Development of Biotechnological Tools for the Genetic Improvement of selected elite clones of *Eucalyptus tereticornis* Sm.

DISSERTATION

By

Jagriti Sharma

Roll No.3040008

Submitted in partial fulfillment of the requirement
for the award of the degree of
Masters of Science in Biotechnology

Department of Biotechnology and Environmental Sciences
Thapar Institute of Engineering and Technology
(Deemed University)
Patiala –147004

CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled "Development of Biotechnological Tools for the Genetic Improvement of selected elite clones of *Eucalyptus tereticornis* Sm." in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology (Deemed University), Patiala, is an authentic record of my own work carried out during the period of five months from January 2006 to May 2006,under the supervision of Dr. Anil Kumar Datta, Research Scientist, **TIFAC-CORE (Center of Relevance and Excellence) in Agro and Industrial Biotechnology,** Thapar Institute of Engineering and Technology (TIET), Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

Place: Patiala
Date: Jagriti Sharma

CERTIFICATE

This is to certify that the thesis entitled "Development of Biotechnological Tools for the Genetic Improvement of selected clones of *Eucalyptus tereticornis* Sm." submitted by Ms Jagriti Sharma in partial fulfillment of the requirements for the award of the degree of Master of Science in Biotechnology, to Thapar Institute of Engineering and Technology (Deemed University), Patiala, is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

Dr. Anil Kumar Datta
Supervisor
TIFAC-CORE, TIET, Patiala.

Dr. Niranjan Das
Head
DBTES, TIET, Patiala.

Dr. T.P. Singh
Dean (Academic Affairs)
Thapar Institute of Engg. & Tech.,
Patiala

Abstract

Eucalypt is the most important plantation hardwoods in the world (Turnbull, 1991), yielding industrial wood, fuel wood, essential oils and important raw material for pulp and paper industry. In this study micropropagation for selected clones of *Eucalyptus tereticornis* was developed using nodal explants. Murashige and Skoog (MS, 1962) medium was used throughout the experimentation. It was supplemented with varying concentrations of BA and NAA. Cultures were established using nodal segments taken from freshly coppiced shoots and about 50% of nodal explants sprouted on medium supplemented with BA (2.5 μM) and NAA (0.5 μM). Shoot multiplication was found to be better on MS medium supplemented with 2.5 μM BA and 0.5 μM NAA in both the clones namely Y8 and T1. Incorporation of a steroid compound (referred to as 'Compound A' : earlier not known for its activity on plant tissues) into the basal MS medium showed beneficial effect on shoot proliferation as well as rooting in clone Y8 at concentration of 0.25 mg/l. In medium supplemented with BA (2.5 μM) and NAA (0.5μM) 'Compound A' was found to be beneficial for shoot proliferation only. Leaves segments taken from *in vitro* grown shoots were used as explants to establish the regeneration protocol. The explants were inoculated on modified MS medium supplemented with different concentrations of BA and 2,4-D. Shoots regeneration was observed on MS medium supplemented with 5.0 μM BA and 1.0 μM 2,4-D in both the clones. For rooting, 1-2 cm long shoots were cultured on root induction medium containing different concentrations of NAA, IBA and IAA (1 μM, 2.5 μM, 5.0 μM and 10.0 μM). Maximum rooting was obtained on medium supplemented with NAA followed by IBA and IAA. Plantlets were then successfully transferred to green house conditions with a survival rate of 70%. Attempts were made to develop a protocol for genetic transformation of *Eucalyptus tereticornis* clone Y8 using *Agrobacterium tumefaciens* and Particle gun bombardment. Callus was obtained from *Agrobacterium* infected as well as particle gun bombarded tissues on selection medium. Further efforts are being made to achieve regeneration from these calli.

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Date: Jagriti Sharma

Place:

ABBREVIATIONS

%	- Percent
°C	- Degree centigrade
μg	- Microgram
μl	- Microlitre
μM	- Micromolar
BA	- 6-Benzyladenine
cm	- centimeter
g	- Gram
GUS	- β – glucuronidase
h	- hour
IBA	- Indole-3-butyric acid
IAA	- Indole-3-acetic acid
M	- Molar
mg	- Milligram
min	- minute
ml	- Mililitre
mm	- Millimeter
mM	-Millimolar
MS	- Murashige and Skooge (1962) medium
NAA	- α- Naphtalene acetic acid
Npt II	- neomycin phospho transferase
O.D.	- optical density
PGR (s)	- Plant growth regulator (s)
SE	- Standard error
w/v	- Weight by volume

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Fig. 1 *Eucalyptus tereticornis* clone T1 growing in the TIET campus.

Introduction

Eucalyptus (family Myrtaceae), a fast growing short duration tree, used in forestry and agro-forestry. Globally, area under its cultivation is increasing with present estimates of about more than 17.8 M ha (Tournier et al., 2003). It is an enormous and fascinating genus, which comprises over 700-800 species, and accounts for more than two-thirds of Australia's vegetation. *Eucalyptus* is popularly known as gum tree, red iron tree, nilgiri or safeda. Its plantations contribute 25% of the total wood consumed in the developed countries. Most of the forest plantation estates are for industrial use. *Eucalyptus tereticornis* is most productive forest crop in the tropical region (Sharma and Ramamurthy, 2000), which includes most of the part of India. In India large area is under *E. tereticornis* plantation, but its productivity is very low (4-6 m^3/year/h) (Tewari, 1992). Over the last twenty years, large-scale planting of *Eucalyptus*, as a fast growing, exotic, has occurred in India, as part of a drive to reforest the subcontinent, and create an adequate supply of fuel and timber for rural communities under the augur of 'social forestry'.

Eucalyptus is a fast growing, medium- sized to tall tree attaining 20-50 m height and upto 2 m in diameter (Fig. 1). Most of them appear more or less similar at first sight. They are distinguished by general habitat and dimensions; the type of adult bark; seedling, juvenile and adult leaf characters, and also at times by the type of transitional leaves; young branches; inflorescence and the shape of buds, stamens, fruits and leaves. The tree has deep tap root system with mycorrhizal associations which increases its ability to draw nutrients and water. The tree has a smooth silvery white stem. The leaves are leathery in texture, hang obliquely or vertically and are studded with glands containing aromatic oil. Flowering takes place during July-August. Flowers in bud are covered with a cup- like membrane (hence the name of the genus, derived from the Greek 'Eucalyptos' meaning- 'well covered'), which is thrown off as a lid when the flower expands. The fruiting occurs during September - October. The fruits are surrounded by a woody, cup-shaped receptacles and contain numerous minute seeds.

It is versatile, fast growing and strongly coppicing tree possessing a wide range of soil and climatic adaptability. *E. tereticornis* has the most extensive latitude range (9 -38 ^{0}S) of any species in the genus. Basically it is a light demander, the growth of the species is very much reduced under shade. *Eucalyptus* is known for its drought hardiness (Chudnoff, 1971), although annual rainfall of 800 mm is preferred. The species is also moderately salt tolerant (Adams et al., 2005) and relatively fire resistant. The species grows under a wide range of climatic/soil conditions from warm to hot, sub humid to humid and from good to degraded soils.

In general, *E. tereticornis* has proved fairly free of pests and diseases. In many areas termites attack young plants, if insecticide is not used while planting. The most serious disease has been the canker caused by the fungus *Corticium salmonicolo*. (Sharma et al., 1988).

Eucalyptus are the most important plantation hardwoods in the world (Turnbull, 1991), yielding industrial wood, fuelwood, essential oils, shade and shelter. It is used worldwide as a raw material for pulp and paper industry. Estimated demand of hardwood requirement for the pulp and paper industry is 3.18 million tons per year. *Eucalyptus* wood is also used for light and heavy construction, railway sleepers, bridges, piles, poles and mining timber. Leaf extracts of the species have pesticidal properties and can be promoted as a biopesticide and are rich in essential oils, that have many medicinal uses. *E. tereticornis* is a major source of pollen in apiculture and produces a medium amber honey of distinctive flavour. The wood and bark of the tree have a tannin content of 6-12% and 3-15% respectively, though not used as a commercial source of tannin. This plant is a large ornamental tree suitable for parks and avenue plantations. Since ancient times trees have been an integral part of human life and a vital component of biodiversity. Forest trees in particular are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products. Due to the rapid growth of population and human desire to progress, there has been a tremendous reduction in forest cover from earth's surface. As a aforestation programme *Eucalypts* have become by far the most widely planted hardwoods in the tropical and subtropical regions of the world (Eldridge et al., 1993).

The ever increasing demand for *Eucalypt* wood for industrial uses has necessitated better and faster methods of propagation on the one hand and genetic improvement on the other. It is traditionally propagated through the seedlings route. However in such genetically diverse stocks, trees with the better qualities, such as straight and clear bole, disease and pest resistance, drought tolerance, fast growing, etc., occur at a very low frequencies. Due to extensive cross-pollination, seedlings of these superior trees do not maintain these characteristics and a lot of heterogeneity exists amongst seed raised populations. For most species, however, multiplication of the mature trees through conventional vegetative propagation is beset with many problems (McComb, 1995). Therefore, in such cases there is a need for the alternative methods of vegetative propagation. Importance of using *Eucalyptus* in the pulp and paper industry is due to its suitability for making high grade of paper with high opacity, resistance to collapse and intrinsic fibre stiffness, smoothness, etc., which makes paper usable for many important purposes. These characteristics are imparted to the paper due to its fibres that are slender in nature, resistant to collapse, small & low coarse. Further, based on these characters and fast growth, certain elite clones has been selected which are required to be propagated at faster rate.

Biotechnological interventions for *in vitro* regeneration, mass propagation and gene transfer methods in forest species have been practised with success, especially in the last decade. The *in vivo* clonal propagation of plants is often difficult, expensive and sometimes even unsuccessful (Razdan, 2003). Tissue culture methods offer an alternative means of plant vegetative propagation which can be achieved in a shorter time frames and lesser space (Razdan, 2003). The significant advantage of the micropropagation of tree plants is that the shoot multiplication is very fast resulting in logarithm increase in the number of shoots.

