BIOCHEMICAL STUDIES IN PLANT TISSUE CULTURE WITH REFERENCE TO HYPERHYDRATION

A THESIS SUBMITTED

TO

THE UNIVERSITY OF PUNE

FOR THE DEGREE OF

MASTER OF SCIENCE (P.P.P.R.)

IN BIOCHEMISTRY

(Ref. No. M. Sc./ Research/ 809 ,1993)

BY

MR. RAMLING HARIBHAU TABE
B. Sc.

TISSUE CUTLURE PILOT PLANT
NATIONAL CHEMICAL LABORATORY
PUNE - 411 008

MARCH 2000

DEDICATED TO

MY

DIVINE MOTHER

CONTENTS

ACKNOLEDGEMENT

I am grateful to **Dr. (Mrs.) Rajani S. Nadgauda** , my research guide, who agreed to accept me as her student and for suggesting this research problem and giving her valuable suggestions from time to time.

I am thankful to Dr. (Mrs.) I. S. Poonawala for her painstaking help in preparation of this manuscript. I am also thankful to Mr. S. V. Shirke for the help rendered by him during the histology work.

I wish to express my sincere thanks to Dr. A. F. Mascarenhas, Mrs. S. V. Kendurkar, Mrs. V. A. Parasharami, Mrs. M. V. Shirgurkar, Dr. S. V. Paranjpe, Dr. C. H. Phadke. Dr. M. M. Jana, Mr. A. L. Nadgir, Mr. V. M. Kulkarni and Dr. C. K. John for their constant encouragement during the course of this research work.

I would like to thank all my colleuges working in the Tissue Culture Pilot Plant Division, at National Chemical Laboratory, for their encouragement and timely help during the course of this research work, without which it would have not been possible for me to complete this task.

I wish to appreciate the efforts taken by Mr. P. R. Akkadkar for the photography work.

I am greatly indebted to my wife Anita and my daughters Anuprita and Apurva for their efforts to spare me from the domestic problems and their constant encouragement for this research work.

And last but not least, I am thankful to **Dr. Paul Ratnasamy** for granting me permission to submit this research work in the form of a thesis to the University of Pune, for my masters degree.

RAMLING H. TABE

DECLARATION

This is to certify that the research work incorporated in this thesis entitled **"BIOCHEMICAL STUDIES IN PLANT TISSUE CULTURE WITH REFERENCE TO HYPERHYDRATION"** submitted by **Mr. Ramling H. Tabe** was carried out by him under my guidance at the Tissue Culture Pilot Plant Division, National Chemical Laboratory, Pune- 411 008. Such material as has been obtained from other sources has been duly acknowledged in this thesis.

RSNadgauda 30/3/2011

Dr. (Mrs.) Rajani S. Nadgauda
(Research Guide)

LIST OF ABBREVATIONS

BA ---- 6-Benzylaminopurine

CH ---- Casein hydolysate

IAA ---- Indole-3-acetic acid

IBA ---- Indole-3-butyric acid

IPA ---- Indole-3-propionic acid

KIN ---- Kinetin (6 Furfurylaminopurine)

MS ---- Murashige and Skoog basal medium

ABSTRACT

ABSTRACT

The concept of Plant Tissue Culture, one of the important areas of Plant Biotechnology started way back in 1838-39 when Schleiden and Schwann put forth the cell theory. At the beginning of the 20th century a German scientist G. Haberlandt, put forth the theory of cell totipotency, which in turn gave birth to various plant tissue culture techniques. Plant cells are totipotent, they have a full potential or inherent capacity to develop in to an entire plant, when provided with a suitable environment.

Based on this hypothesis, since 1902, several attempts were made world wide to grow plant tissues under culture conditions. the important discoveries such as the effect of the auxin:cytokinin ratio on the growth of the cultured tissue (Skoog & Miller, 1957), formulation of various culture media (Gamborg et al, 1968; Murashige & Skoog, 1962 White, 1963) and meristem tissue culture (Morel G. 1960, Murashige T. 1974 &1978) led to several published reports on successful culture of plant tissues belonging to various genera and species. In 1960 G. Morel used plant tissue culture technique, for clonal multiplication of orchid species. This technique, now known as"Micropropagation" has been applied to many plant species of agricultural, horticultural and socio-economic importance during recent years.

Ideally micropropagation means in vitro propagation of plants using apical meristem tissue or axillary bud explants. The process of micropropagation takes place in following stages:

Stage -1: In vitro regeneration and elongation of shoots from suitable explants, mostly apical bud or anxillary bud.

Stage -2: Multiplication of shoots in vitro.

Stage -3: Root initiation and root elongation on cultured shoots in vitro or ex vitro.

Stage -4: Acclimatization and hardening of plantlets in the green house.

The first two or three phases are developed under laboratory conditions, i.e. the explants are cultured on synthetic growth medium under controlled conditions of light, temperature and relative humidity, also called in vitro conditions.

One of the serious problems faced by the micropropagation industry is the hyperhydration of the cultures, which can lead to substantial economical losses affecting the economical viability of the technology (Debergh and Maene 1984).

Hyperhydration, also called as vitrification is a physiological state which appears as an effect of prolonged growth under in vitro conditions and is characterized by the production of shoots with thick, succulent stem and glassy and transparent leaves. Such shoots give very low percentage of survival during following phases i.e. rooting, acclimatization and hardening and subsequent

transfer to soil. This results in high mortality of plants leading to severe financial losses.

Various factors such as the chemical constituents of the culture medium namely minerals, growth regulators and gelling agent and environmental factors namely light, temperature and relative humidity play active part in the process of hyperhydration.

The present research work was undertaken with a view to understand the role of various chemical constituents of the growth medium in hyperhydration and to explore the possibilities of reversion of hyperhydric cultures to normal state in two economically important tree species *Eucalyptus tereticornis* and *Eucalyptus camaldulensis* belonging to Family Myrtaceae

For the sake of convenience the dissertation is sub-divided in four chapters as follows:

Chapter 1: Introduction

This chapter gives a brief account of an introduction to the problem of hyperhydration. The various factors actively participating in the phenomenon of anatomical, and physiological aspects of hyperhydration are discussed.

Chapter 2: Materials and Methods

This chapter deals with the materials and methods followed during this investigation such as glassware preparation, media preparation, autoclaving, incubation, *ex vitro* rooting of shoots etc. and are discussed in details.

Chapter 3: Effect of Media Composition

In this chapter the effect of modification of the culture medium on the reversion of hyperhydric shoots to normal ones are discussed with the help of the results obtained during this investigation.

Chapter 4: *Ex vitro* Rooting of Microshoots

This chapter is devoted to discuss the comparative studies on *ex vitro* rooting of the non-hyperhydric normal shoots, hyperhydric shoots and the reverted normal shoots produced during this investigation.

Conclusions

The conclusions drawn based on the observations made during this investigation are discussed.

CHAPTER 1

GENERAL INTRODUCTION

1.1 PLANT TISSUE CULTURE - A BRIEF INTRODUCTION

The concept of plant tissue culture, one of the important areas of Biotechnology started way back in 1838-39 when Schleiden and Schwann put forth the cell theory stating that cell is a basic unit of life. The cell totipotency in plant cell is "the inherent capacity of plant cell to give rise to a complete plant under favourable growth conditions". The idea of cell, tissue culture was put forth by the German botanist Gottlieb Haberlandt in 1902. In 1934, White could grow tomato roots in a liquid medium. The possibilities of culturing plant tissues for indefinite periods was independently reported by White, Nobecourt and Gautheret in 1939.

After the discovery of auxins viz. IAA and cytokinin zeatin and ribosyl zeatin etc. Skoog and Miller demonstrated a crucial role of auxin:cytokinin ratio in regulation of plant morphogenesis *in vitro* in 1957. The concept of somatic embryogenesis as an alternative pathway of plant production was first demonstrated by Reinert in late 1950's in carrot.

In 1962, Murashige and Skoog, proposed a revised medium for nutritional requirements of tobacco tissue culture. This medium formulation proved to be very useful for culturing a wide variety of plants. Since then many scientists proposed various mineral requirements for different plant species. Vasil and Hildebrandt (1965) were the first to demonstrate the theory of totipotency by isolating a tobacco cell and

producing a whole plant out of it. This has resulted in the development of various branches of Plant Tissue Culture. The major ones are listed below-

Micropropagation

Haploids and triploid culture

Embryo culture

Somaclonal variation

Secondary metabolites

Protoplast fusion

Genetic engineering

Today plant tissue culture has become a centre piece of plant biotechnology (Nadgauda. 1998) and micropropagation is the area which has been exploited commercially.

1.2 MICROPROPAGATION:

Ideally, micropropagation means clonal propagation of plants through shoot tip or axillary bud cultured *in vitro*, on mass scale. Micropropagation has many advantages over conventional methods of vegetative propagation such as budding, grafting etc. These are :

1) Production of significantly greater number of plants in less time and less space compared to other methods of vegetative propagation.
2) Round the year production of plants irrespective of the seasonal variations.

3) Production of disease/virus-free plants.

4) Production of true to type plants.

5) Vegetative propagation of plant species where it is difficult or impossible by conventional methods.

During recent years, micropropgation has been widely used in clonal multiplication of many plant species of agricultural, horticultural and socio-economic importance. This cover several plant genera viz. ornamentals, plantation crops, fruits and vegetables, medicinal and aromatic plants and forestry species etc.

1.3 THE BASIC STEPS IN MICROPROPAGATION :

There are four basic steps generally required for micropropagation of plant species. These are

1) Initiation

2) Multiplication

3) Rooting

4) Hardening or acclimatization.

In this process, an explant is collected from an identified clone of a plant species and the cultures are initiated on a culture medium after surface sterilization. Once the cultures are established, they are multiplied to get a large number of shoots. These shoots are then rooted under *in vitro* or *ex vitro* conditions and are taken to the polyhouse for further hardening and acclimatization.

1.4 MICROPROPAGATION INDUSTRY:

Micropropagation industry is one of the important contributions of plant tissue culture to commercial plant propagation. Several Micropropagation industries are developing day by day and are gaining importance considering the advantages of plant propagation by this method. However, amongst the problems which are faced by the micropropagation industries, the major once are -

1) Contamination

2) Health of the shoots.

These problems are directly related to final survival of the plants. In any of these problems, the ultimate result is the financial losses. Lots of experiments have been carried out to overcome these problems and have been discussed at different fora. The solutions of which being varying from industry to industry and species to species. During the present investigations, the problem of health of the shoots with special reference to hyperhydricity has been dealt with.

1.5 HYPERHYDRATION:

Hyperhydricity (previously known as vitrification) is a phenomenon of abnormal morphological appearance and physiological function of tissue cultured plant material (Debergh, 1992). The hyperhdricity is acquired as a result of the chemical and

environmental stress caused to the growing tissue for a prolonged period under culture conditions (Ziv, 1986).

The plants, which are propagated *in* vitro, are exposed to various culture conditions, both chemical as well as physical. If not properly controlled, these can lead to, morphological and anatomical disorders. For a very long time, these disorders in the tissue cultured plantlets were termed as **"vitrification"**. But the term vitrification is misleading since it refers to a physical and not a biological process. Hence these anamolies are now being termed as **"hyperhydration"**(Debergh *et al.*, 1992).

In other words, a hyperhydric plant can be a vitrified one but vice versa is not necessarily always true. Physically, a hyperhydric shoot can be identified as having thin, glassy leaves and thick water-soaked and translucent stem (Debergh and Maene, (1984). Hyperhydric shoots appears watery as they contain more water in their intracellular space and contain less lignin than normal shoots (Gasper, 1985). The hyperhydricity is mainly seen in leaves and stem and to a lesser extent in the roots (Ziv, 1986).

1.6 PHYSIOLOGICAL AND BIOCHEMICAL CHANGES OCCURING DURING HYPERHYDRICITY:

Symptoms characterizing hyperhydricity are not identical in all the plant species. Development of the morphological symptoms of hyperhydricity depends upon multiple factors expressed over time. Hyperhydric cultures show abnormalities with

respect to the anatomy of the shoot parts. The different anatomical and physiological defects are the results of disorders in the metabolic pathways. Morphological expression in hyperhydric shoots under *in vitro* conditions is a very late symptom. The anatomical and physiological changes initiate much earlier before hyperhydration become apparent. Change of leaf anatomy is considered to be the first precursor of hyperhydric state. As stated by Paques and Boxus (1987a) and Ziv (1986), vitrifiction involves multiple factors such as culture conditions and the species under study.

The anatomical and biochemical features of hyperhydric tissue mainly include:

- lower cellulose and chlorophyll content (Gasper, 1985).
- more ethylene production (Gasper,1985 and Leonhardt and Kandeler, 1987).
- appearance of abnormal or non-functional stomata (Werker and Leshem, 1987, Mohammed Yaseen *et al.*, 1992 and Miguens *et al.*, 1993).
- discontinuous cuticle (Miguens *et al.*, 1993).
- abnormal epidermis (Leshem 1983 and 1988 and Jones *et al.*, 1993).

