

In vitro clonal propagation in *Tecomella undulata* (Sm.) Seem

BY

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requirements for the degree of:*

MASTER OF SCIENCE

in

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I



Dedicated to
My
Beloved Parents

Acknowledgement

With regardful memories.....

Would it be all envisaging to offer salutations at the feet of the Lord, who kindly imbued the energy and enthusiasm through ramifying paths of my thick and thin of the efforts.

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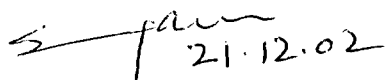
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CERTIFICATE-I

This is to certify that this thesis entitled, "*In vitro* propagation in *Tecomella undulata* (Sm.) Seem", submitted for the degree of **Master of Science** in the subject of **Agroforestry** of the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Shri Rakesh Poria** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.


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CERTIFICATE-II

This is to certify that this thesis entitled, "*In vitro* propagation in *Tecomella undulata* (Sm.) Seem", submitted by Shri Rakesh Poria to the Chaudhary Charan Singh Haryana Agriculture University, Hisar in partial fulfillment for the degree of **Master of Science** in the subject of **Agroforestry**, has been approved by the student's Advisory Committee after an oral examination on the same.

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ABBREVIATIONS USED

%	:	Per cent
°C	:	Degree celsius
μM	:	micro molar
2,4-D	:	2,4-dichlorophenoxy acetic acid
BAP/BA	:	6-benzylaminopurine/benzyladenine
cm (s)	:	Centimetre (s)
EDTA	:	Ethylene dinitrilotetracetic acid
<i>et al.</i>	:	el alia = and others
Fig.(s)	:	Figure (s)
g (s)	:	Gram (s)
HCl	:	Hydrochloric acid
HgCl ₂	:	Mercuric chloride
i.e.	:	id est = that is
IAA	:	Indole acetic acid
IBA	:	Indole-3 butyric acid
<i>in vitro</i>	:	under aseptic conditions
Kin	:	Kinetin (6-furfurylamino purine)
mg l ⁻¹	:	Miligram per litre
Min. (s)	:	Minute (s)
ml	:	Millilitre (s)
MM	:	Millimolar
MS	:	Murashige and Skoog's (1962) basal medium
N	:	Normal solution
NAA	:	α-napthaleneacetic acid
NaOH	:	Sodium hydroxide
no.	:	Number
pH	:	Negative logarithm of hydrogen ion concentration
Sec.	:	Second
v/v	:	Volume/volume
viz.	:	Namely
w/v	:	Weight/volume
wt.	:	Weight

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Word 'Fig.' may be read as 'Plate'

CHAPTER-I

INTRODUCTION

INTRODUCTION

Forests are the main source of biodiversity. It is estimated that about 25 per cent of the world's plant species might be lost by the year 2025 A.D., if the current rate of plant genetic erosion continues (Ravikumar, 2000). Several important medicinal plants are under threat of extinction. The cause of diminishing forest cover and declining productivity of forests are due to heavy pressure of rapidly increasing human population on land, growing demand of timber, fuelwood, fodder and grazing, encroachments, shifting cultivation, urbanization, industrialization and improper management of land.

Tree species are propagated mainly through seeds or by conventional vegetative means (stem cutting, air layering, grafting) or by non-conventional *in vitro* technique. Clonal propagation through tissue culture offers an alternative to vegetative practices and has the potential to provide high multiplication rates of uniform genotypes, resulting in short term gains (Beck and Dunlop, 2001).

With the advent of the new millennium, biotechnology promises new technologies for sustainable development and utilization of the natural resources. Biotechnology will provide immense prospect in tree

improvement programme via micropropagation and genetic engineering in future. Plant tissue culture offers an opportunity for rapid clonal multiplication of desired tree species (Chalupa, 1988; Ahuja, 1991). Tissue and organ culture methods are being increasingly developed for the propagation of many forest tree species. Clonal forestry through tissue culture is receiving increased recognition as an alternative to conventional vegetative practices (Han *et al.*, 1997). In want of basic tissue culture regeneration protocols, work on protoplast culture (Saxena and Gill, 1987) is almost lacking in several tree species. Extensive work has been done on *Acacia* spp., *Dendracalamus longispathus*, *D.strictus*, *Eucalyptus tereticornis*, Leucaena hybrids, Poplars (*Populus ciliata* and *P.deltoides*), *Tectona grandis*, *Actinidia* spp., *Eugenia* spp., *Feijoa* spp., *Malus* spp., *Musa* spp., *Santalum* spp., *Salix* spp., *Swietenia* spp. and *Tamarindus* spp. (Dhawan, 1993). In some cases, plantlets regeneration has been achieved; however, the hardening and nursery is still being standardized. Protocol of nearly 20 species has been perfected (Anonymous, 2000). This technique has now proved to be an efficient tool for rapid clonal propagation of several important plant species. However, tissue culture of forest trees has logged behind that of many agricultural crops probably due to the recalcitrant nature of woody plants.

Tecomella undulata (Sm.) Seeman selected for present study is an evergreen valuable medicinal multipurpose small tree. It is restricted to the drier parts of north-west India and southern Pakistan. It provides man with

very hard wood for making toys, small furniture, fuelwood, leaves and raw fruits provide fodder. *Tecomella undulata* is also known as 'Kapha pitta nashak' and useful in medicine preparation for problems related to blood, spleen and liver. The tree is also planted for its lovely flowers. Natural regeneration of this tree species is mainly occur by wind dispersed self seedling if there is good rainfall distribution in monsoon season. Artificial propagation is also the easiest by direct sowing with fresh seeds, and best if done under a 'nurse' bush for protection. Seeds loose viability at a faster rate after harvest which causes complete loss of viability within six months. This is the main problem in artificial propagation through seeds. Vegetative propagation with cutting is difficult as rooting is poor. This tree is slow growing and suffers from several diseases. So, germplasm with superior traits like fast growth and resistance to salinity, high temperature, water stress, disease and pests have to be identified and multiplied. Plant tissue culture technology has the viable potential in producing thousands of plants, which are true to type, and, therefore, offers an alternative for multiplying elite germplasm. Keeping this in view, the present study was planned with the following objectives:

1. To develop efficient protocol for mass multiplication *in vitro*.
2. To attempt hardening of plants in green house/field conditions.

CHAPTER-II

REVIEW

OF

LITERATURE

REVIEW OF LITERATURE

The first report of organogenesis in tree tissue (Cambium) culture was made by Gautheret (1940). Fully organized plantlets of tree species propagated *in vitro* and capable of subsequent transfer to soil were first obtained in *Populus tremuloides* (Winton, 1968) and among gymnosperms in *Pinus* (Winton, 1978). Since then micropropagation techniques have been successfully used to regenerate plantlets of a large number of tree species. Tissue culture of fruits and forest trees and its importance in clonal improvement has been covered in several reviews (Reinert and Bajaj, 1977; Bajaj, 1986; Litz *et al.*, 1986; Zimmerman, 1986; Bonga, 1987; Bhojwani, 1990; Thorpe and Harry, 1990; Thorpe, 1991; Ahuja, 1991; Dhawan, 1993; Haines, 1993; Vasil, 1994; Minocha and Minocha, 1995).

PLUS TREE SELECTION

The criteria employed for the selection of plus trees vary with the species. However, in general, these trees should possess many desirable characteristics or traits that the foresters value. For example, some of the traits looked for in plus trees are: (1) good stem form, (2) cylindrical bole with less of taper, (3) long clear bole, (4) good height growth, (5) good diameter growth, (6) active leader, (7) narrow crown, (8) good self pruning.

As it is to be expected, such superior individual trees occur in low frequency. Selection of plus trees should be assigned a high priority and infact, should be a continuing process. Selection has to be based on multiple traits so as to bring about genetic upgrading in a number of traits (Kedharnath, 2000).

METHODS OF MICROPROPAGATION

Three patterns of differentiation leading to plantlets formation have been recognized (Rao and Lee, 1986).

1. Explant → axillary bud → multiple shoots → roots → plantlets
(axillary shoot multiplication)
2. Explant → callus → meristemoids → shoots and roots → plantlets
(de novo shoot formation)
3. Explant → callus → cells → embryoids → plantlets
(somatic embryogenesis)

Firstly, axillary meristems are formed in the leaf axils and each is capable of reproducing the main shoot. The plant is thus a natural branching systems that has unlimited proliferation potential. Secondly, adventitious shoot buds may arise from single cells in callus development as a result of wound reaction from cut or injured tissue. Thirdly, somatic embryo developed from a single cell in callus and the germination lead to the plantlet formation. Although different types of explants have been employed for micropropagation of woody plants. the direct adventitious

shoot regeneration from either mature or immature source has been possible only in few woody species (Rout *et al.*, 1998).

The process of micropropagation proceeds through a series of steps, each with a specific set requirement steps commonly involved in tree propagation are:

1. Initiation of aseptic cultures
2. Shoot multiplication
3. Rooting of *in vitro* formed shoots
4. Hardening and transplantation of plants to soil

The above steps were listed by Murashige (1974) but later modified by Debergh and Maene (1981), who included another step: stage 0: preparation of mother plant.

PREPARATION OF MOTHER PLANT

Explants are directly taken from the selected plus tree(s) growing at *in situ*. Alternatively, cutting of the selected individuals are grown in a sterilized potting mix maintained under green house conditions, which favours fresh growth. The plants are carefully watered directly on the soil and, if required, periodically sprayed with fungicides/antibiotics. In hard to root species, which are difficult to establish in pots, the growing branches are loosely covered with polythene bags and later explants are excised from the shoot portion which has grown inside the polybag (Dhawan, 1993). Pre-treatments techniques include spraying of buds with cytokinin before

incubation *in vitro* repeated grafting of mature buds on juvenile root stocks before excision for *in vitro* culture (Bajaj, 1986).

INITIATION OF ASPETIC CULTURES

Explant or choice of explants

Micropropagation can be achieved by culturing different explants. Choice of explants play an important role in achieving morphogenesis. Explants ranging from cotyledons and vegetative meristems to reproductive tissues could be induced to differentiate into new plantlets.

Physiological state

Younger trees provide more suitable explant material for regeneration than the old and aged trees (Bonga, 1982 a,b; Sommer and Wetzstein, 1984). The success in propagation from mature tree is dependent on careful selection of explant (Murashige, 1974; Sommer and Caldas, 1981). Usually, explants from fresh growth of mother plant give better results (Anderson, 1980). Different types of explants (Young v/s old) obtained from mature trees of *Prosopis cineraria* showed different response on the same medium, and old explants produced higher number of shoots (Nandwani and Ramawat, 1993). Shekhawat *et al.* (1993) also observed similar differences in response of different explants obtained from mature trees.