Genetic transformation in plants requires an efficient protocol to regenerate whole plant from isolated cells or tissues which have been genetically transformed. This regeneration is achieved under *in vitro* condition. In addition to a high frequency of regeneration, such cells must be accessible to gene transfer by some technique. The primary aim is therefore to produce, as easily and as quickly as possible, a large number of regenerable calli that

can be used for gene transfer. The subsequent regeneration is often the most difficult step in plant transformation studies. However, it is important to remember that a high frequency of regeneration does not necessarily correlate with high transformation efficiency (Slater et al., 2003).

Plants can be regenerated from cultured cells/tissue via somatic embryogenesis and organogenesis (reviews in Zimmerman, 1993; Thorpe, 1995; Zhou et al., 2000; Thorpe and Stasolla, 2001). The present study shall focus on the regeneration of plant via organogenesis and/or somatic embryogenesis.

Asexual transformation methods provide a means for introducing new traits that are difficult to obtain via traditional breeding and allow modification of valuable clones without the genetic recombination that occurs during sexual reproduction (Slater et al., 2003). Transformation requires that DNA be inserted into plant cells, incorporated into chromosomes, and expressed in cells that can be induced to regenerate into plants. Although the transfer of DNA into plant cells via *Agrobacterium*, biolistics, and other physical methods is now routine (reviews in Han et al., 1996; Jouanin et al.,1993; Kim et al., 1997), coupling transformation with the selection of transgenic cells and plant regeneration is still difficult and costly in many economically important species, therby limiting the use of transgenic technology (Birch, 1997).

Plant transformation has become widely adopted as a method to both understand how plants work and to improve desired characteristics. Plant transformation depends on the stable introduction of transgene(s) into the genome of the plant. Plant transformation can be done either by *Agrobacterium* (*A. tumefaciens* and *A.rhizogenes*) or by Direct uptake of DNA through electroporation and PEG – mediated DNA uptake by protoplast or by introducing DNA into cell using particle gun.

In the present study attempts will be made to develop a transformation protocol using *Agrobacterium tumefaciens* and particle gun bombardment.

Keeping in view the need for the improved quality planting material, the present study was designed on the following objectives:

- Development of micropropagation protocol for the selected elite germplasm with emphasis on development of an efficient acclimatization protocol.

- To develop an efficient regeneration protocol.

- Attempts will be made to develop genetic transformation protocol.

Review of literature

Importance of *in vitro* clonal propagation in *Eucalyptus* is evident from the amount of work carried out on the genus (Sharma and Ramamurthy, 2000; Barrueto Cid et al., 1999; Mullins et al., 1997). Further, there are clonal differences which affect the commercial traits of the wood; therefore, it is very important to develop efficient micropropagation and regeneration protocols for different species as well as clones of same species. Clones from the same species are reported to respond differently in culture, therefore, clone specific protocol for micropropagation and regeneration is required to be developed (Mullins et al., 1997). Depending on the end use, many clones of *E. tereticornis* has been selected. Science, it is one of the most important raw materials for the paper and pulp industry, many clones has also been selected with superior characters such as clear bole, self pruning, fibre length etc. from the plantations by paper and pulp industry. Therefore, to undertake improvement programmes for these clones, biotechnological interventions like clone specific in vitro propagation protocols (including regeneration) and genetic transformation protocols are required to be developed.

In order to undertake biotechnological interventions for its improvement, it is important to develop efficient regeneration protocols. It is not only the most important component in the package to develop the transgenics in trees as it also help in regeneration of the transformants and assist in the faster multiplication of the transformants that are required in the millions for commercial plantations.

Success has been achieved in raising micropropagated plants from mature trees of a few species through enhanced-axillary-branching (Gupta and Mascarenhas, 1987; Gupta et al., 1983; Mc Comb and Bennett, 1986), it has opened up possibilities for large scale clonal propagation. Broader application of micropropagation including meristem culture, however, has been limited to some degree by accompanying somaclonal variation (Ahmad et al., 1987; Hwang, 1986; Rani et al., 1995; Schoofs, 1992; Swartz, 1990; Wakasa, 1979). Thus, before the enhanced-axillary-branching method can be fully exploited for clonal propagation of *Eucalypts*, the genetic integrity of such plants must be evaluated (McComb and Bennett, 1986). Perusal of the earlier literature shows that no serious attempts has been made so far in this direction although thousands of tissue

culture derived *Eucalyptus* plants have been produced (Anon, 1994; Keil and Griffin, 1994; McComb and Bennett, 1986; Rockwood and Warrag, 1994) for reforestation programmes and other economic benefits. A few preliminary reports on the analysis of tissue-culture-raised plants derived through methods other than enhanced-axillary branching culture indicate that *Eucalypts* show low vulnerability to phenotypic and numerical chromosomal variations induced by *in vitro* processes or added biochemicals and other stresses (Piton, 1969; Rockwood and Warrag, 1994; Sussex, 1965).

Sharma and Ramamurthy (2000) had developed a protocol for the micropropagation from mature *Eucalyptus tereticornis* using coppiced shoots. The trees were selected on the basis of their better growth, physical and phenotypic characteristics and freedom from diseases. Effect of hormones and media composition on regeneration and growth were studied. It was noticed that phytagel induced vitrification while calcium chloride dihydrate reduced vitrification and induced the elongation of shoots. In this study best rooting response ws observed on half strength MS medium supplemented with IBA (1.0 mg/l). They observed 84 -100% survival rate while hardening the plantlets in non sterile potting mix.

Wachira (1997) induced *in vitro* shoot multiplication from nodal segments of orthotropic shoot sprouts. Regrowth of 25-year-old *Eucalyptus grandis* using a regeneration medium supplemented with several levels and combinations of different growth regulator, different carbon sources, pH at the time of inoculation was achieved. They obtained optimum shoot regeneration on MS medium supplemented with 2 mg/l BA and 1.0 mg/l NAA. It was found that 8 week subculture period was suitable. Higher concentrations of both NAA and BA decreased the shelf life of *E. grandis* shoot cultures by encouraging callus growth and proliferation. Sucrose gave superior growth in comparison to maltose, glucose and fructose. These cultures also showed that pH of the medium affected the growth of shoot cultures with a pH of 5.8 was found to be opyimum for growth.

Direct regeneration via shoot organogenesis and/or somatic embryogenesis is a preffered mode. Protocol for shoot regeneration (Mullins et al., 1997; Subbaiah and Minocha, 1990; Warrag et al., 1991; Laine and David, 1994; Tibok et al., 1995; Serrano et al., 1996; Murlidharan et al., 1989; Termignoni, 1996) and somatic embryogenesis

(Murlidharan et al., 1989; Watt et al., 1995; Ternigoni, 1996) have been developed for some of the species of *Eucalyptus*. Reports exist on the variable regeneration ability of the different species of some of the clones of *E. tereticornis*, clone specific work needs to be carried out as per the requirement. Subbaiah and Minocha (1990) reported regeneration of shoot buds from stem and leaf callus of the *E. tereticornis*, however, it is important to develop direct and efficient regeneration protocol, which will help in taking up genetic manipulation work in this species. Mullins et al. (1997) reported regeneration from leaf explant taken from *in vitro* grown shoots of *E. cameldulensis* and reported variations in response from clone to clone.

Barrueto Cid et al. (1999) reported an efficient regeneration system in hybrid *Eucalyptus grandis* x *E. urophylla* through organogenesis in hypocotyls tissue of young seedlings, however such protocols are required to be developed for explants taken from mature elite plants with desired characteristics. These authors could induce shoot buds from calli of hypocotyls and cotyledonary explants on basal MS medium supplemented with 5.0 µM BA or 10.0 µM Zeatin and 0.5 µM NAA. In this study higher frequency of regeneration was observed from cotyledonary explant. Earlier it has been established that regeneration from young embryonic tissues is easier as compared to mature tissues taken from old plants, which are relatively recalcitrant to manipulations in the culture (Alhawat et al., 2002).

Subbaiah and Minocha (1990) is perhaps the only report on regeneration of *E. tereticornis*. They obtained adventitious shoots from leaf and stem callus of *E. tereticornis.* Callus was induced on B5 medium with 0.1 mg/l benzyladenine (BA) and 3 or 5 mg/l naphthalene acetic acid (NAA) in the dark. They used modified Woody Plant medium (mWP) containing 0.5 mg/l BA, 500 mg/l polyvinylpyrrolidone and 10% (v/v) coconut milk for shoot initiaion. Multiple shoots were also regenerated directly from hypocotyl segments of 4 to 6 week old seedlings on B5 medium with 0.5 mg/l BA. Regenerated shoots were then rooted with 100% efficiency on mWP medium containing 0.5 mg/l IBA and transferred to soil in the greenhouse. Suspension cultures were also obtained from the callus using B5 medium with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4- D). Callus clumps grew from less than 1 mm to 4–6 mm in diameter within two

weeks on transfer to shoot regeneration medium but failed to form shoots or somatic embryos.

With the initial report on regeneration of *E. camaldulensis* (Mullins, 1997), the optimization of the regeneration process had been done by Dibax et al. (2005). These authors cultured cotyledonary leaves on MS medium supplemented with NAA and BA combinations. It was noticed that when the explants were kept in the dark during first 30 days, the percentage of the explants forming calli increased and explants necrosis was reduced in comparison with light cultured explants. They showed that half strength MS mineral medium was as efficient as full strength medium producing 54% and 47% of explants with buds respectively.

