epidermis with double palisade layer in three species of *Cassia* (*glauca, siamia* and *spectabilis*). In *siamia* and *spectabilis* the characteristic presence of hourglass cells were noted (Fig. 31-39). Corner (1951), in describing the structure of legume seeds, stated that the outer columnar epidermal cells (palisade cells) characteristically occurred in a single row. There are of course, counterpalisade cells in the hilum area of papilionoid seeds which form a double layer. However, there are no reports in the literature of the occurrence of the double palisade cell layer elsewhere. Bragg et.al (1984) reported the presence of a double palisade layers first in *Cassia fasciculata*. Bragg examined the seed coats of *C. alata* and *C. romeriana* seeds and found the presence of double palisade layers as well and suggested further sampling be taken to determine whether this double palisade layers is a generic character for *Cassia*.

I observed the distribution of stomata in the storage tissue of seeds of *A. falcata ria* and *D. sissoo*. Cross sections of these seeds appeared green under a dissecting scope. Rugenstein and Lersen (1981) observed stomata on the surfaces of
eight different species of *Bauhinia* including *B. varigata*. That was the first report of stomatal occurrence in leguminous seed. Bragg et al (1984) confirmed the presence of stomata in those species. They also reported that the size of the stomata varied widely and the stomata were absent near the hilum. In my study I found stomata in storage tissue which was not reported earlier. These stomata were more or less even in shape and were organized in a particular pattern (Fig. 48). The function of these stomata is unclear, but the transections of the testa obtained by Bragg et al (1984) clearly showed the presence of large areas for gaseous exchange. In my study the association of green tissue with the stomata may indicate occurrence of active photosynthesis during the early period seed germination in these species having stomata in the storage tissue.

The endosperm pattern was different in the different species of the same genera. The endosperm pattern was different in *Albizia falcataria* and *lebbeck*, (Fig. 1,2). *Dalbergia assamica, latifolia*, (Fig. 3,4). Again, in the two species of *Cassia*, viz., *siamia, spectabilis* (Fig. 5,6) the endosperm pattern was very species specific and therefore useful in distinguishing one from the others.

