

BIOCHEMICAL STUDIES ON  
PLANT TISSUE CULTURE  
Studies with Eucalyptus citriodora

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A  
THESIS

COMPUTERISED

SUBMITTED TO THE  
UNIVERSITY OF POONA

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TH-363

FOR  
THE DEGREE OF  
DOCTOR OF PHILOSOPHY  
IN  
BIOCHEMISTRY

578.083:582.883(043)

GUP

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"BIOCHEMICAL STUDIES ON PLANT TISSUE CULTURE: Studies  
with Eucalyptus citriodora" submitted by Shri PRAMOD  
KUMAR GUPTA was carried out by the candidate under my  
supervision. Such material as has been obtained from  
other sources has been duly acknowledged in the thesis.

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## ACKNOWLEDGEMENT

The author owes a deep debt of gratitude to **Dr. A. F. Mascarenhas** under whose inspiring and encouraging guidance this work was successfully carried out. He is also profoundly grateful to Dr. V. Jagannathan for the keen interest he evinced in the progress of the work and the valuable suggestions he made from time to time.

He expresses his sincere thanks to Miss Sreelata Nair who helped him in checking the type script and correcting the typographical and other errors. He is also thankful to his other colleagues in the Plant Tissue Culture group for their willing cooperation at all times. Thanks are also due to Miss Usha Deshpande for her expert and efficient typing of the manuscript, the Principal, Law College, Poona, for permission to collect buds from the **Eucalyptus** trees, Mr. Mohan Rao of the Indian Drug Research Laboratory, Poona for his generous gift of Eucalyptus seeds, Dr. S. S. Khuspe for conducting the field trials and last but not least Mr. V. A. Padale for his skilful photography.

Finally he is thankful to Dr. C. SivaRaman, Head Biochemistry Division and Dr. L. K. Doraiswamy, Director, National Chemical Laboratory for permission to submit this work in the form of a thesis.

**P. K. GUPTA**

### LIST OF ABBREVIATIONS

|                  |                                                        |
|------------------|--------------------------------------------------------|
| ABA              | Abscisic acid                                          |
| Adv.             | Adventitious bud                                       |
| BAP              | 6-Benzyl amino purine                                  |
| Bio.             | Biotin                                                 |
| B <sub>5</sub> M | Gamborg B <sub>5</sub> mineral solution                |
| 2-BTOA           | 2-Benzothiazole-2-oxyacetic acid                       |
| Ca.P.            | Calcium pantothenate                                   |
| CCC              | (2-chloroethyl) trimethyl ammonium chloride            |
| CH               | Casain hydrolysate (Casamino acids)                    |
| CM               | Coconut milk                                           |
| CPA              | Chlorophenoxy acetic acid                              |
| DPU              | 1,3-Diphenyl urea                                      |
| Ed               | Edamin-S                                               |
| EDTA             | Ethylene diamine tetra acetic acid                     |
| GA <sub>3</sub>  | Gibberellic acid                                       |
| GB <sub>5</sub>  | Gamborg B <sub>5</sub> basal medium                    |
| H                | Heller basal medium                                    |
| HM               | Heller mineral solution                                |
| IAA              | Indole-3-acetic acid                                   |
| IBA              | Indole-3-butyric acid                                  |
| Inos.            | meso-inositol                                          |
| 2iP              | N <sup>6</sup> - $\gamma$ -dimethyl allyl amino purine |
| IPA              | Indole-3-propionic acid                                |
| K                | Knop basal medium                                      |
| Kin.             | Kinetin (6-furfuryl amino purine)                      |



|                                        |                                                         |
|----------------------------------------|---------------------------------------------------------|
| KM                                     | Knop mineral solution                                   |
| MA                                     | Murashige A mineral solution                            |
| MB                                     | Murashige B vitamin solution                            |
| ME                                     | Malt Extract                                            |
| Mor.A                                  | Morphactin CME 74050 P.                                 |
| Mor.B                                  | Morphactin EMO 7311 W.                                  |
| MS                                     | Murashige and Skoog basal medium                        |
| NAA                                    | $\alpha$ -Naphthalene acetic acid                       |
| NOA                                    | $\beta$ -Naphthoxy acetic acid                          |
| S                                      | Smith basal medium                                      |
| S <sub>1</sub> , S <sub>2</sub> , etc. | Subculture numbers 1, 2 etc.                            |
| SM                                     | Smith mineral solution                                  |
| 2,4,5-TCP                              | 2,4,5-Trichlorophenoxy acetic acid                      |
| TIBA                                   | 2,3,5-Tri iodobenzoic acid                              |
| W                                      | White basal media                                       |
| WB                                     | White B vitamin solution                                |
| WM                                     | White mineral solution                                  |
| YE.                                    | Yeast Extract                                           |
| Zeatin                                 | 6-(4-hydroxy-3 methyl but-trans-2 enyl)<br>amino purine |

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# **CHAPTER I**

## **INTRODUCTION**

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## INTRODUCTION

### Section 1: General

Recent advances made in the field of plant, cell and tissue culture have resulted in the utilization of this technology for basic research in the chemical and biochemical sciences and also for practical application to several agricultural and horticultural problems.

The term "plant tissue culture" broadly refers to the cultivation in vitro of all plant parts, whether a single cell, a tissue or an organ on a defined nutrient medium (Biondi and Thorpe, 1981). Any given tissue composed of living cells is a suitable explant for the initiation of a plant tissue culture.

### History and some landmarks

The idea of culturing plant tissues was conceived in 1902 by Haberlandt (Haberlandt, 1902). He prophesied that plant cells, if given the proper environment and nutrition have the capacity to regenerate into entire plants. Unfortunately he failed in his attempts. In 1921 Molliard demonstrated limited success with the cultivation of plant embryos, and subsequently Kotte (1922a; 1922b) and Robbins (1922a; 1922b) were successful in the establishment of excised plant root tips in vitro. It was only in the 1930s that White (1932a; 1932b; 1933a; 1933b) reported his success on the establishment of an actively growing culture of tomato roots. At the same time Gautheret (1934; 1935) reported successful callus proliferation from cambium

explants of some woody trees and Nobecourt (1937) obtained similar results with carrot and potato. Steward et al. (1952) carried out detailed studies on the effectiveness of coconut milk as a nutrient both for growth and embryogenesis. He established that a process similar to that which occurs in zygotic embryogenesis also occurs in somatic cell cultures (Steward et al., 1958). In 1953 Miller and Skoog demonstrated bud formation in tobacco pith explants followed by the discovery of kinetin, and its role, in shoot and root formation when used at a correct ratio with auxins (Skoog and Miller, 1957). The growth of single cells by placing them on an actively growing "nurse" tissue culture, the so called "paper raft nurse technique" was an important observation by Muir et al. (1954) in the culture of single cells. Nickell (1956) reported the growth of fragments of callus cultures as suspensions by agitation on a shaker or by growth in fermentors, which could be grown by serial subculture like bacteria. In 1960 Cocking reported the release of protoplasts (cells without cell walls) from root tip cells by using a fungal cellulase (Cocking, 1960). Guha and Maheshwari (1964) obtained for the first time haploid plants by culture of Datura anthers. This discovery has now resulted in obtaining haploids from several different economically important plants (Nitsch, 1981).

It is now possible to obtain entire plants from almost any part of the parent plant, from small segments of roots, stems, leaves, flowers, embryos, pollen, isolated cells and



even from protoplasts.

Morel was also the first to demonstrate the effectiveness of shoot tip cultures as a rapid cloning, procedure (Morel, 1960). His effort to recover virus-free Cymbidium plants from infected plants resulted in exclusion of virus, as well as in clonal increase of plants, at rates that were substantially higher than those achieved by traditional propagation methods. Today a wide variety of crops, especially ornamentals, are being propagated commercially through an extension of Morel's finding (Murashige, 1974; 1978). Furthermore in the last two decades this technique has found wide commercial application in several areas as mentioned below:

1. Clonal propagation or rapid multiplication of high yielding or 'elite' plants.
2. Isolation of disease-free plants.
3. Growth of immature hybrid embryos.
4. Breeding programmes and for genetic improvement of plants by isolation of haploids, protoplast fusion etc.
5. Production of pharmaceutical and other natural products, for biotransformation.
6. For isolation of protoplasts and somatic hybridization.
7. For basic studies.

#### Types of cultures

Plant tissue culture can be divided into five classes (Gamborg and Shyluk, 1981). These classes are based primarily on the type of material used.

1. Callus culture The culture and growth of unorganized cell masses generally on agar media.
2. Cell culture or suspension culture The culture of free cells or cells clumps in liquid media.
3. Organ culture The culture of embryos, anthers, ovaries, roots, shoots or other plant organs.
4. Meristem culture Culture of shoot meristems for the purpose of growing complete plants, used to isolate virus-free plants.
5. Protoplast culture The aseptic isolation and culture of plant protoplasts.

#### Nutrition of plant callus (General)

Plant tissue culture media To obtain a plant tissue culture successfully a correct choice of the nutrient medium is necessary. Cells of many plant species can now be grown on completely defined media. The inorganic salt solutions used to supply essential elements to plant tissue cultures have been developed by modifying the solution used initially by Gautheret (1934) and by White (1939) in their pioneering work.

Heller (1953) medium was earlier widely used. The salts content is relatively low and potassium and nitrate are added as two separate compounds.

Among the media formulation developed later are the following: Hildebrandt and Riker (1949); Burkholder and Nickell (1949); White (1939a); Nitsch and Nitsch (1956); White and Risser (1964); Murashige and Skoog (1962); Eriksson (1965); Linsmaier and Skoog (1965); Schenk and

Hildebrandt (1972); Gamborg B<sub>5</sub> (1968) and Y<sub>3</sub> mineral salts medium (Eeuwens, 1976). Murashige and Skoog (1962) medium often with suitable modifications has now found very wide use for culture of a large number of different plants. This medium designed initially for tobacco cells (Murashige and Skoog, 1962) differs from earlier media in the high content of nitrate, potassium and ammonium relative to other nutrient media.

The micronutrient levels in Eriksson medium is much lower than Murashige and Skoog (1962). Gamborg B<sub>5</sub> (1968) medium has been tested and found suitable with a wide range of callus and suspension cultures (Ohira et al., 1973). This medium contains relatively low amounts of ammonium. Schenk and Hildebrandt (1972) medium resemble B<sub>5</sub> Gamborg (1968). The concentration of mineral salts are slightly higher in Schenk and Hildebrandt (1972) with ammonium and phosphate supplied as ammonium phosphate.

In general the nutrient medium for most plant tissue cultures comprises of five groups of ingredients: inorganic salts in macro and micro amounts, a carbon source, vitamins, growth regulators and organic supplements (Thorpe, 1981).

#### Inorganic nitrogen sources

Inorganic nitrogen has been supplied in two forms such as nitrate or ammonium for the growth of plant cells in vitro. With many species rapid growth occurs generally in media containing nitrate as the sole nitrogen source viz. Paul's scarlet rose (Nash and Davies, 1972); carrot (Reinert et al., 1967); tobacco and Populus (Matsumoto et al., 1971).



However, there are species which grow very poorly in the presence of nitrate alone in the medium such as rice (Yatazawa and Furuhashi, 1968). In fact, nitrate alone is inhibitory at the same nitrogen concentration at which ammonium nitrate supports rapid growth in rice (Yatazawa and Furuhashi, 1968).

Reduced nitrogen in the form of ammonium, used to supplement the nitrate media is usually stimulatory to growth and morphogenesis (Sharp and Crocomo, 1975).

Halperin (1966) was the first to recognise the importance of reduced nitrogen in the form of  $\text{NH}_4^+$ . Wetherell and Dougall (1976) showed that as little as 0.1 mM ammonium chloride added to nitrate significantly enhanced embryogenesis in carrot, the optimum being  $\text{KNO}_3$  (40 mM) and  $\text{NH}_4\text{Cl}$  (10 mM). Dougall and Verma (1978) showed that  $\text{NH}_4\text{Cl}$  (10 mM) could serve as the sole source of nitrogen provided that KCl was present and the pH maintained at 5.4. Widely used plant tissue culture media contain both  $\text{NO}_3^-$  and  $\text{NH}_4^+$  as nitrogen sources (Dougall, 1981).

#### Organic nitrogen sources

Reduced nitrogen in the form of organic compounds is usually stimulatory to growth and morphogenesis when added as a mixture of different amino acids such as casein hydrolysate. For rice, yeast extract (which also serves as a source of vitamins) and casein hydrolysate were the best nitrogen sources for callus growth (Yatazawa and Furuhashi, 1968). Casein hydrolysate increased gametophyte formation in fern root cultures (Mumroe and Sussex, 1969). An

inhibitory effect of a mixture of amino acids was found in the induction of buds on Plumbago tissue cultures by Nitsch and Nitsch (1967). Kamada and Harada (1979) found that alanine (5 - 10 mM) was effective in increasing embryo frequency during subculture. Furthermore, they found that reduced nitrogen was needed during embryo development and not during induction. In Pinus callus, glycine inhibited growth whereas asparagine and aspartic acid were found to be stimulatory (Brown and Lawrence, 1968). Arginine and tyrosine also stimulated callus growth of conifers (Harvey and Grasham, 1969).

Lee and Yatazawa (1976) tested the effect of urea and ureide nitrogen (arginine, citrulline, allantoin, allantoic acid and uric acid). They reported that urea (200 mg/l) was the most effective in promoting growth of rice callus, uric acid being the least effective.

#### Phosphorus sources

Phosphorus is supplied mainly as the phosphate. Studies on the optimum concentration of phosphate showed that best growth of potato callus was obtained with 20 mg/l  $\text{KH}_2\text{PO}_4$  from a concentration range of 10 - 100 mg/l which was tested (Lingappa, 1957). Veki and Sato (1971) reported that phosphatase activity is controlled by inorganic phosphate concentration present in the nutrient medium, but amylase and  $\beta$ -galactosidase activity are not effected by inorganic phosphate concentration.

### Potassium sources

Potassium is often the accompanying cation for nitrate and phosphate and as such its concentration is not controlled separately. In some media, potassium chloride is added as an additional potassium source. Brown et al. (1976) showed that the number of embryos produced from carrot suspension cultures was maximal when  $K^+$  in the medium was at a concentration between 10 - 50 mM. This effect of potassium could not be mimicked by sodium. Lavee and Hoffmann (1971) reported that optimum growth of apple callus occurred at 3.5 mM  $K^+$ . The peroxidase activity in these cultures was directly proportional to the  $K^+$  concentration, reaching a toxic level at high potassium concentrations. An increase of potassium in the medium for Vinca rosea cells can substitute various organic compounds, normally required in media (e.g. glutamine) is required with low potassium but not with higher levels of potassium in the medium (Wood and Braun, 1961).

### Sulfur sources

Sulfur is usually provided as the sulfate or with sulfur containing amino acids such as cysteine, methionine etc. and other sources such as glutathione. The effect of different sulfur sources on the growth of Rumex acetosa tumor tissue has been studied by Nickell and Burkholder (1950). They showed that sulfate, bisulphite and cysteine are excellent for growth of Rumex acetosa tumor tissue and cystine and methionine are toxic. Cysteine was used in the medium for tissues of willow and poplar by Gautheret (1933) and for carrot

and tobacco by Nobecourt (1937). Reinert and White (1956) reported that Picea glauca cultures could not grow on media containing cysteine or glutathione.