In vitro cloning of mature trees is generally preferred over that from juvenile tissues, in that it is not always possible to determine whether the juvenile tissues will have the desired qualities when they mature (Bonga,

1987). Establishment of cultures *in vitro* from mature tissues is often problematic. Mature plant materials show recalcitrance, which is a maturation effect (Jones and Van Staden, 1997) and inherent problems like contamination and browning are associated with these explants. Failures to regenerate hardwood trees are frustrating. Therefore, more publications appear about regeneration from juvenile explants. But there are examples of successful cloning from mature explants in some tree species like *Aegle marmelos* (Hossain *et al.*, 1993), *Commiphora wightii* (Barve and Mehta, 1993), *Cocculus pendulus* (Gaur *et al.*, 1995), *Zizyphus mauritiana* (Mathur *et al.*, 1995), *Azadirachta indica* (Easwara *et al.*, 1998), *Aegle marmelos* (Ajithkumar and Seeni, 1998). Uneven response of explants and production of limited number of shoots from mature explants were common features in such tree species, e.g. *Tecomella undulata* (Rathore *et al.*, 1991) and *Commiphora wightii* (Barve and Mehta, 1993). Since it is easier to propagate juvenile tissues (Thorpe *et al.*, 1991), rejuvenation and the establishment of juvenile characteristics prior to *in vitro* culture have proved to be important (Jones and Van Staden, 1997). It was only with juvenile explant that the success was achieved in rapid mass multiplication of hardwood species like (*Anogeissus pendula* (Joshi *et al.*, 1991), *Commiphora wightii* (Singh *et al.*, 1997), *Tecomella undulata* (Nandwani *et al.*, 1995, 1996). Hypocotyls, Cotyledonary nodes and apical buds have provided excellent material in tree species to induce multiple shoots. Cotyledons were found to be more suitable for somatic embryogenesis.

Explants of mature branches exhibit higher contamination than juvenile branches for *Acacia nilotica* (Singh *et al.*, 1993), and *Wrightia tomentosa* (Purohit *et al.*, 1998).

Different angles of branches play a very crucial role in the multiplication through differentiation of shoot buds. In *Eucalyptus citriodora*, the calli of only lignotuber origin gave rise to shoot buds (Aneja and Atal, 1969). Similarly, in *Coffea canefora* differentiation of plantlets was from the calli of orthotropic shoots (Staritsky, 1970; Monaco *et al.*, 1977). For pre-existing meristem proliferation, axillary buds are more responsive than apical bud, which generally does not show any response than apical bud, which generally does not show any response in *Morus australis* (Pattnaik *et al.*, 1996), *Aegle marmelos* (Ajithkumar and Seeni, 1998), *Syzygium cumini* (Jain and Babbar, 2000) and *Alnus nepalensis* (Thakur *et al.*, 2001). Various explants show polarity effect in their response as in bud regeneration of citrus epicotyles and root (Burger and Hackett, 1986), flax hypocotyle axis (Bretagne *et al.*, 1994) and internodes (Cuenca *et al.*, 2000) axillary buds of *Ilex paraguariensis* (Sansberro *et al.*, 1999) and roots of *Populus tremula* (Vinocur *et al.*, 2000).

Season for taking explants

It is well documented that establishment of aseptic cultures of trees is influenced by the season during which explants are taken (Hu and Wang, 1983). The best seasons of bud initiation of most trees are spring, coinciding with bud break and late summer (Bonga, 1987; Welander, 1983).

Seasonal variation in axillary bud proliferation has been reported for *Arthocarpus* (Amin and Jaiswal, 1993), *Crataeva nurvala* (Sharma and Padhya, 1996), *Eucalyptus tereticornis* (Sharma and Ramamurthi, 2000). Implantation of explants on the medium influences the response significantly. Bud breaking of vertically placed explants was earlier and good (Shekhawat *et al.*, 1993). In contrast, Katase (1993) reported that nodal explants when placed horizontally on the culture medium gave more shoots than vertical placement. Shoot cultures have also been raised from mature leaves, cotyledonary leaves, hypocotyl, stem segments, petiole, immature embryo and root segments or root coppices.

Culture medium

The most extensively used medium formulation is that of MS (Murshige and Skoog, 1962). It has been considered ideal for establishing the culture of tropical and sub-tropical tree species (Litz and Jaiswal, 1991) e.g. *Pittosporum napaulensis* (Dhar *et al.*, 2000), *Eucalyptus tereticornis* (Sharma and Ramamurthy, 2000), *Phoenix dactylifera* (Taha *et al.*, 2001) and *Paulownia fortuneii* (Venkateswarlu *et al.*, 2001). There are several basal media (modified MS) and B₅ (Gamborg *et al.*, 1968), which have been used for the regeneration of adventitious shoot buds and development of embryogenic cultures of woody species. Mc Cown and Lloyd (1981) pointed out that tissues of woody species would grow better in medium with a lower salt content. As that of MS medium is by far the highest of all the formulations in this respect, it was found beneficial to reduce its

strength by half (Skirvin, 1980; Griffis *et al.*, 1981). Increasing the concentration of reduced nitrogen in the medium also promoted better organogenesis (Kohlenbach, 1977).

Gelling agents

The role of the solidifying agent in the medium designed for shoot regeneration and elongation has been evaluated. Many authors used agar-solidified media for the induction and maintenance of shoots, and somatic embryos, a culture media, which in itself can produce inconsistencies resulting from variance in agar type and purity. The development of adventitious shoots is also affected by the agar concentration in the culture medium (Von Arnold and Eriksson, 1984). Increasing the agar concentration decreases vitrification, but at the same time reduces growth. In some cases a combination of agar and gelrite is preferable (Russel and Mc Cown, 1988). Phytogel was superior to agar in promoting shoot but regeneration in apple (Welander and Maheswaran, 1992), *Dianthus caryophyllus* (Miller *et al.*, 1991), manosteen (Goh *et al.*, 1994), *Eucalyptus tereticornis* (Sharma and Ramamurthy, 2000), date palm (Taha *et al.*, 2001) and *Syzygium travancoricum* (Anand *et al.*, 1999). However, the shoots growing in phytogel showed vitrification, while shoots growing in agar did not (Sharma and Ramamurthy, 2000).

Growth regulators

Various types of growth regulators have been used for culture initiation and maintenance. High shoot multiplication has been obtained by

the use of BAP in a number of trees like *Madhuca longifolia* (Rout and Das, 1993), *Capparis decidua* (Tyagi and Kothari, 1997), *Aegle marmelos* (Ajitkumar and Seeni, 1998), *Paulownia* spp. (Venkateswarlu *et al.*, 2001), *Acacia* species (Beck and Dunlop, 2001). Higher concentration of BAP causes shunting of shoots as reported by Aitken Christie and Connett (1992) and Vitrification in *Alnus* (Thakur *et al.*, 2001). Combined cytokinins (BAP + Kn) have improved the shoot length as well as proliferation in *Commiphora wightii* (Barve and Mehta, 1993) and these cause no improvement in *Sterculia urens* (Purohit and Dave, 1996). In many cases cytokinin alone was optimal for shoot multiplication but addition of an auxin proved harmful (Gharyal and Maheshwari, 1982; Bhojwani and Rajdan, 1983; Duhoux and Davies, 1985; Beck and Dunlop, 2001). With axillary buds or nodes taken from adult plant material, the shoot initiation medium can be additionally supplemented with gibberellic acid (GA₃) to stimulate elongation of nodes in *Acacia* species (Ide *et al.*, 1994; Reddy *et al.*, 1995).

Carbon sources

The type and concentration of carbohydrates and reduced nitrogen supplements in the medium have been reported to influence morphogenesis in woody plants (Friend *et al.*, 1994). Sucrose (3%) provided a good source of carbon in plant tissue culture of woody plants. Further, increase or decrease in sucrose level reduced the shoot bud number. Similar response of 2 per cent sucrose in combination with 2 per cent mannitol has been

reported in *Crataeva nurvala* (Sharma and Padhya, 1996). The highest number of shoots were observed with 4 per cent sucrose with some evidences of vitrification in date-palm (Taha *et al.*, 2001).

Establishment of aseptic cultures

The isolation protocol for tissues from woody plants does not differ greatly from that used for most herbaceous plants. However, one recurring problem with woody plants is a lack of complete decontamination of the tissue. *In vitro* culture systems of trees are relatively difficult to establish from most plant organs. In general, most explants are washed with 5-10 per cent detergent solution "Teepol" for 10-20 minutes depending upon the type of explants. The explants are disinfected with 0.1-0.15 per cent mercuric chloride solution for 5-8 minutes followed by several rinses (2-5 times) with sterile distilled water. Rinsing the tissue with 70 per cent ethanol serves a good surfactant. Effect of season on explant contamination has been noticed in *Tectona grandis* (Gupta *et al.*, 1980), sweet-gum (Sutter and Barker, 1985) and guava (Jaiswal and Amin, 1987).

Callus culture and adventitious shoot formation

Organogenesis or adventitious shoot formation is a process of inducing shoots directly on explants in locations other than the normal region (Murashige, 1977). Hick (1980) classified organogenesis into the types (1) indirect organogenesis which is mediated by callus stage, (2) direct organogenesis, in which adventitious shoots are formed directly on explants. Leaves, embryos, shoot tips internodes, endosperm have been

used as explants for the adventitious bud formation on MS medium (Tables 5a & 5b). In most of the trees, 2,4-D alone or in combination with BAP has been used for the induction of callus. NAA was used in apple (Caboni *et al.*, 2000) and IAA in beech (Cuenca *et al.*, 2000) along with cytokinins, direct organogenesis has been reported in mangosteen (Goh *et al.*, 1994), *Elaeagnus augustifolia* (Athanasios and Maloupa, 1995), apple (Modgil *et al.*, 1999). In cultures, isolated plant cells, tissues may be induced to form an activated growing mass of cells called callus, from which meristemoids are induced by the treatment with plant growth regulators. The meristemoids then develop further into bud primordia, buds, shoots and plantlets. Callus can be multiplied for an indefinite period by routine sub-culturing. Variations among explant types with respect to callus induction has been reported in several woody species such as *Albizia lebbek* (Lakshmana Rao and De, 1987), *Lonicera japonica* (Georges *et al.*, 1993), *Dalbergia latifolia* (Pradhan *et al.*, 1988). A meristemoid is an active locus of growth, a nodule of undifferentiated tissue from which adventitious buds arise. Meristemoids which develop further into adventitious buds are induced when the explants are treated with cytokinin, but for further development of these buds, the explants have to be transferred to media with low cytokinin concentration or lacking cytokinin. The time for transfer is crucial. Several transfers (two to six) on media lacking growth regulators are usually necessary for shoots to elongate. Mostly BAP has been used for the induction of shoot buds on callus in most

of the woody plants but TDZ has been used in beech (Cuenca *et al.*, 2000), mulberry (Thomas *et al.*, 2000), *Acacia mangium* (Xie and Hong, 2001). Auxins (IAA and NAA) have also been used in the induction medium along with the cytokinins. Addition of activated charcoal (0.05 to 1.5 % w/v) has resulted in more elongation of shoots in *Caesalpinia pulcherrima* (Rahman *et al.*, 1993), *Ficus religiosa* (Deshpande *et al.*, 1998).

