Studies of somatic embryogenesis in selected clones of *Eucalyptus tereticornis*

Dissertation

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CERTIFICATE

This is to certify that the dissertation entitled "**Studies of somatic embryogenesis in selected clones of *Eucalyptus tereticornis***" being submitted by **Ms. Anupriya Gupta (601504003)** towards the partial fulfillment of the award of the degree of **Master of Technology in Biotechnology** of the **Department of Biotechnology, Thapar University, Patiala**, is a bonafied work carried out by her under my supervision. The matter embodied in this thesis has not been submitted in part or full to any other Institute or University for the award of any degree.

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CANDIDATE's DECLARATION

I hereby declare that the project work entitled "**Studies of somatic embryogenesis in selected clones of *Eucalyptus tereticornis***" is an authentic record of my work carried out at TIFAC-CORE as requirement of one year project work for the award of degree of Master in Technology, Department of Biotechnology, Thapar University, Patiala, under the guidance of **Dr. Anil Kumar**, Associate Professor, Department of Biotechnology. The matter embodied in this thesis has not been submitted in any part or full to any other university or institute for the award of any degree in India or abroad.

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ABSTRACT

Eucalyptus tereticornis is an important woody tree species belonging to family "Myrtaceae". It is commercially valuable as it is used as a source of raw material for pulp and paper industry due to its important wood characteristics for the manufacture of high grade paper. The present study was focused on development of somatic embryogenesis protocol for selected elite clone of *Eucalyptus tereticornis*. The effect of plant growth regulators (BA, NAA and 2, 4-D) on somatic embryos induction from leaf and internodal explants taken from microshoots of *E. tereticornis* was evaluated. Maximum frequency of somatic embryo induction was observed on MS medium supplemented with NAA (5µM) and BA (5µM). It was noteworthy, that all combinations of BA with 2,4-D and NAA with 2,4-D resulted in callus induction. This callus was not found to be embryogenic in nature. The explants showing embryogenic structures were transferred to MS medium supplemented with 1µM GA_3 and 1µM BA for germination. Various developmental stages of somatic embryo were observed through histological and scanning electron microscopic studies. The present study indicated the importance of plant growth regulators in embryo development and germination in woody plants.

ABBREVIATIONS

2,4-D	Dichlorophenoxy acetic acid
ABA	Abscisic acid
ANOVA	Analysis of variance
B5	Gamborg's medium
BA	6- benzyl adenine
Conc.	Concentration
CH	Casein Hydrolysate
DMRT	Duncan's multiple range test
DPX	Distyrene, a plasticizer and a xylene mixture
FAA	Formalin-Acetic Acid- Alcohol
g	Gram
GA_3	Gibberellic acid
hr	Hour
IAA	Inodole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin (N^6- furfuryladenine)
m	Meter
mg	Milligram
min	Minute
ml	Milli liter
mM	Milli molar
MS	Murashige and Skoog medium
N	Normality
NAA	Naphthalene acetic acid
PG	Polyethylene glycol
PGR	Plant Growth Regulator
pH	Potential hydrogen
psi	Pounds per square inch
s	Second
SE	Standard error
SEM	Scanning electron microscope

TBA	t-butyl alcohol
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
WPM	Woody plant medium

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

CHAPTER 1 INTRODUCTION

Eucalyptus is the most primarily grown species for trading purposes among all other tree species (Eldridge et al. 1994). *Eucalyptus* genus was named by a French botanist I'Heritier in 1788. It belongs to family Myrtaceae, indigenous to eastern Australia and nearby islands. Genus *Eucalyptus* is known for its fast growing rate, large biomass production and their good quality of wood. It is also used for reforestation programme due to its easy adaptability (Eldridge et al. 1994). Since last twenty years India become the most important part of the reforestation drive initiated all over the world (Brooker, 2000; Lal et al. 1993; Laclau et al. 2013). Apart from its role in reforestation, genus *Eucalyptus* serves as an important source of raw material for paper and pulp industry (Clarke, 2009; Cotterill and Brolin, 1997; Paues, 1999). Its wood is also used for poles, timber construction and fuel. Out of various species, *Eucalyptus tereticornis* is one of (Brooker et al. 2000) the most primary species grown for trading purpose (Potts and Dungey, 2004; Grattapaglia and Kirst, 2008). *E. tereticornis* is known by many names such as forest red gum, queensland blue gum, slaty gum, mysore gum all over the world (Sharma and Ramamurthy, 2000). It is a fast growing plant that can achieve 30 to 45 m of height and can be 1 to 2 m in breadth. It is one of the main source of firewood, raw material for paper and pulp (mainly used), essential oil, a source of nectar for caramel-flavoured honey (Clarke, 2009). Few of the important uses of the tree are listed below:

a) Paper industry raw material: The use of *E. tereticornis* in paper and pulp industry is widely accepted as its pulp contains tannin (6-12%) which can be easily removed by enzymatic treatments and lead to the production of high grade paper with high opacity, smoothness, good capability to collapse, inherent fibre stiffness (Turnbull, 1999). Other properties such as high cellulose content make it a good source for manufacturing paper, fibre board and several other important industrial products (Bolza and Keating, 1972; Clarke, 2009; Cotteril and Brolin, 1997; Lal et al. 1993, 2005). Moreover, the cost of wood is less due to higher production rate and pulp yield of the tree (Patt et al. 2006; Laclau et al. 2013).
b) Pharmaceutical industry raw material: The wood of *E. tereticornis* contains essential oils which have anti-bacterial, insecticidal and anti-fungal properties. Moreover it also has

hasanalgesic, muscle relaxing, anti-hyperglycaemic and anti-inflammatory properties (Boer, 1997; Bolza and Keating, 1972).

Due to above mentioned properties of the tree, demand of cultivating *E. tereticornis* is increasing. Apart from its use as raw material for pulp and paper industry, it is also used to fulfil the need of wood for fuel. Its timber has also gained importance gained for commercial uses like furniture making, construction etc.

Fig 1: Elite plants of *Eucalyptus tereticornis* growing at TIFAC-CORE Thapar University, Patiala (Punjab).

Conventionally, *E. tereticornis* is propagated through seeds or cuttings (Tewari, 1991). However, the germination potential of these seeds is low andseedlings shows much variability attributed to its diverse genetic base (Hogberg et al. 1998; Lakshmi et al. 1986; Merkle, 1995).Thus, in order to meet the increased demand of uniform planting material, techniques for mass vegetative propagation such as *in vitro* propagation are adopted (Aggarwal et al. 2010, 2012; Kumar et al. 2010). While evaluating the suitability of *E. tereticornis* for industrial use, the quality of wood is as important as the production rate (Boer, 1997; Kumar and Bangarwa, 2006). More is the consistency of the wood, greater will be the efficiency of producing industrial products with improved quality (Zobel, 1993; Zobel and Van, 1989). Traditionally, tree improvement is carried out through breeding (Eldridge et al. 1994). But due to extended maturation period and high level of heterozygosity (Corredoira et al. 2015) of tree, selection of hybrids with improved characters becomes extremely difficult (Bonga and Von, 1992). Thus, techniques such as genetic transformation are adopted to improve several characters such as lignin, cellulose and tannin content (Aggarwal et al. 2011, 2013). To undertake genetic transformation programmes, development of plant

regeneration protocol through shoots organogenesis and/or somatic embryogenesis is one of the initial requirements (Hajari et al. 2006; Dibax et al. 2005; Barruetoet al. 1999; Nugent et al. 2001; Mullins et al. 1997). In *E. tereticornis*, regeneration through shoot organogenesis is widely reported (Aggarwal et al. 2010; Hajari et al. 2006; Dibax et al. 2005; Barruetoet al. 1999; Nugent et al. 2001; Mullins et al. 1997; Muralidharan and Mascarenhas, 1987; Pinto et al. 2008b; Andrade et al. 2011). However, the use of somatic embryogenesis is promoted over shoot organogenesis due to the production of non-chimeric plants (Giri et al. 2004). In several species of *Eucalyptus* such as *E. grandis, E. saligna* Smith, *E. dunnii*somatic embryogenesis has been reported genotypes (Qin and Kirby, 1990; Termignoni et al. 1996) but in case of *E. tereticornis,* the work is very limited (Prakash & Gurumurthi, 2010).