#### Calcium sources

Calcium salts are among the least soluble salts used in tissue culture, thereby limiting the choice of calcium compounds to those which are more soluble. Stonier and Yang (1971) reported that  $\text{Ca}^{2+}$  present in the medium could inhibit the process of lignification and senescence.

#### Micro elements

Micro elements play an important role in nutrition. Even though they are required in very small amounts, they are necessary for growth. The essential nutrients required in micromolar concentrations include Fe, Mn, Zn, B, Cu and Mo (Gamborg et al., 1976). Ohira et al. (1975) reported that omission of Mn, Cu, Mo or B from the medium had much less effect on growth than omission of Fe or Zn in a test involving 6 passages with cultures of rice cells. In the formulae of Murashige and Skoog (1962) and Gamborg (1968), the concentration of Mn is similar to that of Fe and is several fold higher than the amount of Zn and Cu. Plant tissue culture media often also contain cobalt, iodine, nickel, aluminium etc. (Ojima and Ohira, 1978).

#### Carbon sources

The majority of higher plants are completely autotrophic being able to synthesize all the organic carbon compounds necessary for growth and survival. In vitro, however, the

isolated tissues of these plants are heterotrophic even if the tissues remain green, as there is usually insufficient photosynthesis to maintain growth, making it necessary to provide an energy source and a carbon source in the medium. The standard carbon source is sucrose or glucose. Fructose also can be used but in general is much less suitable (Gamborg and Shyluk, 1981; Thorpe, 1981). Other carbohydrate sources have also been used to support cell growth, for example, maltose for Pinus callus (Konar, 1974); sorbitol for Malus (Chong and Taper, 1974) and calli from genera of the Rosaceae (Coffin et al., 1976); trehalose for Paul's scarlet rose cell suspension (Nash and Ball, 1975) and stachyose for carrot cell culture (Verma and Dougall, 1977).

Sugars have also been reported to have striking effects on morphogenesis. High sugar concentrations increase embryoid formation from anthers of tobacco (Sharp et al., 1971) while media devoid of sucrose increase gametophyte formation from fern roots (Munroe and Sussex, 1969). Sucrose is the most widely used sugar and this was found to be the best sugar for growth of Populus and tobacco callus (Matsumoto et al., 1971). Sucrose is generally used at a concentration of 2 - 3% (Murashige, 1974; Dougall, 1981).

#### Growth hormones and growth regulators

Plant hormones (Phytohormones) are growth regulators produced by the plant that, in low concentrations control physiological processes (Nickell, 1978). Plant growth regulators are usually defined as organic compounds other than

nutrients, that, in small concentrations affect the physiological processes of plants (Nickell, 1978). They are divided into six classes - (1) Auxins, (2) Cytokinins, (3) Gibberellins, (4) Ethylene, (5) Abscissic acid and (6) Growth retardants or inhibitors.

### Auxins

Auxins are compounds required for cell enlargement, cell proliferation and root initiation. Normal plant tissue cultures require auxins for maintenance of their growth in the culture medium (Gautheret, 1955). IAA the recognised natural auxin, is sensitive to degradation by oxidation (Sharp and Crocomo, 1975) and in many cases proves ineffective or less effective than the synthetic auxins, 2,4-D and NAA. IBA alone or in combination with NAA has proved very effective with some tissues (Street and Shillito, 1977). Most of the crown gall tissues not only proliferate freely in culture in the absence of an external supply of auxins, but their growth is not enhanced by such additions (de Ropp, 1977a; 1977b; Gautheret, 1948; Ferguson, 1963). Differences exist in the effectiveness of these auxins in callus induction for different species. For example, species specificity for NAA, IAA or 2,4-D was seen in 12 species of conifers (Harvey and Grasham, 1969). 2,4-D was better than IAA or other auxins for callus growth in Pinus (Brown and Lawrence, 1968); rice (Yamada et al., 1967); Wu and Li, 1971); Dioscorea (Rao, 1969); 39 species of Nicotiana and 9 species of Populus (Matsumoto et al., 1971). 2,4-D in general is a very effective auxin in callus induction.



Bayliss (1975; 1977) reported that 2,4-D at higher concentrations may promote cytological instability by interfering with normal spindle formation. However, with certain plants as in Plumbago, other auxins such as IAA are more useful (Nitsch and Nitsch, 1967).

### Cytokinins

Cytokinins is a 'generic' term used for substances, which promote cell division of plant cells (Laloue, 1978). Miller et al. (1956) isolated a powerful plant cell division factor from autoclaved herring sperm DNA which they identified as 6-furfurylaminopurine and named it kinetin. Most naturally occurring cytokinins are N<sup>6</sup>-substituted adenine derivatives. The cytokinin with trivial name, zeatin (6-(4-hydroxy-3-methyl-trans-2-butanylamine)-purine) has been found in a wide variety of plant tissues and organs where it occurs free as the base riboside, and in macromolecular form, as the cis-isomer in transfer RNA (Miller, 1965; Miller et al., 1956). Cytokinins interact with other growth factors to initiate or modify a wide range of developmental and physiological events in higher plants. These include senescence, cell elongation, morphogenesis, cell differentiation and many others (Kende, 1971; Skoog and Armstrong, 1970; Fosket and Tepfer, 1978). Skoog and Miller (1957) reported that a basic regulatory mechanism underlying organogenesis involves a balance between auxin and cytokinin; a relatively high level of auxin to cytokinin favoured root formation, the reverse favoured shoot formation, and an intermediate ratio favoured callus formation.

Most of the commonly used cytokinins are artificial in the sense that they do not occur in plant tissues. An exception is zeatin, a cytokinin from Zea mays. The effect of endogenous production by cultures can be seen with regard to the cytokinin requirements also. Tissue from the cotyledons of avocado produces cytokinin in vitro and does not require an exogenous supply for callus growth, while tissue from mesocarp, which does not produce cytokinin, grows only on a medium containing cytokinin (Blumenfeld and Gazit, 1971).

There are many tissues which require cytokinin for callus growth, but in general, the absolute requirement is not as clear as the requirement for auxin, which applies to practically all tissues other than tumor tissues. Matsumoto et al. (1971) reported that of 39 species of Nicotiana cultured, only one, N. glutinosa required a cytokinin in addition to an auxin. In the case of cytokinin requirements, it is almost always a case of a cytokinin plus an auxin for callus growth. The balance between two hormones is important in controlling DNA synthesis and cell division, with IAA implicated in the synthesis of DNA and cytokinin in cytokinesis (Patau et al., 1957; Leshem, 1973). Torrey (1961) reported that kinetin will induce cytokinesis of polyploid cells in vitro while with an auxin alone the DNA synthesis proceeds to form polyploid cells but they do not undergo cell division.

Gibberellins

Gibberellins can be defined as a group of compounds which possess a gibbane skeleton and stimulate cell division or cell

elongation or both. The best known member of this group is gibberellin A<sub>3</sub> (GA<sub>3</sub>), which is isolated from culture filtrates of a fungus Gibberella fujikuroi. Netien (1957; 1958) tested gibberellin for normal tissue cultures of Helianthus tuberosus, Rubus spp. and Daucus carota, and for crown gall tissue of Scorzonera. Gibberellin was without effect or was slightly inhibitory to growth. Various types of auxins used in conjunction with gibberellin did not change the response. Nickell and Tulecke (1959) found that 59 strains of callus derived from 25 different species belonging both to the monocotyledonous and dicotyledonous groups were inhibited by incorporating into their culture medium 10 mg/l of gibberellin. Straus and Epp (1960) reported that an external supply of gibberellic acid (0.5 - 2.0 mg/l) was essential for the growth in culture of tissues derived from Cupressus funebris. Murashige and Skoog (1962) reported that callus growth of Nicotiana tabacum was not enhanced by addition of gibberellic acid.

There are now several instances of the stimulation of culture growth by GA due mainly or entirely to its promotion of cell expansion (Helgeson and Upper, 1970; Lance et al. 1970).

### Ethylene

It is a gaseous plant hormone which controls a number of physiological activities such as ripening of fruit, promotion of leaf abscission etc. Morgan and Hall (1962; 1964) showed in work with cotton, that the production of ethylene is closely

linked to the level of exogenous auxin applied, and later (Burg and Burg, 1968; Kand et al., 1974), it was demonstrated that rates of ethylene production in tissues are related to their endogenous auxin level. These investigators could not replace the need for an exogenous auxin by supplying ethylene. In recent work of Mattoo and Lieberman (1977) an ethylene synthesising system could be located at the cell membrane - cell wall complex.

#### Abscisic acid

The role of abscisic acid in the control of growth and development in several plant systems has been well documented (Addicott et al., 1964; Milborrow, 1974). With few exceptions ABA inhibits the growth of whole plants or excised parts of the plants. Many of these inhibitory effects are manifested at the level of biosynthesis of essential macromolecules such as proteins and nucleic acids in the tissue (Chrispeels and Varner, 1967; Villiers, 1968; Khan and Heit, 1969). Yamaguchi and Nakajima (1974) reported shoot bud induction by ABA in sweet potato and potato cultures. Collett (1970) showed that ABA at very low concentration (0.0026 mg/l) enhanced lateral root initiation in cultured tomato roots. Gresshoff and Doy (1972) in preliminary reports have shown the requirement of abscisic acid for the induction of embryogenesis in tomato pollen.

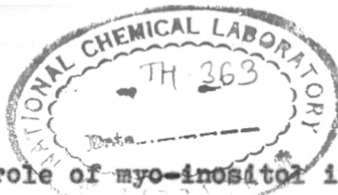
#### Vitamins

Normal plants synthesize the vitamins required for growth and development. However, when cells of higher plants

are grown in culture, some vitamins may become limiting. For instance thiamine is reported to be essential for callus cultures of Nicotiana tabacum (Linsmaier and Skoog, 1965); for continuous growth of tomato roots (White, 1934); for soybean callus (Ikeda et al., 1976). Growth is also improved by the addition of nicotinic acid and pyridoxine. Pyridoxine was found to be essential for Nicotiana tabacum callus (Murashige and Skoog, 1962) and for citrus tissue culture (Murashige et al., 1968). Risser and White (1964) showed a requirement of niacin for callus growth of Picea glauca. Pantothenic acid was found essential for crown gall callus of Datura innoxia (Morel, 1946). Ascorbic acid enhanced growth of tumor tissue of Picea glauca (Reinert and Schrandolf, 1959).

### Inositol

Myo-inositol is a standard constituent of bacterial and animal cell culture media. The discovery by Pollard et al. (1961) that the neutral fraction, isolated from coconut milk contained myo-inositol, scyllo-inositol and sorbitol and that myo-inositol could fully substitute the growth promoting activity of this fraction was assayed by their carrot system (Steinhart et al., 1962). It was subsequently shown that myo-inositol was stimulatory to callus growth, usually at concentrations of 50 - 100 mg/l, in Elmus campestris (Jacquiot, 1964b); Pelargonium hortorum (Narayana, 1963); tobacco (Linsmaier and Skoog, 1965) and Catharanthus roseus (Wood and Braun, 1961) and that it was essential at 10 mg/l for growth of Fraxinus pennsylvanica callus (Wolter and Skoog,



1966). The role of myo-inositol in tissue culture, however remains uncertain, although it is involved in the synthesis of phospholipids and pectins (Anderson and Wolter, 1966).

### Purines and Pyrimidines

Nucleotides are often added to the media for growth of different plant tissues and cell cultures. Ribonucleotides are necessary for mitosis and maturation of microspores in anther culture (Vasil, 1965). Adenine is effective in stimulating growth of callus, an effect which is not entirely due to its cytokinin properties. An absolute requirement for adenine or adenine sulfate was found for growth of potato callus in the presence of coconut milk (Lingappa, 1957). Similarly, adenine was required for bud induction in Plumbago (Nitsch and Nitsch, 1967); Funaria, a moss (Szweykowska et al., 1971). Anderson (1980) reported the adenine requirement for shoot induction from Red raspberry.

### Natural products

Natural products are extracts of plants or cells which are added in the medium, which provide some unknown factors to facilitate the growth of tissue. YE, ME, CH and CM are widely used in plant tissue culture media as a source of amino acids and vitamins (Yatazawa and Furuhashi, 1968). The most widely used and a very effective plant extract is coconut milk.

Originally coconut milk was used by VanOverbeek et al. (1932) for the culture of small Datura embryos. Since then it has found wide application in induction of callus and in

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morphogenesis of many species and has been added at concentrations ranging from 0.5 - 30%. Optimum fresh weight (growth) was found in potato and sweet potato callus with CM added at 5% v/v (Lingappa, 1957); Coconut milk was the only treatment that stimulated embryoid formation in carrot (Steward, 1964); Datura (Guha and Maheshwari, 1967) and in pollen grains of Brassica (Kameya and Hinata, 1970). Coconut milk contains myo-inositol, which stimulates the growth of callus, often in a synergistic way with auxins. Coconut milk also possesses cytokinin activity, which can also interact with auxin to give enhanced growth (Sharp and Crocomo, 1975). Other plant extracts which have been used are carrot root for carrot callus (Syono, 1965); potato juice for potato callus (Lingappa, 1957); potato extract for anther culture of rice and wheat (Hu Han et al., 1978) and tomato juice for tomato fruit culture (Nitsch, 1949). Various other extracts have been used from time to time such as watermelon juice and prune juice (Guha and Maheshwari, 1967); orange and banana juice (Murashige, 1974).

#### Physical factors

There are many physical factors that can influence the growth of callus and organized development in vitro. These include the physical form of the medium i.e. whether it is semisolid or liquid, incubation temperature, light intensity and pH of the medium (Thorpe, 1981).

#### pH

The pH of the nutrient media is also a critical factor



although media for plant tissue cultures are usually adjusted to slightly acidic pH values being between 5 and 6. The optimum pH for Marigold crown gall callus growth was 6.1 (Muir et al., 1958) and for Picea glauca callus between pH 5.5 to 6.0 (White and Risser, 1964).

#### Agar concentration

Media can be left in the liquid state or solidified with agar (gelatin is toxic to plant cells, Street, 1966). Agar is a polysaccharide, purified from seaweeds (Barry and Dillon, 1944), which dissolves when warmed and upon cooling forms a gel. Agar exists in different grades of purity, which influence the growth of tissue. Romberger et al. (1970) reported better growth of Picea meristem on Difco purified agar than on the more highly purified Difco special Noble agar. This could be due to the presence of impurities in the agar, which stimulate the growth of the meristems. The concentration of the agar is another important factor which influences growth in vitro. White and Risser (1964) tested the effect of Difco Noble agar concentration varying from 0.125 - 2% on the growth of Picea glauca callus and obtained best growth at 0.5% agar concentration.

#### Temperature

Temperature is also a very important factor for the growth of tissues in vitro. Temperatures between 25 - 28°C are considered optimum for achieving maximum growth. Some tissues however have different temperature requirements. An early study by Skoog (1944) showed that shoot production in

tobacco callus was highest at 18°C. A temperature of 33°C was too high and 12°C was too low. White and Risser (1964) showed optimal growth of Picea glauca callus at 20°C in the dark whereas Gupta et al. (1980) reported maximum shoot and plantlet formation in teak at 30°C.

#### Light requirements

A major factor of the tissue culture environment is light intensity and length of the daily exposure period. This is particularly important for growth of organized shoots in culture. In experiments with the moss Physcomitrium turbinatum, Nebel and Naylor (1968) discovered that shoot bud formation was related linearly to light intensity, at least in the range 300 - 700 lux. They used incandescent lamps and provided light continuously. Maximum root initiation in Helianthus tuberosus tuber culture occurred at an intensity of 5,000 lux, using white light and daily exposure period of 12 h (Gautheret, 1969).