Uptill now there is no sole opinion on the causes of hyperhydricity. The main factors responsible for inducing hyperhydration are the physical and biochemical environment provided for the growth of the culture under *in vitro* conditions. These include high humidity, superfluous supply of nutritional factors, both minerals and carbohydrates, high levels of growth regulators and low light intensity (Borkowska and

Oplikowska, 1988, Bottcher *et al.*, 1988, Debergh *et al.*, 1981,Gasper *et al.*, 1987,Paques and Boxus 1987, Leshem and Sachs, 1985, Pasqualetto *et al.*,1986 and 1988). It has been suggested that all these exogenous factors act as signals inducing an excessive ethylene production, which through a definite sequence of biochemical reactions, leads to hyperhydricity (Kevers *et al.*, 1984). The visual symptoms of hyperhydration can be avoided by controlling the composition of the culture medium, the environmental conditions in which the culture containers are incubated and the quality of the cultured explants.

1.7 NEED FOR CONTROLLING HYPERHYDRATION:

Hyperhydricity has been known as a serious problem in many plant tissue culture systems because, the hyperhydric material are unable to grow into normal plant and once the plant material become hyperhydric, it is hard for it to come back to a normal state (Bottcher *et al.*, 1988, , Leshem *et al.*, 1988 and Ziv, 1986). Many studies have dealt with overcoming hyperhydricity by raising the medium rigidity, increasing ventilation and controlling temperature (Dillen and Buysens,1989 Selby *et al.*, 1989 and Williams and Taji, 1991). In *Pinus radiata*, hyperhydricity resulted in upto 90% multiplication losses and failure to survive *ex vitro* acclimatization (Nairn *et al.*, 1995).

Hyperhydric tissue have poor water regulation and as a result, when transplanted *ex vitro*, they easily dessicate and die (Daguin and Letouze, 1986 and Pasqualetto *et al.*, 1988). They require a gradual transition period to acquire normal morphology enabling their survival. In several species, a few of the anamolies persisting *in vitro* in

organs before transplanting can not be repaired even after surviving *ex vitro*. In apple and other woody species, hyperhydric leaves can survive but do not expand to full size (Ziv, 1991). Also, vitrified plantlets *ex vitro* are very susceptible to fungal attack and are sensitive to application of antifungal chemicals. Hyperhydricity, therefore decrease the production efficiency of micropropagation and increase the final cost of the plants (Navatel, 1982 and Gribble *et al.*, 1996). What therefore needed is to control the conditions *in vitro* so as to revert the hyperhydric cultures and to promote normal morphogenesis. According to Gasper (1995), in most cases vitrification is reversible, which means that some apices of vitrified shoots placed in nonvitrifying conditions can function normally and give rise to normal plants.

An attempt was therefore made in the present investigation to revert the hyperhydric shoots of two *Eucalyptus* species viz. *E. tereticornis* and *E. camaldulensis* by controlling the chemical environment under *in vitro* conditions.

AIMS OF THE PRESENT INVESTIGATION

In the present investigation, the problem of hyperhydration in the micropropagation of two economically important species of ***Eucalyptus*** viz. ***Eucalyptus tereticornis*** and ***Eucalyptus camaldulensis*** has been studied.

Eucalyptus species are universally known for rapid production of woody biomass. Their wood is excellent for pulp and paper production and as raw material for rayon industries. The wood is also a good source of fuel. Leaves from several ***Eucalyptus*** species yield useful essential oils (John *et al.*, 1998).

In nature, ***Eucalyptus*** is regenerated by seeds. The conventional methods of clonal propagation failed to give promising results in most of the ***Eucalyptus*** species. Therefore, micropropagation is being used for clonal propagation of elite genotypes of ***Eucalyptus*** species (Gupta and Mascarenhas, 1987).

At the National Chemical Laboratory, Pune, a tissue culture pilot plant facility has been established for micropropagation of three economically important forest tree species viz. ***Eucalyptus, Teak and Bamboo.***

During the course of time it has been observed that after prolonged growth under *in vitro* conditions, the cultures of ***Eucalyptus*** species show the symptoms of hyperhydration, charecterised by appearance of shoots with thick, water-soaked and transluscent stem and thin, glassy and transparent leaves under *in vitro* conditions. An attempt was therefore made to revert back such shoots to their normal morphology.

This investigation was carried out to study:

1) Reversion of hyperhydrated cultures of *Eucalyptus tereticornis* and *Eucalyptus camaldulensis* to normal cultures, by modifying the chemical composition of the culture medium.
2) Comparison of anatomy of the leaves and stem of the shoots from hyperhydrated cultures reverted to normal cultures with those from the normal cultures.
3) *Ex vitro* rooting and hardening of the shoots from normal, hyperhydrated and reverted cultures.

CHAPTER 2

MATERIALS AND METHODS

2.1 GLASSWARE:

Autoclavable 400 gm. capacity jam jars (M/S Excel Glass Works Ltd., Kochin) capped with polypropylene screw type caps (M/S Laxbro Ltd., Pune) were used as the culture vessels.

The culture vessels and the caps were first soaked in the diluted solution of detergent labolene (M/S Glaxo India Ltd., Mumbai) and rinsed repeatedly under running tap water. Washed bottles and caps were allowed to dry at ambient temperature on a draining rack.

2.2 CHEMICALS:

The inorganic chemicals were of AR grade manufactured by M/S S.D. Fine Chemicals, Mumbai. The fine chemicals such as vitamins, plant growth regulators, amino acid as well as antivitrifying agent manufactured by M/S Sigma-Aldrich Ltd. U.S.A. were used. Sucrose and gelling agent agar-agar manufactured by M/S Glaxo India Ltd., Mumbai were used.

2.3 STOCK PREPARARTION:

Stock solutions of appropriate concentrations of the inorganic salts, vitamins, amino acid and the plant growth regulators were prepared in double distilled water and stored in the refrigerator at 4 °C . (Table 2.1).

TABLE 2.1 STOCK SOLUTIONS

Mineral Salts:

Stock code No.	Chemical	mg/lit.	Strength	ml/ lit. of medium used
M 1	Ammonium nitrate (NH_4NO_3)	33000	20X	50
M 2	Potassium nitrate (KNO_3)	38000	20X	50
M 3	Calcium chloride ($CaCl_2.2H_2O$)	8800	20X	50
M 4	Magnesium sulphate ($MgSO_4.7H_2O$)	7400	20X	50
M 5	Potassium dihydrogen orthophosphate (KH_2PO_4)	3400	20X	50
M 6	Boric acid (H_3BO_3) Potassium iodide (KI) Sodium molybdate (Na_2MoO_4) Copper sulphate ($CuSO_4.5H_2O$) Cobalt chloride ($CoCl_2.4H_2O$)	1240 166 50 5 5	200X	5
M 7	Manganese sulphate ($MnSO_4$) Zinc Sulphate ($ZnSO_4$)	4460 1720	200X	5
M 8	Ferrous sulphate ($FeSO_4.7H_2O$) Ehtylene-diamino-tetraacetic acid-disodium salt (Na_2EDTA) (As chelate)	5560 7460	200X	5

Vitamins:

Stock code No.	Chemical	mg/lit.	Strength	Ml /lit. of medium used
V 1	Nicotinic acid Pyridoxine-HCl Thiamine-HCl Inositol	25 25 5 5000	50X	20
V 2	Calcium Pantothenate	10 mg/100 ml	200X	0.05
V 3	D-Biotin	10 mg/100 ml	200X	0.05

Growth Regulators:

Stock code No.	Growth regulator	Mg/100 ml
H 1	6-Benzyl-amino purine (BA)	10
H 2	Kinetin (KIN)	10

Amino Acid:

Stock code No.	Amino Acid	mg/100 ml	Strength	ml/ lit. of medium used
A 1	Glycine	100	50x	2

2.4 MEDIA PREPARATION:

During media preparation, appropriate aliquots of the stock solutions were mixed with double distilled water, required quantities of sucrose was dissolved separately, mixed with above solution and the volume was made upto the half of the desired volume. The pH of the medium was adjusted to 5.7 ± 0.1 with 1N NaOH or 1N HCl before autoclaving. The gelling agent agar-agar was weighed, mixed with remaining half of double distilled water and was steamed to melt. This melted gelling agent was then mixed to make the final volume.

50 ml of the medium, thus prepared was then poured into each preautoclaved culture vessels. The culture vessels were capped and then sterilized by autoclaving.

2.5 AUTOCLAVING:

The glassware and the planting substrate viz. the mixture of sand, soil and farm yard manure were autoclaved in an autoclave at 121 °C at 15 lbs/ $inch^2$ for 1 h., whereas the media were autoclaved for 20 mins.

2.6 MEDIA USED:

Control medium: Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) containing different hormones was used for the multiplication of both the *Eucalyptus* species (Gupta and Mascarenhas, 1987). This medium served as the control medium, henceforth referred to as MS I. Composition of MS I is given in Table 2.2.

TABLE 2.2 COMPOSITION OF CONTROL MEDIUM (MS I)

Eucalyptus multiplication Medium (Gupta and Mascarenhas, 1987).

Ingredients	Concentration (mg/l)
Ammonium nitrate (NH_4NO_3)	1650.000
Potassium nitrate (KNO_3)	1900.000
Calcium chloride ($CaCl_2.2H_2O$)	440.000
Magnesium sulphate ($MgSO_4.7H_2O$)	370.000
Potassium dihydrogen orthophosphate (KH_2PO_4)	170.000
Boric acid (H_3BO_3)	6.200
Potassium iodide (KI)	0.830
Manganese sulphate ($MnSO_4$)	22.300
Zinc sulphate ($ZnSO_4$)	8.600
Sodium molybdate (Na_2MoO_4)	0.250
Copper sulphate ($CuSO_4.5H_2O$)	0.025
Cobalt chloride ($CoCl_2.6H_2O$)	0.025
Ferrous sulphate ($FeSO_4.7H_2O$)*	27.800
Ethylene- diamine-tetraacetic acid* (Disodium salt) (Na_2EDTA)	37.300
Thiamine hydrochloride	0.100
Nicotinic acid	0.500
Pyridoxine hydrochloride	0.010
Myo-inositol	100.000
Glycine	2.000
6-Benzylaminopurine (BA)	0.100
Kinetin (Kin)	0.050
Calcium Pantothenate	0.050
D-Biotin	0.050
Sucrose	20000.000
Agar-agar	7000.000

* Used as chelate.

Following modifications were done in MS I medium by addition or deletion of different chemicals, to see their effect on the reversion of the hyperhydric shoots to normal ones.

Effect of nitrates: To see the effect of nitrates on the reversion of hyperhydric shoots, ammonium nitrate was reduced to half the strength in MS I medium viz. 825 mg/l --- MS II.

Effect of Hormones: The concentration of BA in MS I was reduced to half the concentration viz. 0.05 mg/l. --- MS III.

Effect of other additives:

a) Use of antivitrifying agent: An antivitrifying agent manufactured by M/S Sigma-Aldrich U. S. A. was used in MS I medium at the concentration of 5.0 gm/l --- MS IV.

b) Use of organic components:

100 and 200 mg/l casein hydrolysate was added to MS I medium --- MS V and MS VI respectively.

2.7 EXPLANT SOURCE:

Cultures of *E.tereticornis* and *E. camaldulensis* with hyperhydric shoots were selected from the cultures growing for the mass scale production of *Eucalyptus*, at Tissue Culture Pilot Plant Facility at National Chemical Laboratory, Pune.,

2.8 MAINTENANCE:

The cultures were regularly subcultured on the control medium viz. MS I after every 45 days of incubation at 25 ± 2 °C under 1500 lux light intensity provided by cool white fluroscent tube lights with 16:8 h photoperiod. Only the hyperhydric shoots from these cultures were then used every time to see the effect of different chemicals for the reversion to normal growth.

2.9 EXPERIMENTATION:

For the experimentation, the hyperhydric shoots from the control medium MS I were subcultured on the experimental media viz. MS II to MS VI. Eight explants, each containing a clump of 2-3 hyperhydric shoots of 1 cm height were inoculated in each bottle with a total of 10 bottles (i.e. 10X8 = 80 explants) and repeated three times. After an incubation period of 45 days, the hyperhydric clumps from these cultures were again subcultured on the same medium for further two cycles. During each subculture the hyperhydric and normal shoots of the height more than 3 cm. were removed out for ex *vitro* rooting and the remaining hyperhydric clump was used for further subculturing.

2.10 INCUBATION CONDITIONS :

All the cultures were incubated in a growth room at 25 ± 2^0 C. under a photoperiod of 16 h light and 8 h dark with a light intensity of 1500 lux provided by cool white fluoroscent light. The observations were made after 45 days of incubation.

2.11 OBSERVATIONS :

The shoots were called normal or hyperhydric based on the following criteria –

1. Leaf colour
2. Leaf consistency
3. Stem colour
4. Stem consistency
5. Internodal distance (cm)

For any chemical treatment, the normal and hyperhydric shoots in each culture bottle were counted and the results were recorded as the average number of normal or hyperhydric shoots obtained per culture bottle (average of 30 bottles: 10 bottles for each treatment and repeated thrice).

2.12 *EX VITRO* ROOTING:

The normal and hyperhydric shoots after removing from the culture bottles were kept in a plastic container with water just sufficient to cover the base of the container to maintain the humidity. The container was covered with a lid till the shoots were planted.

Once brought to polyhouse, the shoots were trimmed from base to a height of 3 cm with a sharp blade and the leaves were removed upto two nodes from downwards. The shoots were then treated with 1% solution of a fungicide, Bavistin (BASF,

Mumbai) for 1 min., followed by a quick dip in an auxin mixture containing IAA, IBA and IPA 1 mg/l each.(Sigma-Aldrich, USA). The shoots were then planted in a polybag of 2'X5' size containing a mixture of soil, sand and FYM in 1:1:1 proportion.