Somatic embryogenesis is a complex form of asexual plant regeneration where embryo is developed from a somatic cell (Verdeil et al. 2007; Merkle et al. 1995, Dodeman et al. 1997). Developmental stages of somatic embryos resembles with zygotic embryos in terms of no vascular connection with parent tissue (Bong and Von, 1992; Von et al. 2002; Wicart et al. 1984). It is basically divided into two stages:

a) Induction stage: The differentiated somatic cells adopt embryogenic competence with or without involving callus phase.
b) Development stage: Proembryos develop into mature embryos by undergoing various stages such as proliferation, histodifferentiation, maturation and germination/conversion (Merkle et al. 1995). Various stages involved in maturation of somatic embryos are globular, heart-shaped, torpedo-shaped and cotyledonary stages (Jimenez, 2005).

Somatic embryogenesis is an important approach for plant regeneration which shows great advantages in case of forest tree species with increased industrial demands. The advantages of somatic embryogenesis involve clonal mass propagation, cryopreservation of valuable germplasm and genetic transformation (Corredoira et al. 2015; Merkle and Dean, 2000; Thorpe, 2000;Lal et al. 1993; Giri et al. 2004). The success of somatic embryogenesis is dependent on proper interaction between physical (light intensity, pH, incubation temperature, and photoperiod) and chemical factors (medium composition and plant growth regulators) (Merkle et al. 1995; Thorpe, 2000; Phillips, 2004; Gaj, 2004). Among all the factors, plant growth regulator plays a major role in inducing somatic embryogenesis (Williams and Maheshwaran, 1986; Haagen-smith, 1951; Jimenez, 2005). These help in stem elongation, tropism and apical dominance. Plant growth regulators regulate re-entry of cells

in mitosis and also determine their embryogenic state (Thrope, 2000). In addition to PGRs, many other factors such as type and age of explant, medium compositions etc. are also reported to influence induction of somatic embryos (Corredoira et al. 2015; Subbaiah and Minocha, 1990;Dibax et al. 2005; Yu and Wei, 2008). The present study was focussed to examine the effect of plant growth regulators on embryogenic callus induction and differentiation of somatic cells of leaf and internodal explants into somatic embryos. The pathway of somatic embryogenesis was confirmed through histological studies.

CHAPTER 2 REVIEW OF LITERATURE

Every year the use of wood is increasing as itsuse for timber, fuel wood, pulp and paper production is increasing and for these uses wood was taken from forest or is commercially cultivated. To fulfil the need of wood and to maximize biomass yield on a sustainable basis it is critical to search for a method of improvement for development and production of wood. The genus *Eucalyptus* is the most widely grown plant throughout the world especially in India to provide wood for industrial use and to meet the need of wood products in the region of scarcity (Eldridge et al. 1994). For the propagation of *Eucalyptus* commonly used techniques are *in-vitro* micropropagation and vegetative propagation (Le Roux and Van Steden 1991; Nugent et al. 2001; Hajari et al. 2006; Bonga and Von 1992). These methods are limited because of low frequency of roots and shoots (Bennett andMcComb, 1986;Giri et al. 2004;MacRae and van Staden 2000; Bennett et al. 1994; Barrueto et al. 1999).

In plant tissue culture, methods like shoot proliferation using auxiliary buds and adventitious shoots has been widely used for *in-vitro* propagation yet research work on inducing somatic embryogenesis in *Eucalyptus* species is limited (Zobel, 1993; Watt et al. 2003; Lal et al. 1993). This method ensures to provide a valuable approach to clonal propagation for elite genotypes and leads in the development of synthetic seed (Redenbaugh et al. 1993; Gray andPurohit, 1991; Kaur et al. 2017). As it was reported that limitations in plant regeneration limits wider applications of genetic engineering techniques in improving *Eucalyptus* species (Lal et al. 1993; Oller et al. 2004). Therefore, regeneration through somatic embryogenesis provides an efficient ways of mass propagation in species as well as aid in gene transfer for genetic improvement (Tournier et al. 2003; Potts and Dungey, 2004; MacRae and Van Staden, 2000).

In *in-vitro* conditions when de-differentiation of somatic cell occurs into a totipotent embryogenic cell, it leads to the induction of embryo like structures (Verdeil et al. 2007). It is considered as one of the best method for trees improvement (Hogberg et al. 1998; Park et al. 1998; Park, 2002; Canhoto et al. 1999). In wide range of species somatic embryos were reported as a predominant means of plant regeneration (Thorpe, 2012; Vasil, 1999).

The first evidence of somatic embryogenesis was observed in carrot (*Daucuscarot*) cell cultures (Steward et al. 1958). In most reports it was reported that morphologically somatic

embryos resembles zygotic embryos during different developmental stages (Muralidharan et al. 1989; Watt et al. 1991, 1999). In *Eucalyptus* species, firstly somatic embryogenesis was observed from seedlings of *'E. x Liechow'* (Ouyang et al. 1980, 1981). In *E. nitens*(Watt et al. 1991), *E. globulus* (Trindade, 1996), and *E. grandis* (Lakshmi et al. 1986) histological studies was observed during developmental stages of somatic embryos. In *E. nitens* ultra-structures were studied and were compared with mature zygotic embryos and similarities were reported (Bandyopadhyay et al. 1999). It was observed that the features which help in distinguishing organogenesis from somatic embryogenesis are as follows: at cotyledonary stage somatic embryos lack root poles as well as starch grains in the basal region and vascular tissue connection is absent between explant and its regenerating structure (Wicart et al. 1984; Watt et al. 2003). Explant has its specific role in formation of somatic embryos. The plant material mostly meristematic tissues like tips of shoots, roots and auxiliary buds are good explants due to their rapid rate of cell division and are basically virus free and these tissues have higher ability to uptake large amount of nutrients. In *Eucalyptus* species induction of somatic embryogenesis was cultured using: seedlings and their fragments, leaves, internodes, roots, petioles, cotyledons, mature and immature zygotic embryos and seeds as explant (Thorpe, 2012; Le Roux and Van Staden, 1991; Watt et al. 1999, 2003; Hajari et al. 2006; Pinto et al. 20008a; Gaj, 2004). Explant such as zygotic embryos were used to develop somatic embryos in *Eucalyptus species: E. citriodora* (Muralidharan and Mascaranhas, 1987; Muralidhara et al. 1989), *E. globulus*(Pinto et al. 2002, 2008a, 2008b; 2008c; Trindade, 1996), *E. tereticornis* (Prakash and Gurumurthi, 2005). In *E.grandis* leaves from microshoots were used as explant (Watt et al. 1991) whereas 3-days old seedlings were used in *E. dunnii* (Termignoni et al. 1996) for somatic embryogenesis on MS medium supplemented with NAA and 2,4-D. In *E. globulus*explants in different phases of development were examined and it was observed that seeds were more suitable than leaves and cotyledons for somatic embryogenesis (Trindade, 1996). Boulay (1987) used hypocotyls and internodes of *E. gunni,* Bandyopadhyay et al. (1999) used seedling of *E. globulus*and *E. nitens* and Nugent et al. (2001) used cotyledons of *E. globulus* for inducing somatic embryos. The results showed that embryo formation takes place but induction rate was low.