## Section 2: Studies on woody trees - a review

Studies on the growth of callus and differentiation in tissue cultures of trees has recently been reviewed (Winton and Huhtinen, 1976; Bonga, 1977; Winton, 1978; Abbott, 1978; Sommer and Brown, 1979; Sommer and Caldas, 1981).

This Section briefly covers the tissue culture literature on different studies carried out with tree species. The literature on reports of callus differentiation and plantlet formation from forest trees, belonging to the Gymnosperms and Angiosperms is given in Tables 1a - 1g. The references included in these tables have been grouped as follows:

- Table 1a    Differentiation from Gymnosperms - from embryo and seedlings.
- Table 1b    Differentiation from Gymnosperms - plants over 1 year old.
- Table 1c    Differentiation from Angiosperms (forest trees).
- Table 1d    Differentiation from Angiosperms (Populus species).
- Table 1e    Callus formation from Eucalyptus species.
- Table 1f    Plantlet regeneration from Eucalyptus species.
- Table 1g    Studies on Eucalyptus citriodora.

The work on tissue cultures of Eucalyptus species have been referred to in greater detail because of their direct relevance to the thesis.

These tables do not include the media used since this aspect of the work has been described in the respective Chapters in the general discussion.

### Callus culture studies

A callus is defined as an organized mass of cells in which the process of cell division and cell enlargement continues as long as nutritional conditions permit (Sharp and Crocorno, 1975). Primary explants taken from any part of the plant can generally be induced to form viable callus capable of indefinite growth by subculture on medium supported with growth regulators, and given the correct physical conditions (Narayanaswamy, 1977) as described in the earlier Section.

### History

Gautheret (1934) was the first to obtain calli from cambial explants of Pinus maritima, Abies pectinata, Acer pseudoplatanus, Populus nigra, Salix caprea, Alnus glutinosa, Quercus robur, Fagus sylvatica, Ulmus campestris and Fraxinus excelsior. These cultures did not survive on subculture. In 1936 La Rue grew callus from embryos of Pinus resinosa, Thuja occidentalis, Picea canadensis, Tsuga canadensis and Pseudotsuga menziesii. In 1940 Gautheret (1940a; 1940b) reported growth of callus from cambial tissue of buds of Ulmus campestris.

The first angiosperm tree callus cultures capable of subculture were obtained from Salix caprea (Gautheret, 1948a), Syringa vulgaris, Crataegus monogyna (Morel, 1948) and Castanea vesca (Jacquot, 1950). The first gymnosperm to be maintained successfully in continuous culture was of Sequoia sempervirens (Ball, 1950). Wetmore (1954) reported callus

growth of Syringa vulgaris apices in culture using Knop basal medium with sucrose. Konar and Oberoi (1965) reported embryoid formation from cotyledon callus of Biota orientalis. Reinert and White (1956) developed a medium for Picea glauca that consisted of White salts, vitamins, amino acids, auxin and sucrose. White and Risser (1964) carried out detailed studies of several chemical and physical parameters for obtaining optimal growth of normal and tumor tissues of Picea glauca. Brown and Lawrence (1968) developed a medium based on Murashige and Skoog salts with vitamins, asparagine and 2,4-D that was suitable for subculturing Pinus elliotti callus for a period of 5 years.

In the early years of tree tissue culture, most cultures were isolated from cambium explants. The technique for obtaining such explants was originally developed by Gautheret (1934) and has been described in detail (Gautheret, 1959; White, 1963). Grasham and Harvey (1970) reported these techniques specifically for conifer cambial explants. Cambial explants of woody species contain a high content of endogenous cytokinins as a result of which callus can often be induced and maintained through several subcultures on a nutrient medium containing only an auxin as the growth hormone, (Nitsch, 1963). Jacquot (1967; 1972) reported that callus of Castanea vesca and Betula verrucosa could be initiated and maintained on a medium containing mineral salts supplemented only with glucose or sucrose. Another advantage of cambial explants especially if taken from the

trunk of a big tree (White and Risser, 1964; White, 1967) is that many replicates can be made from one large area of the bark thus reducing morphological and physiological variation among the explants. Winton and Mathes (1973) routinely used cambial explants from succulent juvenile shoots. Venverloo (1973) and Chalupa (1974) used cambium from young internodes of Populus. Narasimhan et al. (1970) obtained callus growth from cambial regions of teak, mulberry and Populus on Murashige and Skoog (1962) medium and of Jack callus tissue on Blaydes (1966) medium. Hricova et al. (1974) used twigs and young stems of Picea excelsa and Huhtinen and Yahyaoglu (1974) used twigs of young and mature Betula pendula.

The cambium and phloem tissues of the trunk and branches however, are not the only parts of the tree suitable for the induction of callus. In Tilia americana (Barker, 1959) no differences in growth rates and morphology were detected in calli from explants from various parts of the tree. Sato (1978) reported the induction and maintenance of callus tissues in Japanese black pine (Pinus thunbergi) from hypocotyl segments.

Callus initiation is often affected by season. Sussex and Clutter (1959) found best proliferation using explants of several gymnosperm and angiosperm trees when the explants were collected in spring. Bytchenkova (1963) reported similar results except that generally callus growth was poor shortly after bud break. Trippi (1963) studied callus

proliferation from internode explants of juvenile and adult branches of Robinia pseudoacacia and Castanea vulgaris and found no seasonal effect in explants from the juvenile zone of the tree, those from the adult zone proliferated less if collected during the flowering season. Grasham and Harvey (1970) reported delayed callus development from conifer explants collected in mid winter.

Most tree callus cultures are grown at a constant temperature and generally grow well within a wide range around the optimal temperature (White and Risser, 1964), although some cultures are temperature sensitive (Staritsky, 1970). Pine callus cultures occasionally go dormant if continuously exposed to an optimal constant temperature (Bonga, 1980). Placing the culture in cold storage (0 - 4°C) for about 6 weeks breaks this dormancy and rapid growth resumes when cultures are returned to a higher temperature (27°C).

#### Differentiation studies

Differentiation occurs in plant tissues either by organogenesis or embryogenesis. Organogenesis can be described as the formation of shoot buds or roots from tissue culture (Winton and Huhtinen, 1976) whereas embryogenesis is a process in which plantlets are formed from a single cell or a group of cells (Street and Withers, 1974). This is called an embryoid or adventive embryo, since it is formed from a somatic cell. Its developmental pathway closely resembles the normal embryology of a zygote (Reinert, 1973).



Haccius (1971) distinguished between a developing shoot and an embryoid. According to him an embryoid in the earlier stages is bipolar. It has at the opposite ends, a shoot and a radicular pole. It is not connected with the vascular tissue of the mother plant. In contrast to this a shoot bud or a root primordium is monopolar and is connected with the vascular tissue of the mother plant. Torrey (1966) observed that some small cells in the culture, which have dense cytoplasm, less vacuoles and prominent nuclei form a group of cells known as the meristemoid. These are capable of forming either shoots, roots or embryo like structures. He further stated that the inability of callus to differentiate is due to the lack of such organized structures.

The stages for regeneration and morphogenesis leading to plantlet formation in trees is more difficult to achieve than the continuous culture of callus (Bonga, 1977). The high auxin concentrations commonly used for callus induction and maintenance are generally inhibitory to morphogenesis and in most cases have to be replaced by a precise auxin - cytokinin balance to obtain proper root and shoot primordia. Initially with its discovery, kinetin was the most commonly used cytokinin. Now it is being replaced by other cytokinins especially BAP for the growth of Populus species and Picea glauca tissues (Jacquiot, 1968; Winton, 1971; Chalupa, 1974; Campbell and Durzan, 1975). After the classical discovery of Miller and Skoog, cytokinin-auxin ratios proved effective for differentiation of many plant species.

However, with many plants this system does not work. Lee and de Fossard (1974) tested 175 different auxin - cytokinin combinations on callus cultures of Eucalyptus bancroftii but found no distinctive morphogenetic response. Many chemical factors other than auxin and cytokinins have been found to affect morphogenesis (Murashige, 1974). Bud formation in callus of Ulmus campestris depended on the proper balance of auxin and meso-inositol (Jacquiot, 1951; 1966).

One of the most important factors in obtaining morphogenesis is a correct choice of the explants. Baker (1969) isolated equally fast growing calli from explants taken from different parts of Tilia americana. The morphogenetic potential of these calli was found to vary with the origin of the explant. In explants from the cambial regions of Picea glauca and presumably in cambial explants of other trees, callus arises first mainly from cambial cells, a few cells arising later from the phloem parenchyma and xylem parenchyma cells (White, 1967).

Mathes (1964) and Winton (1968) obtained shoot and root formation from cambial callus of Populus tremuloides. Berbee et al. (1972) reported regeneration from shoot tip callus of Populus canadensis hybrid. In contrast callus obtained from cambial explants failed to form shoots and roots. Huhtinen and Yahyaoglu (1974) obtained plantlet regeneration from cambial callus of Betula pendula.

One major problem in propagation from callus cultures is

genotype variation between the propagules resulting from genetic instability of the callus cultures (Sunderland, 1973; Murashige, 1974). Chromosome abnormalities are common in callus culture of gymnosperms. Sticky and clumping chromosomes were found in callus cultures of Pinus strobus, P. densiflora and P. resinosa (Gautheret, 1956; Tominager and Oga, 1970; Bonga, 1974). Polyploid and aneuploid cells were found in cultures of Ginkgo biloba (Tulecke, 1957) and Picea glauca (Torok de and Roderick, 1962; Torok de, 1968). Partanen (1953) found a few tetraploid cells in cultures in Pinus lambertiana cells while less than the haploid number of chromosomes appeared in cultures of tumors of Picea glauca (Torok de and White, 1960) and microsporo-phylls of Pinus resinosa (Bonga, 1974b).

Differentiation studies carried out on forest trees (Gymnosperm and Angiosperm) from embryonic tissue, seedlings or mature tree explants have been given in Tables (1a - 1g).

From a tree improvement point of view for clonal multiplication, regeneration from sections of embryos or young seedlings has one distinct disadvantage (Lakshmisita et al., 1982). Though these embryos and seedlings may have been obtained from seeds of selected superior trees, their genotype is different from that of the parent tree and the superior characteristics of the latter are not necessarily retained by the propagules (Mehta-Palta et al., 1978; Lakshmisita et al., 1982)

**Table 1a** Tissue culture of forest trees (Gymnosperms - embryo, seedling)

| Tree species                   | Explants                       | Results                              | References                               |
|--------------------------------|--------------------------------|--------------------------------------|------------------------------------------|
| 1. <u>Biota orientalis</u>     | Cotyledons                     | Embryoids                            | Konar and Oberoi (1965)                  |
| 2.                             |                                | Adv. buds                            | Thomas et al. (1977)                     |
| 2. <u>Cryptomeria japonica</u> | Hypocotyl segments             | Adv. buds and roots                  | Isikawa (1974; 1978a; 1978b)             |
| 3. <u>Picea abies</u>          | Embryos                        | Adv. buds                            | Von Arnold and Eriksson (1978)           |
|                                | Needles (20 week old seedling) | Shoot buds                           | Jansson and Bornman (1980)               |
| 4. <u>Picea glauca</u>         | Hypocotyl segments             | Plantlets                            | Campbell and Durzan (1975; 1976a; 1976b) |
|                                | Epicotyl                       | Plantlets                            | Rumary (1981)                            |
|                                | Seedling segments              | Callus-cell suspension embryogenesis | Durzan (1982)                            |
| 5. <u>Pinus gerardiana</u>     | Cotyledons                     | Callus, shoots and roots             | Konar (1975)                             |
| 6. <u>Picea mariana</u>        | Epicotyl                       | Plantlets                            | Rumary (1981)                            |
| 7. <u>Pinus palustris</u>      | Embryos                        | Plantlets                            | Sommer et al. (1975)                     |
|                                | Seedling explants              | Plantlets                            | Sommer et al. (1980)                     |
| 8. <u>Pinus pinaster</u>       | Cotyledons                     | Plantlets                            | David and David (1977)                   |

| Tree species                     | Explants                                                                               | Results                                              | References                                                                                                            |
|----------------------------------|----------------------------------------------------------------------------------------|------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|
| 9. <u>Pinus radiata</u>          | Embryos and seedling shoot tips.<br>Embryonic axis.<br>Seedling explants<br>Cotyledons | Plantlets<br><br>Plantlets<br>Plantlets<br>Plantlets | Horgan and Aitken (1981)<br><br>Reilly and Washer (1977)<br>Smith <u>et al.</u> (1982)<br>Aitken <u>et al.</u> (1981) |
| 10. <u>Pinus strobus</u>         | Embryos, cotyledons                                                                    | Adv. buds, shoots and roots                          | Minocha (1980)                                                                                                        |
| 11. <u>Pinus sitchensis</u>      | Shoot apices                                                                           | Plantlets                                            | Webb and Street (1978)                                                                                                |
| 12. <u>Pinus sylvestris</u>      | Needles                                                                                | Adv. buds and shoots                                 | Bormann and Jansson (1980)                                                                                            |
| 13. <u>Pinus contorta</u>        | Embryos                                                                                | Plantlets                                            | Von Arnold and Eriksson (1981)                                                                                        |
| 14. <u>Pinus taeda</u>           | Needles, Embryos                                                                       | Plantlets                                            | Mott <u>et al.</u> (1977)<br>Mehra-Palta <u>et al.</u> (1978)                                                         |
| 15. <u>Pinus thunbergii</u>      | Hypocotyl, Embryos                                                                     | Callus, Plantlets                                    | Sato (1978a; 1978b)                                                                                                   |
| 16. <u>Pinus wallichiana</u>     | Hypocotyl                                                                              | Shoots                                               | Konar and Singh (1980)                                                                                                |
| 17. <u>Pseudotsuga menziesii</u> | Excised embryos<br>Cotyledons, stem<br>Hypocotyl<br>Cotyledons                         | Plantlets<br>Plantlets<br>Plantlets<br>Plantlets     | Winton and Verhagen (1977)<br>Cheng (1975)<br>Cheng (1977)<br>Cheng (1979)                                            |
| 18. <u>Tsuga heterophylla</u>    | Cotyledons                                                                             | Plantlets                                            | Cheng (1976)                                                                                                          |
| 19. <u>Thuja plicata</u>         | Shoot tips (14 day old seedling)                                                       | Plantlets                                            | Coleman and Thorpe (1977)                                                                                             |