Hussey (1983) reported that adventitious regeneration direct from organs generally gives a limited number of propagules. This number may be increased several folds by an intervening callus phase that regenerates an increased supply of *de novo* shoots. Although *de novo* shoot formation is generally undesirable for clonal micropropagation, because it can result in somaclonal variation, it presents an opportunity to regenerate plants from genetically transformed clones (Cuenca *et al.*, 2000).

Rooting of in vitro formed shoots (microshoots)

After the development of shoots buds, their multiplication and elongation, the next stage is to induce the roots, which are adventitious in origin, arising from stem bases when incubated in suitable medium (Romberger *et al.*, 1993). Rooting of microshoots of tree species is an intricate phenomenon. *In vitro* produced shoots are obtained on media containing high concentration of cytokinins. Recalcitrant nature of these species to regenerate and high cytokinin concentration further complicate the situation where the genotype and physiological conditions of the explants interact with the rooting medium.

Juvenile shoots of many plants usually rooted easily than mature shoot. Very high frequency (50-100%) rooting has been achieved in several tree species in shoots of juvenile explant origin e.g. *Aegle marmelos* (Ajithkumar and Seeni, 1998), *Anogeissus acuminata* (Rathore *et al.*, 1993), *Azadirachta indica* (Eeswara *et al.*, 1998) and *Zizyphus mauritiana* (Mathur *et al.*, 1995).

Delay in the root formation from cytokinin containing medium (particularly TDZ) has been observed in *Albizia julibrissin* (Sankhla *et al.*, 1996). The promotary effect of reducing the salt concentration of MS (1/2) on *in vitro* rooting of shoots has been described in several woody species e.g. *Albizia procera* (Kumar *et al.*, 1998) and *Dendrocalamus strictus* (Mishra *et al.*, 2001). Purohit *et al.* (1998) reported good rooting of *Sterculia urens* on 1/4th strength of MS mineral salts. Minocha (1987) has reported superiority of synthetic auxins like NAA, IBA and 2,4-D over IAA for rooting. Combined presence of IAA and IBA favoured root induction in several tree taxa such as *Cinnamomum zeylanicum* (Rai and Chandra, 1987), *Morus* species (Pattnaik and Chand, 1997) and *Dalbergia sissoo* (Pradhan *et al.*, 1998). Rooting of shoots by a dip treatment of auxin recommended by Harry and Thorpe (1991) has been demonstrated in many woody plants like *Wrightia tomentosa* (Purohit *et al.*, 1994) and *Prosopis cineraria* (Shekhawat *et al.*, 1993). *Ex vitro* rooting in micropropagation may be desirable for reducing the cost and time involved in rooting and acclimatization stages. *Ex vitro* rooting has been reported in *Paulownia*

tomentosa (Burger *et al.*, 1985), *Lagerstroemia indica* (Zhang and Davies, 1986) and *Anacardium occidentale* (Sardinha *et al.*, 1993). Failure of *ex vitro* rooting has been reported by Falcone and Leva (1987).

Somatic embryogenesis

Somatic embryogenesis or asexual embryogenesis is cultured plant cells is the best demonstration of totipotency in plant cells. The first plant somatic embryos were obtained from somatic tissues (secondary phloem) of carrot roots cultured *in vitro* (Reinert, 1958; Steward, 1958). Since then an increasing number of plant species has been found amenable to this mode of regeneration. Somatic embryogenesis has been proved to be a potential tool for rapid clonal propagation of tree species (Attree and Fowke, 1993, 1995; Gupta *et al.*, 1993). There are many reports of this process in woody species and the list is growing (Bajaj, 1986).

Kohlenbach (1978) distinguished three types of somatic embryogenesis: (i) somatic embryos developing from vegetative cells and tissues of mature plants, (ii) somatic embryos arising from cells of reproductive tissues and structures like nucelli, immature and mature embryos, and (iii) somatic embryos originating as adventitive ones without an intervening callus on hypocotyls, cotyledons, embryos and young plantlets.

Sharp *et al.* (1980) described two types, these are (i) direct embryogenesis in which embryos are formed from pre-embryonic determined cells (PEDC) and indirect embryogenesis which is callus

mediated and embryo formation occurs through induced embryonic determined cells (IEDC). Direct somatic embryogenesis does not go through a callus or dedifferentiation phase, thereby, providing genomic stability of regenerated plants (Luthar and Bohance, 1999).

In most of the species, the explants used have either been zygotic embryos or different parts of young seedlings. Zygotic embryogenic tissues is more amenable to somatic embryogenesis because it contains the pre-embryogenic determined cells, which undergo cell division and expression of embryogenesis (Chand and Singh, 2001). In *Camellia sinensis* somatic embryos were obtained from immature leaves (Kato, 1996), in citrus lemon from styles (Carimi *et al.*, 1994), from stamen filaments in *Aesculus hippocastanum* (Capuana and Debergh, 1997), anthers in *Albizia lebbbeck* (Gharyal *et al.*, 1983), *Populus* species (Ho and Ray, 1985), from stem in *Quercus robur* (Cuenca *et al.*, 1999) and leaves of mature oak trees (Wilhelm, 2000).

The most extensively used medium formulation in plant tissue cultures has been the Murashige and Skoog (MS) medium. The use of MS another related media such as Linsmaier and Skoog (LS) and woody plant medium (WPM) accounts for few examples of somatic embryogenesis with woody species. Induction of somatic embryogenesis with many woody species has resulted predominantly from the use of 2,4-D. 2,4-D and NAA are effective synthetic growth regulators for the induction of somatic

embryogenesis in a number of crops ranging from herbaceous species to woody perennials (Deb, 2001).

The requirement of cytokinin in addition to auxin has been observed in many woody taxa such as *Crataeva nurvala* (Inamdar *et al.*, 1990), *Terminalia arjuna* (Kumari *et al.*, 1998), *Citrus* (Bhansali and Arya, 1978), *Santalum* (Rao and Ozais-Akins, 1985). Sequential transfer of cultures from an auxin based media with the addition of exogenous reduced nitrogen in the form of amino acids or ammonium salts have been conducive for embryogenesis.

Somatic embryogenesis has been regarded as the *in vitro* system of choice with the potential for mass propagation of superior and genetically engineered forest tree genotypes in both coniferous and hardwood species (Gupta and Durzan, 1991; Attree and Fowke, 1993). In fact, most of the reports on somatic embryogenesis in woody species describe “embryo cloning” in which the zygotic embryo is induced to replicate itself indefinitely, and the material being propagated is of unproven genetic value (Merkle *et al.*, 1997). Induction of somatic embryos from mature tree tissues or at least from non-seed tissues such as leaf parts or shoot segments is an important objective to be achieved in forestry species (Cuenca *et al.*, 1999).

Hardening and acclimatization of *in vitro* raised plantlets

Transferring plants from the culture vessel to the potting mix requires a careful stepwise procedure. The plantlets formed in culture are

deficient in photosynthesis efficiency and mechanism to control water loss. Therefore, if the plants are transferred directly to the potting mix, they immediately show signs of wilting and die. A careful acclimatization is thus required. Plantlets with 2-3 leaves and 1-2 cm roots are usually removed from the culture. Healthy shoots with gregarious leaf expansion and mist conditions are pre-requisite for efficient establishment of plantlets in soil. Selection of proper season for rooting followed by transfer to fields should be envisaged for successful field establishment. Plantlets roots gently washed so as to remove the medium sticking to them and these are then planted carefully in the potting mix. A period of humidity acclimatization is necessary for newly transferred plantlets to adapt the outside environment (Kanwar *et al.*, 1991; Sehgal and Handa, 1991). The plants should be maintained under diffuse light and about $\pm 5^{\circ}\text{C}$ of the culture room temperature. At this stage, the plant is deprived of only the organic nutrients and within a couple of weeks it starts photosynthesizing efficiently. Also, wax starts appearing on leaves. At this stage, plants are transferred to comparatively low humidity (about 70%) and the temperature in the room usually $\pm 10^{\circ}\text{C}$ of ambient temperature. After a couple of weeks plants are transferred to a shade house and are finally maintained in nurseries for 4-8 months.

Various methods of hardening have been tried to raise healthy plants which can sustain harsh conditions *ex vitro* (Ziv, 1995; Kurata and Kozai, 1992). These system include scaled-up chambers, continuous flow of the

medium using floating rafts, bottom cooling in growth chambers, use of growth retardants and use of biological and physical agents (Ziv, 1995; Dhawan and Bhojwani, 1987).

Several protocols for large scale production of tree species *in vitro* are available but their successful transfer to the field for performance evaluation remains the major bottleneck. Successful acclimatization is one of the primary factors limiting commercial tissue culture production systems (Hartmann *et al.*, 1990). When woody micropropagated plantlets (from various environments) are transferred to green house or field, this change in environment often results in low survival and reduced growth rates (Eliasson *et al.*, 1994). Recently, long term field performance evaluation of micropropagated trees has been reported in apple, banana, strawberry (Fernando, 1994); *Eucalyptus grandis* (Rockwood and Warrag, 1994; Watt *et al.*, 1995); Butela (Jones *et al.*, 1996); Peach (Hammerschlag and Scorza, 1991); douglas fir, loblolly pine, monterry pine, redwood, bamboo, birch, *Eucalyptus*, tamarind, teak and willow (Gupta *et al.*, 1991).

