CHAPTER - 1

INTRODUCTION

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Forests are being exploited to disastrous proportions in recent decades due to the pressures of growing population. The increasing demand of biomass for fuelwood, timber and the pulp for paper industry can no longer be met with the existing natural forest alone. To overcome this shortage, there needs plantation of large number of fast growing trees (Thorpe and Biondi, 1984). However, this requirement will be difficult to accomplish by conventional methods alone. Besides, plants produced by seeds induce considerable variation in them (Krikorian *et al.*, 1981) and also require long duration for the manifestation of juvenile growth phase (Geier, 1986).

Thus, employing tissue culture techniques, rapid propagation of uniform plantation of superior 'elite' trees can be achieved (Bonga and Durzan, 1987; Hanover and Keathley, 1988). Characterestics such as wood quality, disease and pollution resistance, drought and frost resistance etc. can be selected as 'elite' traits and plants possessing them could be produced on commercial scale. (Thorpe and Hasnain, 1988).

The present investigation has been conducted on two multipurpose tree legumes *viz.*, *Dalbergia sissoo* Roxb. and *Albizzia lebbeck* Benth.

MEDICINAL IMPORTANCE OF *DALBERGIA SISSOO* ROXB.

Dalbergia sissoo (Family:Leguminosae;Subfamily : Papilionaceae) is a large deciduous tree. Stem-bark of it is used in the treatment of cholera (Chopra *et al.*, 1958) while leaf decoction is given in acute stage of gonorrhoea. Dutt (1982) reported the presence of quercetin in its leaves.

MEDICINAL IMPORTANCE OF *ALBIZZIA LEBBECK* BENTH.

Albizzia lebbeck Benth. (Family : Leguminosae; Subfamily : Mimosae) is a large, deciduous ornamental tree, good for avenues. Leaves of the tree are used to cure night blindness and opthalmia (Kirtikar and Basu, 1975). Stem-bark of it is used to cure piles, diarrhoea, cough and cold (Chopra *et al.*, 1958). Decoction of stem-bark is also used in the treatment of bronchial asthma and eczema (Tripathi *et al.*, 1979). Varshney and Khan (1961) reported the presence of quercetin in its leaves.

MEDICINALLY IMPORTANT PLANT PRODUCTS

During the life span, plants synthesize certain compounds which could be classified as primary and secondary metabolites, being essential for their growth. These secondary metabolites are complex chemical compounds produced by plants as a chemical interface between plants synthesizing them and their surrounding environment. Medicinally important secondary metabolites fall under the categories of alkaloids, flavonoids, tanins, anthocyanins, coumarins etc. mainly based on their structure. Flavonoids are a class of secondary metabolites with basic structure of two aromatic rings and an oxygen. They are water soluble so that they can accumulate in cell vacuoles, due to hydroxyl groups present in them. Furthermore, one of the hydroxyl groups is often combined to a sugar making them glycosidal forms. However, quantification of flavonoid is carried out in aglycone form as their authentic samples are available in that form. (Hasler *et al.*, 1989).

Dittman and co-workers (1972) reported inhibitory effect of quercetin and its glycosides on malignant cells of brain tumour. Antiviral activity of quercetin was observed by Van Hoof

and co-workers (1984) in the whole plant extracts of *Euphorbia* species. Laekman and co-workers, 1986 assigned cardiovascular activity to 3-methyl quercetin while Soicke and Peschlow (1986) reported anti-hepatotoxic activity to it. Antibacterial activity of quercetin was shown by its capacity to inhibit the growth of *Staphylococcus aureus* while it exhibited viricidal activity against *Herpes simplex* (Vlientinck *et al.*, 1988).