Initially, the polybags were kept under a tunnel covered with a polythene sheet to maintain the humidity of approximately 90% and a temperature of 22 ± 2 °C. Watering was done intermittently to avoid drying.

2.13 OBSERVATIONS FOR *EX VITRO* ROOTING :

Observations for rooting were taken everyday after 15 days of transplanting. The shoots were called rooted based on the visual appearance of root. Percent rooting was calculated based on the total number of shoots planted and that rooted and survive after 30 days. Negative results were confirmed after 30 days of planting.

2.14 HISTOLOGICAL STUDIES :

For histological studies various specimens such as leaves and stem parts from the normal shoots, hyperhydric shoot and the hyperhydric shoot reverted to normal shoot after the experimental chemical treatments were directly taken for microtomy. The sections of 20 µM thickness were cut on fridge microtome (Leica RM 2155, Germany) The sections were then collected on the slide and were observed under microscope (Zeiss, Axioplan-2, Germany) and the microphotographs were taken.

CHAPTER 3

EFFECT OF MEDIA COMPOSITION

RESULTS AND DISCUSSIONS :

In a micropropagation system, hyperhydration can occur at all the stages during initiation, elongation and multiplication. It may occur immediately after the establishment of primary cultures or may arise spontaneously after a number of culture passages.

In the Tissue Culture Pilot Plant at National Chemical Laboratory, Pune, for large scale micropropagation of two *Eucalyptus* species viz. *E. tereticornis* and *E.camaldulensis*, the cultures were initiated from the terminal and axillary buds collected from the identified plus trees located in the forests of different states of India. During their micropropagation, we found that the cultures were normal at the initiation and establishment phase. However, a problem of hyperhydration was faced after many subcultures on the same culture medium. The subculture number at which hyperhydration of shoots was observed varied from species to species and from clone to clone. This indicated that the hyperhydration problem in case of *Eucalyptus* were perhaps due to number of culture passages. Hyperhydration of the cultures, especially at the active multiplication stage creates serious problems, compelling to discard the

cultures. This in turn creates set back to the total planned process and also the cost of the plant. It was therefore decided to study in detail the problem of hyperhydration and the work was initiated to see if such hyperhydric shoots can be reverted back to normal shoots by altering the media components instead of discarding the cultures. A representative of one clone each of *E. tereticornis* and *E. camaldulensis* was selected for these studies.

The shoots were observed for their morphological and anatomical features for the differentiation of hyperhydric and normal shoots.

Morphological Features:

Hyperhydric cultures differed greatly in appearance from normal cultures (Fig.3.1 and 3.2). The parameters used for identifying the normal and hyperhydric shoots are given in table (Table 3.1).

Overall colour of normal shoot (leaves and stem) was dark green with bigger leaves, less number of internodes and more internodal distance. The shoots of the hyperhydric cultures were thicker and pale green with transparent to transluscent stem. Although the total number of leaves were more in hyperhydric shoots as compared to normal one, their leaves were glassy, and transparent. A few of the hyperhydric shoots also showed callus at the base and /or on the stem and leaves.

Fig. 3.1 : Normal and hyperhydric cultures on MS I
A) *E. tereticornis* Left - Hyperhydric Right - Normal
and
B) *E. camadulensis* Left – Normal Right - Hyperhydric

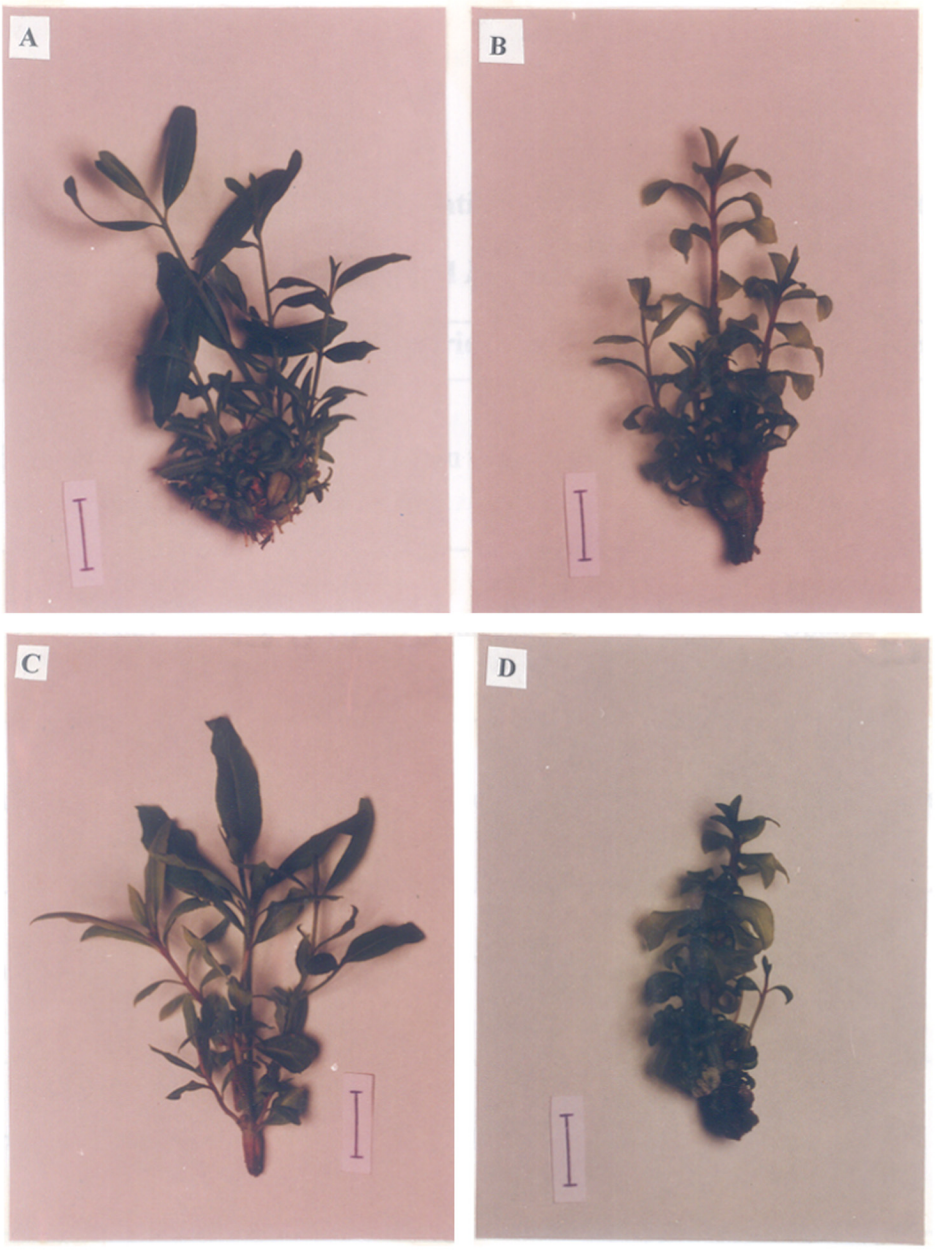


Fig. 3.2 : Normal and Hyperhydric clumps

A) *E. tereticornis* - Normal **B) *E. tereticornis* - Hyperhydric**

C) *E. camaldulensis* - Normal **D) *E. camaldulensis* - Hyperhydric**

TABLE 3.1 Parameters used for differentiating normal and hyperhydric shoots of *Eucalyptus tereticornis* and *Eucalyptus camaldulensis*.

Parameter	Hyperhydric cultures	Normal cultures
1. Leaf Colour	Pale green to green	Dark green
2. Leaf consistency	Glassy, Transparent	Opaque
3. Stem Colour	Reddish to pale green	Green to dark green
4. Stem consistency	Transparent to Transluscent	Opaque
5. Internodal Distance (cm.)	0.5 – 0.8	1.5 – 2.5

The hyperhydric shoots which had reverted to the normal growth were compared morphologically and were found indistinguishable from normal control shoots as far as morphological features were concerned.

Anatomical Features :

Leaves and stem parts of the hyperhydric and normal shoots of both the *Eucalyptus* species studied were taken for the anatomical observations. The leaves from nonhyperhydric shoot showed a well defined upper and lower epidermis. Upper epidermis followed by thick and well organized pallisade tissue with dense chlorophyll. The vascular tissue was well organized with definite arrangement of xylem and phloem cells (Fig. 3.3 A and 3.4 A).

The leaves from hyperhydric shoots showed abnormal epidermal layer with large and open stomata (Fig.3.5), palisade tissue was very thin or was almost absent with mesoplyll tissue consisting of spongy parenchyma rich in intracellular spaces. The vascular tissue was distorted. (Fig.3.3 B and 3.4 B).

The stem from nonhyperhydric shoots showed well defined epidermis follwed by parenchyma, procambial strands, xylem and phloem and pith in the centre. (Fig. 3.6 A and 3.7 A).

The stem from the hyperhydric shoots showed thin epidermis followed by vacuolated and thin parenchyma tissue having large cells with thin cell wall. The vascular bundles lack normal organization and the procambial strands were absent (Fig. 3.6 B and 3.7 B).

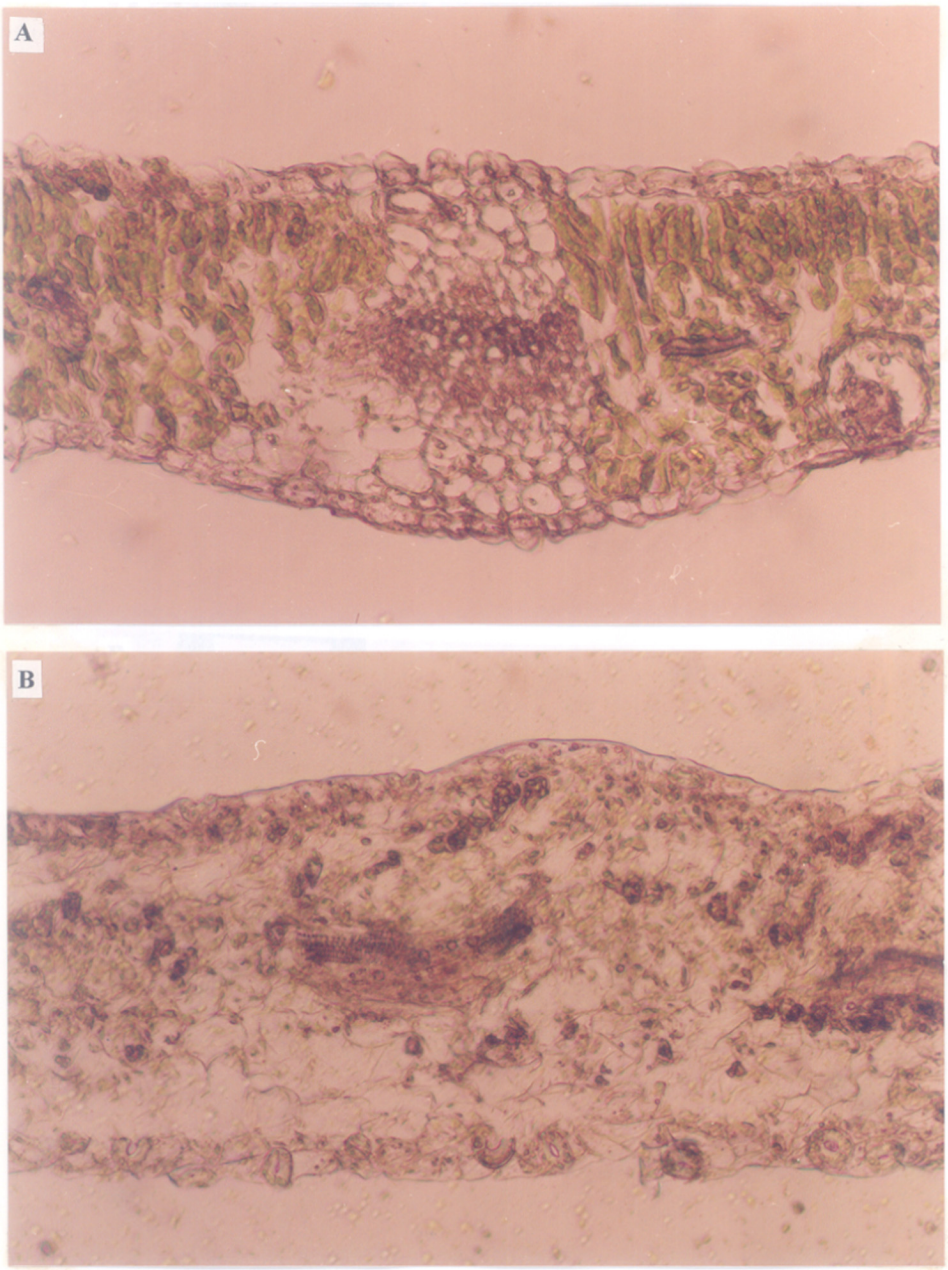


Fig. 3.3 : T.S. of *E. tereticornis* leaf A) Normal B) Hyperhydric (X250)