The transformation of selected clones is a powerful tool in developing new genotypes containing desirable traits, such as insect and herbicide resistance, male-sterility and reduction of lignin content (Jouanin et al., 1993; Haines, 1994; Teulieres et al., 1994). Although, *Eucalyptus* is mainly planted for the purpose of providing raw material for the paper and pulp industry, higher lignin content of this tree is the major constraint, and requires expensive, cumbersome and polluting treatment for the removal of lignin from cellulose. Its productivity, like most forest trees, might be increased by the incorporation of genetic transformation into improvement programs. Generally paper industry require plant species with higher cellulose content (cellulose is mainly required for the manufacture of paper) and lower lignin contents (lignin is mainly removed during pulping of wood). Improvement of *Eucalyptus* with respect to reduction of lignin and enhancement of cellulose content, increased yield, etc., through plant genetic manipulation is the need of the day for the paper and pulp industry.

Mullins et al. (1997) developed a reliable regeneration protocol for *E. cameldulensis* using leaf explants from *in vitro* grown plants. They also achieved success in transformation using *Agrobacterium tumefaciens* strains carrying a kanamycin resistance gene and the reporter gene β – glucuronidase (*GUS*). For selection kanamycin at 9 mg/l was used on which selection of transformed shoots was allowed. The transformation of the plants were verified by staining for the *GUS* enzyme in various plant tissues, *Npt II* assays and by southern blotting on isolated DNA using specific probes for both *GUS* and selectable marker genes.

Transgenic *Eucalyptus globulus* plantlets were generated through *Agrobacterium tumefaciens* mediated transformation (Moralejo et al., 1998) using young seedlings obtained from mature seeds as target material. It was observed that the most important factors affecting transformation efficiency were the nature of the *A. tumefaciens* strains, precultivation of the seedlings and wounding of the tissues. About 1.2% of inoculated seedlings showed regeneration of shoots which were able to grow on a selection medium containing kanamycin and were able to express the *GUS* gene.

An efficient protocol for *Agrobacterium*-mediated transformation of *Eucalyptus camaldulensis* and production of transgenic plants was developed by Chang et al. (1998). Hypocotyl segments were co-cultivated with *A. tumefaciens* containing a binary Ti-plasmid vector harbouring chimeric neomycin phosphotransferase and β - glucuronidase (*GUS*) genes. They used modified Gamborg's B5 medium which was effective for both callus induction and regeneration of transgenic shoots. The transgenic shoots were cultured on MS medium supplemented with 0.1 mg/L BA prior to rooting. Histochemical assay revealed the expression of the *GUS* gene in leaf, stem and root tissues of transgenic plants.

Reports also exist on the use of biolistic for transformation of the hybrid *Eucalyptus grandis* x *E. urophylla* using hypocotyls and cotyledonary derived calli as target material (Sartoretto et al., 2002) . The target was grown on SP medium supplemented with 10 µM 2,4-D and 2.5 µM BA. Tungsten particles were coated with the plasmid pBI 426 harbouring a β-glucouronidase (*GUS*) and neomycin phosphotransferase II (*npt II*) gene fusion controlled by a double 35S cauliflower mosaic virus (CaMV) promoter. They determined the transient transformation efficiency after four days of bombardment by the expression of the *GUS* gene. They observed fully *GUS* positive calli after 105 days in MSM medium supplemented with 2,4-D, BA and the selective agent kanamycin at 200 mg/l. However they were unable to regenerate these transgenic shoots, suggesting that the conditions for regeneration are unsuitable.

Although work has been carried out for thee development of micropropagation, regeneration and transformation for some of the clones (Mullins et al., 1997); but the variation exist from clone to clone. Therefore, similar work is required for the development of these protocols for selected clones important for paper and pulp industry.

Materials and methods

Plant material, Chemicals and Glasswares:

Explants were taken from freshly coppiced shoots from mature selected elite clones of *Eucalyptus tereticornis* Sm. or actively growing young plants (about 6 months old tissue culture raised plants). These were excised and kept in water and brought to the laboratory. Nodal segments from these shoots were used as explants. Unless otherwise mentioned all chemicals used in this study were obtained from HiMedia Laboratories Ltd., Mumbai. Vitamins & plant growth regulators were purchased from Sigma Chemical Company (St. Louis, USA). Unless otherwise mentioned all experiments were conducted in jam bottles covered with polypropylene (pp) caps.

Cleaning of glasswares:

All contaminated material (cultures, glasswares etc.) were sterilized in an autoclave (Equitron, Medica instruments, India) before cleaning. Glasswares used were cleaned following soaking in a soap solution, washed repeatedly with water and finally rinsed with distilled water and dried in an oven (set at 60 °C).

Preparation of medium and culture conditions:

MS Medium supplemented with sucrose (3%, w/v) and 0.65% agar (Bacteriological grade, HiMedia laboratories, Mumbai, India) was used as basal medium for tissue culture experiments. The pH of medium was adjusted to 5.8 with 1N KOH or 1N HCl (Cyberscan 510, Eutech Instruments, Singapore) and sterilized in an autoclave (121 °C; 15 psi; 20 min).

Growth hormones like N6-benzyladenine (BA), Indole-3-butyricacid (IBA), Indole acetic acid (IAA), 2,4-Dichliriphenoxyacetic acid (2,4-D) were added to the basal medium either singly or in various combinations.

The concentrated stock solutions of all the ingredients were prepared which are then used to prepare the medium. Stock solutions of all plant growth regulators (PGR's) (2.5

mM) were prepared by dissolving these in 70% alcohol. All the stock solutions were stored in refrigeration. Basal MS medium (Annexure 1) was prepared from the stocks and used for all the tissue culture studies. The pH of the medium was adjusted to 5.8 and sterilized in an autoclave (121 °C, 15 psi).

All cultures were incubated at 25 ± 2 °C under 2000-2500 lux light intensity from fluorescent lights (Philips India Pvt Ltd.) in a 16h light/ 8h dark cycle.

Preparation of explants:

After removal of leaves, the shoots were excised into smaller sizes (each piece with 3-4 nodes) to facilitate proper cleaning during the disinfestation. After excision, the explants were washed thoroughly under running tap water for 30 minutes followed by treatment with Bavistin solution (0.1% w/v) for 10 min. These were then washed thoroughly with water. Subsequent operations were carried out in a clean air laminar flow cabinet under aseptic conditions. These were then taken for surface disinfection, which was carried out by treating explants with an aqueous solution of mercuric choride (0.1%, w/v) containing few drops of Tween 80 for 6 min. Explants were then washed with sterile distilled water (3 times equal volume) till the traces of disinfectant were removed. These were then trimmed into individual nodes and cut ends (2-3 mm) exposed to mercuric chlorde were also trimmed.

Establishment of aseptic cultures:

Following disinfection and washing, both the ends of nodal segments were trimmed with the help of forcep and scalpel on sterilized glass plate, then the explants (approximately 20-25 mm) were placed vertically on MS medium, gelled with 0.65% (w/v) agar and supplemented with BA (2.5 μM) and NAA (0.5 μM). Experiments were carried out to study the effect of different concentrations of BA (0.5 -5.0 μM) and NAA (0.5 -1.0 μM) in different combinations in MS medium on sprouting of nodal explants of clone Y8.

Shoot multiplication:

Initially shoot multiplication was tried on MS medium supplemented with 2.5 μM BA and 0.5 μM NAA. Effect of different concentrations of BA (0 – 2.5 μM) and NAA (0 –0.5 μM) was studied on shoot multiplication in clone Y8.

In another experiment effect of a compound (yet not known for its activity on plant tissue) was also studied (referred to as a 'compound A'). Basal MS medium containing BA (2.5 μM) and NAA (0.5 μM) was further supplemented with different concentrations of 'compound A' (0 -1.5 mg/l) and three shoot clumps (10 – 15 shoots each) were inoculated on the medium.

Shoot Elongation :

In order to achieve rooting, shoots which are growing on multiplication medium are required to be elongated. For this, the actively growing shoot clumps containing 5 -10 shoots were inoculated on MS medium containing lower concentrations of BA (0 - 1.5 μM) in combination with NAA (0.5 μM). Followed by incubation for 4 weeks, number of shoots elongated and shoot length were recorded.

Rooting :

Elongated shoots (1-2 cm) were excised and were inoculated on MS medium containing different auxins namely α- naphthalene acetic acid (NAA), Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA) at various concentrations (0 – 10 μM). Data with respect to rooting were recoded after 12 days of inoculation.

Hardening:

The *in vitro* raised plants were taken out from culture bottles, washed thoroughly with water to remove agar and then transferred to pots containing various soil mixes having vermicompost, perlite, FYM and soil in varying ratio and the pots were kept in the misting chamber of the polyhouse with 80% humidity for 20 days. Then the plants were shifted to

shade house with less humidity level and indirect sunlight under the net (covered with agronet which cuts 50% light).

Regeneration:

Expanded leaves from shoot cultures of *Eucalyptus* clones (Y8 and T1) were used as explants for regeneration studies. The *in vitro* grown plants generally had very small leaves. Leaves (about 3-8 mm long) were taken and cut into segments and cultured with their adaxial surface touching with the culture medium [MS medium containing 2% sucrose and supplemented with different concentrations of BA (0 -12.5 μM) and 2,4-D or NAA (0 -12.5 μM)]. Data for the regeneration (callusing/shoot differentiation) were recorded following 5-6 weeks of cultures. This experiment was also conducted in petriplates (90 mm).

Testing of antibiotic sensitivity of tissue

Leaf expansion was achieved by prior culture on shoot elongation media for 4 – 6 weeks. Leaves (explants) of the same clone were also tested for their sensitivity towards various antibiotics (kanamycin, cephotaxime, sporadix and carbenicillin). These antibiotics (these were added into the medium after autoclaving when the temperature has lower down to 40-45 °C) were incorporated at different concentrations (0 – 400 µg/ml) into the MS medium supplemented with BA (12.5 µM) and NAA (1.0 µM). Following incubation (3 weeks) data with respect to explant survival, growth and morphogenesis were recorded.

Transformation:

Attempts were made to develop genetic transformation protocols with clone Y8 and T1 either using *Agrobacterium tumefaciens* (LBA 4404) containing binary vector pBI 121 (this plasmid contains a reporter gene β-glucuronidase (*GUS*) and selectable marker gene *npt II* imparting resistance to kanamycin) or using particle gun bombardment.