Seabrook(1980) reported that the plant cells and tissues in culture could be manipulated so that specific chemicals could be extracted from the cultured tissues or from the medium in which tissues were grown. The accumulation of secondary metabolites at any stage in the callus culture is the reflection of the dynamic balance between the rate of their biosynthesis and biodegradation. The type of explant used for the initiation of callus culture plays key role in the production of high yields of desired compounds. Each explant consists of cell population which is heterogenous, cell types with different growth rates, ploidy levels including physiological and biosynthetic activities (Holden *et al.,* 1988). In *Ruta graveolens*, it was observed that shoot derived callus cultures produced shoot specific essential

oils whereas those derived from the roots produced root specific oil (Nagel and Reinhard, 1975).

HISTORY OF PLANT TISSUE CULTURE

Plant tissue culture is a technique of growing plant cells, tissues and organs *in vitro* in a sterile nutrient medium under controlled environmental conditions. This idea of growing plant cells in cultures was conceived as early as 1902 by Haberlandt, a German Botanist while working on staminal hair of *Tradescantia* flower. Later in 1922, Robbins and Kotte working independently reported that for establishment of *in vitro* culture there was an absolute requirement of meristematic tissue.

The first report of organogenesis in tree tissue culture was made by Gautheret (1940), who obtained adventitious buds in cambium callus cultures of *Ulmus campestris*, stating the essentiality of exogenous source of sugar for the process. Later, Ball (1946) developed the concept of shoot-tip culture by raising complete plants of *Lupinus* and *Tropaeolum* by this technique. However, Morel and Martin (1952) are credited with the

demonstration of practical usefulness of this technique by raising virus free *Dhalia* plants from infected stock.

Miller and co-workers in 1955 separated the first cytokinin from DNA of herring sperm and named it kinetin. Two years later, Skoog and Miller reported the concept of hormonal control of organ formation which was regulated by the auxin-cytokinin ratio in the medium. This concept is now applicable to most plant species. Wickson and Thimann (1958) stated that with the exogenous application of cytokinins, growth of axillary buds could be induced even in the presence of a terminal bud.

Morel (1960) realized the potential of shoot-tip culture for rapid propagation of orchids. By this technique he achieved production of an estimated 4 million genetically identical orchids from a single bud in a period of one year. This contribution of Morel gave impetus to the utilization of shoot-tip culture for rapid cloning of other plant species. Thus, the field of commercial plant tissue culture originated with a potential to produce desired superior 'elite' plants in large numbers. Murashige (1974, 1977) was successful in propagating a large number of plants ranging

from ferns to foliage, flower and fruit plants employing this technology.

Another development of plant tissue culture technique was the production of haploid plantlets from immature anthers of *Datura innoxia* by Guha and Maheshwari (1964). Later, they traced the origin of embryos to pollen grains and found the plants to be haploid. Bourgin and Nitsch (1967) confirmed the totipotency of pollen grains by raising complete haploid plants of *Nicotiana tabacum*. These haploid plants are useful in breeding a number of species.

Somatic hybridization is an important rapidly developing area in the field of plant tissue culture. In 1972, Carlson and co-workers produced the first somatic hybrid between *Nicotiana langsdorfii* and *N. glauca*. Later, Melchers *et al.*, 1978 realised that a true escape from sexual incompatibility was possible by fusion of protoplast from potato and tomato.

At this time the idea of gene transfer or genetic manipulation emerged out. This consists of insertion, integration and replication of foreign DNA into a host cell.

Utilization of cell cultures for the production of desired secondary metabolites is another landmark in the field of tissue culture research. Seabrook (1980) reported that the plant cells and tissues in culture can be manipulated so that specific chemicals can be extracted from the cultured tissues. These chemicals *viz.*, secondary metabolites accumulate in higher quantity in tissue cultured plants in comparison to parent plants (Fowler, 1986).