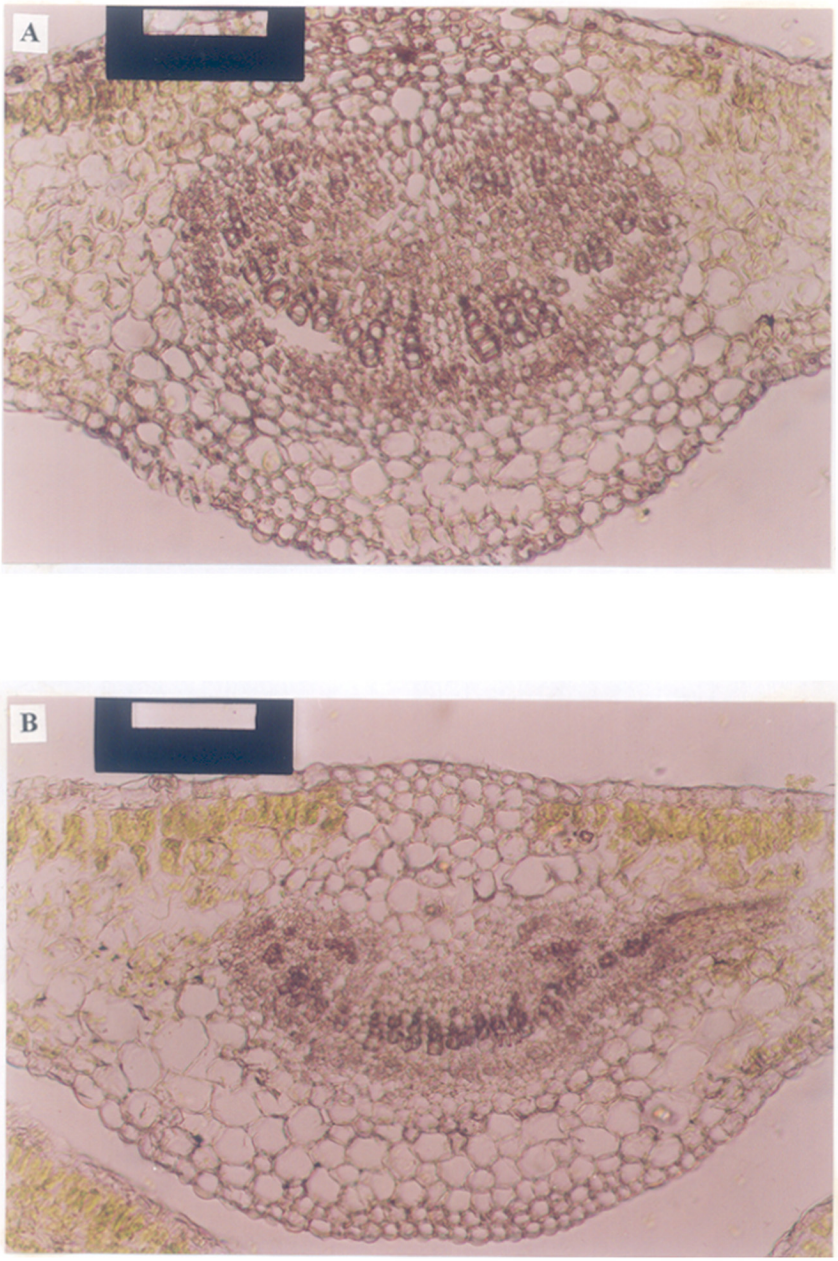


Fig. 3.4 : T.S. of *E. camaldulensis* leaf A) Normal B) Hyperhydric (X250)

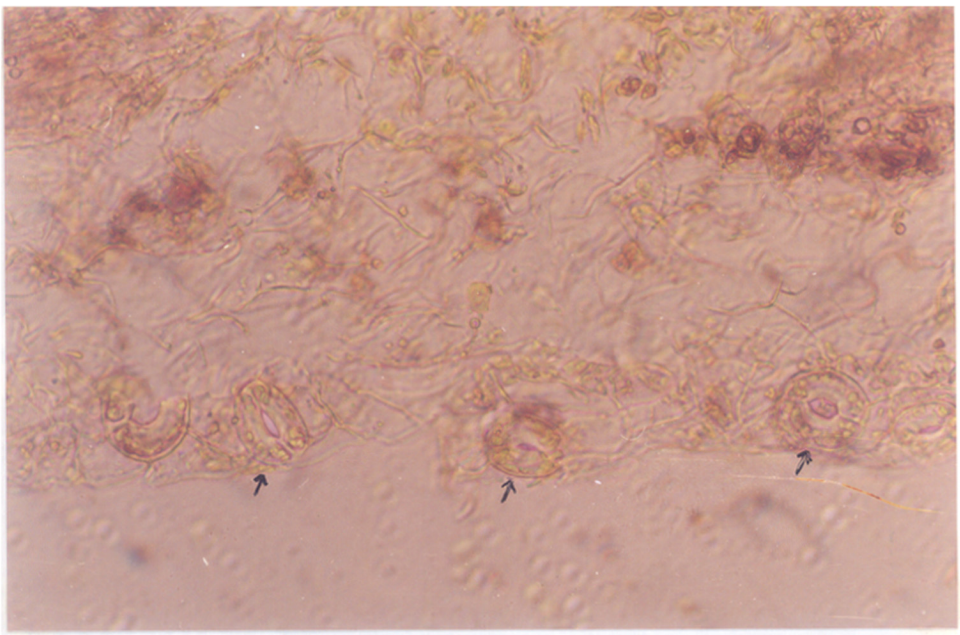


Fig 3.5 : T.S. of *E. tereticornis* hyperhydric leaf showing abnormally large and open stomata (X500)

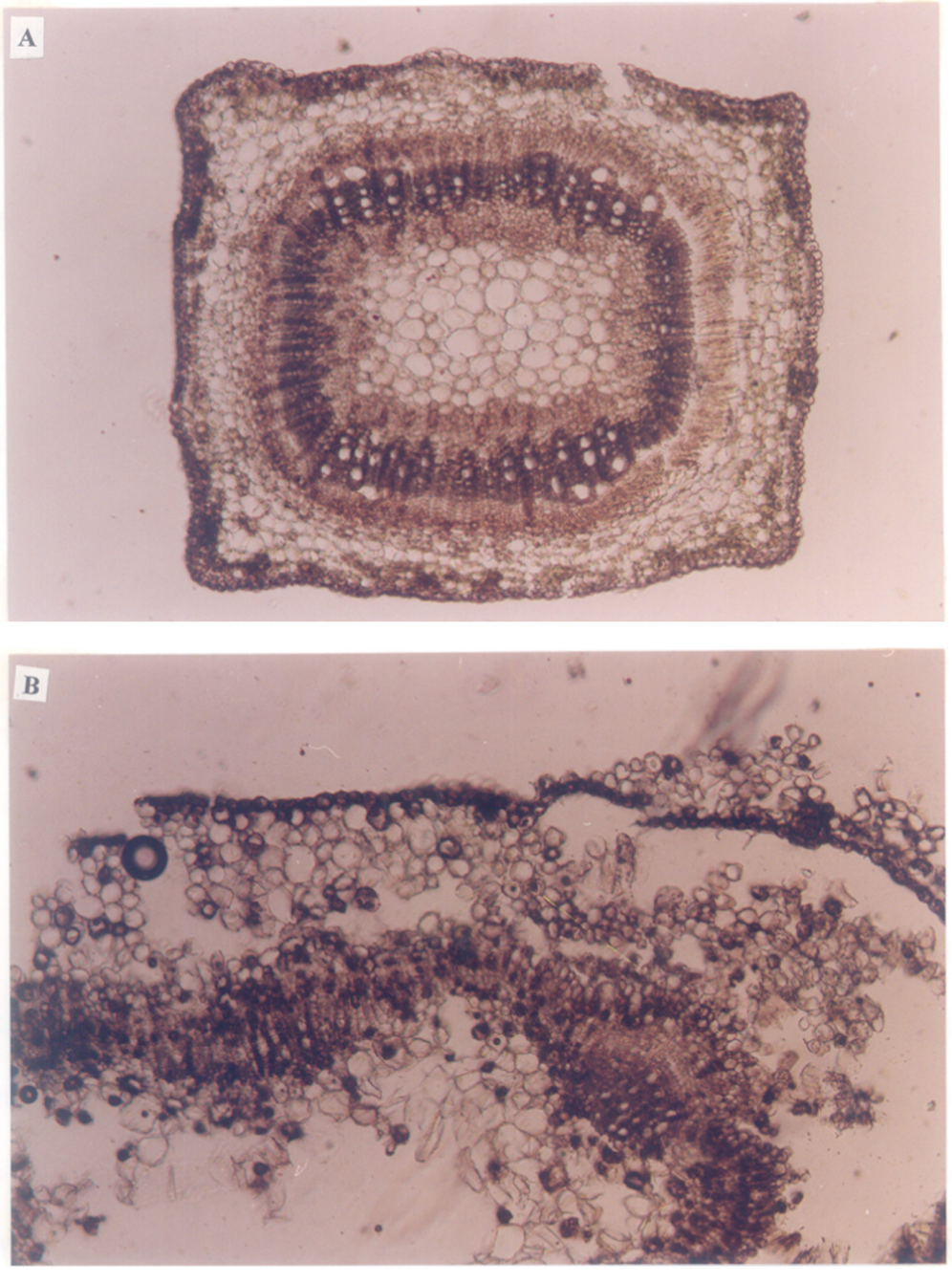


Fig. 3.6 : T.S. of *E. tereticornis* stem - A) Normal B) Hyperhydric (X100)

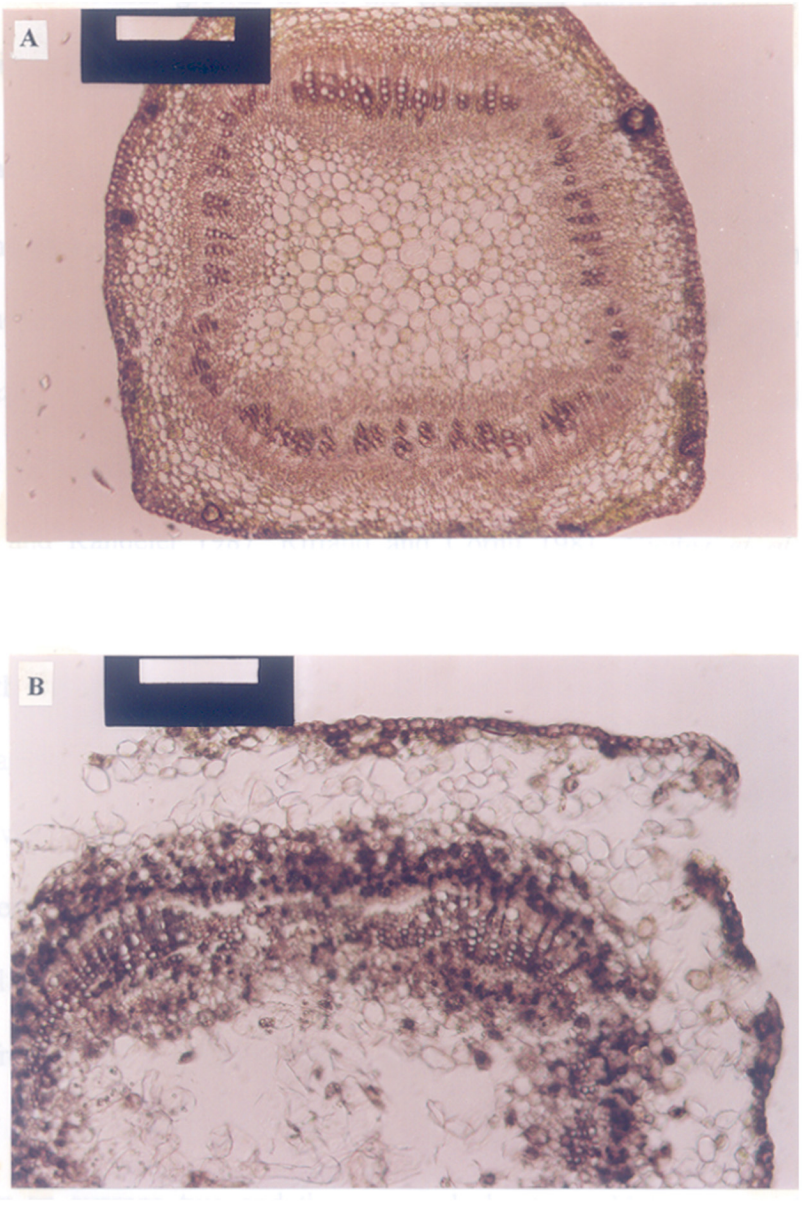


Fig.3.7 : T.S. of *E. camaldulensis* stem - A) Normal B) Hyperhydric (X100)

The leaves and stem parts from the shoots which had reverted from hyperhydric to normal growth in all the experiments showed identical anatomical features with those of the normal shoots.

Effect of ammonium nitrate :

Increasing nutrient levels in the culture medium used for *in vitro* multiplication leads to increased growth and hyperhydric conditions (Paques and Boxus 1987 and Vieitez et *al*. 1985). Many researchers implicate elevated ammonium ion concentrations as a cause of hyperhydricity (Beauchesne 1981, Daguin and Letouze 1985 and 1986 Leonhardt and Kandeler 1987, Riffand and Cornu 1981, Vieitez *et al*. 1985). An attempt was therefore made during the present investigations to see the effect on lowering the concentration of ammonium nitrate in the MS I medium used for micropropagation and large scale production of the two *Eucalyptus* species.