**Table 1b Tissue culture of forest trees (Gymnosperms - plants over one year old)**

| Tree species                     | Explants                                                | Results                                | References                      |
|----------------------------------|---------------------------------------------------------|----------------------------------------|---------------------------------|
| 1. <u>Abies balsamea</u>         | Terminal, axillary buds (15 - 20 year old trees)        | Embryoids                              | Bonga (1977)                    |
|                                  | Young vegetative shoots with young needles, mature tree | Adv. buds and shoots                   | Bonga (1981)                    |
| 2. <u>Araucaria cunninghamii</u> | Stem segments with dormant buds (18 month old tree)     | Plantlets                              | Hainer and de Fossard (1977)    |
| 3. <u>Picea abies</u>            | Resting vegetative buds (75 year old tree)              | Adv. buds                              | Von Arnold and Eriksson (1979a) |
|                                  | Isolated needles (5 year old tree)                      | Adv. buds                              | Von Arnold and Eriksson (1979b) |
|                                  | Mature trees                                            | Adv. shoots                            | Von Arnold (1982)               |
| 4. <u>Picea glauca</u>           | Young vegetative buds with young needles (mature trees) | Adv. buds and shoots                   | Bonga (1981)                    |
| 5. <u>Pinus banksiana</u>        | Juvenile tissues                                        | Callus, cell suspension, embryogenesis | Dorman (1982)                   |
| 6. <u>Pinus pinaster</u>         | Needles (2 - 3 year old trees)                          | Plantlets                              | David and David (1977)          |

| Tree species                    | Explants                                                 | Results                    | References                                                |
|---------------------------------|----------------------------------------------------------|----------------------------|-----------------------------------------------------------|
| 7. <u>Pinus taeda</u>           | Needles (5 year old trees)                               | Plantlets                  | Mott et al. (1977)<br>Mehra-Falta et al. (1978)           |
| 8. <u>Pseudotsuga menziesii</u> | Needles (1 - 2 years old trees)                          | Plantlets                  | Winton and Verhagen (1977),<br>Cheng (1976), Cheng (1979) |
|                                 | Young vegetative shoots with young needles, mature trees | Adv. buds and shoots       | Bonga (1981)                                              |
|                                 | Dormant buds, mature trees                               | Adv. buds                  | Boulay (1979b)<br>Thompson and Zaerr (1981)               |
| 9. <u>Sequoia sempervirens</u>  | Terminal buds and stem segments, mature trees            | Plantlets                  | Ball et al. (1978), Boulay (1979a)                        |
| 10. <u>Tsuga heterophylla</u>   | Stem and needles (2 year old trees)                      | Plantlets                  | Cheng (1976)                                              |
| 11. <u>Thuja plicata</u>        | Shoot tips from lateral branches (4 - 10 year old trees) | Plantlets                  | Coleman and Thorpe (1977)                                 |
|                                 | Shoot tips (mature trees)                                | Induction of male strobili | Coleman and Thorpe (1978)                                 |

Table 1c Tissue culture of forest trees (Angiosperm)

| Tree species                     | Explants                                        | Results                         | References                                 |
|----------------------------------|-------------------------------------------------|---------------------------------|--------------------------------------------|
| 1. <u>Acacia kao</u>             | Cambial callus                                  | Plantlets                       | Skolman and Wapes (1976)                   |
| 2. <u>Aesculus hippocastanum</u> | Anthers                                         | Plantlets                       | Radojevic (1978)                           |
| 3. <u>Betula alleghaniensis</u>  | Seedling segments                               | Plantlets                       | Minocha (1980)                             |
| 4. <u>Betula papyrifera</u>      | Seedling segments                               | Plantlets                       | Minocha (1980)                             |
| 5. <u>Betula pendula</u>         | Explants of 3 - 4 year old trees                | Callus, embryogenesis plantlets | Srivastava and Steinhauer (1981)           |
|                                  | Stems                                           | Plantlets                       | Karnosky (1981)                            |
|                                  | Anthers                                         | Plantlets                       | Huhtinen (1978)                            |
| 6. <u>Betula verrucosa</u>       | Nodal segments mature trees                     | Plantlets                       | Chalupa (1981)                             |
| 7. <u>Aesculus carnea</u>        | Cambial explants                                | Embryoid, plantlets             | Saito (1980c)                              |
| 8. <u>Coffea arabica</u>         | Anthers endosperm embryos, leaf                 | Embryoid, plantlets             | Monaco et al. (1977)<br>Rao and Lee (1982) |
| 9. <u>Corylus avellana</u>       | Embryonic axes                                  | Plantlets                       | Jarvis et al. (1978)                       |
| 10. <u>Dalbergia latifolia</u>   | Nodal segments root segments (5 year old trees) | Plantlets                       | Mascarenhas et al. (1982)                  |



| Tree species                           | Explants                                                                                   | Results                                          | References                                                                                  |
|----------------------------------------|--------------------------------------------------------------------------------------------|--------------------------------------------------|---------------------------------------------------------------------------------------------|
| 11. <u>Dalbergia sissoo</u>            | Root segments<br>from seedlings                                                            | Embryoids                                        | Mukhopadhyay and Mohan Ram<br>(1982)                                                        |
| 12. <u>Drimys winteri</u>              | Young shoots<br>(3 year old trees)                                                         | Callus,<br>shoots                                | Jordan and Cortes (1981)                                                                    |
| 13. <u>Cassia fistula</u>              | Seedling segments                                                                          | Plantlets                                        | Rao and Lee (1982)                                                                          |
| 14. <u>Calophyllum<br/>inophyllum</u>  | Seedling segments                                                                          | Plantlets                                        | Rao and Lee (1982)                                                                          |
| 15. <u>Eugenia aquea</u>               | Seedling segments                                                                          | Plantlets                                        | Rao and Lee (1982)                                                                          |
| 16. <u>E. cumini</u>                   | Seedling segments                                                                          | Plantlets                                        | Rao and Lee (1982)                                                                          |
| 17. <u>E. grandia</u>                  | Seedling segments                                                                          | Plantlets                                        | Rao and Lee (1982)                                                                          |
| 18. <u>E. jambos</u>                   | Seedling segments                                                                          | Plantlets                                        | Rao and Lee (1982)                                                                          |
| 19. <u>Pavaneae fragrans</u>           | Seedling segments                                                                          | Plantlets                                        | Rao and Lee (1982)                                                                          |
| 20. <u>Liquidambar<br/>styraciflua</u> | Hypocotyl segments                                                                         | Embryoids                                        | Sommer and Brown (1980)                                                                     |
| 21. <u>Hevea brasiliensis</u>          | Embryos<br>Seedling segments<br>Anthers<br>Nodal segments<br>(1 year old budded<br>stumps) | Plantlets<br>Plantlets<br>Plantlets<br>Plantlets | Paranjothy (1974)<br>Carron and Enzalrik (1982)<br>Chen (1982)<br>Mascarenhas et al. (1982) |
| 22. <u>Paulownia<br/>taiwaniana</u>    | Seedling segments                                                                          | Plantlets                                        | Fu (1978)                                                                                   |

| Tree species                   | Explants                                         | Results   | References                    |
|--------------------------------|--------------------------------------------------|-----------|-------------------------------|
| 23. <u>Paulownia tomentosa</u> | Embryos, fertilized ovules                       | Plantlets | Radojevic (1979)              |
| 24. <u>Santalum album</u>      | Hypocotyl                                        | Plantlets | Bapat and Rao (1979)          |
|                                | Cambial callus (mature trees)                    | Plantlets | Lakshmisita et al. (1979)     |
|                                | Cell suspension (mature trees)                   | Plantlets | Lakshmisita et al. (1979)     |
|                                | Endosperm                                        | Plantlets | Lakshmisita et al. (1980a)    |
| 25. <u>Sweitenia mahoganii</u> | Seedling segments                                | Plantlets | Rao and Lee (1982)            |
| 26. <u>Tamarindus indica</u>   | Seedling segments, nodal segments (mature trees) | Plantlets | Mascarenhas et al. (1981)     |
| 27. <u>Tectona grandis</u>     | Seedling segments, buds (100 year old trees)     | Plantlets | Gupta et al. (1980)           |
|                                |                                                  |           | Mascarenhas et al. (1981)     |
| 28. <u>Ulmus americana</u>     | Hypocotyl callus, cell suspension                | Plantlets | Durzan and Lopushanski (1975) |
| 29. <u>Ulmus campestris</u>    | Stem                                             | Plantlets | Chalupa (1975)                |

Table 1d Tissue culture of forest trees (Angiosperms - Populus)

| Populus species                                   | Explants       | Results           | References                 |
|---------------------------------------------------|----------------|-------------------|----------------------------|
| 1. <u>Populus canescens</u>                       | Cambial region | Plantlets         | Chalupa (1975)             |
| 2. <u>P. ciliata</u>                              | Leaf, buds     | Plantlets         | Mehra and Cheema (1980)    |
| 3. <u>P. deltoides</u>                            | Anthers        | Plantlets         | Sato (1974)                |
| 4. <u>P. deltoides</u>                            | Cambial region | Callus, shoots    | Saito and Kawanobe (1981)  |
| 5. <u>P. deltoides</u> x <u>P. nigra</u>          | Axillary buds  | Plantlets         | Whitehead and Giles (1976) |
| 6. <u>P. euroamericana</u>                        | Cambial region | Callus, shoots    | Saito (1980e)              |
| 7. <u>P. maximowiczii</u>                         | Anther         | Plantlets         | Sato (1974)                |
| 8. <u>P. nigra</u>                                | Cambial region | Plantlets         | Chalupa (1975)             |
| 9. <u>P. nigra</u>                                | Stem           | Callus, Plantlets | Venverloo (1973)           |
| 10. <u>P. nigra</u>                               | Axillary buds  | Plantlets         | Whitehead and Giles (1976) |
| 11. <u>P. sieboldii</u> x <u>P. grandidentata</u> | Anthers        | Plantlets         | Sato (1974)                |
| 12. <u>P. sieboldii</u> x <u>P. grandidentata</u> | Cambial region | Callus, shoots    | Sato (1981)                |
| 13. <u>P. tremula</u>                             | Root           | Callus, Plantlets | Winton (1971)              |
| 14. <u>P. tremuloides</u>                         | Stem           | Callus, Plantlets | Winton (1970)              |
| 15. <u>P. tristis</u> x <u>P. balsamifera</u>     | Shoot tip      | Callus, Plantlets | Thompson and Gordon (1977) |

| <u>Eucalyptus species</u>                                                                     | <u>Explants</u>        | <u>Results</u> | <u>References</u>                           |
|-----------------------------------------------------------------------------------------------|------------------------|----------------|---------------------------------------------|
| 1. <u>Eucalyptus alba</u>                                                                     | Hypocotyls             | Callus         | Kitahara and Caldas (1975)                  |
| 2. <u>E. bancroftii</u>                                                                       | Stem, lignotuber       | Callus         | de Fossard (1974); de Fossard et al. (1974) |
| 3. <u>E. camaldulensis</u>                                                                    | Cambial tissue         | Callus         | Jacquot (1964)                              |
| 4. <u>E. cladocalyx</u>                                                                       | Cambial tissue         | Callus         | Jacquot (1964)                              |
| 5. <u>E. gomphocephala</u>                                                                    | Cambial tissue         | Callus         | Jacquot (1964)                              |
| 6. <u>E. grandis</u>                                                                          | Stem, leaf blades      | Callus         | de Fossard (1974)                           |
|                                                                                               | Hypocotyls             | Callus         | Goncalves (1975; 1979)                      |
|                                                                                               | Anthers                | Callus         | Kitahara and Caldas (1975)                  |
| 7. <u>E. gunnii</u>                                                                           | Cambial tissue         | Callus         | Jacquot (1964)                              |
| 8. <u>E. laevopinea</u>                                                                       | Stem, leaf, lignotuber | Callus         | de Fossard (1974)                           |
| 9. <u>E. melliodora</u>                                                                       | Stem, leaf, lignotuber | Callus         | de Fossard <del>et al.</del> (1974)         |
| 10. <u>E. nicholii</u>                                                                        | Stem, leaf, lignotuber | Callus         | de Fossard et al. (1974)                    |
| 11. <u>E. obliqua</u>                                                                         | Lignotuber             | Callus         | Blake (1972)                                |
| 12. <u>E. robusta</u>                                                                         | Stem, Leaf, lignotuber | Callus         | Goncalves (1975)                            |
| 13. <u>E. rostrata</u>                                                                        | Cambial tissue         | Callus         | Pine (1968)                                 |
| 14. <u>E. terreticornis</u>                                                                   | Cambial tissue         | Callus         | Jacquot (1964); Goncalves (1975)            |
| 15. <u>E. x trarutii (hybrid)</u> Stem segment<br><u>botryoides x</u><br><u>camaldulensis</u> |                        | Callus         | Macavillaca and Montaldi (1964)             |
| 16. <u>E. viminalis</u>                                                                       | Lignotuber             | Callus         | Blake (1972)                                |

| <u>Eucalyptus species</u>   | <u>Explants</u>                        | <u>Results</u> | <u>References</u>                                      |
|-----------------------------|----------------------------------------|----------------|--------------------------------------------------------|
| 1. <u>Eucalyptus alba</u>   | Hypocotyl                              | Plantlets      | Kitahara and Caldas (1975)                             |
| 2. <u>E. camaldulensis</u>  | Nodal segments, seedling               | Plantlets      | Goncalves (1977); Hartney and Barker (1980)            |
| 3. <u>E. ficifolia</u>      | Nodal segments, seedling               | Plantlets      | de Fossard and Bourne (1976)                           |
|                             | Mature tree explant                    | Plantlets      | de Fossard (1978)                                      |
| 4. <u>E. curtisii</u>       | Seedling segments                      | Plantlets      | Hartney and Barker (1980)                              |
| 5. <u>E. globulus</u>       | Nodal segment (mature tree)            | Plantlets      | Mascarenhas et al. (1982)                              |
|                             | Seedling segments                      | Plantlets      | Hartney and Barker (1980)                              |
| 6. <u>E. grandis</u>        | Seedling segment, mature trees         | Plantlets      | Hartney and Barker (1980); Cresswell and Nitsch (1975) |
| 7. <u>E. nitens</u>         | Seedling segments                      | Plantlets      | Hartney and Barker (1980)                              |
| 8. <u>E. nova-anglica</u>   | Seedling segments                      | Plantlets      | Mehra-Palta (1982)                                     |
| 9. <u>E. marginata</u>      | Nodal segments, seedling, mature trees | Plantlets      | McComb and Bennett (1982)                              |
| 10. <u>E. obtusiflora</u>   | Seedling segment                       | Shoots         | Hartney and Barker (1980)                              |
| 11. <u>E. orcadeae</u>      | Seedling segment                       | Shoots         | Hartney and Barker (1980)                              |
| 12. <u>E. pauciflora</u>    | Seedling segment                       | Shoots         | Hartney and Barker (1980)                              |
| 13. <u>E. regnans</u>       | Seedling segment                       | Plantlets      | Hartney and Barker (1980)                              |
| 14. <u>E. robusta</u>       | Seedling segment                       | Plantlets      | Goncalves (1975)                                       |
| 15. <u>E. terreticornis</u> | Nodal segments, mature trees           | Plantlets      | Mascarenhas (1982)                                     |
| 16. <u>E. viminalis</u>     | Seedling explants                      | Plantlets      | Mehra-Palta (1982)                                     |

| <u>Eucalyptus species</u>    | <u>Explants</u>                                | <u>Results</u>                         | <u>References</u>                                    |
|------------------------------|------------------------------------------------|----------------------------------------|------------------------------------------------------|
| <u>Eucalyptus citriodora</u> | Lignotuber                                     | Plantlets                              | Aneja and Atal (1969)                                |
|                              | Seedling segments                              | Plantlets                              | Gupta et al. (1978)                                  |
|                              | Seedling segments                              | Plantlets                              | Lakshmisita and Vaidyanathan (1979)                  |
|                              | Cotyledons                                     | Callus, shoots and roots               | Lakshmisita (1979)                                   |
|                              | Shoot apices (2 year old trees)                | Multiple shoots                        | Grewal et al. (1980)                                 |
|                              | Apical and axillary buds (20 year old trees)   | Plantlets                              | Gupta et al. (1981); Mascarenhas et al. (1981; 1982) |
|                              | Callus, seedling explants, (20 year old trees) | Field data and essential oil synthesis | Gupta and Mascarenhas (1981)                         |

### Cell suspension culture

Callus tissues can be dissassociated, in shaken liquid cultures into suspensions of single cells and small cell aggregates which grow and divide rapidly like bacterial cells. Multiplication rates of upto 20-fold in cell number in 10 days are easily achieved (Abbott, 1978). Some of these cells can be induced to form embryoids so that virtually unlimited number of potential plantlets can be produced from shake flasks. The embryoids can be separated and in a few cases grown on agar medium into plantlets. (Bonga, 1977). There are some reports on herbaceous species which respond in this way but reports on tree species are still rare, with Santalum album (Bapat and Rao, 1979; Lakshmisita et al., 1980), with Liquidambar styraciflua (Sommer and Brown, 1980). Suspension cultures have been grown of cells of Acer pseudo-platanus (Street, 1973), Pseudotsuga menziesii (Winton, 1972), Picea abies (Winton and Huhtinen, 1976). Durzan and Lopushanski (1975) grew Ulmus americana cells for five months as cell suspension, which when plated on agar medium regenerated apparently normal plantlets. Somatic cell suspension from callus of juvenile tissues of Picea glauca, Pinus banksiana, and Pseudotsuga menziesii showed the potential of embryo formation upto the torpedo stage (Durzan, 1982).