PRESENT STATUS OF PLANT TISSUE CULTURE FOR THE RAPID PROPAGATION OF PLANTS

The most widely used and commercially successful application of plant biotechnology is the rapid and large scale clonal multiplication of plants by meristem culture (Vasil, 1986; Debergh and Zimmerman, 1990). This process is used for mass production of many ornamental plants and increasingly for fruit, vegetable, medicine and tree species. Hence, the most useful

application of this technology can be in the production of uniform trees with accelerated growth rates. In addition to it, early reproductive cycles of tissue cultured trees can be used to raise seed orchards and in breeding program to capture the best genetic trait. Thus, rapid multiplication of adult trees selected on the basis of 'elite' traits is a promising method to harness the goals of clonal forestry for which efforts have been made continuously during the last fifteen years (Hanover and Keathley,1988; Bajaj,1989; Dhawan,1989). Some of the recent reports published on clonal multiplication of mature angiosperm trees are *Azadirachta indica* (Ramesh and Padhya, 1990); *Eucalyptus tereticornis* (Das and Mitra, 1990); *Populus deltoides* (Coleman and Ernst, 1990); *Acer saccharinum* (Preece *et al.*, 1991); *Eucalyptus robusta* (Boxus *et al.*, 1991); *Eucalyptus macarthurii* and *E.saligna* (Le Roux and Van Staden, 1991); *Crataeva nurvala* (Sharma and Padhya, 1996).

As the present investigation is focussed on two economically important leguminous trees, a summary of the recent

literature on clonal propagation of mature leguminous trees is cited in Chart II

Thus, from the above literature it is evident that micropropagation methods could help in the rapid large scale production of propagules for afforestation programmes.

De fossard (1976) and Conger (1981) have cited few advantages of clonal propagation which are summarised under the following heads:

(a) Production of plantlets at high frequency from selected 'elite' plant,

(b) Genetic uniformity to an acceptable level,

CHART II : Summary of recent litreature on clonal propagation of mature leguminous tree species

Sr. No.	Species Name	Source of explant	Results	References
1.	*Caesal'pinia pulcherrima* L.S.W. (Flamboyant tree)	Axillary buds	Multiple shoots, rooted	Rahman *et al.*, (1993)
2.	*Bauhinia variegata* L . (orchid tree)	Axillary buds from mature trees	Shoot-proliferation & rooting	Mathur & Mukuntha Kumar (1992)
3.	*Parkinsonia aculeata* (Jerusalem thorn)	Axillary buds from mature trees	Shoot proliferation & rooting	Mathur & Mukuntha Kumar (1992)
4.	*Gymnocladus dioicus*	Axillary buds & shoot-tips	Nodular tissue, shoots formed & rooted	Smith & obeidy (1991)
5.	*Prosopis alba* Griseb clone B2V50	Axillary buds	Shoot multiplication-depend on nitrogen source, shoot - tip necrosis	Green *et al.*, (1990)
6.	*Prosopis juliflora* (SW) D.C. clones 8001, 8004	Axillary buds	Shoot growth, some basal callus	Wain wright & England (1987)
7.	*Sophora secundiflora (Ort.)* Lg. ex DC (Texas Mountain laurel)	Shoot -tips & axillary bud sections	Multiple shoots,rooted (20%) plants	Froberg (1986)
8.	*Cercis canadensis*	Axillary buds	Shoot multiplication	Bennett & Davies (1985)
9.	*Leucaena leucocephala* (Lam) Dew.	Axillary buds of mature tree	Shoot proliferation, rooted *in vitro*	Goyal *et al.*, (1985)

In addition to these, Zimmerman (1985) reported that the plants produced through tissue culture were more vigorous in their vegetative growth than the ones produced by conventional methods. Working on a similar line, Mascarenhas and co-workers (1988) reported that tissue culture raised plants grown in fields have greater heights, diameter and biomass values. Ishii and Kanzashi (1992) pointed out that micropropagated *Betula platyphyla* plants were greater in height, diameter and biomass values than seedling controls. Thorpe and Patel (1984) outlined the governing principle for micropropagation of woody species.

Plant regeneration *in vitro* can be achieved by any of the following ways :

(a) Regeneration from existing meristems

This is most common and primary method used for hardwoods (Dunstan and Thorpe, 1986). It utilises shoot-tips, axilliary buds and small nodal cuttings. Shoot multiplication by this method occurs as a result of release of axillary buds from apical dominance (Lineberger, 1983; Sutter and Barker, 1985).