In plant tissue culture the plant growth regulators and medium composition are important factors for inducting somatic embryos (Williams and Maheswaran, 1986; Haagen-smith, 1951; Jimenez, 2005). The basal medium which isused for culturing consists of mineral salts, amino acids, carbon sources and vitamins (Ramage and Williams, 2002; Bonga and Von,

1992). Auxins like 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), indole3-butyric acid (IBA) or indole-3-acetic acid (IAA) help in initiation and formation of embryo by reactivating the cell cycle (Williams and Maheswaran, 1986; Haagen-smith, 1951; Jimenez, 2005). Cytokinins like 6-benzylaminopurine (BA) or kinetin (KIN) have been beneficial for inducing somatic embryos (Thorpe, 2012; Bennett et al. 1994; Jimenez, 2005). Qin & Kirby (1990) reported development of embryo like structures from hypocotyls, young leaves and cotyledons of young seedlings of *E. dunnii, E. botryoides, E. rudis*and *E. grandis*and from the shoots of clones of *E. grandis*. Green protuberances development from the trimmed surfaces of explants were taken and transferred on medium consist of BA (1.1 mgl^{-1}) for the germination of embryo like structures and adventitious shoots.In *E. grandis* somatic embryogenesis was reported using MS medium with KIN (5 mgl^{-1}) and NAA (0.1 mgl^{-1}), where callus was developed from internodal segments of 5 year old tree shoots (Lakshmi et al. 1986). Friable callus was cultured in liquid medium consisting of 1 mgl^{-1} KIN, NAA, BA, 2,4-D which resulted in somatic embryogenesis. In *E. grandis*, somatic embryogenesis was obtained from young leaves of microshoots which were cultured on MS medium containing 2,4-D as plant growth regulator (Watt et al. 1991).

In *E. tereticornis* somatic embryogenesis and plant regeneration were reported where embryogenic calli was obtained on culturing mature zygotic embryos using MS medium containing NAA. Further embryogenic calli were transferred on embryo inducing medium consisting of BA. After transferring, development of somatic embryos within 2 weeks (Prakash and Gurumurthi, 2005) was observed which were successfully germinated. In *E. globulus*embryogenic callus phase was obtained when callus formed in adult leaf was grown on MS medium containing IBA (Oller et al. 2004). In several species of *Eucalyptus*, somatic embryos were induced on MS medium (Pinto et al. 2008a) which is a rich nitrogen source. Gamborg/B5 medium (Gamborg et al. 1968) containing NAA (3 mgl^{-1}) and 5% (w/v) sucrose (Muralidharan et al. 1989; Muralidharan and Mascarenhas, 1987) induces somatic embryos from zygotic embryos of *E. citriodora*. Termignoni et al. (1996) also studied the formation of somatic embryos using seedlings of *E. dunnii*on B5 medium. B5 and MS media among other plant growth mediums proved to be the best for somatic embryogenesis and plant regeneration in *Eucalyptus* species (Pinto et al.2008a). The effectiveness of medium was studied in*E. globulus*for somatic embryogenesis induction and expression. In *E. camaldulensis*, direct somatic embryogenesis was reported using hypocotyl explants cultured on MS medium with BA (0.5 mgl^{-1}). Similarly, it was observed that maturation of somatic

embryos decreases with increase in abscisic acid (ABA) (0-5 mgl^{-1}) and sucrose concentration (1-4%) while studying induction and germination of somatic embryos (Prakash and Gurumurthi, 2010). It was seen that basal medium with cytokinin (BA) and auxin (NAA) in the ratio of 20:1 causes direct somatic embryogenesis which leads to *in-vitro* regeneration of plant from nodal segments (18 months old superior genotype) of *E. camaldulensis* (Girijashankar, 2012). It was also observed that plant growth regulators lacking medium leads to small seedlings as compared to normal seedlings when cultured on medium containing plant growth regulators. Several reports were submitted in which auxins and cytokinins were observed as important components which stimulates embryo germination and conversion (Jimenez, 2005). Significant variations in the composition of culture medium were done for better results and was reported that some species of *Eucalyptus* requires extra components in the medium such as casein hydrolysate (CH) and glutamine for somatic embryogenesis (Von Arnold et al. 2002) which leads to more and good quality of embryos. In *Eucalyptus* species the presence of auxins usually does not lead to maturation in somatic embryos (Pinto et al. 2002, 2004). It was studied that plant regeneration takes place in auxin free medium or especially on medium, which is supplemented with gibberlic acid (GA_3) and/or cytokinins (Pinto et al. 2004; Watt et al. 1991; Muralidharan and Mascarenhas, 1987). In *E. citriodora* a report was submitted in which germination of embryos took place on medium free of auxins (Muralidharan and Mascarenhas, 1987). Embryos which were cultured in liquid B5 medium containing $20gl^{-1}$ sucrose were transferred into germination medium which resulted in 52% embryos germination and further regenerated into healthy plantlets. Successful study on maturation and germination of embryos in *E. grandis* and *E. dunnii* was reported by Pinto et al. (2004) but induction was low. Whereas, on adding ABA and polyethylene glycol (PG), low rate of embryo conversion was observed, which depends upon the characteristics of explant (Watt et al. 1999). It was also reported that MS medium without any growth regulators was more effective than B5 medium for the formation, maturation and germination of cotyledonary embryo (Pinto et al. 2008a, Muralidharan et al. 1989; Bandyopadhyay et al, 1999). At lower level of auxins (NAA), proliferation of globular shaped somatic embryos is favoured and it also helps in maintaining the somatic embryogenesis competence on medium without any growth regulators. For germination and conversion of globular shaped embryo BA and KIN were added. These cytokinins improved proliferation rate of embryos. Recently, it was reported that picloram was more effective than NAA for the growth of nodular embryogenic structures (Corredoira et al. 2015). Moreover, it was reported that somatic embryogenesis from leaf and shoot apex explants of mature *E. globulus* and hybrid *E.*

salignax E. maidenii plants was induced using 40 µM picloram. Picloram resulted in higher frequency of *in-vitro* culturing when compared with 2,4-D plant growth regulator (Corredoira et al. 2015). Trindade (1996) observed globular and heart shaped somatic embryos on woody plant medium containing BA and 10% coconut milk in *E. globulus*. In *E. dunnii*, somatic embryos were formed when mediumsupplemented with NAA and casein hydrolysate (CH) was used (Termignoni et al. 1996).

Several studies show that important role is played by carbon sources in growth and multiplication of plant cells. In *Eucalyptus* species carbon source i.e. carbohydrates also plays important role in maintaining osmotic potential of cells and water conservation. As *in-vitro* grown plantlets have less accessibility to carbon dioxide in culture bottles, so for that sugar is added in medium and it acts as a carbon source for *in-vitro* growth and multiplication of plant cells. In *Eucalyptus*, for somatic embryogenesis the concentration of sucrose recommended varies from species to species, but usually sugar concentration ranges from 2% to 5% (w/v). Muralidharan and Mascarenhas (1987) added 5% sucrose in medium for maturation of somatic embryos in *E. citriodora*. Lakshmi et al. (1986) added 2% sucrose in medium for embryogenesis in *E. grandis*. Except sugar there are several other carbohydrates like glucose, mannitol, maltose, cellulose, fructose, lactose, and myo-inositol, which were used for inducing somatic embryos in other species (Canhoto et al. 1999; Lipavska and Konradova, 2004). Pinto et al. (2002) reported that induction medium supplemented with mannitol inhibits callus formation on the surface of the explants in *E. globulus*.Vasil (1999) reported that somatic embryogenesis pathway which leads to plant regeneration is mostly developed from a single progenitor cells or through pro-embryogenic cells. The plants which were regenerated tend to be genetically uniform. From the data of somatic embryogenesis reported till now on *Eucalyptus* species it was seen that indirect somatic embryogenesis was observed which leads to plant regeneration (Cuenca et al. 1999; Gaj, 2004; Montalban et al. 2012; Corredoira et al. 2015).

In this study somatic embryogenesis was studied in cultures raised from mature tissues taken from selected elite clones.