It was observed that the reduction in the strength of ammonium nitrate to half of its concentration in the control medium helped in reverting the hyperhydric shoots to the normal healthy shoots (Table 3.2). The reversion was found to be increasing gradually from first to the third subculture cycle. This gradual change was more obvious in case of *E. tereticornis* as compared to *E. camaldulensis*, where the change was sudden. On an average two and three normal shoots could be removed from each control bottle of *E. tereticornis* and *E. camaldulensis* respectively. On the other hand, the normal shoots per bottle were increased from 2 to 3,5 and 7 by three consecutive subcultures on the medium with reduced ammonium nitrate concentration (MS II) in

TABLE 3.2 EFFECT OF DECREASING CONCENTRATION OF AMMONIUM NITRATE ON REVERSION OF HYPEHYDRIC SHOOTS OF *EUCALYPTUS* SPECIES

No. of Subculture Cycle	Medium	Avg.* No. of Normal shoots/Bottle		Avg.* No. of Hyperhydric shoots/Bottle	
		E. t.	*E. c.*	*E. t.*	*E. c.*
1.	MS I	1.8	2.7	6.0	5.1
	MS II	3.2	6.0	4.1	1.7
2.	MS I	1.6	2.6	6.2	5.2
	MSII	5.2	6.8	2.5	1.2
3.	MS I	1.6	2.8	8.1	5.0
	MSII	7.0	6.9	0.9	1.0

MS I – Control medium.

MS II – MS I with half the concentration of Ammonium nitrate.

* - Figures indicate average number of shoots taken from three replicates each with 80 explants (8-explants/ bottle).

E. c. – *E. camaldulensis*

E. t. – *E. tereticornis*

case of *E. tereticornis*. In the case of *E. camaldulensis* however, the reversion was almost immediate in the first cycle itself.

It has been shown by Givan (1979) that ammonium can become toxic to plant tissues when the concentration in the tissue becomes elevated. Toxic ammonium concentrations are believed to increase glutamate dehydrogenase activity, which in turn, brings about a diversion of the carbohydrate pool from lignin synthesis to amino acid synthesis (Beauchesne 1981, Miffin and Lee 1980) resulting in hyperhydricity (Letouze and Daguin 1983). Reducing NH_4^+ ions in the growth medium helps in increasing lignification of cell walls resulting in decrease in the water uptake because of increased cell turgor pressure, this in turn helps in reducing the hyperhydration of the tissue (Beauchesne, 1981; Daguin and Letouze, 1986; Leonhardt and Kandeler, 1987 and Ziv and Ariel, 1988).

Reducing ammonium ions level have been proved to be reducing hyperhydricity in the case of Salix (Beauchesne 1981 and Letouze and Daguin 1983), Willow, Plum and Cacti species (Beauchesne 1981, Daguin and Letouze 1986 and Leonhardt and Kandeler 1987).

In the present investigation, similar results were obtained in which hyperhydricity was reduced to a considerable extent in case of *E. tereticornis* and *E. camaldulensis* by reducing the concentration of ammonium nitrate to half of its original concentration.. However, it is difficult to ascertain here that reduction in the

hyperhydricity is associated with the ammonium or nitrate ions because both have been reduced simultaneously in the culture medium.

Effect of Benzyl aminopurine (BA) :

Shoot regeneration under *in vitro* conditions necessiates a continuous supply of growth regulators in the culture medium. The most commonly used growth hormones are either cytokinins or auxins. These constituents are not provided exogenously to the growing plants where the plant itself synthesise its own hormones in the required quantities under natural environments. Since the medium used for micropropagation of *Eucalyptus* species (MS I) contained cytokinins, an attempt was made to see the effect of decreasing the benzyl aminopurine (BA) concentration in MS I medium.

From the table (Table 3.3) it was clear that benzyl- aminopurine (BA) plays an important role in reducing hyperhydricity under *in vitro* conditions. Reduction of BA concentration to half of its original concentration (viz. 0.1 mg/l) in MS I gave rise to a gradual increase in number of reverted normal shoots viz. from average of two shoots per bottle in MS I medium to 5,6 and 7 per bottle respectively with 1^{st,} 2 nd and 3rd subculture cycle in case of *E. tereticornis* and a sudden rise viz from an average of 3 shoots per bottle in MS I to 7 per bottle in case of *E. camaldulensis* suggesting that decreasing the BA concentration in the multiplication medium (MS I) will help to circumvent the problem of hyperhydration.

TABLE 3.3 EFFECT OF REDUCED BA CONECENTRATION ON REVERSION OF HYPERHYDRIC SHOOTS OF *EUCALYPTUS* SPECIES

No. of Subculture Cycle	Medium	Avg.* No. of Normal shoots/Bottle		Avg.* No. of Hyperhydric shoots/Bottle	
		E. t.	*E.c.*	*E. t.*	*E. c.*
1.	MS I	2.03	2.56	5.73	5.23
	MS III	4.80	6.83	2.76	1.43
2.	MS I	3.2	2.76	5.90	5.00
	MS III	6.23	7.23	1.36	0.90
3.	MS I	2.00	2.50	5.80	5.33
	MS III	7.20	7.13	0.63	0.96

MS I – Control medium.

MS III – MS I with BA reduced to half the strength.

* - Figures indicate average number of shoots from three replicates each with 80 explant (8 explants/bottle).

E. c. – *E. camaldulensis.*

E. t. – *E. tereticornis.*

There are reports on the involvement of cytokinins in hyperhydricity indicating that high levels of cytokinins in liquid or semisolid media with a high water potential induced abnormal shoot, reducing the morphogenesis (Debergh 1983, von Arnold and Eriksson, 1984). In several species, cytokinin levels have shown to be improving the health of the shoots. In Norway spruce, BA induced hyperhydration, but when the agar level was increased, BA uptake was impeded and hyperhydration was decreased (von Arnold and Eriksson, 1984). In globe artichoke, BA was interpreted to evoke hyperhydration under adverse conditions such as high relative humidity and a high matrix potential (Debergh, 1983). In melon also, cytokinins induced hyperhydration which could be reversed by reducing the cytokinin level (Leshem *et al.*, 1988). In apple, normal plants transferred from solid medium to a liquid medium with BA became hyperhydric (Paques and Boxus, 1987). Removal of BA from liquid medium induced reversal to normal growth and the reversal was gradual (Gasper *et al.*, 1987).

Similar results have been obtained in the present investigation in case of *E. tereticornis*, where the number of shoots reverting to normal increased with increasing the cycles on the same medium. In conifers and carnations, lowering the cytokinin levels led to decreased hyperhydration, which was stimulated more by high BA than high KIN (Dencso, 1987). Nobre (1996) also found BA to be responsible for hyperhydricity in case of micropropagation of *Lavandula stoechas*.

It was further observed during the present investigation that when the BA concentration was reduced, the shoot elongation was faster along with reversion to

normal morphology. A shoot height of 6-7 cm could be obtained in 30-35 days of incubation as against 45 days required in MS I medium. However, these shoots were with less number of nodes and more internodal distance when compared with that on MS I medium. Such shoots when subcultured continuously on the same reduced BA showed a decrease in multiplication ratio. This suggests that although, a decrease in BA concentration help in reducing the problem of hyperhydration, this concentration can not be used.continuously for large scale micropropagation due to the fact that it will reduce the rate of multiplication. In such a situation, it is advisable to reduce the concentration of BA for three cycles when hyperhydricity is observed and revert back to the original medium, once the shoots are normal.

Effect of Other Additives:

An important factor that affect the efficiency of a micropropagation system is the rate of shoot multiplication. One of the ways to increase the rate of multiplication is by increasing the cytokinin level. However, increasing the cytokinin further increases the problem of hyperhydration of the cultures. It has also been widely reported that hyperhydricity of the cultures can be reduced by increasing the concentrations of gelling agents in the growth medium (Debergh *et al.* 1981 and 1983; Ghashghaie *et al.*, 1991; Monsalud *et al.*, 1995; Selby *et al.*, 1989 and Zimmerman and Cobb, 1989). However increasing the gel concentration decreases the availability of the nutrients in the medium to the cultures, creating a stress condition and thereby reducing their growth and increasing the final cost of production. (Vieitez *et al.*, 1985). One of the objectives of

this work was therefore to determine whether the induction of hyperhydricity by high cytokinin levels can be reduced by manipulation of other factors or additives in the growth medium.

Experiments for this were therefore conducted by adding i) an antivitrifying agent and ii) an organic compound casein hydrolysate in the culture medium (MS I) for micropropagation of these two species without reducing any other nutrient concentrations.

i) Effect of antivitrifying agent:

When the interaction of an antivitrifying agent supplied by M/S Sigma-Aldrich Chemicals, U.S.A. was examined at the concentration of 5.0 g/l as recommended by the manufacturer, hyperhydricity decreased almost immediately in both the *Eucalyptus* species studied (Table 3.4). All the explants in a culture bottle showed a reversion of hyperhydric shoots to normal shoots.

The exact effect of the antivitrifying agent used is not known as the chemical structure of it could not be obtained since it is a patented product. However, it has been shown that extracellular mucoid component – a polysaccharide, of *Pseudomonas* species reduces hyperhydricity in tissue cultures of *Origanum vulgare* (Shetty *et al.*, 1996).

TABLE 3.4 EFFECT OF ANTIVITRIFYING AGENT ON REVERSION OF HYPERHYDRIC SHOOTS OF *EUCALYPTUS* SPECIES

No. of Subculture Cycle	Medium	Avg.* No. of Normal shoots/Bottle		Avg.* No. of Hyperhydric shoots/Bottle	
		E. t.	*E. c.*	*E. t.*	*E. c.*
1.	MS I	1.90	2.30	5.80	5.53
	MS IV	5.66	7.26	2.23	0.66
2.	MS I	1.96	2.43	5.86	5.43
	MS IV	6.40	7.06	1.46	0.90
3.	MS I	2.10	2.53	5.73	5.20
	MS IV	7.33	6.93	0.70	0.96

MS I – Control medium.

MS IV – MS I + 5.0 gm/l antivitrifying agent.

* - Figures indicate average number shoots taken from three replicates each with 80 explants (8 explants/bottle).

E. c.- E. camaldulensis.

E. t. – E. tereticornis

ii) Effect of Casein-hydrolysate (CH):

An organic additive casein hydrolysate was added to MS I medium to see its effect on hyperhydricity of *Eucalyptys* cultures at 100 and 200 mg/l concentration. There was an increase in number of normal shoots from 2 to 6 per culture bottle in case of *E. tereticornis* and from 2 to 5 in case of *E. camaldulensis* at both the concentrations. There was no significant increase or decrease in the reversion of hyperhydric shoots to normal shoots when casein hydrolysate was increased from 100 to 200 mg/l (Table 3.5 and 3.6).

Tian Su Zhou (1995), have reported in his preliminary experiments that adding potato juice to the culture medium was one of the most effective and economical ways for overcoming hyperhydricity in *Doritaenopsis*. Some non starch substrate may be responsible for this recovery. In the present investigation, casein hydrolysate also helped in reverting the hyperhydric shoots to normal. However, the overall total number of normal shoots that could be obtained were considerably less when compared with that obtained during the addition of antivitrifying agent. Adding Bacto peptone to the medium was described as effective for carnation in order to recover from hyperhydricity (Sato *et al.*, 1993). According to them, only the basic peptides with molecular weight less than 10,000 were effective in reducing hyperhydricity.

An interesting observation was noted during these studies that in case of *e. tereticornis* there was always a gradual increase in the total number of reverted shoots obtained with increase in the subcultures on the same medium (Fig. 3.8). this was not

TABLE 3.5 EFFECT OF CASEIN HYDROLYSATE AT 100 MG/L CONCENTRATION ON REVERSION OF HYPERHYDRIC SHOOTS OF *EUCALYPTUS* SPECIES

No. of Subculture Cycle	Medium	Avg.* No. of Normal Shoots/Bottle		Avg.* No. of Hyperhydric shoots/ Bottle	
		E. t.	*E. c.*	*E.t.*	*E. c.*
1.	MS I	2.20	2.50	5.63	5.30
	MS V	4.33	4.20	3.60	3.56
2.	MS I	1.93	2.70	5.93	5.13
	MS V	5.23	5.13	2.60	2.80
3.	MS I	1.80	2.63	5.86	5.10
	MS V	6.06	4.86	1.76	3.23

MS I – Control Medium.

MS V – MS I + 100 mg/l Casein hydrplysate

* - Figures indicate average number of shoots from three replicates each with 80 explants (8 explants/bottle).

E. c. – *E. camaldulensis.*

E. t. – *E. tereticornis.*

TABLE 3.6 EFFECT OF CASEIN HYDROLYSATE AT 200 MG/L CONCENTRATION ON REVERSION OF HYPERHYDRIC SHOOTS OF *EUCALYPTUS* SPECIES

No. of Subculture Cycle	Medium	Avg.* No. of Normal shoots/Bottle		Avg.* No. of Hyperhydric shoots/Bottle	
		E. t.	*E. c.*	*E. t.*	*E. c.*
1.	MS I	1.73	2.43	6.23	5.53
	MS VI	4.63	3.63	3.20	4.20
2.	MS I	1.63	2.56	6.10	5.30
	MS VI	5.06	4.90	2.83	2.90
3.	MS I	1.83	2.50	6.03	5.23
	MS VI	5.96	4.60	1.80	3.26

MS I – Control medium.

MS VI – MS I + 200 mg/l Casein hydrolysate.