### Shoot tip and axillary bud cultures

Shoot tips (meristem) and axillary buds have proved the most successful tissues for propagating plants in tissue

culture. The normal organization of the differentiated shoot is maintained and the risk of many mutant cells growing is almost eliminated (Abbott, 1978). Single meristems have been cultured for many years to produce virus-free plants (Morel and Martin, 1952; Quak, 1957). Shoot tips and axillary buds have often been used for mass propagation of many herbaceous species (Murashige, 1974). Some have been commercially used, for example, many orchid genera (Holdgate, 1977). Recently several fruit trees like apple, plum, cherry, almonds have been propagated by apical and axillary bud culture (Cheng, 1979; Hutchinson, 1981). Mascarenhas et al. (1981) reported propagation of forest trees such as teak, Eucalyptus citriodora, and tamarind by apical and axillary bud culture. Giles (1976) and Chalupa (1981) reported plantlet formation from shoot tip culture of Populus spp. and Birch.

#### Embryo culture

One of the oldest applications of tissue culture in plant breeding is embryo culture (Hannig, 1904). The main objective of this technique is to germinate, interspecific hybrid embryos (Raghavan, 1977), that would not normally germinate because of incompatibility between the embryo and maternal tissues, as is common in seeds resulting from interspecific hybridization (Hagman, 1972). Various hybrid embryos in Prunus, Sorbus, Malus and Citrus have been excised and cultured in vitro, most of which have been grown for specific physiological or morphological studies rather than to



circumvent genetic incompatibility (Maheshwari and Rangaswamy, 1965). In one study the second year female strobilus of two pine species (P. strobus and P. peuce), along with several hybrid cones, were grown in vitro to maturity (Kriebel and Shafer, 1972).

#### Haploid cultures

The first successful haploid plants were produced from pollen grains of cultured anthers of Datura (Guha and Maheshwari, 1964; 1966).

The production and use of haploids has not been exploited as a tool in forest tree improvement (Winton and Stettler, 1974; Stettler, 1976). Haploids have been shown to have a potential for the production of homozygous lines of corn (Chase, 1974), in the breeding of improved varieties of barley (Kasha, 1974) and in reducing the production time for new varieties of tobacco by one-half (Nakamura et al., 1974). Haploids might also prove useful in a similar way in forest tree improvement (Bonga, 1977; Abbot, 1978).

Sato (1974) obtained haploid callus with some differentiation from anthers of Populus and haploid plantlets were derived from G. biloba (Tulecke, 1967). Haploid callus, but not plants, has been produced from Pinus, Taxus, Juniperus and Picea (Winton and Stettler, 1974). Recently, plantlets have been obtained from anther cultures of rubber (Chen, 1982).

#### Endosperm cultures

Callus of triploid endosperm from angiosperms has been

reported with herbaceous plants, and was reviewed by Johri and Srivastava (1973), and Johri et al. (1980).

Woody species which have been cultured as triploid callus include Putranjiva roxburghii (Johri, 1971) Johri and Srivastava, 1973; Srivastava, 1973), Jatropha panduraefolia (Srivastava, 1971), in the Euphorbiaceae family, as well as Santalum album (Rangaswamy and Rao, 1963) in the Santalaceae family. Recently Lakshmisita et al. (1980a) obtained triploid plants from endosperm cultures of Santalum album by embryogenesis.

#### Protoplast and somatic hybridization

Protoplasts are plant cells with cell walls removed, leaving a spherical cell surrounded by a cytoplasmic membrane (Cocking, 1972). Somatic hybridization is a technique in which naked protoplasts of somatic or genetic cells of different plants are fused and plantlets raised from the fused protoplasts (Bajaj, 1977). There are a number of reports on protoplast isolation, regeneration and somatic hybridization from herbaceous plants (Cocking, 1979; 1981).

Relatively little work has been done on the isolation of protoplasts from tree species. Among the forest trees, Huhtinen and Winton (1973) and Chalupa (1974) isolated protoplasts from Norway spruce callus, and Winton (1974a; 1974b) isolated protoplasts from Douglas-fir, loblolly pine and Western hemlock. Saito (1980a) reported the isolation of protoplast from mesophyll cells of Paulownia

and Populus. Othman and Paranjothy (1980) reported protoplast isolation from various tissues of Hevea brasiliensis. Douglas (1982) obtained protoplasts from cell cultures of Populus hybrid. There are very few reports of protoplast fusion in tree species, probably because of the difficulty (as compared with non woody species) in regenerating plants from tree cells. Saito (1980b) obtained fusion between protoplasts isolated from expanding young leaves of Populus euroamericana and Paulownia taiwaniana in the presence of polyethylene glycol. Recently Rodenbaugh (1981) obtained protoplasts from cotyledons, callus and suspension cultures of Ulmus americana and U. pumila and cotyledons and callus of U. parvifolia using various enzyme solutions. He also fused U. pumila protoplasts using a PEG-calcium solution.

#### Mutation breeding

The conventional methods used for isolation of mutants from tree species are mainly by irradiation (Campbell, 1974; Campbell and Lacey, 1973) or application of mutagenic chemicals to pollen, seeds or whole plants (Privaloy, 1972; Durzan and Campbell, 1974a; 1974b). However, these methods are not always applicable because mutation rates are often low, necessitating the use of large populations for effective mutant screening. If mutagens are applied to single cells mutant isolation could be simplified, by selection methods similar to those used in microbial systems.

Auxin autotrophic mutant cell lines were separated from suspension cultures of Acer pseudoplatanus treated with

physical and chemical mutagens (Lescure, 1969). Spiegel-Roy and Kochba (1973) found that gamma irradiation of ovular callus cultures of Citrus sinensis stimulated embryo formation but the effect appeared to be caused mainly by radiation induced chemical changes in the nutrient medium. An arginine requiring mutant cell line, originating from a callus obtained from Ginkgo biloba pollen, has been described (Tulecke, 1960).

#### Gene pool preservation

Impoverishment of the natural gene pool is a problem of great concern to tree breeders. As pointed out by Bajaj and Rienert (1977); Bajaj (1979) tissue culture could have an important function in the establishment of gene banks by using deep freeze storage techniques, particularly for plants that normally are propagated vegetatively i.e. those that are infertile and therefore cannot be stored as seeds. With regards to trees, cells of Acer pseudoplatanus have been stored in liquid nitrogen and have resumed growth after thawing (Sugawara and Sakai, 1974).

### Section 3    Production of secondary metabolites

Plants are used as sources of many compounds and mixtures of compounds that are useful to man. These compounds referred to as secondary products, include drugs, flavours, enzymes, essential oils, pigments etc. (Dougall, 1979). Secondary products do not include compounds such as amino acids or nucleotides that are intermediate in the metabolism of cells essential for survival. In addition secondary products do not include compounds that are ubiquitous in plants such as cellulose or sucrose (Dougall, 1979).

Secondary metabolites are not only sources of reserve materials but also contribute to specific functions in plants as follows (Jalal, 1981):

1. Secondary metabolites like pectin give mechanical strength to plants.
2. Some of the poisonous alkaloids repel insects and herbivores.
3. Compounds like isoflavones help the plants in resisting fungal infections.
4. Coloured pigments such as anthocyanin and some carotenes attract pollinators.
5. Previously it was thought that these secondary metabolites are end products but in some cases it has been shown that they are degraded in plants (Dougall, 1979).

Recent advances in plant tissue and cell culture have introduced a new potential source for studies on the production of secondary metabolites, their biosynthesis

and biotransformation (Stohs, 1980; Overton and Picken, 1977; Kurz and Constabel, 1979; Arya and Ramawat, 1978).

The major advantages of plant tissue culture are as follows: (Dougall, 1979; Crocorno et al., 1981; Misawa and Suzuki, 1982):

1. Since the cells are grown under controlled conditions such as shake flasks, fermentors, chemostats etc. the yield of the desired compounds can be carefully studied.
2. Useful compounds can be produced under sterile conditions irrespective of climatic changes and soil conditions.
3. Once the procedure is established the desired compounds can be produced round the year.
4. The biosynthetic pathway and biotransformation can be easily studied.
5. Cells in tissue culture can be genetically modified and those that accumulate specific intermediates and metabolites isolated.

The vast majority of the secondary compounds produced by plants are classified on the basis of chemical structure and their mode of biosynthesis. There are three main categories of products: Essential oils, glycosides and alkaloids. The essential oils consisting of a mixture of terpenoids are used as flavouring agents, perfumes, and solvents. The glycosides include flavonoids, saponins, phenolics, tannins and cyanogenic glycosides. These compounds are utilized as dyes, food colors and medicinals

(e.g. Steroid hormones, antibiotics and digitalis). The alkaloids are a diverse group of compounds with over 4000 structures. New alkaloid structures are being continually reported. Some examples of alkaloids are morphine, strychnine, nicotine, atropine and cocaine (Shuler, 1981).

Many of these secondary products have also been produced in tissue culture. The work is now extensively covered in several recent reviews (Butcher, 1977; Dougall, 1979; 1981; Shuler, 1981; Misawa and Suzuki, 1982) and will be briefly dealt with here on herbaceous plants, with examples of the forest trees where available.

1. Alkaloids Much attention has been given to cell cultures of Catharanthus roseus because of a wide variety of pharmaceutically important compounds present in this plant (Zenk et al., 1977; James et al. 1981; Constabel et al., 1981). In callus cultures of Rauwolfia serpentina, a producer of serpentine and rescinnamine, accumulation of these alkaloids (0.027% on cell dry weight) was shown (Yoshikawa et al., 1977). Chen and Staba (1965) reported 0.11% alkaloids production on dry weight basis in suspension cultures of Datura stramonium. Tropane alkaloids production and their biosynthesis has been extensively investigated in cultures of Atropa belladonna root (Mitra, 1972), Atropa callus and suspension cultures, cultures of Datura species (Khanna and Khanna, 1976a; 1976b; Staba and Kaul, 1971) and Scopolia species (Tabata et al., 1972).

Nicotine, anabasine, anatabine were isolated from suspension cultures of Nicotiana spp. (Steck and Constabel, 1974); other indole alkaloids from Cannabis (Veliky, 1972), furochromane visnagin from Amni visnaga (Staba, 1977); nor-sanguinarine a new alkaloid from opium poppy callus (Furuya et al., 1972). Harmin, an alkaloid not found in the intact plant but present in Phaseolus callus cultures (Veliky, 1972); caffeine from callus cultures of Coffea arabica (Keller et al., 1972); Camptothecin in both callus tissue and cell suspension cultures Camptotheca acuminata (Misawa, 1977); thebaine in amounts of upto 0.013% of dry weight in cultures of Papaver bracteatum (Khanna et al., 1976) and ephedrine (0.17%) in callus culture of Ephedra gerardiana grown under different physio-chemical conditions (Arya and Ramawat, 1978).

2. Steroids An important steroid diosgenin from Agave cultures was reported by Ehrhardt et al. (1966); from Dioscorea, Yucca and Lycopersicon (Marshall and Staba, 1976); from Momordica charantia (Khanna and Moham, 1973); Solanum xanthocarpum (Heble et al., 1971). Diosgenin with a yield of approximately 1% on dry weight was first isolated from callus cultures of Dioscorea deltoidea (Kaul and Staba, 1968). Suspension cultures of Trigonella foenum-graecum, when grown in medium containing 0.05% cholesterol, produced 3.5% diosgenin (Khanna et al., 1975), gitogenine (0.14%) and tigogenine (0.05%) were found in callus culture of Trigonella occulta (Jain et al., 1977). Production of



higher levels of trigonelline in callus cultures than plants of Trigonella foenum-graecum were reported by Radwan and Kokate (1980).

3. Saponins and Sapogenins Ginsenoside was found in amounts of upto 21.4% in the callus tissue of Panax ginseng (Furuya and Ishii, 1973); Panaxadiol and panaxatriol have been isolated from callus cultures of Panax ginseng (Furuya et al., 1970) and Panax quinquefolium (Jhang et al., 1974); oleanolic acid from Isodon japonicus callus culture (Tomita and Seo, 1973). 19.4 mg/g saponins on dry weight basis was obtained in root cultures of Bupleurum falcatum which was the same as had accumulated in the mother plants (18-5 mg/g) (Tomita and Uomori, 1976).

4. Quinones Pandya et al. (1966) isolated Ubiquinone-40 (0.053 µg/g of tissue) from Parthenocissum spp. normal and crown gall callus. Suspension cultures of N. tabacum accumulated 360 µg of ubiquinone-10/g of dried cells (Ikeda et al., 1977). Ubiquinone-9 was detected in suspension cultures of Carthamus tinctorius yielding 41 µg of compound per gram of dried cells (Hagimori et al., 1978).

5. Anthraquinones Zenk et al. (1975) reported the production of anthraquinones from Morinda citrifolia cultures.

Anthraquinones and dianthrone have also been found in callus cultures of Cassia angustifolia (Friedrich and Bair, 1973).

Dhruva et al. (1972) isolated three anthraquinones from teak callus. One of the compounds was 1,2,5-trihydroxy, 8-methyl-anthraquinone. Another was characterized as digitolutein

Another was characterized as digitolutein (1-methoxy, 2-hydroxy, 3-methylanthraquinone). This compound also shown to be present in teak wood extract. The third, a new anthraquinone (Teak quinone A) was isolated and its structure shown to be 1-methoxy, 2,5-dihydroxy, 3-methylanthraquinone.

6. Anthocyanin Anthocyanin production was observed in Haplopappus gracilis cultures (Constabel et al., 1974); in Catharanthus roseus culture (Carew and Kruger, 1974; Knobloch et al., 1982) and Daucus carota (Alfermann and Reinhard, 1971).

7. Naphthoquinones The presence of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) has been detected in callus cultures of Plumbago zeylanica (Heble et al., 1974). Mizukami et al. (1977) reported naphthoquinone production in cell suspension cultures of Lithospermum erythrorhizon at concentration of about 12% of the dry weight.

8. Tannin Tannin accumulation has been shown in Juniperus communis (Constabel et al., 1974) and Pinus eliotti (Baur and Walkinshaw, 1974). It was found that they constituted about 13% of the total dry weight and included both hydrolyzable and condensed tannins.