The ultimate success of any tissue culture programme depends upon the ability to transfer intact plants to soil.

Durand-Cresswell *et al.* (1982) transferred *in vitro* raised eucalypts plantlets to “melfert balls” which are small unwoven bags containing pine bark, sphagnum peat, brown coal ash and esmocote fertilizer. For *Eucalyptus marginata*, Mc Comb and Bennett (1982) used a mixture of two parts sand to one part sphagnum peat. Vieitez *et al.* (1986) obtained a

survival rate of 34 per cent when rooted chestnut plantlets were transferred to soil after 4 weeks in the root culture medium. For *Quercus acutissima* plantlets, a combination of vermiculite and 5 mg l⁻¹ ancymidol provide the best growing medium and plantlets so propagated became acclimatized (from 78% humidity to lower humidity) in about 9 days (Yamamoto *et al.*, 1991).

PROBLEMS AND LIMITATIONS OF TREE TISSUE CULTURE

Tissue culture of hardwood species is still in the developmental stage. Although the technique is well developed for many fruit crops (Boxus and Druart, 1985), it is still to be optimized for forest tree species. The technique is often in direct competition with the conventional methods. The independence of seasonal supply, the cloning of plants for which no vegetative method is available, freedom from disease and clonal uniformity all combine to increase the value of this technique over its conventional competitors. However, there are several problems which must be solved before the technique can be exploited commercially for hardwood.

Most tree species which are difficult to propagate by conventional methods have also proved recalcitrant to tissue culture techniques. The high cost of plantlets production restricts the usage of these plants as large areas have to be covered. Further, returns from these plants are expected only after a few years. Even the evaluation can not be carried out before half rotation. One major problem in tissue culture is contamination. In many species, the bacteria may remain latent for varying period of time but do

affect the multiplication rates and rooting percentage. Cassells (1991) has reviewed the contamination problem and its control, especially for automated systems.

Numerous laboratories in different parts of the world are engaged in developing protocols for propagating elites of various forest tree species and in the last few years success has been quite encouraging.

THREATS

Strict evaluation of *in vitro* raised plants is essential at morphological, physiological, ecological and genetical level before their release. Even after 10 years, tissue culture raised plants have been known to show abnormal variations (Bisht *et al.*, 1998). The case of oil palm (*Elaeis guineensis*) is an important example. Abnormal flowering was shown by some clones after growth of several years (Corley *et al.*, 1986), especially excessive feminization of male flowers thus leading to androgynous male inflorescence. Similar abnormal flowering is also quite common in maize (Rood *et al.*, 1980).

The above review on various tissue culture aspects of trees envisages tremendous scope to explore certain areas viz., micropropagation. The biotechnological approach will certainly help in overcoming the basic problems of the trees and in improving the genotype.

A brief review of regeneration of plantlets via shoot formation and embryogenesis in tree species has been given in Tables 5a and 5b.

Table 5a: Shoot organogenesis in tree species

Plant	Explant	Medium	PGRs Supplements	Reference
<i>Acacia auriculiformis</i>	Nodes (M)	MS	BAP+NAA	Reddy, <i>et al.</i> , 1995
<i>A.mangium</i>	Embryonal axis, cotyledon, mature zygotic embryo, leaflets	MS	Kn+2,4-ID	Xie and Hong, 2001
<i>Azadirachta indica</i>	Anther	MS	BAP+NAA+PVP	Gautam <i>et al.</i> , 1993
<i>A.indica</i>	SE	MS	BAP+IAA	Salvi <i>et al.</i> , 2001
<i>A.indica</i>	Leaf	MS	BAP+Kn, AdSO ₄	Eeswara <i>et al.</i> , 1998
<i>Bauhinia purpurea</i>	Stem cutting (M)	MS	Kn	Kumar, 1992
<i>B.variegata</i>	Node (M)	MS	BAP	Mathur and Mukunthakumar, 1992
<i>Capparis decidua</i>	Node (M)	MS	BAP+NAA	Deora and Shekhawat, 1995
<i>C. decidua</i>	Cotyledon, cotyledonary node, hypocotyl	MS	BAP	Tyagi and Kothari, 1997
<i>C.decidua</i>	Node, shoot tip (M)	MS	BAP	Tyagi and Kothari, 2001
<i>Casuarina equisetifolia</i>	Inflorescence	MS	NAA+BAP	Duhoux <i>et al.</i> , 1986
<i>Dalbergia laifolia</i>	Hypocotyl	MS	BAP+NAA	Pradhan <i>et al.</i> , 1998
<i>D.latifolia</i>	Node, Internode (M)	MS	BAP+NAA	Lakshmi Sita <i>et al.</i> , 1986
<i>D.latifolia</i>	Internode (M)	MS	BAP	Rao, and Lee, 1986
<i>D.latifolia</i>	Shoot segments (M)	MS	NAA+BAP	Rai and Chandra, 1988
<i>D.sissoo</i>	Hypocotyl	MS	NAA, BAP	Pattnaik <i>et al.</i> , 2000
<i>D.sissoo</i>	Cambium (M)	MS	BAP	Kumar <i>et al.</i> , 1991
<i>Eucalyptus globulus</i>	Node (M)	MS	BAP+NAA	Bennet <i>et al.</i> , 1994
<i>E.grandis</i>	Node (M)	MS	BAP, NAA	Lakshmi Sita, 1986
<i>E.sideroxylon</i>	Bud (M)	MS	Kn+BAP	Arya and Shekhawat, 1986

Continue...

Contd..

Plant	Explant	Medium	PGRs Supplements	Reference
<i>Ficus religiosa</i>	Axillary bud (M)	MS	BAP+AdSO ₄	Deshpande <i>et al.</i> , 1998
<i>G.mangostana</i>	Leaves	MS	BAP	Goh <i>et al.</i> , 1994
<i>Hardwickia binata</i>	Nodal segments, Apical buds	MS	BAP+Kn	Anuradha <i>et al.</i> , 2000
<i>Morus alba</i>	Cotyledon, leaf, hypocotyl, shoot tip	MS	NAA+BAP	Kim <i>et al.</i> , 1985
<i>M.alba</i>	Endosperm	MS	2,4-D	Thomas <i>et al.</i> , 2000
<i>M.alba</i>	Leaf, internode	MS	BAP+2,4-D	Bhau and Wakhlu, 2001
<i>Prosopis cineraria</i>	Axillary bud (M)	MS	IAA+Kn	Arya and Shekhawat, 1986
<i>P.cineraria</i>	Hypocotyl, Cotyledonary node, Node	MS	Kn, Kn+NAA+IAA BAP+IAA	Nandwani and Ramawat, 1993
<i>P.cineraria</i>	Nodal segment (M)	MS	IAA+BAP+ additives	Shekhawat <i>et al.</i> , 1993
<i>P.juliflora</i>	Node (M)	MS	BAP+IAA	Nandwani and Ramawat, 1991
<i>Santalum album</i>	Stem	MS	GA ₃	Lakshmi Sita <i>et al.</i> , 1979
<i>S.album</i>	Endosperm	MS	IAA	Rao and Bapat, 1978
<i>S.album</i>	Hypocotyl	MS	IAA+BAP	Rao and Raghava Ram, 1983

Table 5b: Somatic organogenesis in tree species

Plant	Explant	Medium	PGRs Supplements	Reference
<i>Acacia nilotica</i>	Endosperm	MS	2,4-D+BAP+CH	Garg <i>et al.</i> , 1996
<i>Azadirachta indica</i>	Seed (M)	MS	TDZ	Murthy and Saxena, 1998
<i>C.pubescence</i>	Axillary bud (M)	MN+WPM	2,4-D	Jordan and Velozo, 1996
<i>Dalbergia latifolia</i>	Embryos	MS	BAP	Rao and Lakshmi Sita, 1996
<i>Eucalyptus dunii</i>	Seedling	B ₅	NAA, 2,4-D	Termignoni <i>et al.</i> , 1996
<i>Juglans nigra</i>	Cotyledons	WPM	2,4-D+TDZ	Long <i>et al.</i> , 1995
<i>Tamarindus indica</i>	Cotyledon	MS	BAP	Jaiwal and Gulati, 1991

CHAPTER-III

MATERIAL

AND

METHODS



Fig. 1: Blooming mature tree of *Tecomella undulata*

MATERIAL AND METHODS

MATERIAL

Plant Material:

Tecomella undulata (Sm.) Seeman, a tree of family Bignoniaceae was selected for the present investigation. It is an important valuable medicinal plant of desert. Various explants used for the callus initiation and plant regeneration are as under:

Table 1: Various explants used for the callus initiation and plant regeneration

S. No.	Source of plant material	Explants
1.	*Seedlings (7-10 days old)	Cotyledon
2.	Mature tree (15-20 years old)	Axillary bud, leaves

*For seedlings, seeds were collected from the same tree from where we were getting explants.

Explants were taken from the mature tree (15-20 years old) growing at farmer field near village Kaimari, District Hisar (Haryana). New and tender branches of the tree were cut and experiments were conducted at the Plant Tissue Culture and Genetic Engineering Laboratory, Department of Biotechnology and Molecular Biology, CCS Haryana Agricultural University, Hisar.

Seeds were collected from the tree growing at farmer's field, the trees we used for getting explants i.e. branches of the tree.

Glasswares:

All the glasswares used were of the borosilicate quality and obtained from either Borosil India or Corning Glass Company.

Chemicals:

Throughout the investigation, precaution was taken to use chemicals of high purity only. Fine chemicals such as growth hormones, vitamins, inositol, glycine, etc. were obtained from Sigma Chemical Company (USA), whereas ordinary chemicals were purchased from BDH, Glaxo, E'Merck, Sisco Research Laboratory and Hi-Media.

METHODS

Sterilization of glassware, culture media and explants:

Glassware were thoroughly washed, rinsed with distilled water and dried in an oven for two hours at 140°C. Flasks and test tubes containing media or distilled water were cotton plugged and steam sterilized in an autoclave at 1 kg/cm² pressure at 121°C temperature for 15 min. Petriplates, scalpels, forceps were heat sterilized at 180°C for 2-3 hours in an oven. Scalpels and forceps were also flame sterilized prior to use.

Explants were surface sterilized by washing or dipping in 0.1% HgCl₂ solution for 3-5 min., depending upon the nature of the plant material and finally rinsed several times in sterilized distilled water.

In order to maintain aseptic conditions, all the manipulations were carried out in a Laminar Air Work Station.