Transformation experiments using Agrobacterium tumefaciens:

These experiments were conducted using expanded leaves from shoots growing on elongation medium.

***1. Agrobacterium* cultures:**

Actively growing bacteria maintained on Luria Agar (LA) medium (Annexure 1) containing 15 μg/ml rifampicin and 25 μg/ml kanamycin were cultured Yeast Extract Peptone (YEP) broth (Annexure 1) containing both the antibiotics at the same concentration and kept on shakers at 100 rpm and incubated at 25 °C for 12 – 16 h (till culture attain the O.D. of about 0.6 – 0.8).

2.Co-cultivation:

Bacterial cultures were taken and centrifuged at 8000 rpm for 10 min. The pellet was dissolved with sterile distiiled water and used for tranformation experiments. Leaves were excised from the shoots. These were soaked into the bacterial suspension and injured by one of the following methods: (i) by cross cutting with scalpel, (ii) with needle or with sand paper. Following injury the tissue was blotted on the blotting paper to remove the excess of bacteria . These were then cultured on basal MS medium and incubated in dark for the different time periods (12 – 72 h). These were removed at different intervals and after washing, explants were cultured on the selection and regeneration medium.

3.Washing and culturing:

Following co- cultivation, tissues were repeatedly washed with sterile water and cultured on MS medium supplemented with BA (5 μM) and 2,4-D (1 μM) containing 200 μg/ml carbenicillin (to check the growth of *Agrobacterium)* and 50 μg/ml kanamycin (for the selection of transformed tissue). These were subcultured on the same medium at 4 week interval and regeneration was monitered from these explants which will be further analysed for the integration/ expression of the *GUS* gene.

Direct gene transfer using particle gun

1. Preparation of microcarriers:

10 mg of microparticles (gold) were weighed and 0.5 ml of 70% ethanol (v/v) was added to it and taken in a 1.5 ml microfuge tube. It was vortexed vigorously for 3-5 min and the particles were allowed to soak in 70% ethanol fof 15 min. Now, the microparticles were pelleted by spinning for 10 seconds and the supernatant was discarded. The pellet was washed three times with 1 ml sterile water followed by vortexing for 1 min and were pelleted by briefly spinning in a microfuge. The liquid was then removed. Followed by washing, 167 µl of 50% glycerol was added to bring the microparticle concentration to 60 mg/ml. The microparticles (gold) can be stored at 4 °C for upto 2 weeks.

2. Coating of gold particles:

From the microcarrier suspension, 50 µl was taken and to this 5 µl DNA, 50 µl $CaCl_2$ (2.5 M) and 20 µl spermidine (0.1 M) were added. Vortexed for 3 min and then the microcarriers were allowed to settle for 3 min. Microcarriers were pelleted by spinning 10 seconds in a microcentrifuge at 5000 rpm. Decant the supernatant. Now the pellet was washed with 70% ethanol followed by 100% ethanol. Then the pellet was resuspended in 50 µl of 100% ethanol by taping the sides of the tube. From this, 8.0 µl of the DNA coated microparticles were used for each bombardment.

3. Preparation of Tissue:

For carrying out particle gun bombardment, the plant material (leaves) were inoculated on medium of high osmoticum (MS basal + 2% sucrose + 2.5 µM BA + 1 µM 2,4-D + 0.4 M mannitol) approximately 18-20 hrs before bombardment.

4.Bombardment of tissue with gold particles:

Model PDS - 1000/He Biolistic (Bio Rad) was used and the device was operated as per manufacturer instructions. The rupture disc (1100 psi), stopping screen, macrocarrier assembly were loaded by maintaining the aseptic conditions and the target tissue/cells were then placed at an appropriate distance from the macrocarrier assembly and the bombardment was carried out. The sample was removed after bombardment. One day

after the bombardment, the bombarded tissue was transferred to the medium containing 50 mg/l kanamycin (as a selective marker).

RESULTS

Establishment of axenic cultures

Nodal segments taken from freshly coppiced shoots showed response whereas nodes taken from mature plants did not sprout. About 50% of nodal explants sprouted on MS medium supplemented with BA (2.5 μM) and NAA (0.5 μM) (Fig. 2). Higher concentrations of BA (more than 10 μM) did not favour sprouting from nodal explants. Experiments were carried out to see the effect of different concentrations of BA and NAA on sprouting of explants. Better response was observed when medium was supplemented with 5.0 μM BA and 0.5 μM NAA (Table 1).

Table 1. Effect of BA and NAA in MS medium on sprouting of nodal explants of *E. tereticornis* clone Y8.

BA (μM)	NAA (μM)	Percent of nodal segments sprouted
0.5	0.5	12
1.0	0.5	42
2.5	0.5	50
5.0	0.5	47
0.5	-	5
1.0	-	10
2.5	-	19
5.0	-	16
0.5	1.0	16**
1.0	1.0	44**
2.5	1.0	51**
5.0	1.0	46**

A total of 50 nodal explants were inoculated in each teatment. Data were recorded after 4 weeks of culture.

**Higher callusing and poor growth was observed in the combinations.

Shoot proliferation

Shoots sprouted from the nodal segments were subcultured on the same medium for two subsequent cycles. To investigate the effect of BA and NAA on shoot multiplication of clone Y8 experiments were carried out using different concentrations of BA (0 – 2.5 μM) and NAA (0 – 0.5 μM). Three clumps of about 10 –12 shoots each were inoculated in each bottle. Each treatment consisted of five replicates and experiment was repeated three times and it was observed that BA (2.5 μM) and NAA (0.5 μM) showed maximum shoot multiplication in clone Y8 also (Table 2).

Table 2. Effect of BA and NAA on shoot multiplication and shoot elongation in *E. tereticornis* clone Y8.

BA (μM)	NAA (μM)	Shoot multiplication	Number of shoots elongated per clump
-	-	+	5.8 ± 0.62
-	0.5	+	7.2 ± 0.31
0.1	0.5	++	7.71 ± 0.29
0.5	0.5	++	2.15 ± 0.52
1.5	0.5	+++	0.85 ± 0.43
2.5	0.5	++++	-

Each treatment consisted of 3 bottles and three shoot clumps were inoculated in each bottles and data were recorded after 4 weeks of subculture.

Each + sign indicates the doubling of the original inoculum.

Effect of a 'Compound A' on shoot multiplication

Effect of a compound ('Compound A'), not known earlier for its activity on plant tissues was also tested for shoot proliferation in *Eucalyptus*. Shoot multiplication of clone Y8 was examined on basal MS medium and MS medium supplemented with BA (2.5 μM) and NAA (0.5 μM) (referred to as MS1) with varying concentrations of Compound A. Rapid shoot multiplication, with 2 fold increase over initially inoculated shoot, was observed after 15 days. Increase in shoot proliferation was observed at lower concentrations of 'compound A' (0.25 mg/l) whereas higher concentrations were inhibitory. At lower concentrations (0.25 mg/l) shoot elongation was also observed. However, the increase in shoot length over initial length was maximum on medium supplemented with 0.25 mg/l concentration of the 'Compound A' (fig. 3). At the higher concentration of 'Compound A' (1.5 mg/l) (Table 3), elongation of shoots was reduced. It may, however, be mentioned that these shoots upon transfer to a medium with lower concentration of 'Compound A' reverted to normal shoot growth.

The 'Compound A' has also affected the rooting ability of the microshoots of clone Y8. Rooting was obtained only on basal MS medium and there was no rooting in MS1 medium (Table 3). Maximum rooting was observed when medium was supplemented with 0.25 mg/l 'Compound A' (Fig. 3).

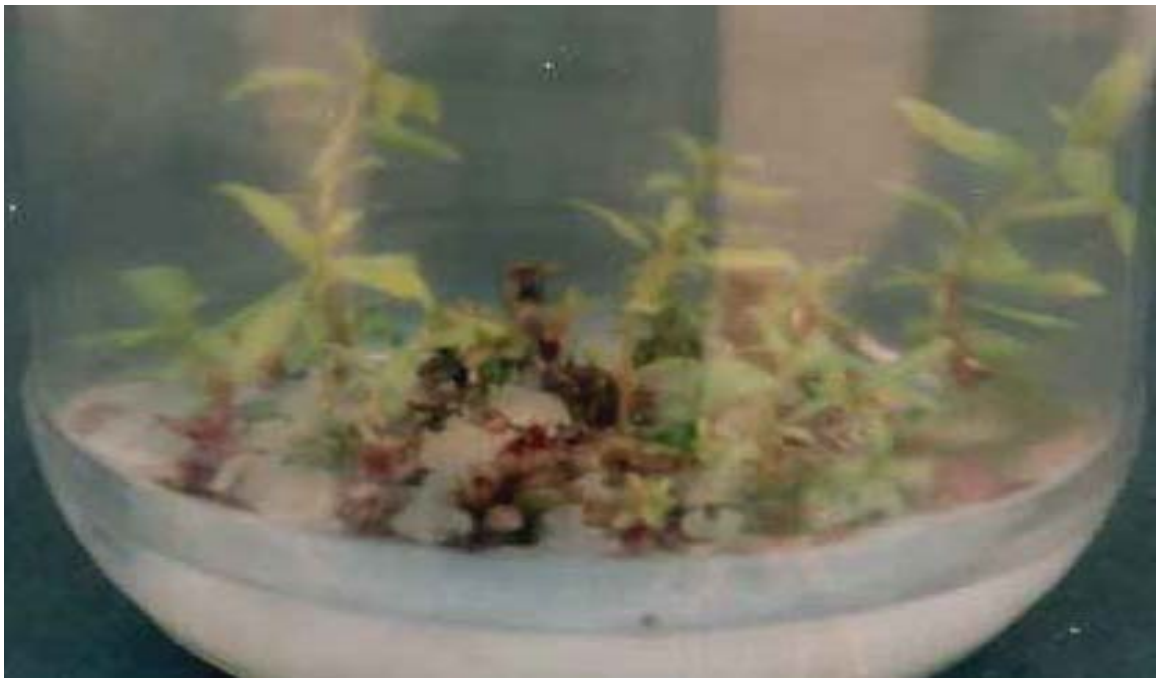

Fig. 4 Rooting of microshoots of *Eucalyptus* clone Y8 inoculated on MS medium supplemented with 5 µM NAA.