This investigation on seed anatomy of the leguminous seeds, especially on seed epidermis revealed useful diagnostic features for distinct identification and taxonomic interpretation.
Table 1: Comparative account of the seeds of 26 Leguminous species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Habitat</th>
<th>Soil type</th>
<th>Seed dimension</th>
<th>Wt. In mg</th>
<th>Imbibition rate/day (w/w in g/g)</th>
<th>Water availability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia arabica</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>7-8x7-8</td>
<td>147.7</td>
<td>1.210</td>
<td>76%</td>
</tr>
<tr>
<td>A. auriculiformis</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>3-4x2-3</td>
<td>21</td>
<td>0.970</td>
<td>71%</td>
</tr>
<tr>
<td>A. catechu</td>
<td>Semi-arid</td>
<td>Sandy-loam</td>
<td>5-6.5x4-5.5</td>
<td>30</td>
<td>0.520</td>
<td>69%</td>
</tr>
<tr>
<td>A. centiales</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>12.5x11-12</td>
<td>168</td>
<td>0.460</td>
<td>77%</td>
</tr>
<tr>
<td>A. farnesiana</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>5-6x3-4</td>
<td>60</td>
<td>0.440</td>
<td>73%</td>
</tr>
<tr>
<td>A. holrisia</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>3-4x2-2.2</td>
<td>13.4</td>
<td>0.690</td>
<td>64%</td>
</tr>
<tr>
<td>A. melanoxylon</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>5-6x2-3</td>
<td>17</td>
<td>0.940</td>
<td>77.5%</td>
</tr>
<tr>
<td>A. tortillia</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>6-7x4-5</td>
<td>77.3</td>
<td>0.340</td>
<td>67%</td>
</tr>
<tr>
<td>Albizia falcatoria</td>
<td>Moist</td>
<td>Alluvial</td>
<td>7x4</td>
<td>30</td>
<td>1.400</td>
<td>88%</td>
</tr>
<tr>
<td>A. lebbeck</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>12-14x6-8</td>
<td>151</td>
<td>1.212</td>
<td>76%</td>
</tr>
<tr>
<td>A. lucida</td>
<td>Moist</td>
<td>Clay-loam</td>
<td>Diameter: 11</td>
<td>120</td>
<td>2.400</td>
<td>88%</td>
</tr>
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<td>A. odoratissima</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>6-7x4-5</td>
<td>80</td>
<td>1.090</td>
<td>74%</td>
</tr>
<tr>
<td>A. proclara</td>
<td>Moist</td>
<td>Alluvial</td>
<td>5-6x4-5</td>
<td>47.6</td>
<td>0.890</td>
<td>70%</td>
</tr>
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<td>A. richardiana</td>
<td>Dry</td>
<td>Laterite</td>
<td>22-25x9-10</td>
<td>834</td>
<td>0.950</td>
<td>75%</td>
</tr>
<tr>
<td>A. stipulata</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>5-6x4-5</td>
<td>26.7</td>
<td>0.510</td>
<td>79%</td>
</tr>
<tr>
<td>Cassia glauca</td>
<td>Moist</td>
<td>Alluvial</td>
<td>6-7.5x4-4.5</td>
<td>35.5</td>
<td>1.442</td>
<td>91%</td>
</tr>
<tr>
<td>C. grandis</td>
<td>Moist</td>
<td>Alluvial</td>
<td>14-15x8-9</td>
<td>353</td>
<td>0.1097</td>
<td>0%</td>
</tr>
<tr>
<td>C. javanica</td>
<td>Moist</td>
<td>Clay-loam</td>
<td>7-8x6-6.5</td>
<td>230</td>
<td>0.2231</td>
<td>87%</td>
</tr>
<tr>
<td>C. marginata</td>
<td>Semi-arid</td>
<td>Clay-loam</td>
<td>10.5x6.5</td>
<td>260</td>
<td>0.1497</td>
<td>79%</td>
</tr>
<tr>
<td>C. multijunga</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>7-8x5-6</td>
<td>150</td>
<td>0.0131</td>
<td>69.3%</td>
</tr>
<tr>
<td>C. nodosa</td>
<td>Moist</td>
<td>Clay-loam</td>
<td>9-10x5.5-6</td>
<td>234.5</td>
<td>0.2396</td>
<td>88.4%</td>
</tr>
<tr>
<td>C. siamea</td>
<td>Moist</td>
<td>Alluvial</td>
<td>7-8x5.5-6</td>
<td>33.6</td>
<td>0.0641</td>
<td>90.4%</td>
</tr>
<tr>
<td>C. spectabilis</td>
<td>Dry</td>
<td>Sandy-loam</td>
<td>6.5-7x4-4.5</td>
<td>27.8</td>
<td>0.5405</td>
<td>69%</td>
</tr>
<tr>
<td>Dalbergia assamica</td>
<td>Moist</td>
<td>Clay</td>
<td>6-7x5-6</td>
<td>40.7</td>
<td>1.5831</td>
<td>99%</td>
</tr>
<tr>
<td>D. latifolia</td>
<td>Semi-arid</td>
<td>Sandy-loam</td>
<td>8-9x4-5</td>
<td>51</td>
<td>0.0231</td>
<td>81%</td>
</tr>
<tr>
<td>D. sissoo</td>
<td>Moist</td>
<td>Alluvial</td>
<td>7-8x3-4</td>
<td>15</td>
<td>1.3822</td>
<td>89.2%</td>
</tr>
</tbody>
</table>
LEGEND TO THE FIGURES

SEM Studies on the seed anatomy of twenty-six leguminous species

Fig.-1 A seed of *Acacia arabica* (X10) under dissecting scope.

Fig.-2 A seed of *Acacia catechu* (X14) under dissecting scope.

Fig.-3 A seed of *Acacia auriculiformis* (X16) under dissecting scope.

Fig.-4 Seed section of *Acacia arabica* showing epidermis at low magnification (X35).

Fig.-5 Seed section of *Acacia catechu* showing epidermis at low magnification (X35).

Fig.-6 Seed section of *Acacia auriculiformis* showing epidermis at low magnification (X100).

Fig.-7 Seed section of *Acacia arabica* showing epidermis at low magnification (X600).

Fig.-8 Seed section of *Acacia catechu* showing endosperm tissue at low magnification (X600).

Fig.-9 Seed section of *Acacia auriculiformis* showing epidermis at low magnification (X600).
LEGEND TO THE FIGURES

Fig.-10 A seed of Albizia falcatoria (X15) under dissecting scope.

Fig.-11 A seed of Albizia lebbeck (X10) under dissecting scope.