Storage of culture media and stock solutions:

The autoclaved media in conical flasks or tubes were stored in shade at 20-25°C in cold room and were used within a week of their preparation. Stock solutions were stored in a refrigerator at 1-4°C in liquid state.

Culture media:

The seeds of *Tecomella undulata* were germinated on a medium containing 0.8 per cent agar and 1.0 per cent sucrose. The various media used for the culture are described in the Table 3. All the media contained the Murashige and Skoog's (1962) medium having components shown in Table 2 and differed only in the kind and concentration of growth hormones. The pH of the medium was adjusted to 5.8, with the help of IN HCl and IN NaOH, before autoclaving. After melting the agar at 1 kg per sq. cm pressure, the media were dispensed either into culture tubes or conical flasks. The media were sterilized as described earlier.

Seed germination:

The two-three hours water pre-soaked seeds were surface sterilized by treating with 0.1 per cent HgCl₂ solution for 5 minutes, followed by four to five rinses in sterile distilled water. Sterilized seeds were aseptically inoculated on agar-sucrose medium for germination after properly removing the seed coat with the help of sterilized blade and forceps.

Table 2: Composition of MS

Constituents	MS (mg l ⁻¹)	Constituents	MS (mg l ⁻¹)
1. Macro elements		3. Iron source	
KNO ₃	1900	FeSO ₄ .7H ₂ O	27.80
NH ₄ NO ₃	1650	Na.EDTA.2H ₂ O	37.30
(NH ₄) ₂ SO ₄	-		
MgSO ₄ .7H ₂ O	370		
CaCl ₂ .2H ₂ O	440	4. Vitamins	
KH ₂ PO ₄	170	m-Inositol	100
NaH ₂ PO ₄ .H ₂ O	-	Nicotinic acid	0.50
CaCl ₂	-	Pyridoxine-HCl	0.50
Ca(NO ₃) ₂	-	Thiamine-HCl	0.10
K ₂ SO ₄	-		
MgSO ₄	-		
2. Micro elements		5. Amino acids	
KI	0.83	Glycine	2
H ₃ BO ₃	6.20		
MnSO ₄ .4H ₂ O	22.30	6. Carbohydrate	
MnSO ₄ .H ₂ O	-	Sucrose	30000
ZnSO ₄ .7H ₂ O	8.60		
Na ₂ NoO ₄ .2H ₂ O	0.25		
CuSO ₄ .5H ₂ O	0.025		
CaCl ₂ .6H ₂ O	0.025		

MS = Murashige and Skoog (1962)

Micropropagation:

Explant and callus cultures

Cotyledons from both mature and immature seeds were excised from 7-10 days old seedlings and surface disinfected explants (axillary bud, young leaves) were cultured into different media (Table 3) for callus initiation and/or plant regeneration. The explants were placed horizontally (cotyledon and young leaves) or vertically (axillary bud) on the culture medium. Each flask contained 5 explants. About 30-35 explants were cultured per variation of medium/explant type. Initially, seedling explants were placed on different media for callus/organogenetic response.

The cultures were assessed for the frequency of explants regenerating shoots, number of shoots per regenerating explant and callus and root formation periodically for two months. Experiments were repeated thrice. The cultures were regularly sub-cultured on fresh medium whenever required. Callus initiated on the cultured explants was separated and recultured on the same media.

Rooting:

In this, regenerated shoots were individually cultured on half strength MS liquid medium containing auxins as shown in Table 4. This treatment was given for 48-72 hrs in darkness and after that again cultured on half MS solid medium without hormones. The cultures were assessed for root differentiation after 20-24 days of incubation.

Table 3: List of media tried for callus induction/shoot regeneration in different explants of *Tecomella undulata*

Media code	Growth regulators (mg l ⁻¹)					
	Cytokinins			Auxins		
	BAP	Kin.	Zeatin	IBA	IAA	NAA
TB ₁	1.0	-	-	-	-	-
TB ₂	2.0	-	-	-	-	-
TB ₃	3.0	-	-	-	-	-
TB ₄	4.0	-	-	-	-	-
TB ₅	5.0	-	-	-	-	-
TB ₆	1.0	-	-	-	0.001	-
TB ₇	2.0	-	-	-	0.01	-
TB ₈	3.0	-	-	-	0.02	-
TB ₉	4.0	-	-	-	0.05	-
TB ₁₀	5.0	-	-	-	0.05	-
TB ₁₁	1.0	-	-	-	0.05	-
TB ₁₂	5.0	-	-	-	1.0	-
TB ₁₃	1.0	-	-	-	-	0.01
TB ₁₄	2.0	-	-	-	-	0.01
TB ₁₅	3.0	-	-	-	-	0.01
TB ₁₆	4.0	-	-	-	-	0.01
TB ₁₇	5.0	-	-	-	-	2.0
TB ₁₈	1.0	-	-	-	-	0.5
TB ₁₉	5.0	-	-	-	-	0.5
TB ₂₀	1	-	-	0.1	-	-
TB ₂₁	2	-	-	0.2	-	-
TB ₂₂	3	-	-	0.3	-	-
TB ₂₃	4	-	-	0.3	-	-
TB ₂₄	5	-	-	0.4	-	-
TK ₁	-	1	-	-	0.05	-
TK ₂	-	2	-	-	0.05	-
TK ₃	-	3	-	-	0.05	-
TK ₄	-	4	-	-	0.05	-
TK ₅	-	5	-	-	0.05	-
TK ₆	-	1	-	-	-	0.05
TK ₇	-	2	-	-	-	0.05
TK ₈	-	3	-	-	-	0.05
TK ₉	-	4	-	-	-	0.05
TK ₁₀	-	5	-	-	-	0.05
TZ ₁	-	-	1.0	-	-	0.25
TZ ₂	-	-	2.0	-	-	0.25
TZ ₃	-	-	3.0	-	-	0.25
TZ ₄	-	-	4.0	-	-	0.25
TZ ₅	-	-	5.0	-	-	0.25

All the media contained Murashige and Skoog (1962) basal medium

Table 4: Root induction media for cultured *Tecomella undulata* shoots

Media code	Basal medium	Auxins (mg l ⁻¹)		
		IBA	IAA	NAA
R ₁	Half MS	1.0	-	-
R ₂	Half MS	2.5	-	-
R ₃	Half MS	3.0	-	-
R ₄	Half MS	-	1.0	-
R ₅	Half MS	-	2.5	-
R ₆	Half MS	-	3.0	-
R ₇	Half MS	-	-	1.0
R ₈	Half MS	-	-	2.5
R ₉	Half MS	-	-	3.0

All the cultures were kept under fluorescent light (4000 lux intensity) /dark cycles of 16/8 h at $25 \pm 1^\circ\text{C}$.

Transfer of plants from test tube to pots:

Once the roots had developed, plantlets were taken out of the culture vessels along with medium and washed in running tap water. Regenerated plants were transferred to half strength MS basal medium/tap water for acclimatization. Thereafter plantlets were transferred to small pots containing a sterile soil: vermiculite: FYM mixture (1:1:1, v/v) and covered initially with beakers to maintain humidity. The beakers were removed from pots periodically for fresh aeration. Upon the first sign of leaf proliferation, plants were taken to a net house. Data were recorded for the percentage success of transfer of plants from test tubes to pots.

STATISTICAL ANALYSIS

The data of all the experiments recorded during the present investigation were subjected to statistical analysis in the following way:

$$(a) \text{ Mean } (\bar{X}) = \frac{\sum xi}{n} = \frac{\text{Sum of } i^{\text{th}} \text{ treatment}}{\text{Number of observations}}$$

$$(b) \text{ Standard deviation } (\sigma) = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n}}$$

$$(c) \text{ Standard error of mean (SEm)} = \frac{\sigma \text{ (SD)}}{\sqrt{n}}$$

CHAPTER-IV

RESULTS

RESULTS

The present investigation was undertaken to develop an efficient protocol for plant regeneration and to attempt hardening of *in vitro* plants under green house/field conditions.

Results of the experiments conducted in the present study have been divided into following sections:

- A. Callus induction
- B. Shoot regeneration

A. CALLUS INDUCTION

Tables 6, 7 and 8 describe the effect of different hormonal combinations and other additives in the media on callus induction in different explants of *Tecomella undulata*.

(a) Callus response of leaf explants in different media:

Callus induction was observed in leaf explants on most of the media tried (Table 8). Initiation of callus took place within 8-10 days of inoculation. Callus formation was seen usually at the cut ends and callus was mostly loose and friable, having light green to creamish yellow in colour (Fig. 5). Browning of callus started 15-17 day after callus initiation.

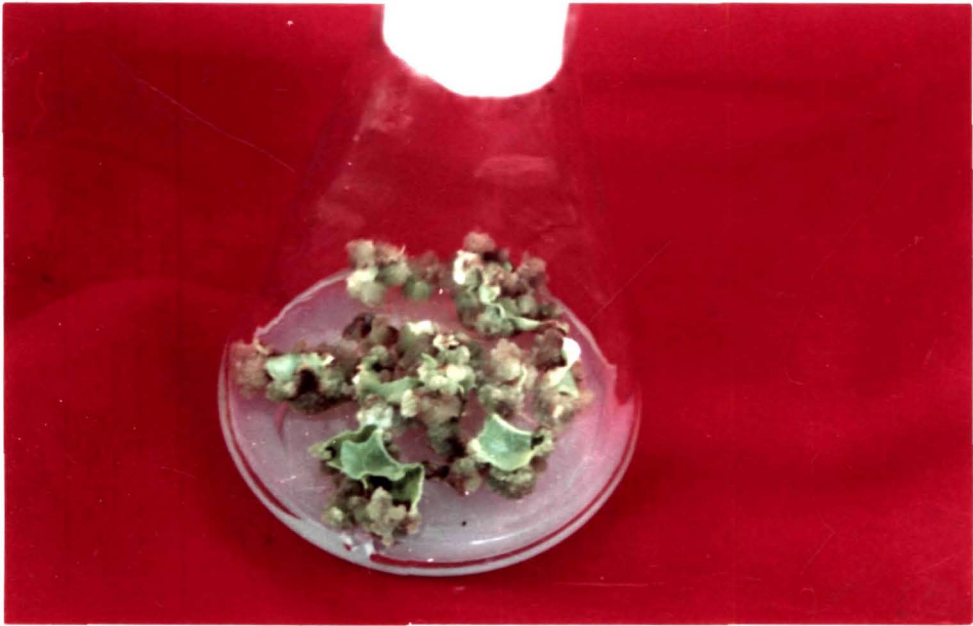


Fig.5: Callus induction from leaf explants of *Tecomella undulata*

Table 6: Percent response of shoot formation/ regeneration from axillary bud on different media combinations