9. Flavonoids Flavonoids have been reported in cultures of Haplopappus (Constabel, et al., 1974), Glycine max (Hahlbrock, 1972) and Petroselinum hortense (Kreazules and Halbrock, 1973).

10. L-Dopa Brain (1976) reported that suspension cultures of Mucuna pruriens produced L-Dopa (3,4-Dihydroxyphenylalanine) and released it to the culture medium in amounts of upto 1% w/v.
11. Proteinase inhibitors Rele et al. (1980) Isolated papain inhibitors from callus cultures of Sorghum vulgare, Triticum vulgare, Lillium sp., Zea mays and Vigna unguiculata. Misawa and coworkers (1975) reported a compound with high antiplasmin activity consisting of five polypeptides in cultures of Scopolia japonica.
12. Antimicrobial substances Antimicrobial substances have been reported in Populus tremuloides and from Phytolacca americana (Mathes et al., 1971).
13. Antiviral substances Misawa et al. (1974) reported accumulation of anti-plant viral substances in a suspension culture of Phytolacca americana. Anti-plant viral substances were also isolated from callus cultures of Agrostemma githago (Takayama et al., 1977).
14. Lipids Lipids were reported in cultures of Artemisia absinthium, Brassica napus, Daucus carota, Phaseolus aureus (Radson et al., 1974; 1975).
15. Cardenolides Production of cardenolides in Digitalis lanata cultures was reported by Garve et al. (1980); in Digitalis purpurea (Karting et al., 1976) in Digitalis mertonensis (Medora et al., 1967).

16. Lignin Lignin was isolated from Pinus strobus callus and was shown to be of the typical conifer lignin type (Hasegawa et al., 1960). Venverloo (1969) also showed lignin production from callus cultures of Populus nigra.

17. Monoterpenes Although a great deal of work has been carried out in this field, much of it has been concentrated on alkaloids and steroids and little attention has been paid to the formation of monoterpenes in such cultures.

Krikorian and Steward (1969) reported that calli derived from Mentha piperita, Mentha spicata and other species do not produce detectable quantities of the monoterpenes characteristic of the intact plants (Becker, 1970). Similar negative results were obtained by Downing and Mitchell (1975) with callus cultures of Nepeta caparia; by Macrae and How, via Jones (1974) with cultures derived from a number of rose species. Takayuki et al. (1980) and Ishii et al. (1982) showed that none of the monoterpenoids characteristic of an essential oil of intact plants of Mentha spicata and Eucalyptus dives respectively were found in leaf callus tissues.

A small amount of monoterpenoids were detected by Hiroshi and Yoshuyuki (1976) from cultured cells of Perilla frutescens and by Purohit and Khanna (1981) from callus cultures of Ocimum basilicum. Recently Tabata et al. (1981) have shown that plants regenerated from callus of lemon grass contained identifiable concentrations of essential oil as in the parent plant.

#### Section 4      Present investigations

##### Tissue culture studies on Eucalyptus

The available literature on callus cultures and an differentiation induced from different parts of the plant viz. seeds, hypocotyl, cotyledons, stem, petiole, leaves, shoot apex, seedling roots, lignotuber, anther and cambial tissues have been given in Tables 1e, - 1g.

The present work deals with studies on plant tissue culture of Eucalyptus citriodora Hook (Fig. 1).

Eucalyptus citriodora Hook is commercially a very important forest tree and is prized both for its wood and the essential oil present in its leaves. It is commonly known as "spotted gum" or "lemon scented gum" or "citron scented gum". It is one of the important Eucalyptus species and was introduced into India at the beginning of the century from Australia. In India this tree grows very well in Tamil Nadu, Karnataka and Uttar Pradesh.

The wood is used for railway sleepers and for general construction purposes and also for fuel, whereas the leaves yield an essential oil, the concentration of which can often vary from 0.5 - 4.0% of the dried leaves (Singh, 1977). The oil contains an aldehyde citronellal and an alcohol citronellol whose concentrations also vary from 60 - 95% and 10 - 25% respectively in different trees (Fernandez, and Suri, 1981).

The oil of E. citriodora is a high grade oil much in demand in the perfumery industry. The oil is colourless,



Fig. 1 20 year old Eucalyptus tree  
growing at Law College, Pune.

with a very pleasant smell. The specific gravity at 15°C varies between 0.8658 to 0.8740 and the refractive index at 20°C varies between 1.451 to 1.456 (Penfold and Willis, 1961). It contains citronellal, citronellol, geraniol, esters, pinene, cineole and isopolegol. The aldehyde citronellal and the alcohol citronellol are the 2 major components of the oil. The ester and other fractions are present in traces (Geunther, 1948).

Citronellal is one of the important monoterpene aldehydes. It has a flowering honey like smell but cannot be used as such because of the unstable nature of the aldehyde group. However, it is a goldmine for the synthesis of high grade perfumery chemicals like citronellol, esters, hydroxy citronellal, menthol and rose oxide (Agarwal et al., 1979). Recently about sixty new compounds have been prepared from citronellal by chemical conversions. Some of the compounds prepared from citronellal possess an aroma which are now of great importance to the perfumery world. Compounds like citronellyl, isopropyl ketone, citronellyl methyl ether and citronelloxy methyl acetate prepared from the citronellal possess new aromas which are quite useful for the preparation of synthetic rose blend (Atal, 1979). A small quantity of oil is also added in germicides and disinfectants to improve their odour.

"Elite" trees have been identified in the forest in Tamil Nadu (South of India) which contain 3.5% oil and 92% aldehyde.

The normal method for propagation of Eucalyptus species is from seeds but seed raised plants show a very wide variation (Hartney and Barker, 1980). To prevent this variation in the progeny a vegetative method would be more useful for raising plants where the 'elite' characters are expressed in the progeny. Except for a few species the conventional methods of budding, grafting, layering and rooting of cuttings have not been very successful (Goncalves et al., 1979; Hartney, 1980). With this objective, the present studies were undertaken in order to multiply the 'elite' trees of E. citriodora which were fast growing and contained high levels of oil and citronellal in the leaves.

A few reports are available on the isolation of callus and plantlet regeneration from lignotubers, cotyledons and shoot apices of E. citriodora (Table 1g), and also from other species of Eucalyptus (Table 1f). These studies however deal only with cultures isolated from embryonic or juvenile seedling tissues and not from the mature trees of proven genotype. These reports also do not give data on the rooting percentages of the cultures and of their survival in the field.

The present work was carried out with two main objectives - (1) to develop a tissue culture method for clonal propagation of 'elite' trees, to grow the plants in field and to compare their oil, citronellal and



citronellol contents with the parent trees, and (2) to determine the conditions for callus and plantlet regeneration in order to determine the stages at which the essential oil and its main components are synthesised in culture.

These studies have been divided into five main Chapters:

1. Studies on shoot regeneration from seedlings and a 20 year old tree.
2. Studies on root initiation and plantlet formation from shoots of seedlings and a 20 year old tree.
3. Monoterpenes of field grown tissue culture raised plants.
4. Studies on the nutritional requirements of callus.
5. Essential oil, citronellal and citronellol concentration in callus and organized cultures.

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## **CHAPTER II**

### **MATERIALS AND METHODS**

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## MATERIALS AND METHODS

### Glassware

Glassware used in all the experiments was of Corning Brand. 25 x 150 mm test tubes and 100 ml and 250 ml Erlenmeyer flasks were used.

### Washing of Glassware

Glassware was first boiled in washing soda (saturated solution of sodium carbonate) for 1 h and then washed with tap water. Following this treatment it was left in 30% nitric acid for 30 min and later thoroughly washed with tap water and finally rinsed with distilled water. It was then allowed to dry on draining racks.

### Sterilization of Glassware

Tubes, flasks, pipettes etc. were plugged with absorbent cotton and petridishes were covered with wrapping paper and then sterilized at 20 lb/sq. inch for 1 h. Sterile glassware was dried at 100°C for 1 h before use.

### Sterilization of plant material

Seeds Eucalyptus citriodora seeds were supplied to us by the Silviculturist, Forest Research Institute, Dehra Dun, India. The seeds were surface sterilized as follows: They were first washed with a detergent 0.1% 'Acinol' solution for 1 min. The detergent was washed off with distilled water. Then they were sterilized with 70% alcohol for 2 min. Alcohol was washed off thoroughly with distilled water. The seeds were then sterilized with 30%

chlorine water for 20 min under vacuum in a desiccator. Finally the chlorine water was washed off thoroughly with sterile distilled water by giving 3 - 4 washings and the seeds were inoculated on W-3 solid medium for germination. The tubes were incubated in dark. Within 6 - 8 days, the seeds germinated and the first leaves emerged. Then they were transferred to a light chamber (1,000 lux and 16 h photoperiod).

Mature tree explants Terminal and axillary vegetative buds along with 15 - 20 mm portions of the stem were excised during the months of September and October from 9 - 12 m tall 20 year old tree, immediately surface sterilized with 0.05%  $\text{HgCl}_2$  for 15 min and then transferred to inoculation cabinet, where they were washed several times with sterile distilled water. At this stage they were dissected and 5 - 10 mm segments containing the terminal and axillary buds, and inoculated into different media.

Stem and leaf explants from seedling 10 - 20 mm stem and leaf explants from one year and 4 months old plants were collected and surface sterilized with 0.05%  $\text{HgCl}_2$  for 10 min and then 5 - 10 mm segments were dissected and inoculated into different media.

#### Chemicals

All chemicals used were of analytical grade. Inorganic salts and carbohydrates were obtained either from British Drug House (BDH) or E. Merck, vitamins from BDH and Bacto-agar, yeast extract, malt extract and casein hydrolyzate

from Difco Laboratories (USA). BAP and kinetin were from Sigma Chemical Co. zeatin from Calbiochem Co. and DPU from Light & Co. Auxins were either from BDH or E. Merck, edamin from Sheffield Chemical Co., USA. Gibberellic acid and ABA were from Sigma, and CCC from American Cyanamid Co. The morphactins were obtained as a gift from Celameark, Germany and 2,4,5-TCP was from Nutritional Biochemical Corporation, USA.

#### Coconut milk

Coconut milk from several tender coconuts was pooled and filtered through a cotton pad. It was autoclaved at 15 lbs/sq inch pressure for 20 min, refiltered and stored at -20°C. When required, it was thawed and used.

#### Media preparation

All the media were prepared with glass distilled water. The composition of Heller (1953), White (1954), Smith (1967), Knop (1856), Murashige and Skoog (1962) and Gamborg B<sub>5</sub> (1968) are given in Table 2a, 2b and 2c. The macrosalts were used according to the original compositions. The alterations made in the micronutrient and organic constituents were as follows: In Smith's medium (Smith, 1967) instead of ferric tartrate, Na<sub>2</sub>FeEDTA was added at the same concentration as in Murashige and Skoog medium (1962). Ferric citrate was added to White medium at 5 mg/l concentration instead of ferric sulfate (White, 1954). In White medium copper and molybdenum were added according to Smith (1967). In Knop (1856) medium (Gautheret, 1959) Smith



trace elements were added. Aluminium and nickel chloride were omitted from Heller (1953) medium.

Solution of  $\text{Na}_2\text{FeEDTA}$  was prepared according to Murashige and Skoog (1962). 5 ml of stock solution of  $\text{Na}_2\text{FeEDTA}$  containing 5.57 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 7.45 g  $\text{Na}_2\text{EDTA}$  in one litre was used per liter of medium.

Glycine, nicotinic acid, pyridoxine hydrochloride and thiamine hydrochloride according to Murashige and Skoog (1962) were added to all media.

In the present studies a modified Murashige and Skoog medium (1962) was used. Edamin and IAA were omitted and sucrose added at 2%. This basal medium of Murashige and Skoog (1962) is hereafter referred to as MS.

After addition of the supplements, the pH of the medium was adjusted to  $5.8 \pm 0.2$  with 0.1 N NaOH or 0.1 N HCl and then made to volume. For semisolid media bacto-agar was added at 0.8%. 20 ml medium (solid or liquid) was distributed to previously sterilized boiling tubes or flasks. Whatman No. 1 filter paper bridges were used in the case of liquid medium in tubes to give the necessary support to the inoculum. The medium was sterilized at 15 lb/sq. inch for 20 min followed by steaming for 30 min on the subsequent day.

Heat labile compounds such as  $\text{GA}_3$ , IAA, IBA etc. were either filter sterilized (Millipore filter 0.22 u) or made in 70% alcohol and added to the sterilized medium aseptically. After cooling the medium was used for

**Table 2a** Macroelement concentrations in different basal media

Concentration expressed in mg/l

| Chemical                                             | Murashige<br>and<br>Skoog<br>(MA)<br>(1962) | Smith<br>(SM)<br>(1967) | White<br>(WM)<br>(1954) | Knop<br>(KM)<br>(1856) | Heller<br>(HM)<br>(1953) | Gamborg<br>B <sub>5</sub><br>(B <sub>5</sub> M)<br>(1968) |
|------------------------------------------------------|---------------------------------------------|-------------------------|-------------------------|------------------------|--------------------------|-----------------------------------------------------------|
| $\text{NH}_4\text{NO}_3$                             | 1650                                        | -                       | -                       | -                      | -                        | -                                                         |
| $\text{KNO}_3$                                       | 1900                                        | 405                     | 80                      | -                      | -                        | 2500                                                      |
| $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ | -                                           | 710                     | 288                     | 144                    | -                        | -                                                         |
| $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$            | 440                                         | -                       | -                       | -                      | 75                       | 150                                                       |
| $\text{KH}_2\text{PO}_4$                             | 170                                         | 70                      | -                       | 25                     | -                        | -                                                         |
| $\text{NaCl}$                                        | -                                           | 60                      | -                       | -                      | -                        | -                                                         |
| $\text{Na}_2\text{SO}_4$                             | -                                           | -                       | 200                     | -                      | -                        | -                                                         |
| $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  | -                                           | -                       | 22                      | -                      | 125                      | 150                                                       |
| $\text{KCl}$                                         | -                                           | -                       | 80                      | -                      | 750                      | -                                                         |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$            | 370                                         | 245                     | 360                     | 51                     | 250                      | -                                                         |
| $(\text{NH}_4)_2\text{SO}_4$                         | -                                           | -                       | -                       | -                      | -                        | 134                                                       |

**Table 2b** Macroelement concentration in various media

Concentration expressed in mg/l

| Chemical                                            | Murashige<br>and<br>Skoog<br>(MA)<br>(1962) | Smith<br>(SM)<br>(1967) | White<br>(WM)<br>(1954) | Knop<br>(KM)<br>(1856) | Heller<br>(HM)<br>(1953) | Gamborg<br>(B <sub>5</sub><br>(B <sub>5</sub> M)<br>(1968) |
|-----------------------------------------------------|---------------------------------------------|-------------------------|-------------------------|------------------------|--------------------------|------------------------------------------------------------|
| Na <sub>2</sub> EDTA                                | 37.5                                        | 37.5*                   | -                       | 37.5*                  | -                        | 37.5                                                       |
| FeSO <sub>4</sub>                                   | 27.8                                        | 27.8*                   | -                       | 27.8*                  | -                        | 27.8                                                       |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                | 8.6                                         | 0.62                    | 2.64                    | 0.62*                  | 1.0                      | 2.0                                                        |
| MnSO <sub>4</sub> ·4H <sub>2</sub> O                | 22.3                                        | -                       | 7.0                     | -                      | 0.1                      | 1.0                                                        |
| H <sub>3</sub> BO <sub>3</sub>                      | 6.2                                         | 0.57                    | 1.50                    | 0.57*                  | 1.0                      | 3.0                                                        |
| Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O | 0.25                                        | 0.25                    | 0.25*                   | 0.25*                  | -                        | 0.25                                                       |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                | 0.025                                       | -                       | -                       | -                      | 0.03                     | 0.025                                                      |
| FeCl <sub>3</sub> ·6H <sub>2</sub> O                | -                                           | -                       | -                       | -                      | 1.0                      | -                                                          |
| CoCl <sub>2</sub> ·6H <sub>2</sub> O                | 0.025                                       | -                       | -                       | -                      | -                        | 0.025                                                      |
| Ferric citrate                                      | -                                           | -                       | 5.0                     | -                      | -                        | -                                                          |
| MnCl <sub>2</sub>                                   | -                                           | 0.4                     | -                       | 0.4*                   | -                        | -                                                          |
| CuCl <sub>2</sub>                                   | -                                           | 0.27                    | 0.27*                   | 0.27*                  | -                        | -                                                          |
| KI                                                  | 0.83                                        | -                       | 0.75                    | -                      | 0.01                     | 0.75                                                       |

\*indicates difference from the original media.