Fig.-12 A seed of Albizia lucida (X6) under dissecting scope.

Fig.-13 Seed section of Albizia falcatoria showing epidermis at low magnification (X50).

Fig.-14 Seed section of Albizia lebbeck showing epidermis at low magnification (X75).

Fig.-15 Seed section of Albizia lucida showing epidermis at low magnification (X350).

Fig.-16 Seed section of Albizia falcatoria showing hilar region at low magnification (X150).

Fig.-17 Seed section of Albizia lebbeck showing epidermis and storage tissue at low magnification (X200).

Fig.-18 Seed section of Albizia lucida showing epidermis at high magnification (X150).
Seed anatomy of *Albizia odoratissima, A. procera and A. stipulata*:

Fig.-19 A seed of *Albizia odoratissima* (X10) under dissecting scope.

Fig.-20 A seed of *Albizia procera* (X10) under dissecting scope.

Fig.-21 A seed of *Albizia stipulata* (X12) under dissecting scope.

Fig.-22 Seed section of *Albizia odoratissima* showing epidermis at low magnification (X35).

Fig.-23 Seed section of *Albizia procera* showing epidermis at low magnification (X35).

Fig.-24 Seed section of *Albizia stipulata* showing epidermis at low magnification (X75).

Fig.-25 Seed section of *Albizia odoratissima* showing epidermis and storage tissue at high magnification (X1500).

Fig.-26 Seed section of *Albizia procera* showing epidermis and storage tissue at high magnification (X3500).

Fig.-27 Seed section of *Albizia stipulata* showing epidermis and storage tissue at high magnification (X3500).

Fig.-28 Seed section of *Albizia odoratissima* showing storage tissue at high magnification (X5000).

Fig.-29 Seed section of *Albizia procera* showing storage tissue at low magnification (X750).

Fig.-30 Seed section of *Albizia stipulata* showing storage tissue at high magnification (X1000).
Seed anatomy of *Cassia glauca*, *C. siamia* and *C. spectabilis*:

Fig.-31 A seed of *Cassia glauca* (X11) under dissecting scope.

Fig.-32 A seed of *Cassia siamia* (X10) under dissecting scope.

Fig.-33 A seed of *Cassia spectabilis* (X12) under dissecting scope.

Fig.-34 Seed section of *Cassia glauca* showing epidermis at low magnification (X350).

Fig.-35 Seed section of *Cassia siamia* showing epidermis at low magnification (X100).

Fig.-36 Seed section of *Cassia spectabilis* showing epidermis at low magnification (X75).

Fig.-37 Seed section of *Cassia glauca* showing storage tissue at high magnification (X2000).

Fig.-38 Seed section of *Cassia siamia* showing epidermis with “hour glass Cells” at high magnification (X530).

Fig.-39 Seed section of *Cassia spectabilis* showing epidermis and storage tissue at high magnification (X350).
LEGEND TO THE FIGURES

Seed anatomy of Dalbergia assamica, D. latifolia and D. sissoo:

Fig.-40 A seed of D. assamica (X10) under dissecting scope.
Fig.-41 A seed of D. latifolia (X10) under dissecting scope.
Fig.-42 A seed of D. sissoo (X10) under dissecting scope.
Fig.-43 Seed section of D. assamica showing epidermis at low magnification (X50).
Fig.-44 Seed section of D. latifolia showing epidermis at low magnification (X150).
Fig.-45 Seed section of D. sissoo showing epidermis at low magnification (X350).
Fig.-46 Seed section of D. assamica showing storage tissue at low magnification (X750).
Fig.-47 Seed section of D. latifolia showing storage tissue with “reniform cells” at low magnification (X350).
Fig.-48 Seed section of D. sissoo showing storage tissue with “stomata” at high magnification (X750).
LEGEND TO THE FIGURES

Variation in storage tissue in the leguminous tree species:

Fig.1 *Albizia falcataaria* storage tissue in higher magnification (X3500).

Fig.2 *A. lebbeck* storage tissue in higher magnification (X2000).

Fig.3 *Dalbergia assamica* storage tissue in low magnification (X350).

Fig.4 *D. latifolia* storage tissue in low magnification (X500).

Fig.5 *Cassia siamia* storage tissue in higher magnification (X5000).

Fig. 6 *C. spectabilis* storage tissue in low magnification (X100).


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