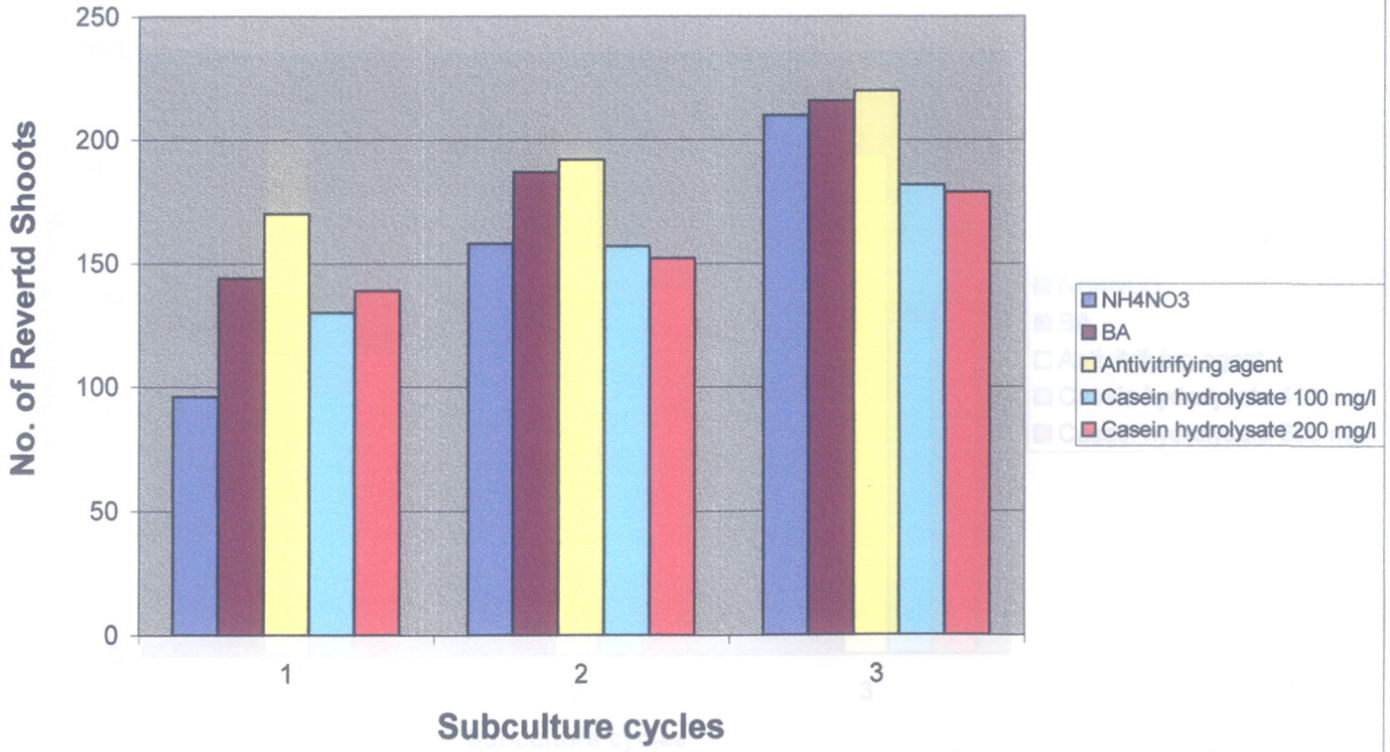
* - Figures indicate average number of shoots from three replicates each with 80 explants (8 explants/bottle).

E. c. – *E. camaldulensis.*

E. t. – *E. tereticornis.*

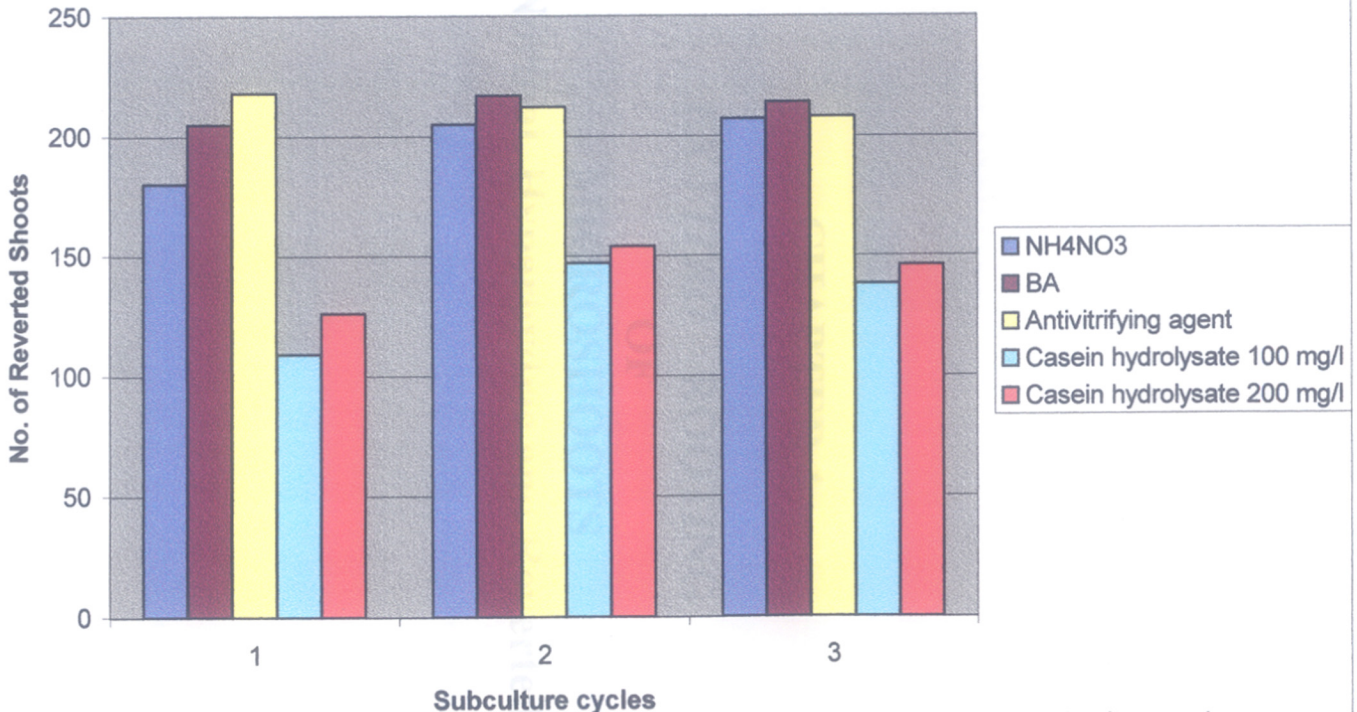
true with *E. camaldulensis*, where the reversion of hyperhydric shoots to normal ones was sudden (Fig. 3.9), indicating the difference in adaptability of the two species, *E. camaldulensis* being more adaptive than *E. tereticornis*.

Fig. No. 3.8 TOTAL NO.* OF REVERTED SHOOTS OF *E. tereticornis* AT DIFFERENT SUBCULTURE CYCLES WITH DIFFERENT CHEMICAL TREATMENTS



* - Total No. of shoots obtained from 30 bottles (10 Bottles x 3 replicates) for each subculture cycle.

Fig. No.3.9 TOTAL NO.* OF REVERTED SHOOTS OF *E. camaldulensis* AT DIFFERENT SUBCULTURE CYCLES WITH DIFFERENT CHEMICAL TREATMENTS



* - Total No. of reverted shoots obtained from 30 bottles (10bottles x 3 replicates) for each subculture cycle

CHAPTER 4

EX VITRO ROOTING OF MICROSHOOTS (Normal, Hyperhydric and Reverted)

RESULTS AND DISCUSSIONS :

Rooting and subsequent survival and acclimatization is the final, but important step in all micropropagation systems. In Tissue Culture Pilot Plant, at NCL, a method of *ex vitro* rooting of the micropropagated shoots has been adopted. In this process, after giving a cut near a node of a microshoot, shoots are given a quick dip in a hormone solution and are transplanted in planting mixture in a polybag. The bags are then kept under 90% relative humidity for rooting and hardening, to occur simultaneously. The rooted and hardened plants are then slowly exposed to an environment of lower relative humidity, higher light intensity and temperatures. The hormone type and concentration varies from species to species and from clone to clone (Jana, 1998).

In general, the intrinsic plant quality, produced *in vitro* is one of the most important factors governing the success of *ex vitro* rooting and subsequent survival of the plants. During the present investigation, many chemicals or reduction in the concentrations of the ingredients were found to be useful in improving the conditions of the shoots under *in vitro* conditions (Chapter 3, this thesis). However, these

experiments can be called successful only if reverted shoots could root and survive. Experiments were therefore, conducted during the present investigation to see and compare the rooting pattern of the nonhyperhydric normal shoots, hyperhydric shoots and reverted normal shoots.

EX VITRO ROOTING OF NORMAL SHOOTS :

Microshoots of two clones, one each of *E. tereticornis* and *E. camaldulensis* selected for the studies have been regularly taken for *ex vitro* rooting and the plants produced are being supplied to different forest agencies and universities for field trials by Tissue Culture Pilot Plant at National Chemical Laboratory, Pune. In general, the rooting percentage of the normal non hyperhydric shoots varied from 80-95% depending on the clone and the species. Results obtained with the hyperhydric and the reverted normal shoots by different chemical treatments is given below.

EX VITRO ROOTING OF HYPERHYDRIC AND REVERTED NORMAL SHOOTS :

Effect of Ammonium nitrate:

After reducing ammonium nitrate concentration to half of its concentration in the control medium, it was observed that there was a gradual increase in the total number of reverted shoots to normality due to the reduction in NH_4NO_3 concentration in the case of *E. tereticornis*. Table 4.1 describes the rooting percentage of hyperhydric and reverted shoots. The total number of shoots taken for *ex vitro* rooting was obtained from 10 bottles (80 explants) from 3 replicates for each subculture upto three

TABLE 4.1 *EX VITRO* ROOTING OF SHOOTS OF *E. tereticornis* PRODUCED *IN VITRO* ON MEDIUM WITH REDUCED AMMONIUM NITRATE (NH_4NO_3) [MS II]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting of Hyprehydric Shoots
1	96	83	86.45	125	23	18.40
2	158	142	89.87	76	19	25.00
3	210	180	85.71	28	00	00.00
Total	464	405	87.28**	229	42	18.34**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

subcultures on the same medium. *Ex vitro* rooting of these reverted normal shoots showed around 87% rooting comparable to the non hyperhydric normal shoots as against 18% average rooting of hyperhydric shoots.

In case of E. *camaldulensis*, there was not significant difference in the total number of reverted normal or hyperhydric shoots obtained in the 1st, 2nd and 3rd cycle (Table 4.2). The reversion was almost sudden. The rooting percentage of reverted shoots were also comparable to that of normal shoots.

Effect of Benzyl-amino purine (BA) :

Like ammonium nitrate, reduction in the BA concentration to half of its concentration in the control medium showed a gradual rise in the total number of reverted shoots and a gradual decrease in the number of hyperhydric shoots with subculture on the same medium upto three cycles in case of *E. tereticornis*. The rooting percentage of the reverted shoots were comparable to that on the control medium.. The hyperhydric shoots showed only 13% survival of the plants (Table 4.3).

In case of *E. camaldulensis*, the reversion was immediate and the total number of reverted shoots that could be obtained from each subculture cycle remained almost the same with a 94% of rooting percentage (Table 4.4).

TABLE 4.2 *EX VITRO* ROOTING OF SHOOTS OF *E. camaldulensis* PRODUCED *IN VITRO* ON MEDIUM WITH REDUCED AMMONIUM NITRATE (NH_4NO_3) [MS II]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting of Hyperhydric Shoots
1	180	148	82.22	53	11	20.75
2	205	187	91.21	38	00	00.00
3	207	176	85.85	32	05	15.62
Total	592	511	86.31**	123	16	13.00**

* - Total no. of shoots from all the three replicates.

** - Average % rooting of all the three subculture cycles.

TABLE 4.3 *EX VITRO* ROOTING OF SHOOTS OF *E. tereticornis PRODUCED IN VITRO* ON MEDIUM WITH REDUCED BENZYL-AMINOPURINE (BA) [MS III]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting of Hyperhydric Shoots
1	144	117	81.25	83	12	14.45
2	187	178	95.72	41	07	17.07
3	216	210	97.22	19	00	00.00
Total	547	505	92.32**	143	19	13.28**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

TABLE 4.4 *EX VITRO* ROOTING OF SHOOTS OF *E. camaldulensis* PRODUCED *IN VITRO* ON MEDIUM WITH REDUCED BENZYL-AMINOPURINE (BA) [MS III]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting of Hyperhydric Shoots
1	205	205	100.00	43	00	00.00
2	217	193	88.94	27	04	14.81
3	214	200	93.45	29	05	17.24
Total	636	598	94.02**	99	09	09.09**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

Effect of antivitrifying agent :

Similar results like ammonium nitrate and BA were obtained by addition of antivitrifying agent in the control medium in case of E. *tereticornis* and *E. camaldulensis* species (Table 4.5 and 4.6). It was further observed in case of *E. tereticornis* that the rooting percentage were comparatively high viz. 25% in hyperhydric shoots as against only 13-18% in case of the reduction in ammonium nitrate and BA concentrations indicating that these hyperhydric shoots if subcultured further for many more cycles perhaps would revert all the shoots to normal ones.