Media code	Percent response	Media code	Percent response
TB ₁	58.33±0.57	TK ₁	25.00±0.57
TB ₂	41.66±1.00	TK ₂	33.33±0.57
TB ₃	63.88±0.00	TK ₃	25.00±0.57
TB ₄	63.88±0.33	TK ₄	50.00±1.00
TB ₅	58.33±0.57	TK ₅	63.88±0.66
TB ₆	50.00±0.57	TK ₆	25.00±0.57
TB ₇	50.00±1.00	TK ₇	25.00±0.57
TB ₈	75.00±0.57	TK ₈	33.33±0.57
TB ₉	88.88±0.66	TK ₉	33.33±0.57
TB ₁₀	63.88±0.66	TK ₁₀	69.44±0.66
TB ₁₁	38.88±0.33	TZ ₁	38.88±0.88
TB ₁₂	63.88±0.33	TZ ₂	38.88±0.66
TB ₁₃	80.55±0.88	TZ ₃	58.33±1.15
TB ₁₄	80.55±0.88	TZ ₄	50.00±1.00
TB ₁₅	75.00±0.57	TZ ₅	63.88±0.66
TB ₁₆	75.00±0.57		
TB ₁₇	80.55±0.88		
TB ₁₈	75.00±0.57		
TB ₁₉	80.55±0.88		
TB ₂₀	63.88±0.66		
TB ₂₁	63.88±0.66		
TB ₂₂	69.44±0.66		
TB ₂₃	63.88±0.66		
TB ₂₄	75.00±0.57		

Table 7: Percent response of callus formation/ shoot regeneration from cotyledons on different media combinations

Media code	Percent response Callus	Percent response shoot regeneration
TB ₂	8.33±0.33	0.00
TB ₇	8.33±0.33	0.00
TB ₈	58.33±0.33	25.0±0.57
TB ₉	8.33±0.33	8.33±0.33
TB ₁₃	16.66±0.33	0.00
TB ₁₉	83.33±0.33	50.0±0.57
TK ₁	8.33±0.33	0.00
TK ₃	33.33±0.33	8.33±0.33
TK ₄	41.66±0.33	16.66±0.33
TZ ₁	8.33±0.33	0.00

Table 8: Percent response of callus formation from leaves on different media combinations

Media code	Percent response Callus
TB ₃	26.66±0.33
TB ₅	36.66±0.33
TB ₆	86.66±0.33
TB ₈	66.66±0.33
TB ₁₃	66.66±0.33
TB ₁₅	53.33±0.33
TB ₁₇	43.33±0.33
TB ₂₀	56.66±0.66
TB ₂₃	33.33±0.33
TK ₁	23.33±0.33
TK ₅	16.66±0.33
TZ ₃	20.00±0.57



Fig.6a: Callus induction and shoot regeneration from cotyledonary explants of *Tecomella undulata*



Fig.6b: Multiple shoot formation from cotyledonary explants of *Tecomella undulata*

Further, sub-culturing of callus on the same media did not favour differentiation. Maximum response for callusing was observed on media (TB₆), containing BAP 1 mg l⁻¹ and IAA 0.001 mg l⁻¹. The media having cytokinin (kinetin) and auxin (IAA) show poor callusing. Similarly, only with BAP low callusing was observed. The other media combinations, which relatively show good results after TB₆ were TB₈, TB₁₃ media.

(b) Induction of callus from cotyledon explants:

Initiation of callus took place within 10-12 days of inoculation in cotyledonary explants. Callusing was observed at the outer end of the petiole. Cotyledon explants developed compact calli (Fig.6a). Further, sub-culturing increased the compactness of calli. Cotyledonary callus turned brown after 20 days of initiation and did not show further response. Callusing was observed on ten media (Table 7) but maximum percentage of callusing (83.33%) was observed on TB₁₉ medium. Relatively, good callusing was observed on TB₈ media and there was no or poor response on media containing only BAP or Kinetin. Response of Zeatin in media was also very poor (Table 7).

(c) Callus response of axillary bud explants in different media

No callusing was observed in these explants as is evident from the Table 6.

B. SHOOT REGENERATION

Shoot regeneration was achieved by the initiation of adventitious buds and the enhancement of the development of axillary bud. Response of

axillary buds and cotyledons for shoot regeneration on different media are presented in Tables 6 and 7.

(i) Shoot regeneration from cotyledon explants in different media:

In cotyledonary explants, initiation of adventitious buds was observed within 10-15 days of incubation (Fig.6a). Highest regeneration frequency (50.00) was realised on media namely TB₁₉ (Table 7). A reasonable good response was from other media also i.e. TB₈. The use of single BAP hormone gave poor results similarly is the case with Zeatine combination with NAA.

(ii) Shoot regeneration from axillary bud explants in different media:

Shoot buds from axillary bud explants were apparently visible within 18-20 days. Shoot regeneration was highest (88.88%) as shown in Table 6, when the media contained BAP (4 mg l⁻¹) and IAA (0.05 mg l⁻¹), along with the percent shoot regeneration on TB₁₃, TB₁₄, TB₁₇, TB₁₉ was not different from TB₉. Figures 2a, 2b and 3 show different stages from initiation to multiple shoot formation.

(iii) Shoot regeneration from leaf bud explants in different media:

Leaf bud explants remained unresponsive for shoot regeneration under the experimental conditions.

Multiple shoot formation:

Multiple shoots were observed from cotyledon and axillary bud on media listed in Table 9.

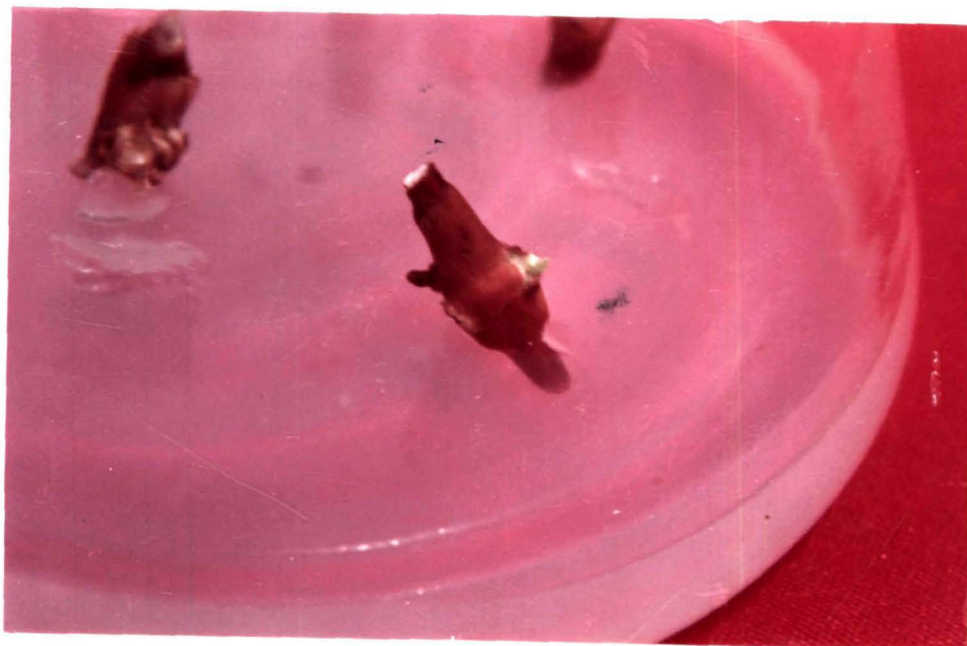


Fig.2a: Axillary bud break in cultured nodal explant on MS modified media



Fig.2b: Regeneration of shoots from nodal explants of *Tecomella undulata*

Table 9: Average number of multiple shoots from axillary and cotyledon explants on different media

Media code	Average no. of multiple shoots from axillary explant	Average no. of multiple shoots from cotyledon explant
TB ₂	10.33±0.33	-
TB ₄	11.66±0.33	-
TB ₁₃	11.00±0.57	-
TB ₁₉	12.33±0.33	10.33±0.33
TK ₁	6.33±0.33	-
TK ₃	6.33±0.33	8.33±0.33
TK ₄	10.33±0.33	10.33±0.33
TZ ₁	10.33±0.33	-

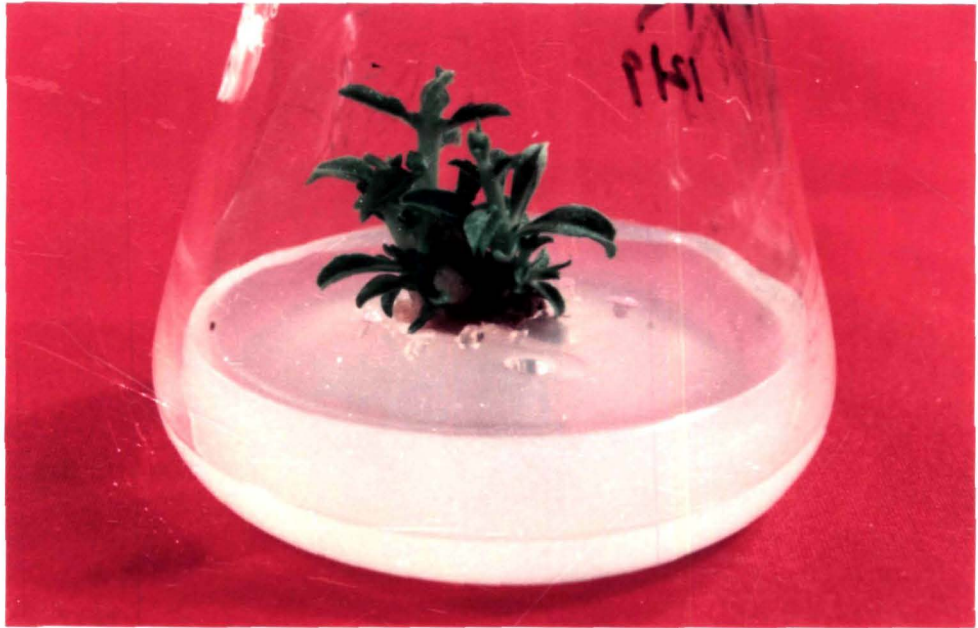


Fig.3: Multiple shoot formation from nodal explants of *Tecomella undulata*



Fig.4: Hormonal differentiation: Variation in leaf shapes in regenerated plants with Kinetin (left) and BAP (right) on MS modified media

Multiple shoot formation was highly dependent on source of explants as no single medium produced multiple shoots in all the explants under study. Cotyledonary explants produced 10 shoots in each explant on TB₁₉, TK₄ (Fig 6b). Axillary bud explants produced 11-12 multiple shoots on media (Fig. 3) TB₂, TB₄, TB₁₃, TK₄, TZ₁. In rest of the media solitary shoots were developed (Table 9).