CHAPTER 3 MATERIALS & METHODS

Plant material, Chemicals and Glassware:

Shoot cultures of an elite clone of *Eucalyptus tereticornis*; clone 'T1' which were already available at Plant tissue culture laboratory of TIFAC-CORE, Thapar University, were multiplied through regular subculture on Murashige and Skoog (1962) medium. Cultures were incubated at 25±1 °C, photoperiod cycle of 16 hrs day and 8 hrs night and light intensity of 50 µmol $m^{-2}s^{-1}$was provided by white-cool fluorescent lamps (Phillips Ltd, Mumbai). Leaves and internodes were excised from 21 days old microshoots and used as explants. All routine chemicals, vitamins & PGRs used in this work were obtained from Hi-Media Laboratories Ltd., (Mumbai, India). Glassware's like measuring cylinders, beakers etc, were purchased from Borosil Glass Works Ltd. (Mumbai, India). Plant tissue culture bottles (300 ml) covered with polypropylene caps were purchased from Kasablanka Corporation (Mumbai, India)

Preparation of medium and culture conditions:

Murashige and Skoog (1962) media containing 3% sucrose (w/v) as well as (0.7%) agar (Plant Tissue Culture grade, Hi-Media laboratories, Mumbai, India) was used as basal medium for tissue culture experiments. The medium pH was adjusted upto 5.8 with 1N potassium hydroxide (KOH)/Sodium hydroxide (NaOH) or 1N hydrochloric acid (HCl) and medium was autoclaved (121 °C; 15 psi; 20 min) for sterilization.

Plant growth regulators like Naphthalene acetic acid (NAA), 6-benzyl adenine (BA) and 2, 4-Dichlorophenoxyacetic acid (2,4-D) were added in MS medium in various combinations before autoclaving. Plant growth regulators stock of 2.5 mM were also prepared by dissolving them in conc. HCl or conc. NaOH/KOH solution and distilled water. The prepared stock solutions were kept in cold store room at 4ºC.

Plant growth regulators effect and somatic embryogenesis on explants

From microshoots of *Eucalyptus tereticornis* (30 days old culture) leaves and internodal explants were used for initiating somatic embryos. Leaf explants with adaxial surface in touch with medium and internodal explants were cut from young microshoots. Both explants were cultured on medium containing (0-5 µM) 6-benzyl adenine (BA), (0-25 µM) 2,4-

Dichlorophenoxy acetic acid (2,4-D) and (0-5 µM) Naphthalene acetic acid (NAA) in 300 ml culture bottles. Explants differentiated into embryo like structure were transferred on MS medium with 2.5 mM of 6-benzyladenine as well as 2.5 mM of Gibberellic acid (GA_3) for germination of somatic embryos into whole plants.

Histological and scanning electron microscopy (SEM)

For histological examination, explants with embryo like structure were selected and fixed for 7 days in a solution mixture of formalin, glacial acetic acid, and 50 % (v/v) aqueous ethanol (FAA; 5:5:90 v/v). Sample was properly dehydrated through a series of t-butyl alcohol according to the given protocol (Table 6). The dehydrated samples were then fixed in paraffin wax (melting point 58–60°C) and 10µm thick longitudinal segments were cut by using rotary microtome (RP-30, Radical Instruments AmbalaCantt. India). Plant tissue sample was stained using toluidine blue and casted with DPX (distyrene, a plasticizer, and a xylene mixture) for observing under light microscope and steps werefollowed by the procedure given Sharma and Millam, (2004). The formed sections which were mounted on slides were observed and photographed under microscope (Nikon Eclipse 50i, Nikon, Japan).

Slide preparation for histological studies (Johansen, 1940)

Fixing and preservation of tissue:

The selected plant sample was firstly fixed in FAA (Formaldehyde: Acetic acid: 50% v/v Alcohol; 5:5:90) for one week and then tissue was stored in 70% ethyl alcohol. Dehydration of the sample was done by t-butyl alcohol (TBA) series (Table 6) and to each grade plant sample is kept for 3-4 hours but at 3rd grade sample can be kept for maximum of 24 hours.

Waxing:

The fixed and dehydrated plant sample was dipped in TBA solution and is kept in oven at 60ºC and pellets of wax was added slowly (Hi-Media Laboratories Pvt. Ltd., Mumbai) in order to penetrate wax in fixed tissue sample.

Block making and section cutting:

Wax blocks of plant sample were formed which is free from TBA and were in proper orientation. After the formation of blocks, 10µM thin sections were cut with the help of rotary microtome.

Mounting, stretching and dewaxing:

The formed thin sections of plant sample were floated over water on glass slide. The sections were stretched on hot plate at 60ºC and allowed to dry for 5-6 days at room temperature. After proper drying the slide were soaked in pure toluene for 15 min (until the wax gets dissolved) and then stained in toluidine blue following procedure described by Sharma and Millam (2004).

Light and scanning electron microscopy (SEM)

According to the protocol given byRoemhildt et al. (2012) the explants which differentiate into somatic embryo like structures were used in scanning electron microscope studies. For SEM, the explants were fixed in FAA for 2 days then dehydrated through ethanol series (Table 6). Dehydrated tissue was casted on metal stab by using double sided carbon tape and surface of explant was covered with gold using automated fine coater (JFC-1600, JEOL Limited, Japan) under vacuum made by ULVAC (GHD030) vacuum pump (J B Systems, Inc, GA). Explants coated with gold were observed at 20 kV through scanning electron microscope (JSM-6510LV, JEOL Limited, Japan).

The effect of plant growth regulators (PGRs) on induction of callus and somatic embryos from leaf and internodal explants taken from microshoots of *Eucalyptus tereticornis* was studied. Further, histological and scanning electron microscopic (SEM) studies were carried out to confirm the pathway of somatic embryogenesis.

Fig 2: Initiation of somatic embryogenesis and regeneration of plant in selected elite clone of *Eucalyptus tereticornis* (**A**) 4 weeks old microshoots of *Eucalyptus tereticornis* cultured on MS medium (**B**) explants (leaves and internodes) taken from 4 week old microshoots were cultured on medium fortified with plant growth regulators (NAA, BA and 2, 4-D) (where IN:

internode and L: leaf) (**C**) explants cultured on MS medium containing NAA and 2,4-D shows compact callus (hard and greenish in colour) (**D**) explants cultured on MS medium with BA and 2,4-D shows friable callus (pale yellowish in colour) (**E**) Explant showing nodular callus when cultured on MS medium with NAA and 2,4-D (**F**) explants showing embryogenic callus with somatic embryo like structure developed on leaf explant of *Eucalyptus tereticornis* (**G**) explant showing early staged globular embryos observed under light microscope (*Bar* 60 µm) (**H**) regeneration of shoots from somatic embryo conversion (embryogenic callus) observed within 10 weeks after induction on MS medium supplemented with BA and GA_3.