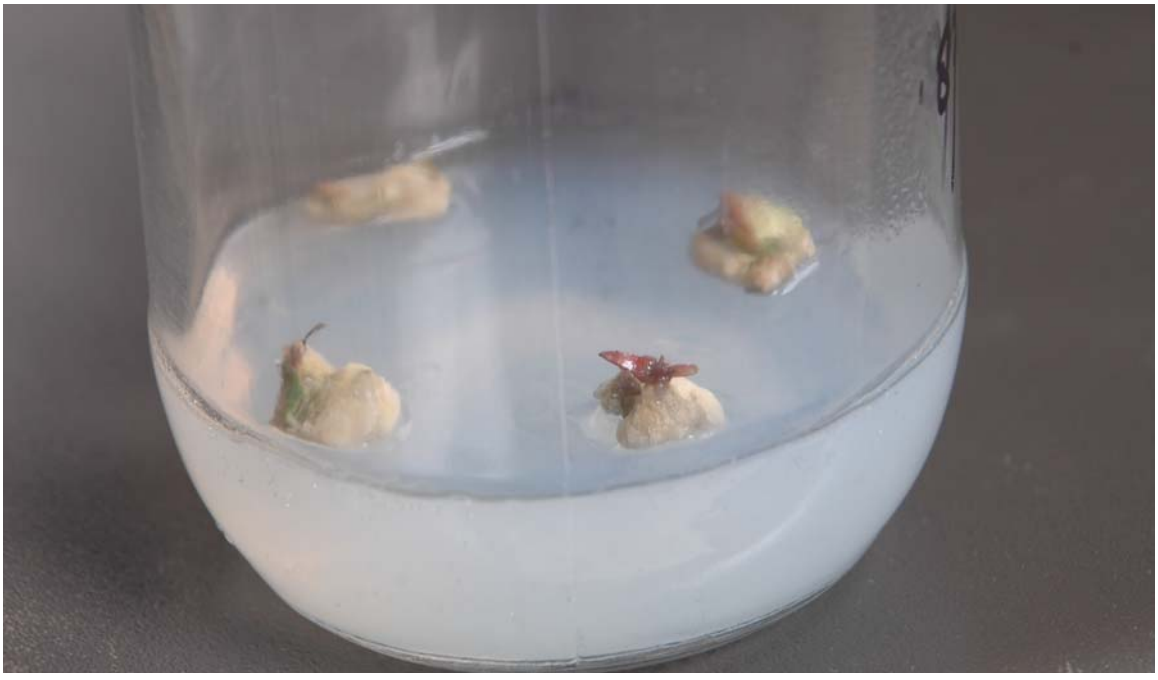

Fig. 5 Regeneration of shoot bud obtained in *Eucalyptus* clone Y8 inoculated on MS medium supplemented with BA (5 µM) and 2,4-D (1 µM).

Table 3. Effect of different concentrations of 'Compound A' on morphogenesis on basal MS medium (MS) and MS medium supplemented with BA (2.5 μM) and NAA (0.5 μM) (MS1) in *E. tereticornis* clone Y8.

Different concentrations of Compound A (mg/l)	Shoot multiplication		Average shoot length		Rooting*	
	MS	MS1	MS	MS1	MS	MS1
control	++	++++	++	++	++	-
0.25	+++	+++++	+++	++	+++	-
0.5	++	+	++	+	++	-
1.0	+	+	+	+	+	-
1.5	+	++	+	+	+	-

Shoot multiplication: each + sign indicates doubling of original inoculum

Average shoot length: + (0.5 –1.5 cm), ++ (1.5 –2.5 cm), +++ (2.5 – 3.5 cm).

* Rooting reflects not those of individual shots but in shoot clumps.

+++ indicates maximum rooting and - indicates no root emergence.

Data were recorded after 21 days of culture.

Rooting of microshoots

Transfer of individual shoots of clone Y8 and T1 (about 1.0 -2.0 cm length) to MS basal medium supplemented with varying concentrations of NAA, IAA and IBA resulted in 73.33%, 66.66% and 66.66% rooting in clone Y8 and 60.00%, 66.66% and 60.00% rooting in clone T1 respectively (with callus formation in some cases) after 12 days of inoculation. It was observed that maximum number of roots per explant were found in 5 μM NAA followed by IBA and IAA (Fig. 4). The root length was found to be maximum at the same concentration (Table 4).

The media supplemented with IBA and IAA exhibited callus formation at the basal end of the shoots in case of clone Y8 only, however, there is no callus formation in case of NAA in both the clones. Maximum callus was found in case of 5 μM IAA. On comparison, best rooting was observed in media supplemented with NAA followed by IAA and IBA.

Table 4. Effect of varying concentrations of NAA, IAA and IBA in MS medium on rooting of *E. tereticornis* clones Y8 and T1.

PGR concentration (μM)	% shoots rooted		No. of roots per rooted shoot Mean ± S.E. n = 3		Average root length Mean ± S.E. n = 3	
	clone Y8	clone T1	clone Y8	clone T1	clone Y8	clone T1
control	26.66	-	0.66 ± 0.334	-	0.9 ± 0.057	-
NAA						
1.0	66.66	60.00	4.0 ± 0.577	2.66 ±0.882	3.16 ± 0.441	0.90 ± 0.208
2.5	60.00	53.33	3.66 ± 0.885	2.00 ± 0.577	2.83 ± 0.168	0.73 ± 0.147
5.0	73.33	60.00	4.33 ± 0.882	3.00 ± 0.577	2.73 ±0.371	1.26 ± 0.147
10.0	66.66	46.66	3.66 ± 0.885	2.33 ± 0.332	1.16 ±0.091	1.16 ± 0.168
IBA						
1.0	60.00	53.33	2.66 ± 0.882	2.00 ± 0.577	1.73 ± 0.147	0.56 ± 0.147
2.5	40.00	46.66	3.0 ± 0.577	1.66 ± 0.334	2.31 ± 0.173	0.66 ± 0.091
5.0	66.66	66.66	5.0 ± 0.577	3.00 ± 0.577	0.73 ± 0.147	0.83 ± 0.168
10.0	60.00	40.00	3.0 ± 0.577	1.33 ± 0.334	1.90 ± 0.208	0.46 ± 0.091
IAA						
1.0	46.66	53.33	2.0 ± 0.577	1.66 ± 0.667	1.73 ± 0.147	0.50 ±0.05
2.5	66.66	53.33	3.66 ± 0.885	1.66 ± 0.334	2.63 ± 0.091	0.53 ± 0.147
5.0	60.00	60.00	4.0 ± 0.577	2.00 ± 0.577	3.23 ± 0.147	0.80 ± 0.152
10.0	53.33	40.00	2.0 ± 0.577	1.33 ± 0.334	2.46 ± 0.147	0.30 ± 0.057

Each treatment consisted of 3 bottles and 20 shoots were inoculated in each bottles.
Data were recorded after 12 days of inoculation.

Fig. 6 Regeneration of shoots obtained from leaf segment of *Eucalyptus* clone Y8 inoculated on MS medium supplemented with BA (12.5 µM) and NAA (0.1 µM).

Fig. 7 Acclimatized plants of *Eucalyptus* clone Y8 in polybags containing various soil mixes (Soil; FYM:soil 1:1; Vermicompost, FYM, Perlite : soil 1:1; Vermicompost : soil 1:1; Perlite : soil 1:1)

Regeneration

In order to standardize protocol for regeneration of shoot/embryo from the leaf explants. Experiment has been carried out in clone Y8 and T1 using *in vitro* grown leaves as explant. Various combinations of BA and 2,4-D were used (Table 5). Callus was observed in each combination but the size of callus and colour differ respectively. Maximum callus was observed on medium supplemented with BA (1 μM) and 2,4-D (5 μM). The regeneration rate although very poor but was observed on medium supplemented with BA (5 μM) and 2,4-D (1 μM) (Fig. 5) in both the clones. In clone T1 shoot regeneration was also observed in other combinations (Table 5).

Table 5. Effect of different concentrations of BA and 2,4-D on regeneration ability of *E. tereticornis* clones Y8 and T1.

Medium	Callus		Shoots formed	
	Clone Y8	Clone T1	Clone Y8	Clone T1
DO(MS basl +2% sucrose)	-	-	-	-
D0+1 μM BA+1 μM 2,4-D	+++	++	-	-
D0+1 μM BA+5 μM 2,4-D	+++++	+++	-	-
D0+1 μM BA+25 μM 2,4-D	++	+	-	-
D0+5 μM BA+1 μM 2,4-D	+++	+++	+	+
D0+5 μM BA+5 μM 2,4-D	+	+	-	-
D0+5 μM BA+25 μM 2,4-D	+	++	-	-
D0+12.5 μM BA+1 μM 2,4-D	+	++	-	+
D0+12.5 μM BA+25 μM 2,4-D	+	+	-	-
D0+12.5 μM BA+25 μM 2,4-D	++	+	-	+

Callus intensity: - (0%), + (6 –10%), ++ (30 –60%), +++ (60 –80%), +++++ (85-90%).

+ sign for shoots formed indicate shoot regeneration

In another experiment, regeneration was tried in clone Y8 and T1 using medium supplemented with different combinations of BA and NAA. In this experiment, also callus was observed in most of combinations. Regeneration was observed only in clone Y8 on medium supplemented with BA (12.5 μM) and NAA (0.1 μM) (Table 6) (Fig. 6).

Table 6. Effect of different concentrations of BA and NAA on regeneration ability of *E. tereticornis* clone Y8 and T1.