Table 2c Vitamins and Glycine

| Chemical       | Murashige and<br>Skoog (MB)<br>(1962)<br>(mg/l) | White (WB)<br>(1954)<br>(mg/l) |
|----------------|-------------------------------------------------|--------------------------------|
| Glycine        | 2.0                                             | 3.0                            |
| Inositol       | 100.0                                           | -                              |
| Nicotinic acid | 0.5                                             | 0.5                            |
| Pyridoxine HCl | 0.5                                             | 0.1                            |
| Thiamine HCl   | 0.1                                             | 0.1                            |

Note - Vitamins and Glycine were common to all the media except W-3 medium where vitamins and glycine, as used by White (1954) were used.

Table 2d List of media used and their concentrations

Figures in parenthesis are mg/l.

|                    |                                                                         |
|--------------------|-------------------------------------------------------------------------|
| MS                 | MA + MB                                                                 |
| MS-1               | MA + MB + CM (5%) + 2,4-D (3) + Ed (0.05%) + Inos (20)                  |
| MS-2               | MA (without nitrogen source) + MB + 2,4-D (3) + Inos. (20)              |
| MS-3               | MA + MB + Kin (0.2) + BAP (0.3) + Ca.P. (0.1) + Bio.(0.1)               |
| MS-4               | MA + MB + Ca.P. (0.1) + Bio. (0.1)                                      |
| MS-5               | MA + MB + BAP (0.2) + Ca.P. (0.1) + Bio. (0.1)                          |
| MS-6               | MA + MB + Kin (0.2) + Ca.P. (0.1) + Bio. (0.1)                          |
| MS-7               | MA + MB + Kin (0.2) + BAP (0.3)                                         |
| MS-8               | MA + MB + Kin (0.2) + BAP (0.3) + Ca.P. (0.1) + Bio.(0.1) + NAA (2.0)   |
| MS-9               | MA + MB + Ca.P. (0.1) + Bio. (0.1) (liquid)                             |
| MS-10              | MA (1/2 strength) + MB (1/2 strength) + activated charcoal (0.25%).     |
| MS-11              | MA (1/2 strength) + MB + Ca.P. (0.1) + Bio.(0.1)(liquid)                |
| W-1                | WM + MB + CM (5%) + 2,4-D (3) + Ed (0.05%) + Inos.(20)                  |
| W-2                | WM + MB + Kin (0.2) + BAP (0.3) + Ca.P. (0.1) + Bio.(0.1)               |
| W-3                | WM + WB (liquid)                                                        |
| S-1                | SM + MB + CM (5%) + 2,4-D (3) + Ed (0.05%) + Inos.(20)                  |
| S-2                | SM + MB + Kin (0.2) + BAP (0.3) + Ca.P.(0.1) + Bio.(0.1)                |
| GB <sub>5</sub> -1 | B <sub>5</sub> M + MB + CM (5%) + 2,4-D (3) + Ed (0.05%) + Inos.(20)    |
| GB <sub>5</sub> -2 | B <sub>5</sub> M + MB + Kin (0.2) + BAP (0.3) + Ca.P. (0.1) + Bio.(0.1) |
| H-1                | HM + MB + CM (5%) + 2,4-D (3) + Ed (0.05%) + Inos.(20)                  |
| H-2                | HM + MB + Kin (0.2) + BAP (0.3) + Ca.P. (0.1) + Bio.(0.1)               |
| K-1                | KM + MB + CM (5%) + 2,4-D (3) + Ed.(0.05%) + Inos.(20)                  |
| K-2                | KM + MB + Kin (0.2) + BAP (0.3) + Ca.P. (0.1) + Bio.(0.1)               |

inoculations.

#### Inoculation

All inoculations were carried out in a sterile cabinet inside a sterile room. Half an hour prior to inoculations the UV light in the cabinet was put on. The sterile cabinet was sprayed with 10% formalin once a week to reduce contamination.

All instruments used for inoculations were sterilized by autoclaving for 1 h at 20 lb/sq. inch pressure before use.

#### Incubation

For the nutritional studies cultures were incubated at  $25 \pm 2^\circ\text{C}$  in darkness. For the differentiation studies incubation of cultures was carried out at  $25 \pm 2^\circ\text{C}$  unless otherwise mentioned, with a photoperiod of 16 h light and 8 h dark for the duration of the experiment. The light intensity was 1,500 lux and was supplied by cool daylight fluorescent tubes. All the experiments were carried out in triplicate.

#### Measurement of growth

Fresh weights were taken separately for 3 tubes after 25 days. Dry weights were taken after drying the tissues to constant weight at  $80 - 100^\circ\text{C}$  for 48 h to constant weight. The wet and dry weights given in the tables are the average wet weight and dry weight per tube.

Standard error was calculated according to the formula (Murashige and Skoog, 1962):

$$\text{Standard error} = \pm \sqrt{\frac{(\Delta_1)^2 + (\Delta_2)^2 + (\Delta_3)^2}{n(n-1)}}$$

where  $\Delta$  is the deviation in weight from average weight and 'n' is the number of weights taken.

Both wet and dry weights are used as an index for comparative purposes.

The effect of a particular compound is considered significant if it differs from the control by at least 15%.

#### Differentiation studies

In the differentiation studies the number of shoots developed in a particular treatment was used as an index for comparison.

#### Oil analysis

Oil from field grown plants was extracted from fresh leaves by steam distillation, whereas cultured cells and leaves were extracted directly with ether at 5°C. Samples were made to a known volume and 1 µl used for analysis. Estimations were carried out using a Shimadzu Gas chromatography equipment (6 - AMPF Model) in a 2 meter length and 2 mm diameter stainless steel column packed with 10% carbowax, 20 M on chromosorb P. The column temperature of 175°C and the thermal conductivity detector (T.C.D.) temperature at 200°C was maintained during analysis. The concentration of oil, citronellal and citronellol have been calculated on the basis of dry weight. In the present studies the total oil, citronellal and citronellol have been compared.

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## CHAPTER III

### STUDIES ON SHOOT REGENERATION

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## Section 1    Experimental

This Chapter describes conditions for obtaining high rates of shoot multiplication from seedling segments and from buds of a 20 year old tree of Eucalyptus citriodora. Only a few parameters were tested for seedling experiments in order to obtain some indication of the growth requirements, before attempting work on the optimum conditions for mature tree. The composition of different media used are given in Chapter II.

### Seedlings

E. citriodora seeds were supplied by the silviculturist, Forest Research Institute, Dehra Dun. Then seeds were surface sterilized by method described in Chapter II and kept for germination on W-3 solid medium in the dark.

After 20 - 25 days when the seedlings had attained a height of 50 - 60 mm, they were excised and 5 - 10 mm segments from various regions inoculated in different media.

### Effect of different explants

Explants from different regions of E. citriodora seedlings were cultured to determine the most suitable region of the seedling for multiple shoot formation. MS-3 medium was used since from preliminary studies this medium showed promise. The explants were grown on this medium for 30 days (Table 3). Maximum number of shoots were obtained from the hypocotyl region, followed by the nodal and shoot tip segments, Cotyledonary tissue formed callus,

**Table 3** **Effect of different explants from seedlings**

Medium - MS-3

| Explant             | Observations<br>(Shoot formation) |
|---------------------|-----------------------------------|
| 1. Shoot tip        | ++                                |
| 2. Stem (with node) | +++                               |
| 3. Hypocotyl        | ++++                              |
| 4. Cotyledon        | Callusing                         |
| 5. Root             | -                                 |

**Number of Shoots**

|      |          |
|------|----------|
| -    | (None)   |
| +    | (1 - 3)  |
| ++   | (3 - 5)  |
| +++  | (5 - 7)  |
| ++++ | (7 - 10) |





Fig. 2 Effect of different explants from seedlings

- |              |                |
|--------------|----------------|
| 1. Shoot tip | 2. Stem (node) |
| 3. Hypocotyl | 4. Cotyledon   |
| 5. Root.     |                |



and root explants did not proliferate and died after 30 days (Fig. 2).

#### Effect of different basal media

In this experiment in vitro grown shoots obtained from seedling hypocotyl explants were used as inoculum and single shoots were inoculated in different basal media, the composition of which is given in Chapter II (MS-3, GB<sub>5</sub>-2, K-2, W-2, S-2, H-2). The effect of the media are shown in Table 4. Shoot formation was observed on all media but high salt concentration medium MS-3 was superior and produced a maximum number of shoots followed by S-2. With GB<sub>5</sub>-2, W-2, K-2 and H-2 (Fig. 3), the number of shoots formed was about half that found on MS-3 medium.

#### Mature tree explants

Terminal and axillary vegetative buds along with portions of the stem were excised in September and October from a 20 year old tree (Fig. 1) and brought to the Laboratory. They were surface sterilized by method described in Chapter II. The conditions for incubation and illumination of cultures are given in Chapter II. Observations were taken after 30 days.

These preliminary experiments with seedling explants indicated that MS-3 medium was suitable for the development of multiple shoots. This medium was then tested with sterile explants from a 20 year old E. citriodora tree. The cultures were incubated at 25°C (16 h light) and 23°C (8 h darkness) with 1,500 lux light intensity as with seedling explants.

Table 4    Effect of different basal mediaMedia      MS-3, GB<sub>5</sub> - 2, K-2, W-2, S-2, H-2

|    | Basal medium       | Shoot formation |
|----|--------------------|-----------------|
| 1. | MS-3               | ++++            |
| 2. | GB <sub>5</sub> -2 | ++              |
| 3. | K-2                | ++              |
| 4. | W-2                | ++              |
| 5. | S-2                | +++             |
| 6. | H-2                | ++              |

## Number of Shoots

-      (None)

+      (2 - 3)

++     (3 - 5)

+++    (5 - 7)

++++   (7 - 10)

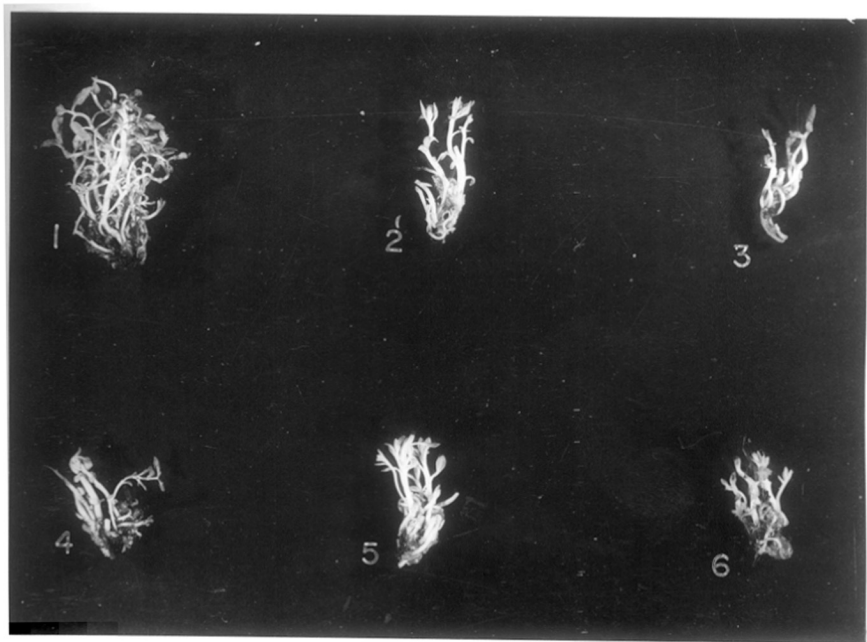


Fig. 3 Effect of different basal media

1. Murashige and Skoog    2. Gamborg B<sub>5</sub>  
3. Knop    4. White    5. Smith    6. Heller.

Under these conditions however buds remained green for 3 - 4 weeks but gradually turned brown. The requirement for adventitious shoot induction from primary explants of 20 year old trees appeared to be different since they did not respond to the media and other conditions used for multiple shoot formation from seedling explants. Detailed studies were then carried out using primary explants from the mature trees.

Effect of cytokinin and auxin concentrations on shoot initiation from mature tree

Different concentrations of two cytokinins, kin (0.1 - 3 mg/l) and BAP (0.1 - 4 mg/l) added either singly or in combination to MS-4 medium were tested, but in all the explants the buds failed to grow (Table 5). At the higher cytokinin concentrations (2 - 3 mg/l) the buds turned brown within a week. Addition of auxins (NAA, 2,4-D, IAA, IBA) resulted in slight callusing of explants at the cut end of the buds with NAA (0.1 mg/l) or 2,4-D (0.1 mg/l) but shoot initiation was not observed. Since it was clear from this experiment that the requirement for shoot formation from mature trees differed from those of seedlings, several other treatments were tested. The effect of temperature was first tried.

The primary explants were inoculated on MS-3 medium and then incubated at different temperatures (5, 15, 20, 25, 30°C) under continuous light (1,000 lux) for varying periods (24, 48, 72, 96, 120 h) before transfer to 25°C. When buds

**Table 5** **Effect of different cytokinin and auxin concentrations on shoot initiation from buds of 20 year old trees**

Medium - MS-4

|     | Cytokinins<br>(mg/l)                | Shoot initiation |
|-----|-------------------------------------|------------------|
| 1.  | Kin (0.1, 0.5, 1.0, 2.0, 3.0)       | -                |
| 2.  | BAP (0.1, 0.5, 1.0, 2.0, 3.0)       | -                |
| 3.  | Kin (0.2) + BAP (0.3)               | -                |
| 4.  | Kin (0.5) + BAP (0.2)               | -                |
| 5.  | Kin (1.0) + BAP (2.0)               | -                |
| 6.  | Kin (2.0) + BAP (1.0)               | -                |
| 7.  | Kin (0.5) + BAP (3.0)               | -                |
| 8.  | Kin (3.0) + BAP (0.5)               | -                |
| 9.  | Kin (0.2) + BAP (0.3) + NAA (0.1)   | Callusing        |
| 10. | Kin (0.2) + BAP (0.3) + 2,4-D (0.1) | Callusing        |
| 11. | Kin (0.2) + BAP (0.3) + IAA (0.1)   | -                |
| 12. | Kin (0.2) + BAP (0.3) + IBA (0.1)   | -                |

Number of Shoots

- (None)

were incubated at 15°C in continuous light (1,000 lux) for 72 h and later transferred to 25°C with a 16 h photoperiod, they gradually elongated and leaves opened within 3 weeks (Fig. 4). This indicated the importance of a cold treatment for breaking dormancy of the bud. Each explant produced only 2 - 3 shoots by this method but no elongation of shoots was observed. Several experiments were performed at this stage to increase the rate of shoot development and also for elongation of the shoots, by changing the media constituents using several cytokinins and auxins at varying concentrations. Different physical parameters were also tested.