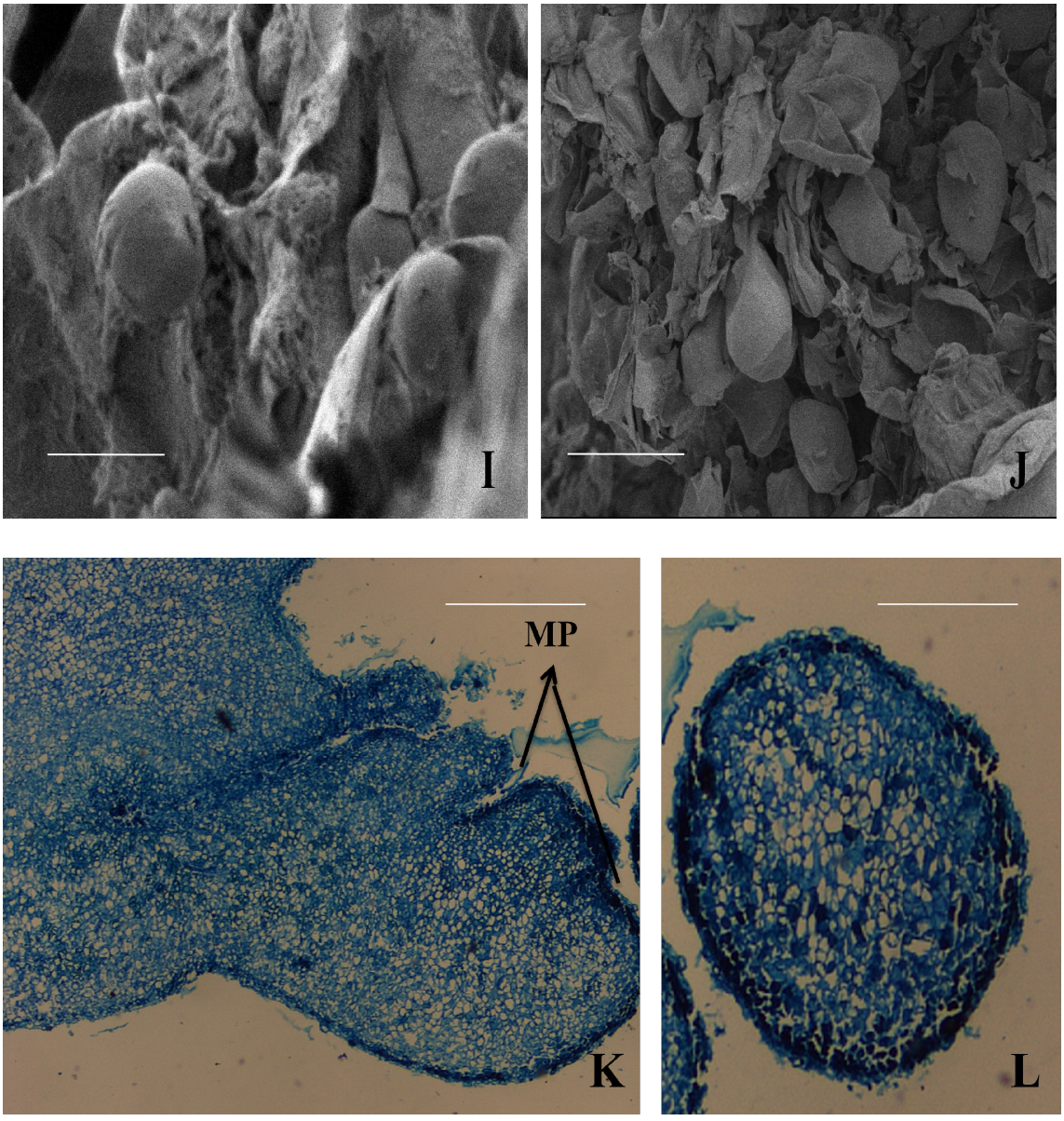

Fig 3: Scanning electron microscope and histological studies shows development of somatic embryo like structures in explants of *Eucalyptus tereticornis*. (**I**) and (**J**) Scanning electron microscopic studies showed early developmental stage of globular embryos (**I**) globular embryo (**J**) cluster of globular embryos. Histological studies showing (**K**) active cell division in meristematic pockets (**L**) developing globular embryo observed under compound microscope (*Bar* 100 µM).

In present work, somatic embryos like structures were formed both in leaf and internodal explants within 30 days of initial culturing on MS medium fortified with NAA and BA singly and in combination with each other. The embryogenic callus progressed to form globular somatic embryos, which have regeneration potential of developing plantlets. Upon transfer of embryo like structures to basal MS medium, shoot regeneration was observed. Least induction of somatic embryos was observed on MS medium consisting of low concentration of NAA, whereas high NAA concentration increased somatic embryo induction (Table1). Maximum frequency of somatic embryos was 78.33% from leaf explant and 75% from internodal segments on MS medium fortified with NAA (5µM) and BA (5µM). Increase in concentration of BA and keeping NAA concentration constant (25µM) causes decrease information of somatic embryos in explants. Maximum initiation of somatic embryos was observed when concentration of NAA and BA was kept same.Thus, for better results the combination of cytokinins (BA) with auxins (NAA) is necessary for initiating somatic embryos.

Table 1: Effect of NAA and BA on somatic embryogenesis in leaf and internodal explants taken from microshoots of *Eucalyptus tereticornis*.

Sr. No.	PGRs combination		Mean explant showing somatic embryogenesis (%)		Mean number of embryos per explant	
	NAA (µM)	BAP (µM)	Leaf	Internode	Leaf	Internode
1	0	0	0^{b}	0^{c}	0^{d}	0^{d}
2	1	0	0^{b}	0^{c}	0^{d}	0^{d}
3	5	0	0^{b}	0^{c}	0^{d}	0^{d}
4	25	0	55.55^{ab}	33.33^{abc}	10.5^{ab}	2.5^{bcd}
5	0	1	22.22^{ab}	33.33^{abc}	2.33^{cd}	1^{cd}
6	1	1	55.55^{ab}	31.11^{abc}	6.17^{abcd}	2.56^{bcd}
7	5	1	33.33^{ab}	61.11^{ab}	6.0^{abcd}	5.17^{abc}
8	25	1	55.55^{ab}	72.22^{a}	8.56^{abc}	6.22^{ab}
9	0	2.5	38.89^{ab}	30.55^{abc}	3.83^{abcd}	2.67^{bcd}
10	1	2.5	8.33^{b}	44.44^{abc}	2.67^{bcd}	8.83^{a}
11	5	2.5	55.55^{ab}	33.33^{abc}	8.17^{abc}	2.83^{bcd}

12	25	2.5	52.78^{ab}	33.33^{abc}	7^{abcd}	3.17^{bcd}
13	0	5	0^{b}	0^{c}	0^{d}	0^{d}
14	1	5	44.44^{ab}	11.11^{bc}	5.44^{abcd}	1.33^{cd}
15	5	5	78.33^{a}	75^{a}	11.45^{a}	4.56^{abcd}
16	25	5	0^{b}	0^{c}	0^{d}	0^{d}

Leaf and Internodes were used as explants from 30 days old microshoots of *Eucalyptus tereticornis.* Data was recorded after incubation of 6 weeks and was then analysed by ANOVA. Mean was compared by DMRT within columns; values followed by same lowercase letter are not significant at P\0.05.

The induction of callus and type of callus developed on explants (leaf and internodes) depends upon certain physical and chemical factors. Among all the factors one of the most important factor is plant growth regulators. In this work effect of auxins and cytokinins was studied for inducing somatic embryogenesis.

MS medium containing NAA (0-25 µM) used to culture explants (Table 2) and friable callus was observed in leaf explant whereas internodal explant shows no result of callus induction. Same results were observed for leaf explants when 2,4-D was supplemented in MS medium whereas friable callus was also observed in internodal explants also. When NAA concentration was kept higher and constant (25 µM) and 2,4-D concentration was keep on increasing (0-5 µM) the frequency of callus induction also kept on increasing. Nodular callus was observed in both explants when MS medium with NAA (1 and 25 µM) and 2,4-D (0-5 µM) was used. The maximum frequency of callus initiation observed was 100% when higher concentrations of plant growth regulators were used.