Medium	Callus		Shoot	
	Clone Y8	Clone T1	Clone Y8	Clone T1
R0 (MS basal +2% sucrose)	-	-	-	-
R0+1 μM BA+0.1 μM NAA	-	++	-	-
R0+1 μM BA+ 0.5 μ M NAA	+	+	-	-
R0+1 μM BA+2.5 μM NAA	++	+	-	-
R0+1 μM BA+5 μM NAA	++	+	-	-
R0+5 μM BA+0.1 μM NAA	+++	++	-	-
R0+5 μM BA +0.5 μM NAA	+	+	-	-
R0+5 μM BA+2.5 μM NAA	+	+	-	-
R0+5 μM BA+5 μM NAA	+++++	++	-	-
R0+12.5 μM BA+0.1 μM AA	+++	+	+++	-
R0+12.5 μM BA+0.5 μM NAA	+++	+	-	-
R0+12.5 μM BA+2.5 μM NAA	+	++	-	-
R0+12.5 μM BA+5 μM NAA	+	+	-	-

Callus intensity: - (0%), + (6 –10%), ++ (30 –60%), +++ (60 –80%), +++++ (85-90%).

+ sign for shoots formed indicate shoot regeneration.

Acclimatization of plants

The rooted plants of clone Y8 were transferred to pots containing various soil mixes consisting of agropeat, perlite, coirpeat, vermicompost and FYM (Farm Yard Manure) in different ratios and kept in the mist chamber of polyhouse (80.0% relative humidity). The plantlets transferred to mixture containing vermicompost:Soil (1:1) showed 90% survival rate (Fig. 7) compared to those transferred to soil alone which showed only 40% survival rate. During hardening of clone Y8, it was observed that Perlite in combination with soil and Agropeat in combination with soil & sand also proved beneficial for the acclimatization of the plants.

Table 7. Survival rate of plantlets of *Eucalyptus tereticornis* clone Y8 during acclimatization.

Different soil mixes	Number of plants transplanted	Percentage of survival (%)
T	15	40
T1	15	60
T2	15	80
T3	15	58.33
T4	15	60
T5	15	66.66
T6	15	73.33
T7	15	90
T8	15	96.66
T9	15	73.33
T10	15	86.66

T – soil; T1 – Soil:Sand 1:1; T2 – Soil:Sand:Agropeat 2:1:1; T3 – Soil:Sand:Perlite 2:1:1;
T4 – Soil:Sand:Coirpeat 2:1:1; T5 – Soil:Sand:Perlite:Coirpeat 4:2:1:
T6 – Soil:Sand:Agropeat:Coirpeat 4:2:1:1; T7 – FYM:soil 1:1; T8 - Vermicompost : soil 1:1;
T9 -Vermicompost, perlite, FYM : soil 1:1; T10 - Perlite : soil 1:1

Hardening experiments were also carried in clone T1 using soil mixtures comprising FYM, perlite and vermicompost. T1 clone showed better survival compared to clone Y8. Hardening was best in soil mixture containing Vermicompost : soil (1:1) (Table 8).

Table 8. Survival of *in vitro* produced plantlets of *Eucalyptus tereticornis* clone T1 during acclimatization.

Different soil mixes	Number of plants Transplanted at Once*	Percentage survival (%)	Number of plants survived Mean ± S.E. n = 6
A	6	75	4.5 ± 0.562
B	6	61.1	3.6 ± 0.671
C	6	86.1	5.1 ± 0.403
D	6	80.1	4.8 ± 0.544
E	6	63.8	3.8 ± 0.479

* Experment was done in 6 replicates.

A – Soil; B - FYM : soil 1:1 ; C - Vermicompost : soil 1:1; D -Vermicompost, perlite, FYM : soil 1:1; E - Perlite : soil 1:1

Antibiotic sensitivity test

Sensitivity of tissue to various antibiotics was tested using *in vitro* grown shoots of clone Y8 from which leaf segments were taken and placed on medium having different antibiotics viz kanamycin, cephotaxime, sporadix and carbenicillin with varying concentrations. Also small clumps of shoots were also inoculated on the same media. The leaves were found to be resistant against all the antibiotics except kanamycin (Fig. 9) as callus formed in all (Table 9). Regeneration was observed in medium having 300 mg/l cephotaxime (Fig. 8). The shoot formed on medium containing 300mg/l cephotaxime was green and healthy. The inoculated shoot clumps showed almost similar response to cephotaxime, carbenicillin and sporadix, howver, growth was severly inhibited in medium cotaining kanamycin (Table 9) (Fig. 10).

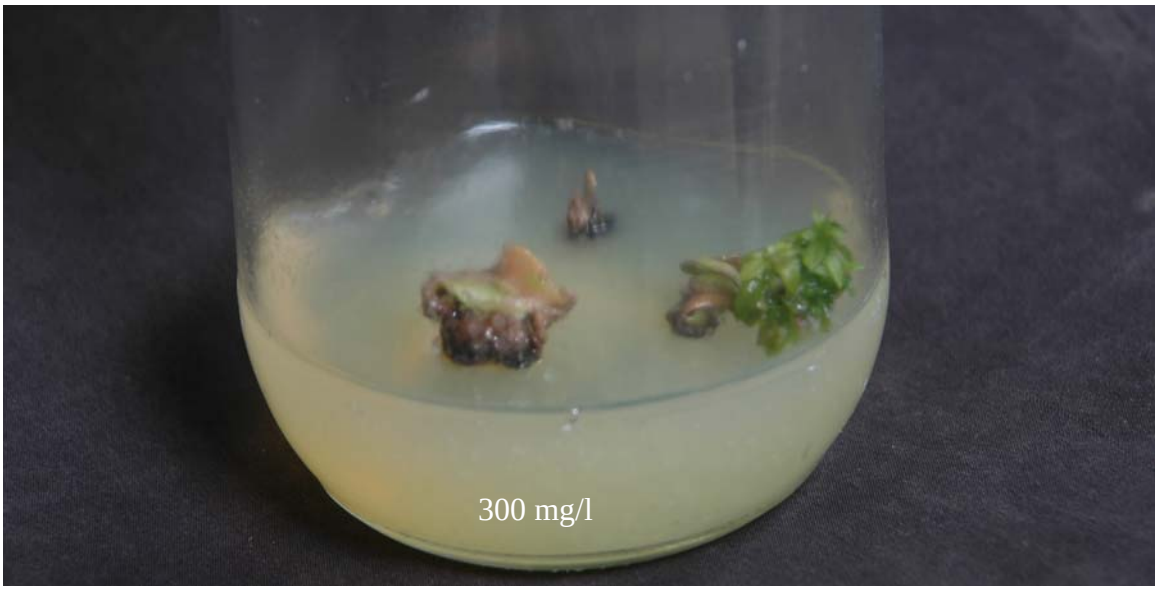

Fig. 8 Regeneration in *Eucalyptus* clone Y8 on MS medium supplemented with BA (5 µM) and 2,4-D (1 µM) containing Cefotaxime (300 mg/l).

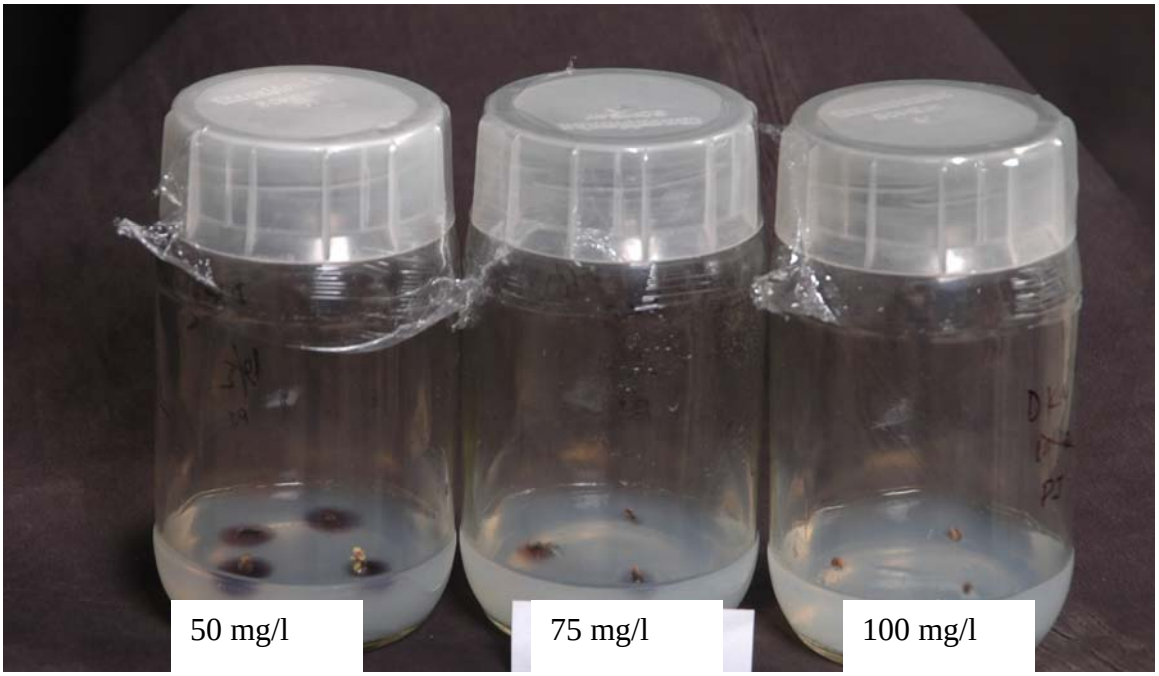

Fig. 9 Restriction in the growth of *Eucalyptus* clone Y8 inoculated on MS medium supplemented with BA (5 µM) and 2,4-D (1 µM) containing different concentrations of Kanamycin.

Table 9. Effect of various concentrations of different antibiotics on leaf tissue and shoot clumps of *E. tereticornis* clone Y8.