Initial multiplication rates are low but increases occur during the first few subcultures and eventually reach a steady state e.g.*Prosopis cineraria* (Goyal and Arya, 1984); *Duboisia myoporoides* (Kukreja and Mathur, 1984); *Lagerstroemia flos-reginae* (Paily and D'souza, 1986); *Prosopis juliflora*(Nandwani and Ramawat, 1991); *Dalbergia latifolia* (Raghava Swamy *et al.*, 1992); *Morus indica* (Mahtre *et al* ., 1985).

Mature trees with superior genetic constitution are most attractive for mass propagation. However,it is difficult to establish them in cultures (Bonga, 1987). Hence, stump sprouts are a favourable juvenile alternative for establishment of mature tree cultures e.g. *Quercus robur* and *Tilia cordata* (Chalupa, 1984).

The tendency of axillary shoots to multiply depends on the genotype, the type of shoot and the number of previous subcultures (Bergman *et al.*,1985). Axillary buds, when cultured on medium supplemented with high levels of cytokinins induced bud break (Von Arnold and Wallin, 1988). Supplementing NAA to BAP containing medium reduced the frequency of axillary bud

formation when compared to medium supplemented with BAP only in *Ponderosa* pine cultures (Lin *et al.*, 1991). A multiplication rate of 20-30 shoots per bud in five weeks was achieved from mature trees of *Lagerstroemia flos-reginae* (Paily and D'souza, 1986) while 6-7 fold multiplication every three weeks was achieved from adult trees of *Leucaena leucocephala* (Dhawan and Bhojwani, 1985).

Evaluating the influence of seasons on axillary bud sprouting, Welander (1988) pointed out that best season was spring as it coincided with bud-break.

In histological aspect of axillary bud sprouting, not much work has been done, so considering the uniformity of clonal propagules, Boxus (1987) pointed out that this method is the safest method of shoot multiplication. Upon testing the uniformity of micropropagated *Liquidambar styraciflua* plants, Sutter and Barker (1985) pointed out that these plants were genotypically similar to the parent plants.

As axillary bud proliferation depends upon the genotype, it was thought desirable to apply this to *Dalbergia sissoo* and *Albizzia lebbeck* plants in order to standardise the protocol for their rapid multiplication and propagation.

(b) Regeneration from adventitious meristems

There are a number of recent reports on adventitious bud induction e.g. *Betula pendula* (a) leaves of *in vivo* juvenile shoots (Leege and Tripepi, 1993) (b) leaves of *in vitro* shoots (Srivastava *et al.*, 1985) (c) leaf discs of mature tree (Valobra and James, 1990). Mehra and Cheema (1980)reported direct shoot regeneration from leaf discs of *Populus ciliata*, while Islam and co-workers, 1993 reported adventitious shoot formation from excised leaf explants of *in vitro* grown seedlings of *Aegle marmelos.* Genotype and age of leaf strongly determines regeneration ability in *Anthurium scherzerianum* (Geier,1986). Generally, BAP is chosen for induction of adventitious buds and ususally it is the only phytohormone required for leaves of *Alhagi camelorum* (Bharal and Rashid, 1981); hypocotyl of *Dalbergia*

latifolia (Rai and Chandra,1989); hypocotyl of *Sesbania bispinosa* (Kapoor and Gupta,1986).

Further, effect of leaf size on shoot-bud formation in *Annona squamosa* was evaluated by Nair and co-workers,(1984). It was found that leaf base with petiole induced maximum number of buds per explant, when leaf was excised into segments. In *Crassula argentea*, maximum number of adventitious buds were induced from proximal side of the intact leaf (Paterson, 1983). This proximal regeneration of bud was possibly due to polar movement of endogenous auxins. Upon comparing shoot-bud regeneration from micro-cross-sections (MCS) and 1 cm explant of hybrid *Populs* leaves, it was found that MCS were 25 times more efficient than 1 cm explant (Lee Stadelman *et al.*, 1989) which might be due to better uptake of nutrients by MCS than 1 cm explant.