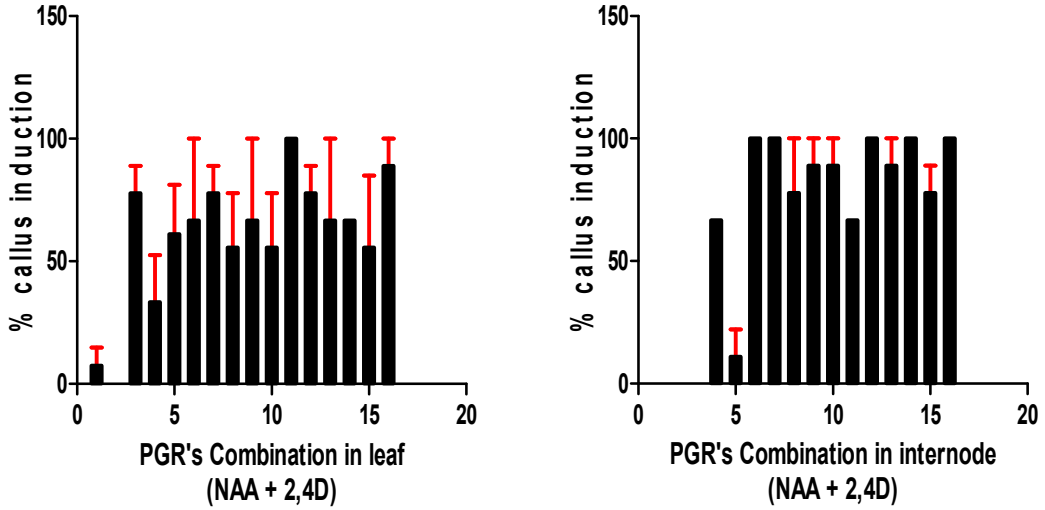

Fig 4: Graphs showing callus induction on explants (leaf and internode) of *Eucalyptus tereticornis* using plant growth regulators (NAA and 2,4-D) supplemented on MS medium.

Table 2: Plant growth regulators effect on callus initiation in *Eucalyptus tereticornis* cultured on MS medium with different concentrations of NAA and 2,4-D.

Sr. No.	PGRs combination		% callus induction from explants (Mean ± SE)		Explants showing friable callus	
	NAA (µM)	2,4-D (µM)	Leaf	Internode	Leaf	Internode
1	0	0	7.407 ± 7.407	0 ± 0	Friable	--
2	1	0	0 ± 0	0 ± 0	--	--
3	5	0	77.77 ± 11.11	0 ± 0	Friable	--
4	25	0	33.33 ± 19.24	66.66 ± 0	Friable	--
5	0	1	61.11 ± 20.03	11.11 ± 11.11	Friable	Friable
6	1	1	66.66 ± 33.33	100 ± 0	Nodular	Friable
7	5	1	77.77 ±11.11	100 ± 0	Friable	Friable
8	25	1	55.55 ± 29.39	77.78 ± 22.22	Friable	Friable
9	0	2.5	66.66 ± 33.33	88.89 ± 11.11	Friable	Friable
10	1	2.5	55.55 ± 22.22	55.55 ± 15.47	Nodular	Nodular

11	5	2.5	100 ± 0	66.66 ± 0	Friable	Friable
12	25	2.5	77.77 ± 11.11	100 ± 0	Nodular	Nodular
13	0	5	66.66 ± 33.33	88.89 ± 11.11	Friable	Friable
14	1	5	66.66 ± 0	100 ± 0	Nodular	Friable
15	5	5	55.55 ± 29.39	77.77 ± 11.11	Friable	Friable
16	25	5	88.88 ± 11.11	100 ± 0	Nodular	Nodular

Leaf and Internodes were used as explants from 30 days old microshoots of *Eucalyptus tereticornis*. The data was recorded after incubating for 6 weeks and was analysed by Graph pad prism.

Explants (leaf and internodal segments) were cultured on medium to study the effect of 2,4-D (0-25 µM) and NAA (0-5 µM) (Table 3) and observation was made after 4 weeks of culture. Increase in concentration of 2,4-D resulted in increase of callus induction from leaf explants, whereas no callus was initiated on internodal explants. The combination of 2,4-D with NAA, increases the frequency of callus induction. It was observed that low auxin concentration resulted in friable callus, nodular callus which was initiated from leaf explants on MS medium supplemented with 2,4-D (1 and 25 µM) and NAA (2.5-5 µM) in leaf explants. The highest frequency of callus was observed on MS medium supplemented with 1µM 2,4-D and 1µM NAA from both leaf and internodal explants.

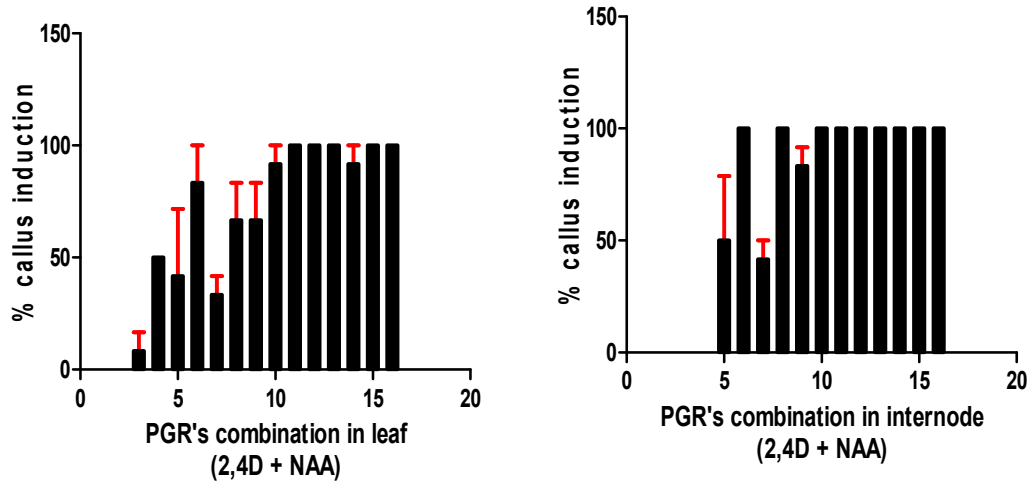

Fig 5: Graphs showing callus induction on explants (leaf and internode) of *Eucalyptus tereticornis* using plant growth regulators (2,4-D and NAA) supplemented on MS medium.

Table 3: Plant growth regulators effect on callus initiation in *Eucalyptus tereticornis* cultured on MS medium with different concentrations of 2, 4-D and NAA.

Sr. No.	PGRs combination		% callus induction from explants (Mean ± SE)		Explants showing friable callus	
	2,4-D (µM)	NAA (µM)	Leaf	Internode	Leaf	Internode
1	0	0	0 ± 0	0 ± 0	Friable	Friable
2	1	0	0 ± 0	0 ± 0	--	--
3	5	0	8.33 ± 8.33	0 ± 0	Friable	--
4	25	0	50 ±0	0 ± 0	Friable	--
5	0	1	41.67 ± 30.05	50 ± 28.87	Friable	Friable
6	1	1	83.33 ± 16.67	100 ± 0	Friable	Friable
7	5	1	33.33 ± 8.33	41.67 ± 8.33	Friable	Friable
8	25	1	66.67 ± 16.67	100 ± 0	Friable	Friable
9	0	2.5	66.67 ± 16.67	8.33 ± 8.33	Friable	Friable
10	1	2.5	91.67 ± 8.33	100 ± 0	Nodular	Friable
11	5	2.5	100 ± 0	100 ± 0	Friable	Friable
12	25	2.5	100 ± 0	100 ± 0	Nodular	Friable
13	0	5	100 ± 0	100 ± 0	Nodular	Friable
14	1	5	91.67 ± 8.33	100 ± 0	Friable	Friable
15	5	5	100 ± 0	100 ± 0	Friable	Friable
16	25	5	100 ± 0	100 ± 0	Nodular	Friable

Leaf and Internodes were used as explants from 30 days old microshoots of *Eucalyptus tereticornis*. The data was recorded after incubating for 6 weeks and was analysed by Graph pad prism.

MS medium containing BA (0-25 µM) as well as 2,4-D (0-5 µM) in combination with each other (Table 4) with sucrose (3%) which to the formation of friable callus. Increase in concentration of both BA and 2,4-D in MS medium increases the percent callus initiation from the explants. On MS medium supplemented with all combinations of BA (0-25 µM), callogenesis was observed but the maximum frequency of callus inductionwas observed at lower concentration of BA in comparative to higher concentration. Differential response from both explants was observed. With increase in 2,4-D in MS medium, callus induction from internodal segments was decreased whereas in leaf explants, increased callogenesis was

observed with increased auxin levels. Maximum frequency of callus induction was observed on medium supplemented with higher concentration of BA (25 µM) and 2,4-D (0-5 µM).