Effect of Casein hydrolysate (CH) :

Addition of casein hydrolysate in the control medium at 100 and 200 mg/l concentrations showed a similar gradual change in E. *tereticornis* and an immediate change in *E. camaldulensis* (Table 4.7, 4.8, 4.9 and 4.10). At both these concentrations there was no significant differences observed in total number of reverted shoots. The total number of reverted shoots was 469 and 470 in case of *E. tereticornis* and 426 and 394 in case of *E. camaldulensis* respectively. However, these numbers were less as compared to that obtained during the previous treatments viz. ammonium nitrate, BA and antivitrifying agent. The average rooting of reverted shoots were also less viz. between 57 and 77 as compared to 80 and 100 in case of normal shoots. This indicates that although, casein hydrolysate help in reverting

TABLE 4.5 *EX VITRO* ROOTING OF SHOOTS OF *E. tereticornis* PRODUCED *IN VITRO* ON MEDIUM WITH ANTIVITRIFYING AGENT [MS IV]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting of hyperhydric Shoots
1	170	127	74.70	67	20	29.85
2	192	185	96.35	44	09	20.45
3	220	195	88.63	21	04	19.04
Total	582	507	87.11**	132	33	25.00**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

TABLE 4.6 *EX VITRO* ROOTING OF SHOOTS OF *E. camaldulensis* PRODUCED *IN VITRO* ON MEDIUM WITH ANTIVITRIFYING AGENT [MS IV]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting of Hyperhydric Shoots
1	218	154	70.64	20	05	25.00
2	212	182	85.84	27	00	00.00
3	208	208	100.00	29	06	20.68
Total	638	544	85.26**	76	11	14.47**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

TABLE 4.7 *EX VITRO* ROOTING OF SHOOTS OF *E. tereticornis* PRODUCED *IN VITRO* ON MEDIUM WITH 100 MG/L CASEIN HYDROLYSATE (CH) [MS V]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting of Hyperhydric Shoots
1	130	83	63.84	108	18	16.66
2	157	119	75.79	78	06	7.69
3	182	160	87.91	53	05	9.43
Total	469	362	77.18**	239	29	12.13**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

TABLE 4.8 *EX VITRO* ROOTING OF SHOOTS OF *E. camaldulensis* PRODUCED *IN VITRO* ON MEDIUM WITH 100 MG/L CASEIN HYDROLYSATE (CH) [MS V]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric shoots Planted*	Rooted	%Rooting of Hyperhydric Shoots
1	126	68	53.96	107	24	22.42
2	154	112	72.72	84	00	00.00
3	146	135	92.46	97	23	23.71
Total	426	315	73.94**	288	47	16.31**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

TABLE 4.9 *EX VITRO* ROOTING OF SHOOTS OF *E. tereticornis* PRODUCED *IN VITRO* ON MEDIUM WITH 200 MH/L CASEIN HYDROLYSATE (CH) [MS VI]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting Of Hyperhydric Shoots
1	139	82	58.99	96	17	17.70
2	152	92	62.50	85	11	12.94
3	179	132	73.73	54	04	07.40
Total	470	309	65.74**	235	32	13.61**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

TABLE 4.10 *EX VITRO* ROOTING OF SHOOTS OF *E. camaldulensis* PRODUCED *IN VITRO* ON MEDIUM WITH 200 MG/L CASEIN HYDROLYSATE (CH) [MS VI]

No. of Subculture Cycle	No. of Reverted Shoots Planted*	Rooted	%Rooting of Reverted Shoots	No. of Hyperhydric Shoots Planted*	Rooted	%Rooting of Hyperhydric Shoots
1	109	57	52.29	126	11	8.73
2	147	90	61.22	87	17	19.54
3	138	78	56.52	98	24	24.48
Total	394	225	57.10**	311	52	16.72**

* - Total No. of shoots obtained from all the three replicates.

** - Average % rooting of all the three subculture cycles.

the hyperhydric shoot to the normal one, its effect in not comparable to that of ammonium nitrate, BA and antivitrifying agent.

With the help of results obtained from all the above experiments, it can be concluded that the rooting percentage of the normal shoots varied from 80-95%. On the contrary, the hyperhydrated shoots showed a very poor rooting of 0-30%. Daguin and Letouze (1986) and Pasqualetto *et al.* (1988) have stated that hyperhydric shoots have poor water regulation and as a consequence when transplanted *ex virto* they easily desiccate and die.

One very interesting observation was noted during this investigation that even though the converted shoots showed morphological and anatomical features comparable to that of normal shoots, their rooting percentage varied between 50-100% depending on the species and the chemical treatment. This indicates that, perhaps the morphologically normal shoots were not normal physiologically always.

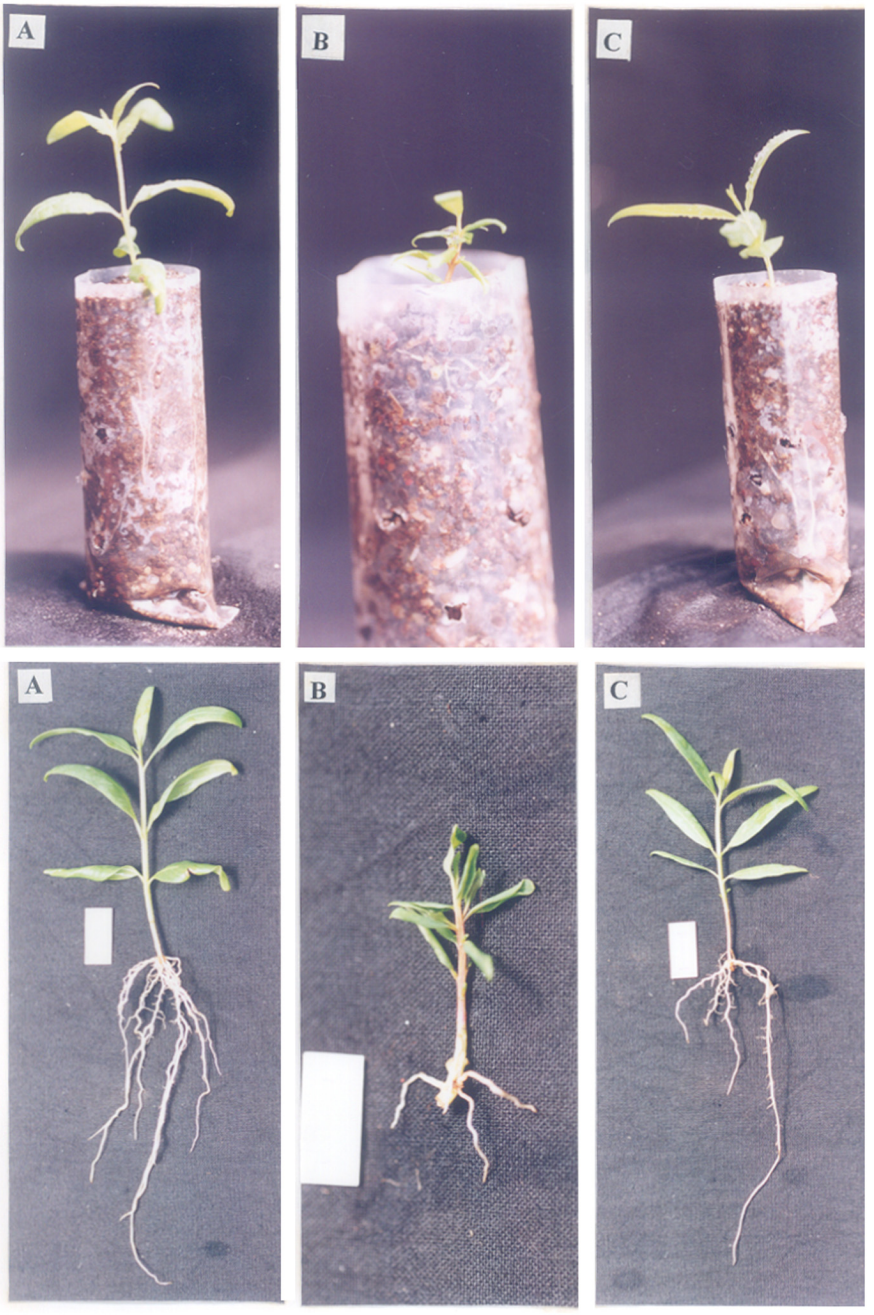


Fig 4.1 : Rooting of *E. tereticornis* shoots
A) Normal B) Hyperhydric C) Reverted

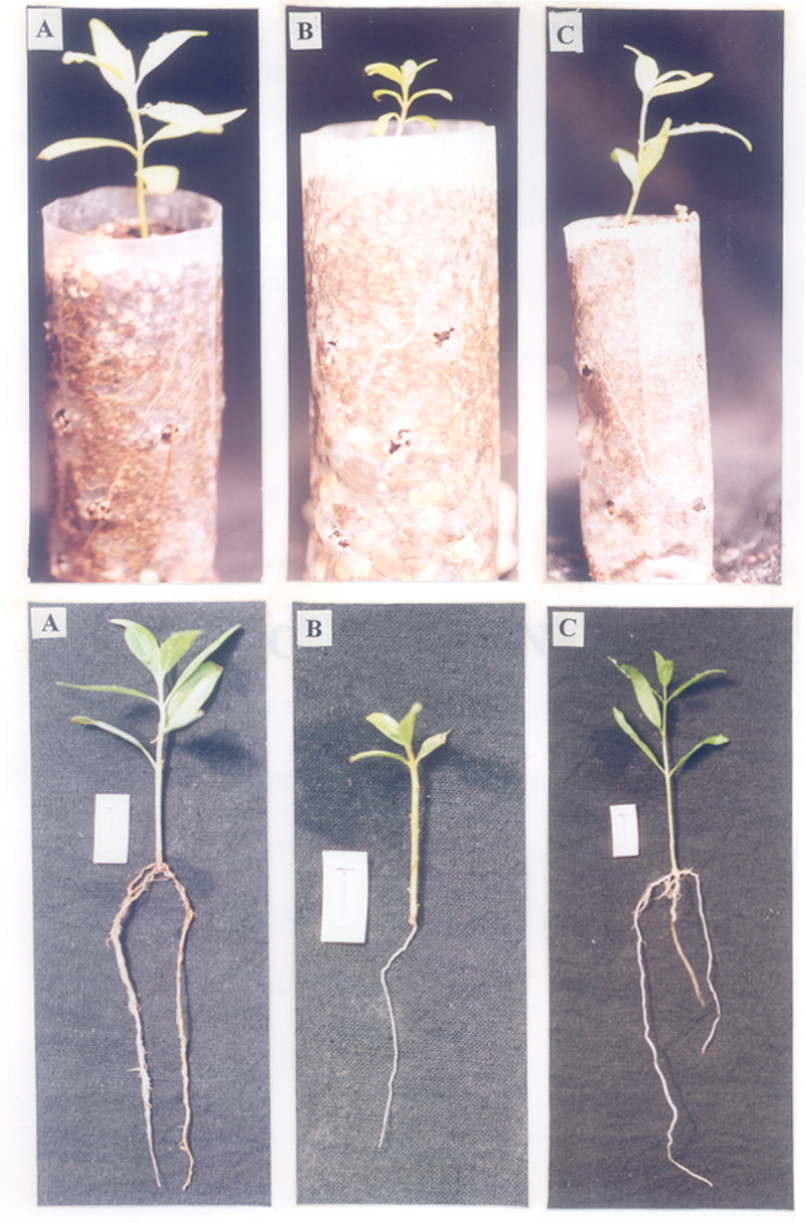


Fig 4.2 : Rooting of *E. camaldulensis* shoots
A) Normal B) Hyperhydric C) Reverted

CONCLUSIONS

CONCLUSIONS

It is possible to revert back the hyperhydric shoots of both the *Eucalyptus* species viz. *E. tereticornis and E. camaldulensis* to normal shoots by altering the media components rather than regarding them as waste material and throwing them away, in a micropropagation laboratory. This is very important if a clone is rare, new and the cultures are very less. Following conclusions can be drawn from the results obtained during this investigation:

1) Several factors are responsible for inducing hyperhydricity in case of the *Eucalyptus* species studied, chemical environment being an important factor.
2) The subculture number at which hyperhydration appears varies from species to species and clone to clone.
3) Hyperhydricity, in case of *E. tereticornis* and *E. camaldulensis* was associated with the morphological and anatomical changes resulting in abnormal growth of the shoots.
4) In a single culture jar, both hyperhydric and normal shoots occur indicating a possible variability among the explants.
5) The control of levels of mineral, growth hormones or addition of organic additives or antivitrifying agent can revert back the hyperhydric shoots to normal shoots.

6) Reduction in the strength of ammonium nitrate to half of that in the production medium (MS I) is effective in reducing the hyperhydricity.

7) Reduction in BA (benzyl-aminopurine) concentration to half of that used in the MS I medium, although reduced the rate of multiplication, helped in reverting the hyperhydric shoots to the normal ones.

8) Addition of an antivitrifying agent from M/S Sigma Chemical, U.S.A. helped in improving the shoot quality without affecting the multiplication ratio. Rather it was found to be the most effective chemical in reverting the hyperhydric shoots to the normal one.

9) There was no significant difference in the reversion at the two concentrations of casein hydrolysate (CH) tested viz. 100 and 200 mg/l. Also the reversion was to a limited extent.

10) The shoots reverted to normal shoots had the same morphology and anatomy like non hyperhydric normal shoots.