According to Douglas (1984) explant orientation, abaxial / adaxial side in contact with the medium was critical for adventitious bud induction. Age of the cultured organ also

decided the level of endogenous hormones and subsequently the number of buds induced per explant. Goldfarb and co-workers(1991) reported induction of 10 fold more adventitious buds from youngest seedling when compared to the oldest one.

Adventitious bud induction when accomplished without an intervening callus phase has been considered as a reliable method for clonal propagation (Von Arnold and Wallin, 1988).

(c) Regeneration via callus phase

Another potential method for rapid propagation is via callus phase. Thus, from single callus culture large number of plants can be obtained.

Incorporation of auxin in the medium facilitates induction of callus from explants. Supplementing the media with 2,4-D facilitates callus induction while for its growth, a cytokinin is always added to the medium in addition to an auxin. Hence, effective concentration of each growth regulator needs to be adjusted according to the genotype of the plant and the type of

tissue or organ to be cultured. With the stimulus of endogenous growth substances and phytohormones added to the medium, metabolism of the cells is changed from quiescent to metabolically active state. Durig this process of callus induction, storage products of the cells disappear and new meristems arise producing undifferentiated parenchymatous cells (Mantell *et al.*, 1985).

Individual cells or groups of cells are said to be determined when they become committed to follow a particular genetically programmed developmental pathway and can continue towards that outcome without further influence of growth regulators (Christianson, 1987). During the formation of new meristems from which organs will arise, the component cells adopt a different inherent programming which decides their subsequent pattern of development. Growth regulators in combination with the presence of correct nutritional factors induce this programming. Lakshmisita and Raghava Swamy (1993) reported organogenesis from leaf disc callus of *Dalbergia latifolia,* on woody plant medium supplemented with BAP and NAA while Jaiswal and Narayan

(1985) reported shoot-bud regeneration from stem callus of *Ficus religiosa* on MS medium supplemented with BAP only. In *Leucosceptrum canum*, shoot-buds were induced from callus of *in vitro* regenerated explants in BAP supplemented media (Pal *et al.*, 1985). Between the cytokinins, Kn and BAP tested, BAP proved to be superior for efficient shoot-bud organogenesis from hypocotyl and leaf callus cultures of Sesbania bispinosa (Sinha and Mallick, 1991).

In most unorganised callus cultures, shoot-buds are produced from meristems arising irregularly while in organised or semiorganised callus, a superficial layer of proliferating shoot meristems with an inner core of vacuolated cells acts as a mechanical and nutritional support. Hunault (1979) termed calluses of this kind as 'organoid colonies'. The presence of outer layer of shoot meristems inhibits proliferation of unorganised central tissue (Hussey, 1983).

(d) Regeneration via somatic embryos

Employing this method, multiplication has been achieved in certain plants (Padhya, 1983; Sharma and Chaturvedi, 1989). Somatic embryos produced by this technique, when encapsulated in calcium alginate can be preserved for desired length of time and hence they are known as artificial seeds. A 100% conversion frequency of somatic embryos into synthetic seeds was achieved in *Santalum album* by Bapat and Rao(1988). While according to Becwar and co-workers (1989), although a large number of somatic embryos could be produced from a small tissue, their recovery into plantlets was low.

Shoots produced by multipliction of axillary bud/adventitious bud / via callus phase have to be elongated and subsequently rooted before they can be acclimatised and transferred to field.

ELONGATION OF SHOOT-BUDS

In order to propagate plants from shoot-buds, they have to be grown and elongated to desired length with adequate

foliage. Before elongation of shoots, they are checked for any abnormality in the form of vitrification or shoot-apex necrosis. Subsequently, isolated or clusters of shoots are transferred to elongation medium devoid of cytokinin or to media with low cytokinin concentration. The time for transfer is critical. Several transfers on media lacking growth regulators are usually necessary for shoots to elongate (Horgan, 1987). Each clump of the shoot is successively cut into smaller pieces and newly cut surfaces are placed in contact with the medium. However, from the economic viewpoint, clusters of shoots are elongated (Debergh and Maene, 1981). In case of adventitious buds, elongation can occur while still attached to the explant although their growth becomes slower when compared with the growth of isolated shoots.