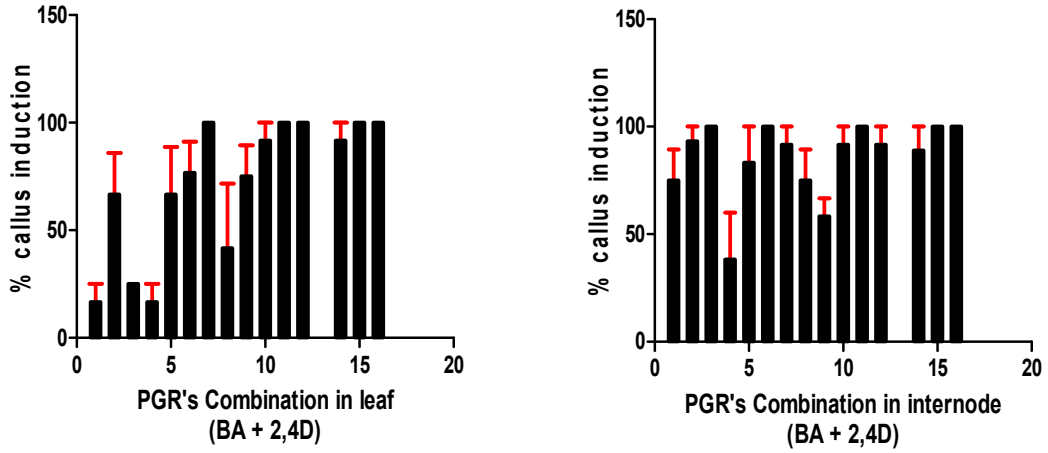

Fig 6: Graphs showing callus induction on explants (leaf and internode) of *Eucalyptus tereticornis* using plant growth regulators (BA and 2,4-D) supplemented on MS medium.

Table 4: Plant growth regulators effect on callus initiation in *Eucalyptus tereticornis* cultured on MS medium with different concentrations of BAP and 2, 4-D.

Sr. No.	PGRs combination		% callus induction from explants (Mean ± SE)		Explants showing friable callus	
	BAP (µM)	2,4-D (µM)	Leaf	Internode	Leaf	Internode
1	0	0	16.68 ± 8.33	75 ± 14.43	Friable	Friable
2	1	0	66.66 ± 19.25	93.33 ± 6.67	Friable	Friable
3	5	0	25 ± 0	100 ± 0	Friable	Friable
4	25	0	16.68 ± 8.33	38.33 ± 21.67	Friable	Friable
5	0	1	66.68 ± 2.05	83.33 ± 16.67	Friable	Friable
6	1	1	76.67 ± 14.53	100 ± 0	Friable	Friable
7	5	1	100 ± 0	91.68 ± 8.33	Friable	Friable
8	25	1	41.67 ± 30.05	75 ± 14.43	Friable	Friable
9	0	2.5	75 ± 14.43	58.33 ± 8.33	Friable	Friable

10	1	2.5	91.68 ± 8.33	91.67 ± 8.33	Friable	Friable
11	5	2.5	100 ± 0	100 ± 0	Friable	Friable
12	25	2.5	100 ± 0	91.67 ± 8.33	Friable	Friable
13	0	5	0 ± 0	0 ± 0	--	--
14	1	5	91.68 ± 8.33	88.89 ± 11.11	Friable	Friable
15	5	5	100 ± 0	100 ± 0	Friable	Friable
16	25	5	100 ± 0	100 ± 0	Friable	Friable

Leaf and Internodes were used as explants from 30 days old microshoots of *Eucalyptus tereticornis*. The data was recorded after incubating for 6 weeks and was analysed by Graph pad prism.

The effect of BA and 2,4-D singly and in combination with each other was also examined on explants of *E. tereticornis.* Although, pale yellow coloured friable callus was observed in all combinations of BA and 2,4-D, but, higher concentration of BA (25µM) and 2,4-D (5µM) resulted in increased callus induction.

The effects of auxins (NAA and 2,4-D) were examined alone and in combination with each other and induction of callus was observed. The callus was friable and pale yellowish in colour whereas in few combinations compact callus was also observed which was greenish in colour and maximum frequency for inducing callus was observed at higher concentration of NAA and 2,4-D.

The best results for initiation of somatic embryos were observed at various combination of NAA with BA. Histological studies proved that the observed samples were somatic embryos as developmental globular shaped stage was observed. Explants differentiated into somatic embryos were transferred on MS medium supplemented with BA (1 µM) and GA_3 (1 µM) for germination of embryos into plantlets. Shoot regeneration was observed from embryo like structures.

CHAPTER 5 DISCUSSION

Ability of a somatic cell to differentiate into somatic embryo is known as somatic embryogenesis (Verdeil et al. 2007). Somatic embryos produced during embryogenesis pathway have the ability to germinate into whole plants. It is the commonly used protocol for conservation of many trees (Jain, 1999; Germana and Lambardi, 2016). Somatic embryogenesis has been widely reported in many plants including *Eucalyptus* (Arezki et al. 2000; Dibax et al. 2005; Barrueto et al. 1999; Mullins et al. 1997; Muralidharan and Mascarenhas, 1987; Prakash and Gurumurthi, 2005; Tournier et al. 2008; Pinto et al. 2008aBandyopadhyay et al. 1999; Merkle et al. 1995).

In many *Eucalyptus* species such as *E. grandis*, *E. nitens, E. globulus, E. dunnii, E. citiordia, Eucalyptus camaldulensis,* initiation of somatic embryos has been reported from various explants such as zygotic embryos, seeds and their fragments, anther filament, shoot meristem, petioles, leaves, roots, seeds, cotyledons (Arezki et al. 2000; Barruetoet al. 1999; Thorpe, 2012;Gaj, 2004; Muralidharan et al, 1989; Prakash and Gurumurthi, 2005, 2010; Pinto et al. 2002, 2008b; Le Roux et al. 1991; Lakshmi et al. 1986; Watt et al. 1991, Kaur et al. 2017). But in case of *E. tereticornis*, somatic embryo induction has been reported only from immature zygotic embryos (Prakash and Gurumurthi, 2005).Generally, somatic embryo induction is achieved in two stages; firstly callus is induced from the explants and then somatic embryos differentiate from callus (Kumar et al. 2010; Vasil, 1999; Canhoto et al. 1996, 1999). Somatic embryogenesis is reported to be affected by many factors (Dibax et al. 2005; Corredoira et al. 2015; Subbaiah and Minocha, 1990; Merkle and Dean, 2000; Thorpe, 2012; Giri et al. 2004).Among all other factors, plant growth regulators (PGRs) play an important role in inducing callus and somatic embryogenesis. They influence morphogenesis and development of tissue/cell under both tissue culture and natural conditions (Nandi et al. 1996; Tiwari et al. 2006; Yu and Wei, 2008). It was well documented that presence of PGRs, especially auxins in the embryogenesis medium result in an increase in endogenous auxin levels, which plays vital role in somatic embryo induction (Gaj, 2004; Jimenez, 2005). It has been reported that auxins and cytokinins present in medium either singly or in combination with each other help in reactivating the cell cycle and initiate somatic embryo formation (Thorpe, 2012). In the present study, the effect of PGRs namely NAA, BA and 2,4-D on induction of somatic embryos from leaf and internodal segments taken from microshoots of

Eucalyptus tereticornis was studied. All the experiments were carried out on MS medium supplemented with various auxins and cytokinins. It was observed that higher concentration of BA and NAA (5 µM) led to the development of somatic embryo like structures from both explants (leaf and internodal segments). Similar results have already been reported in many *Eucalyptus* species such as *E. grandis, E. globulus, E.nitens* and *E. gunnii*where somatic embryos were induced from explants like hypocotyl, leaves, internodes etc. on MS medium supplemented with 0.5 mgl^{-1} concentration of BA (Lakshmi, 1986; Boulay, 1987; Bandyopadhyay et al. 1999).