11) The normal nonhyperhydric shoots showed rooting percentage between 80-95%.

12) Hyperhydric shoots showed a very poor rooting viz. between 0-30% depending on the species and the chemical treatment.

13) The reverted shoots showed 50-100% rooting depending on the species and the chemical treatment.

14) There was always a gradual increase in reversion of hyperhydric shoots to the normal one in case of *E. tereticornis* as a response towards chemical treatment for three subculture cycles tested.

15) This reversion was almost immediate in case of *E. camaldulensis*.

BIBLIOGRAPHY

BIBLIOGRAPHY

Beauchesne G. (1981) Les milieux mineraux utilises en culture *in vitro* et leur incidence sur I'apparition de boutures d'aspect pathologique. C. R. Acad. Agric. Paris. **67** : 1389-1397.

Borkowska B. and Opliowska M. (1988) Influence of BA and other cytokinins on proliferation and metabolic status of sour cherry cultures cultivated *in vitro*. Fruit Sci. Rep. **15** : 147-156.

Bottcher I., Zoglauer K. and Goring H. (1988) Induction and reversion of vitrification of plants cultured *in vitro*. Physiol. Plant. **72**: 560-564.

Daguin F. and Letouze R. (1985). Relations entre hypolignification et etat vitreux chez-*Salix babylonica* en culture *in vitro*- Role de la nutrition ammonicale. Can J. Bot. **63**: 324-326.

Daguin F.and Letouze R. (1986) Ammonium -induced vitrification in cultured tissues. Physiol. Plant. **66**: 94-98.

Debergh PC. (1983) Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant. **59**:270-276.

Debergh PC. (1987) Improving micropropagation. IAPTC Newsletter. **51**: 2-10.

Debergh PC. Aitken-Christie J. Cohen D. Grout B. von Arnold S. Zimmerman R. & Ziv M. (1992) Reconsideration of the term 'vitrification' as used in micropropagation. Plant Cell, Tissue and Organ Culture. **30** : 135-140.

Debergh PC. Harbaoui Y. and Lemeur R. (1981) Mass propagation of globe artichoke (*Cynara scolymus)* : Evaluation if different hypotheses to overcome vitrification with special referrence to water potential. Physiol. Plant. **53**: 181-187.

Debergh PC. and Maene LJ. (1984) Pathological and physiological problems related to the *in vitro* culture of plants. Parasitica **40**: 69-75.

Dencso I. (1987) Factors influencing vitrification of carnation and conifers. Acta Horti. **212**: 167-176.

Dillen W. and Buysens S. (1989) A simple technique to overcome vitrification in *Gypsophila paniculata* L. Plant Cell, Tissue and Organ Cult. **19**: 182-188.

Gamborg OL., Miller RA. And Ojima K. (1968) Nutrient requirements of suspension cultures of soyabean root cells. Expt. Cell Res. **50**: 151-158.

Gasper T. (1995) The concept of cancer in *in vitro* plant cultures and the implication of habituation to hormones and hyperhydricity. Plant Tissue Cult. And Biotech, **1**: 126-136.

Gasper T. and Kevers C. (1985) Cobalt prevention of vitrification process in carnation. Plant Physiol. **77**(suppl.): 13.

Gasper T., Kevers C., Debergh P., Maene L., Paques M. and Boxus P. (1987) Vitrification: morphological, physiological and ecological aspects. In: Bonga JM. and Durzan DJ.(Eds.) Cell and Tissue Culture in Forestry. vol.**1** General Principles and Biotechnology., Martinus Nijhoff Publications, Dordrecht, Holland, pp 152-166.

Gautheret RJ. (1939) Sur ia possibilite de realiser le cultur indefinie des tissus de tubercules de Carote. C. R. Ac. Sc. **208**: 118-130.

Ghashghaie J. Brenckmann F. and Saugier B.((1991) Effect of agar concentration on water status and growth of rose plants cultured *in vitro*. Physiol. Plant. **82**: 73-78.

Givan CV. (1979) Metabolic detoxification of ammonia in tissues of higher plants. Phytochem. **18**: 375-382.

Gribble K., Sarafis V., Nailon J., Holford P. and Uwins P. (1996) Environmental scanning electron microscopy of the surface of normal and vitrified leaves of *Gypsophila paniculata* (Babies Breath) cultured *in vitro*. Plant Cell Rep. **15**: 771-776.

Gupta PK. and Mascarenhas AF. (1987) Eucalyptus. In: Bonga JM. and Durzan DJ. (Eds.) Cell and Tissue Culture in Forestry vol. **3** Case Histories: Gymnosperms, Angiosperms and Palms., Martinus Nijhoff Publications Dordrecht, Holland., pp 385-399.

Haberlandt G. (1902) Kulturversuche mit isolierten Pflanzenzellen. Sitz. Akad. Wiss. Wien. **111**: 69-92.

Jana MM. (1998) Studies on Plant Tissue Culture – A Ph.D. thesis submitted to the University of Pune.

John CK., Nadgauda RS. And Mascarenhas AF.[Eds.] (1997) Eucalyptus. In:Tissue Culture of Economic Plants., Centre for Science and Technology of the Non--Aligned and other Developing Countries, New Delhi, India., pp 81-107.

Jones NB., Drennan PM. And Staden JV.(1993) S. Afr. J. Bot. **59**: 551-555.

Kevers C., Coumans M. Coumans-Gilles MF. and Gasper T. (1984) Physiological and biochemical events leading to vitrification of plants cultured *in vitro* . Physiol. Plant. **61**: 69-74.

Leonhardt pp 385-399 W. and Kandeler R. (1987) Ethylene accumulation in culture vessels- a reason for vitrification. Acta. Hortic. **212**: 223-229.

Leshem B. (1983) Growth of carnation meristems *in vitro*: Anatomical structures of abnormal plantlets and the effect of agar concentration in the medium on their formation. Ann. Bot. **52**: 413-415.

Leshem B. and Sachs T. (1985) Vitrified *Dianthus*- teratomata *in vitro* due to growth factor imbalance. Ann. Bot. **56**: 613-617.

Leshem B., Shaley PD. and Izhar S. (1988) Cytokinin as an inducer of vitrification in melon. Ann. Bot. **61**: 255-260.

Leshem B., Werker E.and Shaley PD (1988) The effect of cytokinins on vitrification in melon and carnation. Ann. Bot. **62**: 271-276.

Letouze R. and Daguin F. (1983) Manifestation spontanee et aleatoire d'une croissance anorma culture *in vitro*. Recherche de marqueurs metaboliques. Rev. Can. Biol. Expt. **42**: 33-38.

Letouze R. and Daguin F. (1987) Control of vitrification and hypolignification process in *Salyx babylonica* cultured *in vitro*. Acta. Hortic. **212**: 185-191.

Miffin BJ. and Lea PJ. (1980) Ammonia assimilation – In: The Biochemistry of Plants P. K. Stumpf and E. E. Conn (Eds.) Vol. **5** pp 169-199. Academic Press, New York.

Miguens FC., Louro RP. And Machado RD. (1993) A scanning electron microscope study of normal and vitrified leaves from *Datura insignis* plantlets cultured *in vitro*. Plant Cell, Tissue and Org. Cult. **32**: 109-113.

Mohammed Yasseen Y., Devenport TL., Splittstoesser WE. and Litz RE. (1992) Proc. Fla. State Hort. Sci. **105**: 210-212.

Monsalud MJ., Mathews H., Litz RE. And Gray DJ. (1995) Control of hyperhydricity of mango somatic embryos. Plant Cell, Tissue and Org. Cult. **42**: 195-206.

Morel G. (1960). Producing virus free cymbidium. Bull. Amer. Orchid Soc. **29**: 495-497.

Murashige T. (1974) Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. **25**: 135-165.

Murashige T. (1978) In: Propagation of Higher Plants Through Tissue Culture. Huges KW., Henke R. and Constsntin M. (Eds.) tech. Info. Centre US Dept. of Energy, Sringfield. Va. Pp 14-24.

Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. **15**: 473-497.

Nadgauda RS. (1998) Plant Tissue Culture: the centre piece of plant biotechnology. Natl. Acad. Sci. Lett. **21**: 1-4.

Nairn BJ., Furneaux RH. and Stevenson TT. (1995) Identification of an agar constituent responsible for hydric control in micropropagation of radiata pine. Plant cell, Tissue and Org. Cult. **43**: 1-11.

Navatel JC. (1982) Problemes lies a la production de porte- greffe d'arbres fruitiers par multiplication *in vitro*. Fruits. **37**: 331-336.

Nobecourt P. (1938) Sur les proliferations spontanees de fragments de tubercules de Carotte et leur culture sur milieu synthetique. Bull. Soc. Bot. Fr. **85**: 1-7.

Nobre J. (1996) *In vitro* cloning and micropropagation of *Lavandula stoechas* from field-grown plants. Plant Cell, Tissue and Org. Cult.**46**: 151-155.

Paques M. and Boxus P. (1987a) Vitrification: review of literature. Acta. Hortic. **212**: 155-166.

Paques M. and Boxus P. (1987b) A model to learn "Vitrification", the rootstock apple M-26 present results. Acta. Hortic. **212**:193-210.

Paques M. and Boxus P. (1987c) Vitrification: a phenomenon related to tissue water content. Acta. Hortic. **212**: 245-252.

Paques M., Boxus P. and Dulos M. (1987) Vitrification an inducible and reversible phenomenon. Acta. Hortic. **212**: 253-258.

Pasqualetto M., Zimmerman RH. and Fordham I. (1986) The influence of gelling agent, growth regulator concentration on vitrification of apple cultivars *in vitro*. J. Amer. Soc. Hort. Sci. **111**: 976-980.

Pasqualetto M., Zimmerman RH. and Fordham I. (1988) The effect of cations and gelling agent concentration on vitrification of apple cultivars *in vitro*. Plant Cell, Tissue and Org. Cult. **14**: 31-40.

Reinert J. (1959) Uber die Kontrolle der morphogeneses and die induktion von advantivaemberyonen and Genebed ulturen aus karotten. Planta **53**: 318-333.

Riffaud JL. and Cornu D. (1981) Utilization de la culture in *in vitro* pour la multiplication de merisiers adultes (*Prunus avium* L.) selectionnes en foret. Agronomic **1**: 633-640.

Schleiden MJ. (1838) Beitrage zur Phytogenesis. Muller Arch. Anat. Und Physiol. 137-176.

Schwann T. (1839) Mikroskopische untersuchungen uber die Ubereinstimmung in der Struktur und dem Wachstum der Thiere und Pflanzen. No. 176: Oswalds, Berlin, Germany.

Selby C., Lee R. and Harvey BMR. (1989) The effects of culture medium rigidity on adventitious bud production and tissue vitrification in needle cultures of Sitka spruce [*Picea sitchensis* (Bong.) Carr.] New Phytol. **113**: 203-210.

Shetty K., Carpenter TL., Curtis OF. And Potter TL. (1996) Reduction of hyperhydricity in tissue cultures of oregano (*Origanum vulgare*) by extracellular polysaccharide isolated from *Pseudomonas* species. Plant Sci. **120**: 175-183.

Skoog F. and Miller CO. (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. **11** The Biological Action of Growth Substances. pp 118-131.

Vasil V. and Hildbrandt AC. (1965) Differentiation of tobacco plants from single, isolated cells in microculture. Science. **150**: 889-892.

Vieitez AM., Ballester A., San-Jose MC. and Vieitez E. (1985) Anatomical and chemical studies of vitrified shoots of chestnut regenerated *in vitro*. Physiol. Plant. **65**: 177-184.

von Arnold S. and Eriksson T. (1984) Effect of agar concentration on growth and anatomy of adventitious shoots of *Picia abies* L. Karst.. Plant Cell, Tissue and Org. Cult. **3**: 257-264.

Werker E. and Leshem B. (1987) Structural changes during vitrification of carnation plantlets. Ann. Bot. **59**: 377-385.

White PR. (1934) Potentially unlimited growth of excised tomato root-tips in a liquid medium. Plant Physiol. **9**: 585-600.

White PR. (1939) Potentially unlimited growth of excised plant callus in an artificial medium. Amer. J. Bot. **26**: 59-64.

White PR. (1963) The cultivation of Animal and Plant Cells. Ronald Press, New York, USA.

Williams RR. and Taji AM. (1991) Effect of temperature, gel concentration and cytokinins on vitrification of *Olearia microdisca* (J.M. Black) *in vitro* shoot cultures. Plant Cell, Tissue and Org. Cult. **26**:1-6.

Zhou T. (1995) *In vitro* culture of Doritaenopsis: Comparison between formation of the hyperhydric protocorm-like-body (PLB) and the normal PLB. Plant Cell Rep.**15**: 181-185.

Zimmerman TW. and Cobb BG. (1989) Vitrification and soluble carbohydrate levels in *Petunia* leaves as influenced by media gelrite and sucrose concentration. Plant Cell Rep. **8**: 358-360.

Ziv M. (1986) *In vitro* hardening and acclimatiztion of tissue culture plants. In: Plant Tissue Culture and its Agricultural Applications Withers LA. and Alderson PG. (Eds.) pp 187-196 Butterworths, London.

Ziv M. (1991) Vitrification : morphological and physiological disorders of *in vitro* plants. In : Micropropagation. Debergh PC. And Zimmerman RH. (Eds.) pp 45-69, Kluwer Academic Publs. Dordrecht, Netherlands.