Browning:

To prevent browning of explants, explants were transferred into fresh medium. Browning of the callus could not be prevented for longer time.

Induction of rooting:

The summarized results obtained with rooting of the isolated shoots are present in Table 10. The roots were visible within 10-12 days of incubation on rooting medium (Fig. 7). Nine media combinations were tried for inducing roots in the isolated cultured shoots (Table 4). Rooting was observed in only one medium. Highest rooting percentage (55.55) was observed on half MS liquid medium treatment of 2.5 mg l⁻¹ IBA (R₅) for 48 hrs and after that transfer to half MS solid medium without hormones. No rooting was observed in single step method i.e. solid MS media with auxins.

Transfer of plants to soil:

Regenerated plants with well developed roots were taken out along with the medium from the culture vessels and rinsed to remove traces of agar, which might provide a substrate for micro-organisms. Maintaining



Fig.7: Root formation in regenerated plants on half MS medium without hormone

Fig.8: Transfer of regenerated plant in the soil



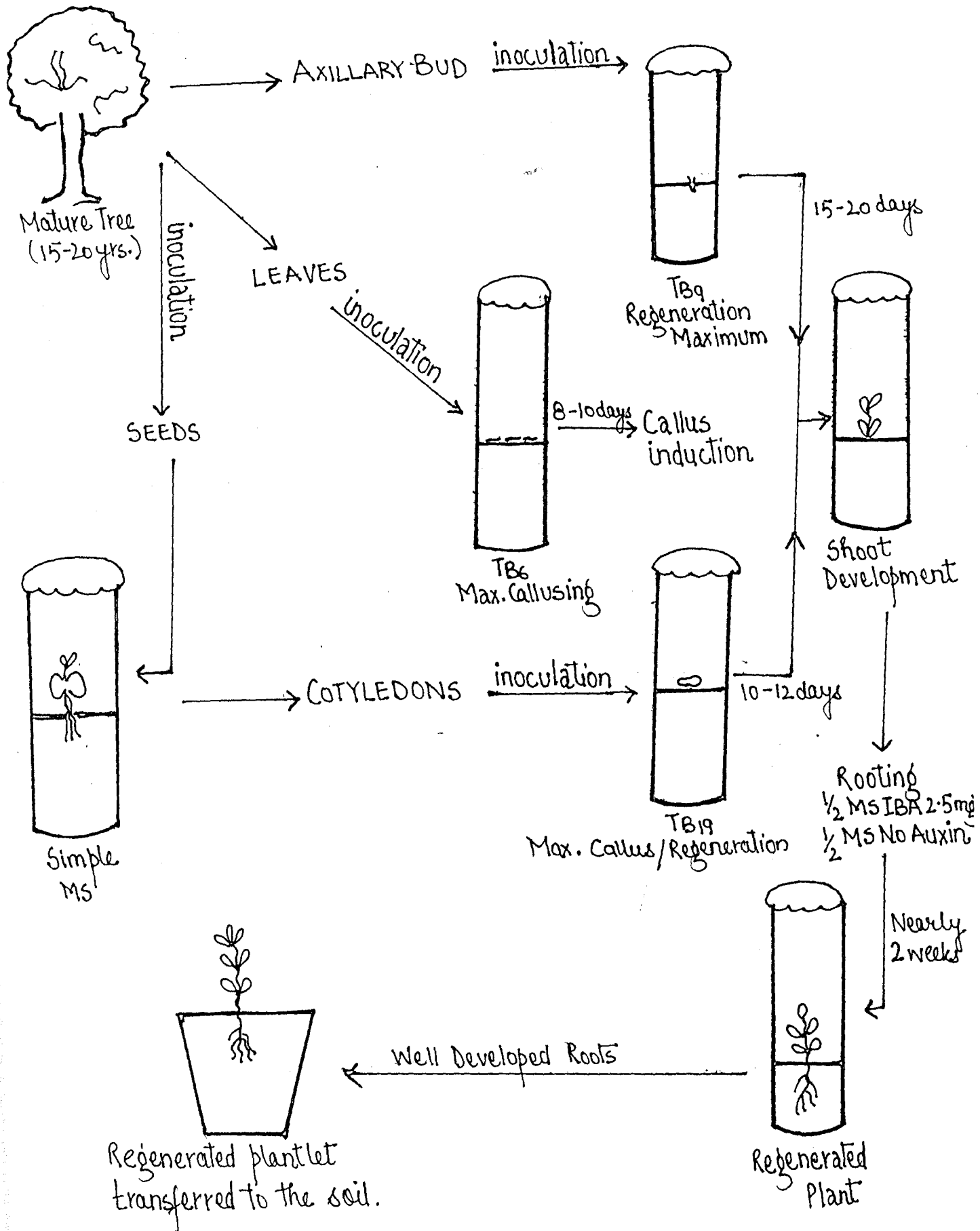
Table 10: Percent rooting response/callus formation from the regenerated shoots from axillary and cotyledon explants

Media code	Percent rooting	Callus formation response
R ₁	0.00	No response
R ₂	0.00	No response
R ₃	0.00	No response
R ₄	0.00	Callus formed
R ₅	55.55	No response
R ₆	0.00	Callus formed
R ₇	0.00	No response
R ₈	0.00	No response
R ₉	0.00	No response

humidity by covering the plant with beaker helped to maintain plant under soil conditions. Observations for plant transfer to soil were recorded after 1 month potted plants as shown in Fig. 8.

On the basis of experimental results, we propose a protocol for *in vitro* multiplication of *Tecomella undulata* from different explants i.e. leaves, cotyledons from mature/immature seeds and axillary bud and suitable media for each explant for shoot regeneration, rooting and conditions for plantlet transfer to soil are illustrated in Fig.9.

Fig.9 Protocol for in vitro multiplication of Tecomella undulata.



CHAPTER-V

DISCUSSION

DISCUSSION

Trees form an important component of forest ecosystem providing food, fuel, timber and other valuable products. In addition, they are very important in maintaining ecological balance at regional and global levels. The use of *in vitro* techniques for clonal and asexual mass propagation is an important application of tissue culture for reforestation programmes. In contrast to the herbaceous species, woody plants have been less amenable to *in vitro* culture. About 150 species showing wide diversity have been successfully micropropagated (Rout *et al.*, 1998). A still smaller number have differentiated plantlets and even plantlets of fewer species have been field-tested.

Tecomella undulata, a member of Bigoniaceae is a multipurpose tree having edible, medicinal and fuel importance. There is little *in vitro* work done on such an important tree. Keeping this in mind, it was selected for the present study.

Explants from 15-20 years old tree were taken in the present study. It was only in juvenile explants that the success was achieved in rapid mass multiplication of hardwood species like *Anogeissus pendula* (Joshi *et al.*, 1991), *Commiphora wightii* (Singh *et al.*, 1997), *Tecomella undulata*

(Nandwani *et al.*, 1995, 1996). But there are examples of successful cloning from mature explants in trees like *Zizyphus mauritiana* (Mathur *et al.*, 1995), *Azadirachta indica* (Eeswara *et al.*, 1998), *Aegle marmelos* (Ajithkumar and Seeni, 1998). Since it is easier to propagate juvenile tissues (Thorpe *et al.*, 1991), rejuvenation and establishment of juvenile characters prior to *in vitro* culture have proved to be important (Jones and Van Staden, 1997).

Best proliferation of buds from the cultured nodal segments occurred on BAP supplement medium as in our present study. The optimum BAP concentration for shoot induction and proliferation was 3-4 mg l⁻¹. Higher concentration of BAP caused stunting of shoots as reported earlier also by Aitken Christie and Connett (1992). High shoot multiplication has been obtained by the use of BAP in many trees like *Madhuca longifolia* (Rout and Dass, 1993), *Capparis decidua* (Tyagi and Kothari, 1997), *Aegle marmelos* (Ajithkumar and Seeni, 1998), *Acacia* spp. (Beck and Dunlop, 2001). By the addition of auxin (IAA, NAA), shoot bud proliferation was improved, as also reported by Gharyal and Maheshwari (1982), Bhojwani and Razdan (1983), Duhoux and Davies (1985). No concentration of combined cytokinins (BAP+Kn) in the medium could improve the shoot length as well as proliferation as also reported in *Sterculia urens* (Purohit and Dave, 1996). These results are in contrast to the results in *Commiphora wightii* (Barve and Mehta, 1993).

It is well documented that establishment of aseptic cultures of field grown trees is influenced by the season during which the explants are taken (Hu and Wang, 1983). In the present study, best results was obtained during August-October. Seasonal variation in axillary bud proliferation has also been reported for *Artocarpus* (Amin and Jaiswal, 1993), *Crataeva nurvala* (Sharma and Padhya, 1996), *Eucalyptus tereticornis* (Sharma and Ramamurthy, 2000). Seasonal variation might be related to the difference in the physiological conditions of the plant grown under natural environmental conditions.

Explants of vertically oriented twigs (orthotropic growth) responded better in comparison to the branches having angular deviation (plagiotropic growth) during the present study. In *Eucalyptus citriodora*, the calli of only lignotuber origin gave rise to shoot buds (Aneja and Atal, 1969). Similarly, in *Coffea canefora* differentiation of plantlets, was from the calli of orthotropic shoots (Staritsky, 1970; Monaco *et al.*, 1977). Similar type of shoot topophysis was also observed in *Taxus mairei* (Chang *et al.*, 2001).