Antiboiotic concentration (mg/l)	Leaf (explant)		Shoot clump (explant)
	Callus	Shoot differentiation	Shoot growth
Control	+	-	-
Kanamycin			
25	-	-	-
50	-	-	+
75	-	-	-
100	-	-	-
Cephotaxime			
100	++	-	++
200	+	-	++
300	+	++	+++
Carbenicillin			
100	++	-	++
200	+	-	++
250	++	-	+++
300	+++	-	++
Sporadix			
100	+	-	++
200	+++	-	++
250	++	-	++
300	++	-	++

Callus intensity: each + sign indicates extent of callus growth.

Regeneration: - (0%), + (10 –15%), ++ (20—40%)

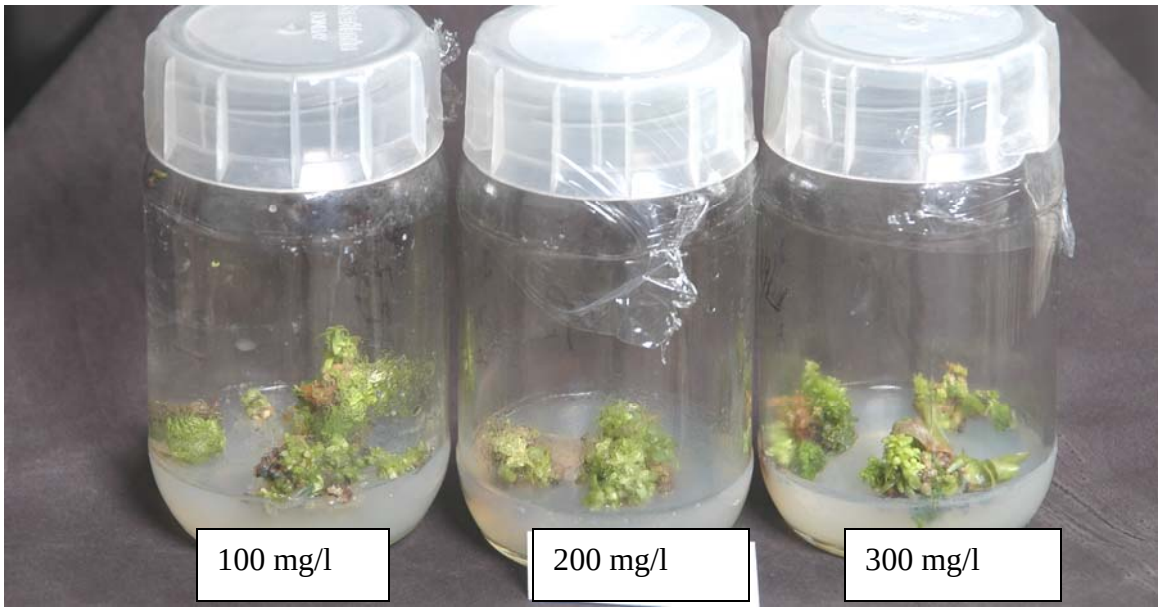

Fig. 10 Shoot clumps of *Eucalyptus* clone Y8 inoculated on MS medium supplemented with BA (5 µM) and 2,4-D (1 µM) containing different concentrations of Carbenicillin.

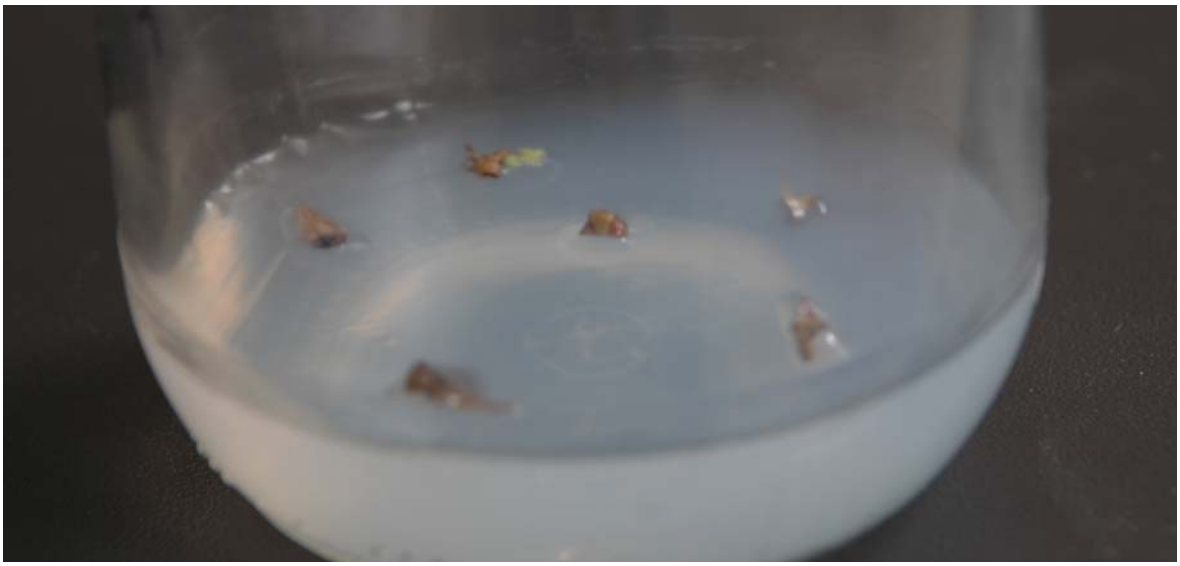

Fig. 11 Regeneration obtained in *Eucalyptus* clone Y8 after 45 days of bombardment followed by inoculation on MS medium supplemented with BA (5 µM) and 2,4-D (1 µM) and kanamycin (50 mg/l).

Antibiotic sensitivity of *A. tumefaciens* LBA 4404

An *Agrobacterium tumefaciens* strain was tested against different antibiotics (carbenicillin, sporadix and cephotaxime). The growth of the bacterium was completely checked by carbenicillin, sporadix and cephotaxime at a concentration more than 100mg/ml. However, it showed tolerance to kanamycin upto 100 µg/ml (highest concentation tested).

Transformation using *Agrobacterium*

Complete elimination of Agrobacterium was observed following distilled water washing and culture of tissue on medium containing 200 mg/l carbenicillin and 50 mg/l kanamycin. Callus growth was observed from the cut/injured areas of the explants in all media combinations. Efforts are in progress to attain the regeneration in these explants.

Particle gun Bombadment

Particle gun bombardment was carried out using leaves taken from *in vitro* grown *E. tereticornis* For the selection of transformants the bombarded tissue was placed on Kanamycin (50 mg/l) containing medium. After about 45 days shoot regeneration was observed from one of the bombarded leaf segments indicating that transformation might have occurred (Fig. 11). Its growth is being monitored and subsequently it will be tested.

Discussion

Plant micropropagation, at present is commercially most efficient and practically oriented plant biotechnology (Altman and Loberant, 1998). The number of species cututred *in vitro* has been steadily increasing over the last two decades (Murashige, 1974; Vasil and Vasil, 1980; Hartman et al., 1990; Bonga and Durzan, 1987). Studies on tree tissue culture have, however, received little attention. Consequently the success of *in vitro* work on forest trees has been rather slow and limited (Bajaj, 1989; Bisht et al., 1998). In India, micropropagation protocols for several trees species have been developed (Datta et al., 1982: Rai and Chandra, 1989; Shekhawat et al., 1993; Sunitabala Devi et al., 1994; Sha Ali Khan, 1997; Kumar et al., 1998) but reproducibility has been achieved only in a few cases.

Therefore, the present study was designed in a way to put in more efforts for optimization and standardization of each step in micropropagation protocol for selected elite clones of *Eucalyptus*, so that large number of plantlets can be obtained in shorter time frames. Earlier there are reports on *in vitro* propagation of various species of *Eucalyptus* (Sharma SK, Ramamurthy V, 2000; Subhaih and Minocha, 1990; Das et al., 1990; Gupta PK, Mascarenhas AF, 1987; Serrano et al., 1996). Since a lot of variation exist from clone to clone in respect of micropropagation, the clone specific protocols are required to be developed.

About 50% of nodal explants taken from freshly coppiced shoots showed sprouting on MS media supplemented with 2.5 μM BA and 0.5 μM NAA.The importance of plant growth regulators (PGR's) in plant tissue culture is well documented. Each cytokinin is tested for stimulation of shoot production. Cytokinins are generally used in plant cell culture at a concentration range of 0.1-10.0 mg/L. When added in appropriate concentrations, they may regulate cell division, stimulate axillary and adventitious shoot proliferation, regulate differentiation, and stimulate protein and enzyme activity (Gross et al., 1995). A range of cytokinins (Kinetin, BA, 2-ip and zeatin) has been used in micropropagation work (Bhojwani and Razdan, 1992). In the present study, BA was used as a cytokinin for achieving shoot multiplication. Choice of BA over other cytokinins has been demonstrated in many other tree species (Chaturvedi and Sharma, 1989; Rout and

Das, 1995; Chand and Singh, 2000; Singh and Chand, 2002). In white clover (Bhojwani, 1981), hybrid willow (Bhojwani, 1980), chickpea (Barna and Wakhlu, 1994) and *Eucalyptus grandis* (Yang et al., 1995). BA was the most effective cytokinin for the shoot tip, meristem and bud culture. Therefore, in the present study, effect of BA and NAA on sprouting of nodal explants was studied and it was found that higher concentration of BA (more than 10 μM) and NAA (more than 1.0 μM) did not favour sprouting. Higher callusing and poor growth was obtained in MS medium supplemented with >10.0 μM BA and 1.0 μM NAA. *In vitro* propagation using nodal segments and cotyledonary node explants has been reported in some tree species such as *Wrightia* spp. (Purohit et al., 1994; Purohit and Kukda, 1994), *Dalbergia latifolia* (Raghwa Swamy et al., 1992; Pradhan et al., 1998), *Dendrocalamus sissoo* (Gulati and Jaiwal,1996; Pradhan et al.,1998) and *Morus laevigata* (Ahlawat et al., 2002). The effect of BA and NAA on shoot multiplication of Clone Y8 was also studied. Shoot multiplication was found to be better on MS medium supplemented with BA (2.5 μM) and NAA (0.5 μM). In this treatment shoots multiplied almost double than the original inoculum Earlier effect of BA on shoot multiplication was documented in many species (Yang et al., 1995; Singh and Chand, 2002).