It was interesting to note that the presence of NAA in MS medium leads to the differentiation of pale yellow coloured friable callus from the explants. It was observed that this callus does not differentiate into somatic embryos. Further, when NAA was present in medium in combination with BA, somatic embryo induction was observed. A similar observation has been reported *E. tereticornis*, where zygotic embryo tissue was used for somatic embryo induction on MS medium supplemented with NAA and BA (Prakash and Gurumurthi, 2005) Like NAA, the presence of 2,4-D in medium also result in callus induction. It was observed that higher concentration of NAA and lower concentration of 2,4-D, when used in combination with each other induces nodular callus from explants whereas other combinations leads to friable callus induction. Difference in response of explants to medium combination was observed during the study. Although, friable callus was observed from leaf explants of *E. tereticornis* on medium supplemented with 2,4-D but no callogenesis was observed from internodal explants on same medium combinations. These results were in line with previous studies where leaf explants were cultured on 2,4-D rich medium to induce callus (JayaSree et al. 2001).

Further, in the present study it was observed that all combinations of BA and 2,4-D induce friable callus. Whereas maximum frequency of induction of friable callus was observed on MS medium supplemented with higher concentration of BA (5µM) and/or in combination with 2,4-D (5-25 µM). However, this callus did not differentiate into somatic embryos. In contrast to the present observations, direct somatic embryogenesis has been reported from hypocotyls of *E. camaldulensis* cultured on MS medium supplemented with BA without callogenesis (Girijashankar, 2012).In the present study, embryo like bodies when transferred on to MS medium containing BA (1 µM) and GA_3 (1 µM) germinated into shoots within 6 weeks. The use of auxin free medium to convert somatic embryos into complete plant has been reported in many plants (Kaur et al. 2017; Khilwaniet al. 2016; Sharma and

Ramamurthy, 2000, JayaSree et al. 2001). It has been reported that gibberlic acid (GA_3) plays important role in cell elongation whereas BA helps in cell division (Pinto et al. 2002, 2008b). In several *Eucalyptus* species, such as, *E. camaldulensis, E. citriodora, E. grandis*and *E. tereticornis* similar studies has been reported where plant regeneration was observed on MS medium containing higher concentration of GA_3 (Girijashankar, 2015; Muralidharan and Mascaranhas, 1987; Watt et al. 1991; Pinto et al. 2002, 2008c; Trindade, 1996; Prakash and Gurumurthi, 2005) . It was reported that the presence of auxins in the medium inhibit the maturation of somatic embryos, thus their germination (Prakash and Gurumurthi, 2010).

Somatic embryos like structures developed from leaf and internodal segments were studied through histological and scanning electron microscopic studies. Somatic embryos were observed to progress through globular, heart shaped, torpedo shaped to cotyledonary stage which can develop into whole plant. The present study showed early developmental stages of somatic embryogenesis. Similar results have been earlier observed in *Bacopamonnieri*(Khilwani et al. 2016).

Present study demonstrates the induction of somatic embryogenesis on MS medium supplemented with BA and NAA. According to best of our knowledge, this is the first study describing somatic embryo induction from tissue taken from mature selected elite plants of *Eucalyptus tereticornis.* The induction of somatic embryogenesis from the explants (leaves and internodes) of *Eucalyptus tereticornis* opens a new road to overcome the problems of slow seed germination. According to Park et al. (1998) somatic embryogenesis is cheaper method than other clonal propagation techniques. Somatic embryogenesis offers possible ways of mass multiplication of plants in less time duration and also helps in preserving large number of genotypes in confined space. Thus, scale up of plant propagation become easier.

CONCLUSION

In this study,the effect of plant growth regulators such as 2,4-D, BA and NAA on somatic embryogenesis from leaf and internodal explants taken from 21 days old microshoots of elite clone 'T1' of *Eucalyptus tereticornis* was studied. Salient observations of the study are given below:

- MS medium supplemented with 5µM NAA and 5 µM BA resulted in maximum somatic embryos induction from both leaf and internodal explants.
- Although, presence of 2,4-D in the medium fails to induce somatic embryos in all explants of *Eucalyptus tereticornis*, but another auxin NAA alone or in combination was able to induce somatic embryos in leaf explants and internodal explants.
- Early developmental stages of somatic embryos were observed on MS medium supplemented with BA and NAA. Upon transfer of these embryos to MS medium supplemented with GA_3 (1µM) and BA (1µM), germination was observed.
- High level of BA (25 µM) and 2, 4-D (5 µM) induces friable callus .Although, higher concentration of NAA and low concentration of 2,4-D leads to the induction of nodular callus from leaf explants whereas for callus induction from internodal explants, low concentration of NAA and higher concentration of 2, 4-D was found to be essential.

Thus, from this study it was concluded that plant growth regulators plays an important role in inducing somatic embryos as well as callus from leaf and intermodal explants of *Eucalyptus tereticornis*. It was observed that an optimised concentration of auxin and cytokinin for somatic embryogenesis was required. Further the developed embryos like structure were transferred to germination medium which was free of auxins or contain very less concentration of auxins which helps in germination of embryos into fully developed plantlets.

APPENDIX

Table 5: Murashige and Skoog (MS) media (1962) composition.

S. No.	Components	Concentration
Macronutrients (mg/l)		
1.	Ammonium Nitrate (NH_4NO_3)	1600
2.	Potassium Nitrate (KNO_3)	1900
3.	Calcium Chloride ($CaCl_2.2H_2O$)	440
4.	Magnesium Sulfate ($MgSO_4.7H_2O$)	370
5.	Potassium Phosphate (KH_2PO_4)	170
Micronutrients (mg/l)		
6.	Manganese Sulfate ($MnSO_4.H_2O$)	16.9
7.	Zinc Sulfate ($ZnSO_4.7H_2O$)	8.6
8.	Boric Acid (H_3BO_3)	6.2
9.	Potassium Iodide (KI)	0.83
10.	Sodium Molybdate ($Na_2MoO_4.2H_2O$)	0.25
11.	Cupric Sulfate ($CuSO_4.6H_2O$)	0.025
12.	Cobalt Chloride ($CoCl_2.6H_2O$)	0.025
Vitamins (mg/ml)		
13.	Nicotinic acid	0.5
14.	Pyridoxine HCL	0.5
15.	Thiamine HCl	0.1
16.	Thiamine HCl	1
Freshly added components (g/l)		
17.	Myo-inositol	0.1
18.	Sucrose	30
19.	Agar	0.03

Table 6: TBA series followed for dehydrating selected plant sample.

Sr. No.	Rectified alcohol (ml)	t-butyl alcohol (TBA) (ml)	Water (ml)	Time (hr)
1.	30	20	50	3-4
2.	50 + few drops of Eosin	20	30	3-4
3.	50	35	15	3-4
4.	45	55	-	3-4
5.	25	75	-	3-4
6.	-	100	-	3-4

CHAPTER 6 REFERENCES

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Thesis

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Barkha Khilwani, Amanpreet Kaur, Ritika Ranjan, Anil Kumar. "Direct somatic embryogenesis and encapsulation of somatic embryos for in vitro conservation of Bacopa monnieri (L.) Wettst", Plant Cell, Tissue and Organ Culture (PCTOC), 2016 Publication	%2
2	Forestry Sciences, 2000. Publication	%2
3	Pinto, Glória, Clara Araújo, Conceição Santos, and Lucinda Neves. "Plant regeneration by somatic embryogenesis in Eucalyptus spp.: current status and future perspectives", Southern Forests a Journal of Forest Science, 2013. Publication	%1
4	Biotechnological strategies for the conservation of medicinal and ornamental climbers, 2016. Publication	%1
5	iufro20902.org Internet Source	