Mature branches exhibited good proliferation but with high rates of contamination in comparison to juvenile branches of *Tecomella undulata*. Similar results were also obtained in *Wrightia tomentosa* (Purohit *et al.*, 1994). Shekhawat *et al.* (1993) also observed similar differences in response of different explants obtained from mature trees. Equal rate in proliferation of both the types of material has been noted in *Acacia mearnsii* (Beck *et al.*, 2000). Highest percentage of explants forming shoots

was obtained when nodal segment number six to ten (counting from the apical meristem) were cultured. Similar difference in the response of nodal segments was noted in *Ilex paraguariensis* (Sansberro *et al.*, 1999). Longer and thick basal nodal explants produced larger shoot buds and leaves in comparison to small and thin apical nodal explants. The greater developmental capacity of larger explants over that of small explants has also been reported earlier (Hu and Wang, 1983). Such type of difference in response was also noted in *Taxus mairei* (Chang *et al.*, 2001).

In the present study agar-agar (0.9%) was found to be better than phytigel for the proliferation of axillary buds. On the contrary phytigel proved superior to agar in mangosteen (Goh *et al.*, 1994), *Eucalyptus tereticornis* (Sharma and Ramamurthy, 2000), date-palm (Taha *et al.*, 2001), *Syzygium travancoricum* (Anand *et al.*, 1999). However, the shoots growing in phytigel showed vitrification, while shoots growing in agar did not. According to Von Arnold and Eriksson (1984), the development of adventitious shoots is affected by the agar concentration in the culture medium. Increasing the agar concentration decreases vitrification, but at the same time reduces the growth.

In this study, bud breaking of vertically placed explants was 1 or 2 days earlier than that of horizontally placed ones. In vertically implantation of explant, bud number was also greater against the horizontally placed ones. Similar results were also observed by Shekhawat *et al.* (1993). In contrast, Katase (1993) reported opposite results in mulberry.

In the present study, callus induction was observed in most of the media from cotyledon and leaves. Cotyledonary explants produced callus in media in Table 7. Rao *et al.* (1988) have found that cotyledonary explants are the best source of callus induction in *Azadirachta indica* followed by leaves. Role of phytohormones in tissue culture studies has been emphasized by many workers (Chalupa, 1975; Durzan and Lopushanski, 1975; Bhargava *et al.*, 1981; Rao *et al.*, 1988). King and Morehart (1987) reported in *Acer rubrum* that optimal growth of callus depended on the ratio of cytokinin/auxin as well as the total amount of plant growth regulators in the medium.

When explants response was compared in different media, no single explant produced callus in all the medium tried. Similarly, no particular medium responded to each and every explant for callus induction. This shows that every explant behaves specifically in each medium under study with respect to percent callus formation. This might be due to difference in internal hormonal levels of the explant and as well as other physiological factors favouring or inhibiting callus formation. Callus formation and its regeneration is important and pre-requisite for any genetic manipulation work for tree improvement. Our studies indicate that callus induction and maintenance is possible from different explants discussed above, however, little attempts to regenerate these calli was successful during present investigation. This aspect was also not seriously tried, as our objective was micropropagation rather than regeneration from callus culture.

Furthermore, regeneration from callus culture is not desirable from micropropagation point of view, as it requires absolute fidelity of genotype during *in vitro* culture.

Leaves and cotyledons explant exhibited variation response for shoot formation in media under consideration. Among seedling explants cotyledons showed maximum regeneration potential on TB₁₉ medium (Table 7). Cotyledonary explants have been found to have higher morphogenetic potential in many trees such as *Tamarindus indica* (Jaiwal and Gulati, 1991) and *Anogeissus pendula* (Joshi *et al.*, 1991). No regeneration has been possible in leaves calli.

The shoot regeneration from axillary bud of *T. undulata* was highest (88.8%) in the medium containing BAP (4 mg l⁻¹) and IAA (0.05 mg l⁻¹) i.e. TB₉ (Table 6). Relatively good regeneration frequency was also observed when the medium containing BAP (1.0 mg l⁻¹) and NAA (0.01 mg l⁻¹) i.e. TB₁₃. Sharma and Chandra (1988) found the combination of BAP and IAA better over kinetin combination for shoot bud differentiation from axillary bud explants in *D. aiso*. Lakshmi Sita and Shobha Rani (1985) also reported the shoot formation from nodal explants on media fortified with BAP (0.5 – 2.0 mg l⁻¹) and NAA (0.1 – 1.0 mg l⁻¹). In *Melia azedarach* nodal bud segments responded on MS medium supplemented with BAP (1.0 mg l⁻¹) (Ahmed *et al.*, 1990). All these reports point out that every genotype/tree species has specific nutritional and hormonal requirement and each genotype has to be standardized to make it amenable for *in vitro*

culture and plant regeneration. Five media namely TB₁₃, TB₁₄, TB₁₇, TB₁₉ were found suitable for shoot regeneration response for axillary bud explants (Table 6), however, no single media was found best for all the explants used in the study and similarly no single explant responded in all the media under study.

Multiple shoot formation from different explants has been reported from nodal explants in *Acacia auriculiformis* (Mittal *et al.*, 1989) cotyledonary and epicotyledonary nodes in *Anogeissus pendula* (Joshi *et al.*, 1991). In our studies, axillary buds as explants showed different response for multiple shoot formation in different media. Axillary multiple shoot buds were observed on different media. Therefore, it suggests that multiple shoot formation depends upon media composition i.e. hormonal balance, auxin-cytokinin interaction which might or might not be favourable for this process.

Regenerated shoots from various explants were multiplied and maintained by regular sub-culturing on fresh medium. Regular transferring onto fresh medium after three weeks was essential to prevent defoliation, culture deterioration and for sustained shoot growth in *Tecomella undulata*. Such type of regular transferring has also been done in *Prosopis cineraria* (Shekhawat *et al.*, 1993). Repeated transferring causes activation of meristems (Boulay, 1984).

In the present study multiplication of elongated shoot was done by transferring them on reduced concentration of BAP and IAA. Continued

presence of higher cytokinin concentration after first sub-culture caused vitrified shoots, which were also observed in *Pistachio* (Onay, 2000) in similar culture conditions. In this study, multiplication of elongated shoot was also done by ratooning as practiced in *Paulwonia* (Venkateswarlu *et al.*, 2001). The shoot bud developed onto induction medium after 3 weeks were of different sizes. Only some of them multiplied in first sub-culture and remaining few shoot buds did not multiply due to their smaller size. It has been earlier reported that axillary buds which have reached a suitable stage of development, were capable of multiplication (Bhojwani and Razdan, 1983). Similar type of shoot bud size-proliferation relationship was observed in *Crataeva nurvala* (Sharma and Padhya, 1996).

Shoot regeneration is of no practical value without proper rooting in regenerated shoots. Importance of auxins in root formation is well documented (Torrey, 1967). However, basal media and auxin (Kind and Concentrations) vary from species to species (Carswell and Locy, 1984; Profumo *et al.*, 1985). Upadhyay and Chandra (1983) have used liquid medium for rooting in *Albizia lebbek*. Goyal and Arya (1984) and Arya and Shekhawat (1986) used WM+BA (3.0 mg l⁻¹) + Kin (0.05 mg l⁻¹) for rooting in regenerated shoots of *Prosopis cineraria*. Rathore *et al.* (1991) used two step method for effective induction for rooting in *Tecomella undulata*.

In the present investigation half MS liquid medium with IBA 2.5 mg l⁻¹ followed by hormone free half MS solid media i.e. R₂ gave

highest rooting percentage (55.55). Other media as shown in Table 4 did not show relatively any good results. In liquid media the regenerated plantlets were dipped from the base for 48 hrs and then transferred to the solid half MS media. In both the media, the plants were placed in a dark room. Root induction was visible in 10-12 days.

Different types of substrates have been used under the acclimatization period before transfer of plantlets to soils. Soil-vermiculite mixture (Goyal and Arya, 1981, 1984), sterilized sand (Bhansali *et al.*, 1988), soil (Kurten *et al.*, 1990). Durand-Cresswell *et al.* (1982) transferred *in vitro* raised *Eucalyptus* plantlets to “melfert balls”. For *Eucalyptus marginata*, Mc Comb and Bennett (1982) used a mixture of two parts sand to one part sphagnum peat. *Acacia koa* plantlets were allowed to develop normal well-branched roots with root hairs by growing in Hoagland’s solution before transplanting to potting mixture (Skokmen and Mapes, 1978).

In the present investigation agar was removed from regenerated shoots and then the plantlets were transferred to pot containing a mixture of Vermiculite + FYM + Sand in 1:1:1 v/v as shown in Fig.8.

CHAPTER-VI
SUMMARY
AND
CONCLUSION

SUMMARY AND CONCLUSION

In the present investigation, efforts were made to optimize conditions for callus induction, plant regeneration in Rohira (*Tecomella undulata*).

Axillary bud, leaves, cotyledons of Rohira were used for different callus induction and shoot regeneration under different media. The results obtained are summarized below:

1. Axillary bud explants were very hard to sterilize and various combination of treatments were used. The combination of Teepol (1-2 drops) + Bavistin (0.5%) for 5 minutes + HgCl₂ (0.1%) for 5 minutes was found to be the best for sterilization. Similar was the case for leaves and seeds.
2. August and October were found to be the best months for collection of explants for culturing.
3. To expedite seed germination the seed coat was removed by soaking the seeds for 2-3 hours in water.
4. Shoot regeneration from axillary bud, cotyledon and leaf was studied using MS media supplemented with different growth regulators. Shoot regeneration was highest in axillary bud explants (88.8%) followed by cotyledon (50.0%). Leaf explants

did not exhibit shoot regeneration. TB9 medium (MS+BAP+mg l⁻¹ + IAA 0.05 mg l⁻¹) was the most responsive for shoot regeneration in axillary bud explants and TB19 medium (MS+BAP 5 mg l⁻¹ + NAA 0.5 mg l⁻¹) for cotyledonary explants.

5. BAP was most effective in comparison to other cytokinin like Kinetin and Zeatin for shoot regeneration from axillary bud/cotyledonary explants.
6. Callus induction was maximum (86.6%) in leaf explants on medium TB6 (MS+BAP 1.0 mg l⁻¹ +IAA 0.001 mg l⁻¹). But in case of cotyledonary explants maximum callus (83.3%) was formed on medium TB19 (MS+BAP 5.0 mg l⁻¹+NAA 0.5 mg l⁻¹). No callusing was observed on axillary bud explants.
7. The two-step method was found to be the best for rooting of regenerated shoots.
8. In the present investigation agar was used in the medium as a gelling agent and showed better response than phytagel.

The present study describes a simple, efficient and reproducible protocol for micropropagation, which can be used for clonal propagation of useful elite genotypes of *Tecomella undulata*.

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