The effect of a steroid compound 'compound A', not known earlier for its activity on plant tissues was also tested on shoot proliferation and morphogenesis in *Eucalyptus* and it was observed that 'compound A' (0.25 mg/l) favoured shoot proliferation to a greater extent while at 1.5 mg/l concentration there was reduction in shoot proliferation. However, persual of literature indicate that there are no earlier reports on the activity of this compound on plants. Further, detailed examinations regarding the effect of this compound are in progress. Possibly the inhibition of growth at concentration more than 1.5 mg/l is due to toxicity of this compound.

In the present investigation, various auxins were tested for the rooting of microshoots. The effect of auxins on rooting of miroshoots of many plant species has been reported (Feito et al., 1995; Monleuuis and Bon, 2000; Haissing, 1974). In the present study, maximum response with respect to percent shoots rooted was obtained on modified MS medium supplemented with 5 μM NAA followed by IBA and IAA. Beneficial effect of NAA on rooting of microshoots over other auxins has also been reported in Eucalyptus

(Sharma and Ramamurthy, 2000), peach (Hammerschlag et al., 1987), *Pinus strobus* (Kaul, 1987).

Beneficial effect of 'Compound A' was also observed in basal MS medium, in this medium perfused rooting was observed from shoot clumps when 25 mg/l was used. (Table 3).

Acclimatization of *in vitro* raised plantlets is a most crucial step in successful micropropagation or clonal propagation. For this, the well rooted plantlets were transferred to plastic pots containing non- sterile soil and its various mixes with FYM, agropeat, perlite and coirpeat. The plantlets were successfully acclimatized by keeping them in a mist chamber (25 °C, 80% relative humidity) for 10 days with a survival rate of 70%. The plants were then transferred to shade house after 10 days, these grew well and appeared healthy. During hardening experiments involving various soil mixtures, it was observed that FYM and vermicompost containing mixtures showed better results. It may be due to the high organic content present in these mixtures that supported the plant growth and improved water holding capacity of soil. Similar work has been done on hardening of *Morus laevigata* (Alhawat et al., 2002); *Quercus floribunda* (Purohit et al., 2002); *Dendrocalamus asper* (Arya et al., 2002); *Bambusa vulgaris* (Arya et al., 2002).

In order to undertake plant genetic transformation work, an efficient regeneration protocol is required which will not only help in regeneration of whole plant from isolated cells or tissues which have been genetically transformed but will also help in multiplication of transformed plants. The primary aim is therefore to develop an efficient and reliable regeneration protocol which can be used for transformation studies. In the present study, regeneration was achieved from leaf segments in both the clones on MS medium supplemented with BA and 2,4-D or NAA. Earlier, shoot organogenesis has been reported from different species of *Eucalyptus* (Barrueto et al., 1999; Tibok et al., 1995; Watt et al., 1991; Serrano et al., 1996). In the present study, 2,4-D was proved to be better auxin for achieving regeneration from the *E. tereticornis* clone T1 whereas, in case of clone Y8 NAA promoted shoot regeneration from explants. This clone related response for regeneration and multiplication has been reported earlier in *Eucalyptus*

(Mullins et al., 1997). Presently, regeneration was found best on MS (2% sucrose) medium supplemented with 5 µM BA and 1 µM 2,4-D or 12.5 µM BAP and 0.1µM NAA.

For *Agrobacterium* mediated transformation, tissue is co-cultivated with the bacterial cultures and subsequently these are required to be rmoved. In order to eliminate bacteria following co-cultivation some antibiotic is required to be incorporated into the medium. Further to select the transformed tissue on the selectable marker such as kanamycin (as in prsent study), the sensitivity of the tissue to these antibiotic is required to be tested. Leaves taken from in vitro grown *E. tereticornis* were also studied for their sensitivity towards various antibiotics (kanamycin, carbenecillin, cephotaxime and sporadix). It was found that the explants showed resistance against all the antibiotics but kanamycin restricted the growth to a greater extent and completely inhibited growth at concentration above 50 mg/l. Shoot regeneration obtained in case of cephotaxime (300 mg/l) was good and the regenerated shoot was green and healthy. This sensitivity test of the tissue to kanamycin will help in further screening of transformants during transformation studies. Earlier, there are many reports on the use of carbenicillin, cephotaxime and sporidex for the eliminating bacteria following co-cultivation (Barrette et al., 1997; Estope et al., 2001; Alsheikh et al., 2002). In the present study kanamycin more than 50 mg/l is required to select the transformed tissue, these results are in line with the earlier reprorts on Eucalyptus (Mullins et al., 1997) and Populus deltoides (Confalonieri et al., 1997).

Development of an efficient and reproducible regeneration protocol and high competency of regenerating cells for the transformation are the two prime requirements for establishment of an efficient genetic transformation protocol for a notoriously recalcitrant species (Christou, 1997). There are several reports on integration of chimeric genes via *Agrobacterium* in Eucalyptus camaldulensis (Mullins et al., 1997); *Leucaena leucocephala* (Rastogi et al., 2002); Populus deltoides (Confalonieri et al., 1997); *Eucalyptus globulus* (Moralejo et al., 1998); *Eucalyptus camaldulensis* (Chang et al., 1998) and particle bombardment mediated transformation in *Pinus radiata* (Tang and Newton, 2003); hybrid *Eucalyptus grandis* x *E. urophylla* (Sartoretto et al., 2002). In the present study, efforts were made to develop an *Agrobacterium*-mediated transformation protocol for *E. tereticornis* clone Y8. For transformation *Agrobacterium tumefaciens* strain LBA4404 carrying binary vector pBI 121 was used. This strain has been used for he

transformation in plants by many workers (Kant t al., 2002; Mullins et al., 1997). Different co-cultivation periods were tried and subsequently cultures were washed and placed on selection and regeneration medium. Callus growth was observed from the injured areas of the explants but the work is in progress to attain regeneration in those explants. Particle bombardment was also carried out and after about 45 days shoot regeneration was observed from one of the bombarded leaf segments indicating that transformation might have occurred. Its growth is being monitored and subsequently it will be tested. If these protocols for regeneration are developed, then these may help in undertaking genetic improvement programmes for these important clones of *Eucalyptus tereticornis*.

CONCLUSION

The availability of reliable techniques for *in vitro* culture and genetic transformation offers the opportunity to undertake tree improvement programmes. Micropropagation represent an alternative, fast and reliable method for mass cloning and germplasm preservation and are ideal for genetic transformation studies. The genetic transformation, avoiding the sexual process, offers opportunities for creating new varieties with important agronomic traits otherwise unavailable such as insect pest resistance, herbicide tolerance, improvement in growth, metabolism and wood quality and in the reduction in expression of endogenous genes that encode undesirable traits. If current progress in tissue culture and genetic transformation combined with biotechnological applications continues the future may witness super trees species tailolored for special agronomic and economic characteristics.

The aim of the present study was to standardize optimum conditions for the establishment of axenic cultures of *E. tereticornis* from elite germplasm, shoot multiplication, rooting of cuttings and hardening of plants and to develop a reliable shoot regeneration protocol which can be further used for transformation purpose. But due to shortage of time period only the regeneration work had been standardized and the transformation work is in progress.

The conclusion drawn from present study are :

1. Surface sterilization with $HgCl_2$ (0.1% for 5-minutes) with 70% alcohol dip was best for the surface sterilization of the explants.
2. For the initiation of the culture, MS medium with BA 2.5μM with NAA 0.5μM was used.
3. About 50% of nodal explants taken from freshly coppiced shoots showed sprouting and it was observed that higher concentrations of BA did not favour sprouting of nodal explants.
4. Shoot multiplication was found to be better on MS medium supplemented with 2.5 μM BA and 0.5 μM NAA.
5. Best shoot multiplication and elongation was obtained on MS medium containing 0.5μM of compound A and supplemented with 2.5μM BA and 0.5μM NAA.

6. shoot regeneration was obtained on MS medium with 2% sucrose and supplemented with 5μM BA and 1μM 2,4-D in both the clones (Y8 and T1).
7. The callus was obtained on MS medium with 2% sucrose in most of combinations. Further efforts are being made to achieve regeneration from these calli.
8. Regeneration was obtained on MS medium with 2% sucrose and supplemented with 12.5μM BA and 0.1μM 2,4-D.
9. It was found that leaves taken from *in vitro* grown *E. tereticornis* showed resistance against antibiotics viz. Cephotaxime, Carbenicillin and Sporadix upto 300 mg/l whereas these were sensitive to kanamycin and growth was completely checked at concentration > 50 mg/l.
10. Best rooting was obtained on MS medium supplemented with 5 μM NAA and 5 μM IBA and 5 μM IAA.
11. Regenerated plantlets showed 70% survival rate during hardening.
12. FYM and vermicompost showed beneficial effect on hardening of *in vitro* plantlets.
13. Callus growth was observed from the *Agrobacterium* infected and particle gun bombarded tissue but the work is in progress to attain regeneration.

ANNEXURE 1

Composition of basal MS medium

MACRO SALTS	(mg/l)
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
MICRO SALTS	
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025
Na_2Fe-EDTA	30.00
ADDITIVES	
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	3000

Composition of Luria Agar

	gm/l
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	5.00
Agar	15.00
Final pH (at 25° C)	7.0 ± 0.2

Composition of YEP (Yeast Extract Peptone)

	gm/l
Yeast Extract	10.00
Peptone	10.00
Sodium chloride	5.00
Final pH	7.5

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