PLANTLET ESTABLISHMENT

In order to obtain complete plantlets, elongated shoots must be rooted. This rooting of *in vitro* cultured shoots is very similar to rooting of small cuttings(Gasper and Coumans, 1987).

Generally, treatment of shoots with a medium containing reduced concentration of mineral salts facilitates rooting (Hyndman *et al.*, 1982). Supplementing diluted mineral fraction with auxins, IAA/NAA/IBA stimulates root initiation. Kukreja and Mathur (1985) reported superiority of NAA over IAA or IBA for root formation in *Duboisia myoporoides* while Gaspar and Coumans (1987) preferred synthetic auxins, IBA and / or NAA for induction of roots. This selection of auxin depends not only on its ability to induce rooting but also on the quality of roots it produces.

Exogenous application of auxin can be (a) 'Chronic', when shoots are cultured for prolonged period on a medium containing an auxin,(b) 'acute' or pulse treatment, when shoots are cultured for a short period with an auxin in the culture medium or in solution, prior to their culture on an auxin free medium (Lane and Mc Dougald, 1982).

Root formation in culture is divided into two stages *viz.*, 'induction' and 'elongation' where each stage requires

different culture conditions. However, this can be accomplished in the same culture condition in chronic application of auxin, where endogenous auxin levels decline due to metabolism and conjugation to permit both root initiation and its elongation in the same medium.

Rooting ability of the shoots is highly influenced by the juvenility of the parent stock. Pronounced variation in the rooting ability among various genotypes has been reported which might be due to comparative ability of the tissues to take up or metabolise auxins .

ACCLIMATIZATION AND TRANSFER OF MICROPROPAGATED PLANTS TO FIELD

Adaptation of *in vitro* cultured shoots to *in vivo* conditions in a conventional growing substrate requires improvement of both shoot and root systems. Leaves of micropropagated shoots and plantlets often have less surface wax, thinner cuticle on epidermis and less vascular tissues than green house grown seedings (Smith *et al.*, 1986). In addition to it

they have low photosynthetic capacity. *In vitro* cultured plantlets are generally grown under high humidity and low light conditions. As such, plantlets removed from culture are highly susceptible to wilting, desiccation and infection. Gradual acclimatisation period with decreasing humidity and increasing light intensity is necessary for plants to survive transition from culture vessels to greenhouse and then to field conditions (Hartmann *et al.*, 1990). A two step process for transplanting and acclimatising *Liquidambar* plantlets was proposed by Sommer (1981). Plantlets from nutrient agar are transferred to perlite in a pot covered with polythene. The covers are gradually removed over a period of two weeks, kept for 6-8 weeks in lab conditions, finally transferred to greenhouse and then to field.

Usually micropropagated plants have a sales breakthrough when they are cheaper or at least not more expensive than the traditional material. This reduction in cost of tissue cultured plants can be achieved by increasing the rate and efficiency of multiplication, simultaneously reducing mannual labour with the introduction of robots.

From this review of literature it became evident that there are only a few tissue culture studies on mature trees under investigation.

Thus, the present study was undertaken with the following objectives :

1) To screen stem-bark/ leaf samples of *D.sissoo* and *A.lebbeck* for flavonoid contents in particular quercetin,
2) to select 'elite' plants of both these trees from their screened population,
3) to standardise procedures for *in vitro* multiplication of the 'elite' selected species of both the plants either through
    a) axillary bud sprouting ,
    b) adventitious bud induction and
    c) regeneration of plants from callus cultures,
4) to carry out histological studies during multiplication and propagation of both these trees,
5) to check the ploidy level of regenerated plants.