A striking effect was observed when buds grown at 25°C for 14 days after receiving a 15°C treatment for 72 h on MS-3 (used for seedling segments) medium were transferred to 100 ml Erlenmeyer flasks containing 10 ml of MS-3 liquid medium and incubated on a rotary shaker (120 rev/min) with continuous illumination (500 lux) at  $27 \pm 2^\circ\text{C}$ . 30% of the buds developed 4 - 6 shoots within two weeks (Fig. 5).

Individual shoots from shake flasks when excised and transferred to MS-3 agar medium formed 6 - 8 shoots within 25 - 30 days (Fig. 6). Subsequent subcultures were carried out by transfer of 25 - 30 mm long single excised shoots to MS-3 agar medium. The initial requirement for incubation at 15°C and agitation on a rotary shaker were not necessary.

Subsequent experiments, to determine the conditions



Fig. 4 Sprouting of buds from explants of a mature (20 year old) tree.



Fig. 5 Shoot formation from explant of mature tree in shake flask.



Fig. 6 Multiple shoot formation on subculture.  
From mature tree. MS-3 medium.



for optimum shoot multiplication were carried out using this inoculum grown on MS-3 medium for 25 - 30 days. Individual shoots were excised and inoculated into the respective test media, and the results noted after 30 days. The basal medium used for these studies was MS-3, and the experiments were conducted after the cultures had undergone 4 subcultures.

The regimen followed for incubation and illumination of cultures was as given earlier in Chapter II.

#### Effect of different cytokinins

Different cytokinins, kinetin, BAP, zeatin, 2iP and DPU were added individually or in combination. All were added at 0.2 mg/l except zeatin which was incorporated at 20 µg/l (Table 6). In the presence of a cytokinin added singly, maximum number of shoots was obtained with BAP followed by Kin, DPU and 2iP. With zeatin just 1 - 3 shoots developed (Fig. 7).

When kinetin (0.2 mg/l) was combined with either of the other cytokinins, maximum number of shoots was obtained with a combination of kin and BAP (Table 6). The other cytokinin combinations of zeatin, 2iP and DPU with kin did not inhibit or stimulate shoot formation (Fig. 7).

#### Effect of kinetin concentrations

To test the optimum levels of the combined effect of BAP and Kin, in the first experiment BAP concentration was fixed at 0.2 mg/l (MS-5) and the kinetin concentration varied from 0.1 - 2.0 mg/l (Table 7). Maximum shoot

**Table 6** Effect of different cytokinins

Medium - MS-4

|     | Cytokinins<br>(mg/l)       | Shoot formation |
|-----|----------------------------|-----------------|
| 1.  | None                       | -               |
| 2.  | 2iP (0.2)                  | ++              |
| 3.  | BAP (0.2)                  | +++             |
| 4.  | Zeatin (0.002)             | +               |
| 5.  | Kin (0.2)                  | ++              |
| 6.  | DFU (0.2)                  | ++              |
| 7.  | Kin (0.2) + BAP (0.2)      | ++++            |
| 8.  | Kin (0.2) + Zeatin (0.002) | ++              |
| 9.  | Kin (0.2) + DFU (0.2)      | ++              |
| 10. | Kin (0.2) + 2iP (0.2)      | ++              |

## Number of Shoots

|      |          |
|------|----------|
| -    | (None)   |
| +    | (1 - 3)  |
| ++   | (3 - 5)  |
| +++  | (5 - 7)  |
| ++++ | (7 - 10) |

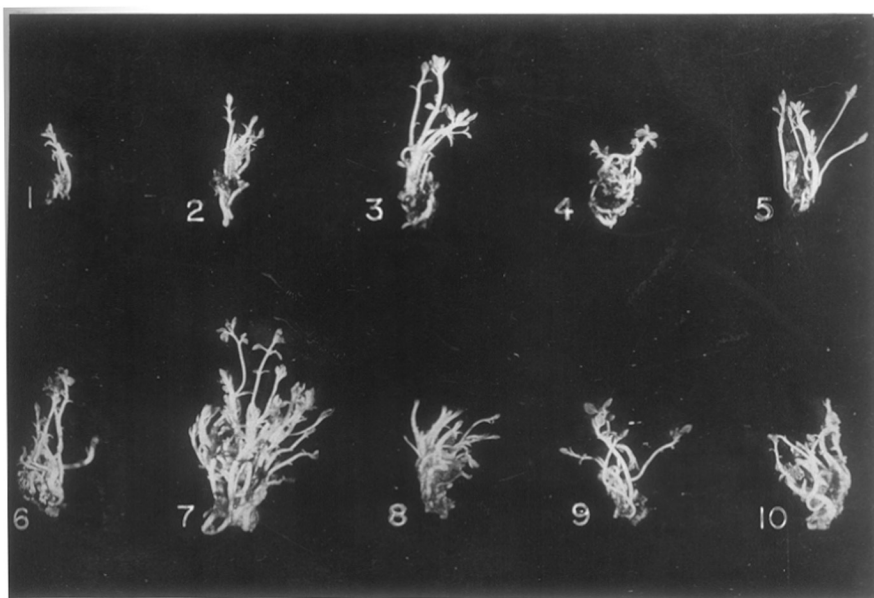


Fig. 7 Effect of different cytokinins.

- |                 |                |              |                 |
|-----------------|----------------|--------------|-----------------|
| 1. No cytokinin | 2. Kin.        | 3. BAP       | 4. Zeatin       |
| 5. 2iP          | 6. DPU         | 7. Kin + BAP | 8. Kin + zeatin |
| 9. Kin + 2iP    | 10. Kin + DPU. |              |                 |

**Table 7** Effect of different kinetin concentrations

Medium - MS-5

|    | Kin<br>(mg/l) | Shoot formation |
|----|---------------|-----------------|
| 1. | Nil           | ++              |
| 2. | 0.1           | +++             |
| 3. | 0.2           | ++++            |
| 4. | 0.3           | +++             |
| 5. | 0.5           | ++              |
| 6. | 1.0           | +               |
| 7. | 2.0           | Callusing       |

**Number of Shoots**

|      |          |
|------|----------|
| -    | (None)   |
| +    | (1 - 3)  |
| ++   | (3 - 5)  |
| +++  | (5 - 7)  |
| ++++ | (7 - 10) |

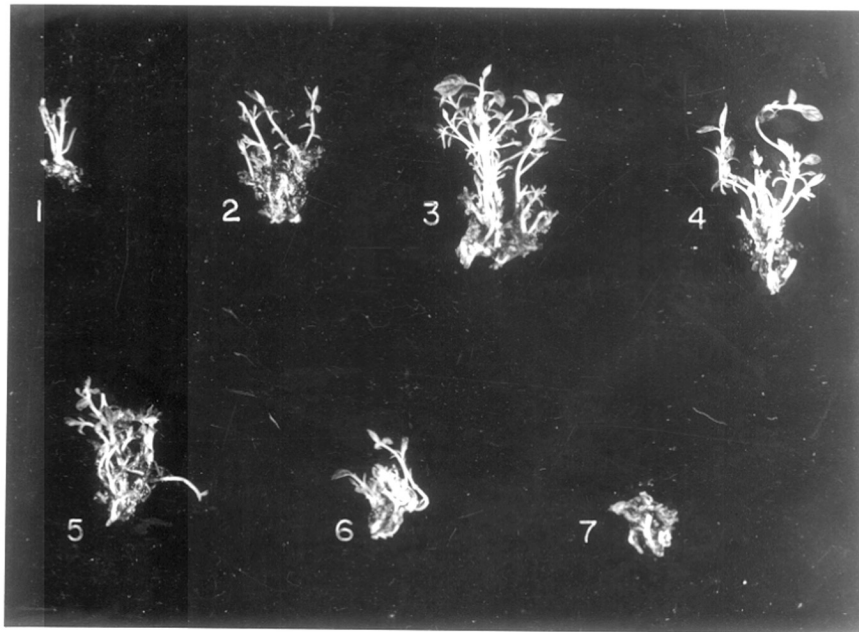


Fig. 8 Effect of kinetin concentration (mg/l)

- |            |        |        |
|------------|--------|--------|
| 1. No Kin. | 2. 0.1 | 3. 0.2 |
| 4. 0.3     | 5. 0.5 | 6. 1.0 |
| 7. 2.0.    |        |        |

formation was observed on medium containing (0.2 mg/l) Kin (Fig. 8). 2 mg/l Kin resulted in inhibition of shoot formation and formation of callus. The number of shoots formed was lower at 0.1 and 0.3 mg/l being reduced further at 0.5 and 1.0 mg/l or in the absence of Kin.

#### Effect of BAP concentrations

In this experiment the Kin concentration was kept constant at 0.2 mg/l in the medium (MS-6) and the BAP levels were varied from 0.1 - 2.0 mg/l (Table 8).

Maximum number of shoots was observed with 0.3 mg/l BAP. With higher or lower levels of BAP the shoot numbers gradually reduced being lowest at 2 mg/l. About 3 - 5 shoots were noticed even if BAP was omitted (Fig. 9).

#### Effect of different auxins

In this experiment different auxins NAA, IPA, UBA, IAA, 2,4-D, B-NOA and 2,4,5-TCP were added to MS-3 medium individually at 0.1 mg/l (Table 9). All the auxins were ineffective in increasing shoot formation. Although different degrees of shoot formation was observed with IAA, 2,4-D, B-NOA and 2,4,5-TCP, this was always accompanied by callusing (Fig. 10).

#### Effect of GA<sub>3</sub> concentrations

The effect of GA<sub>3</sub> was tested by incorporation into MS-3 medium at concentrations ranging from 0.1 - 10 mg/l (Table 10). GA<sub>3</sub> definitely inhibited shoot formation even if added at 0.1 mg/l. This effect was even more marked as the concentrations were raised (10 mg/l) (Fig. 11). However,

Table 8 Effect of different BAP concentrations

Medium - MS-6

|    | BAP<br>(mg/l) | Shoot formation |
|----|---------------|-----------------|
| 1. | Nil           | ++              |
| 2. | 0.1           | +++             |
| 3. | 0.2           | ++++            |
| 4. | 0.3           | +++++           |
| 5. | 0.5           | +++             |
| 6. | 1.0           | ++              |
| 7. | 2.0           | +               |

## Number of Shoots

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |

Table 9    Effect of different auxins

Medium - MS-3

|    | Auxin<br>(mg/l) | Shoot formation |           |
|----|-----------------|-----------------|-----------|
| 1. | NAA             | +               | Callusing |
| 2. | IPA             | ++              |           |
| 3. | IBA             | +++             |           |
| 4. | IAA             | +++             | Callusing |
| 5. | 2,4-D           | +               | Callusing |
| 6. | B-NOA           | ++              | Callusing |
| 7. | 2,4,5-TCP       | +++             | Callusing |
| 8. | None            | +++++           |           |

## Number of Shoots

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |



**Table 10** Effect of different concentrations of Gibberellic acid

Medium - MS-3

|    | GA <sub>3</sub><br>(mg/l) | Shoot formation |
|----|---------------------------|-----------------|
| 1. | Nil                       | +++++           |
| 2. | 0.1                       | +++             |
| 3. | 0.5                       | ++              |
| 4. | 1.0                       | ++              |
| 5. | 5.0                       | +               |
| 6. | 10.0                      | +               |

**Number of Shoots**

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |

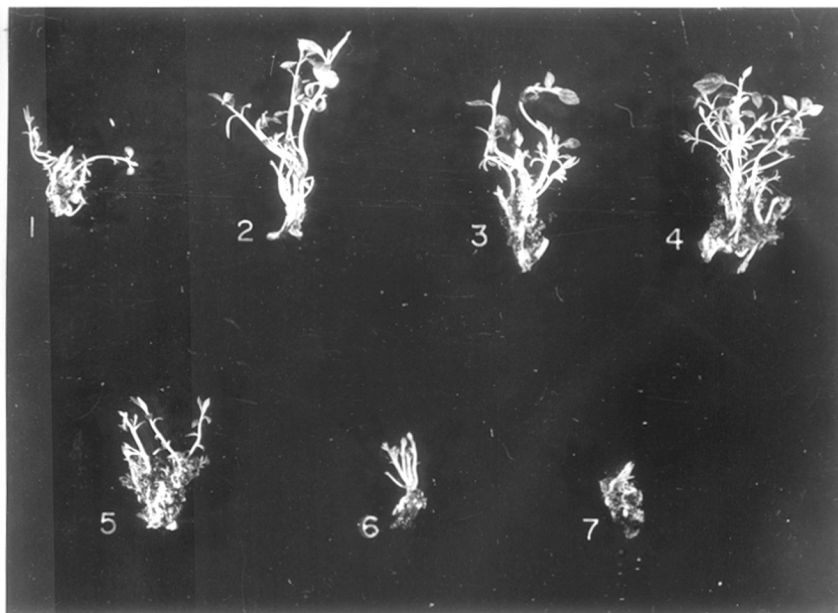


Fig. 9 Effect of BAP concentration (mg/l)

- |           |        |        |
|-----------|--------|--------|
| 1. No BAP | 2. 0.1 | 3. 0.2 |
| 4. 0.3    | 5. 0.5 | 6. 1.0 |
| 7. 2.0.   |        |        |

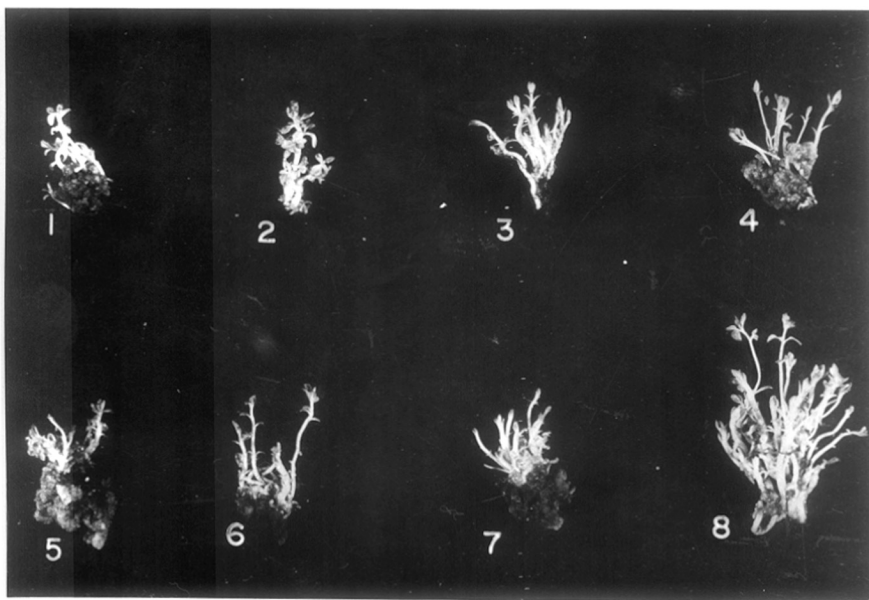


Fig. 10 Effect of different auxins

1. NAA 2. IPA 3. IBA 4. IAA 5. 2,4-D  
6. B-NOA 7. 2,4,5-TCP 8. No auxins.

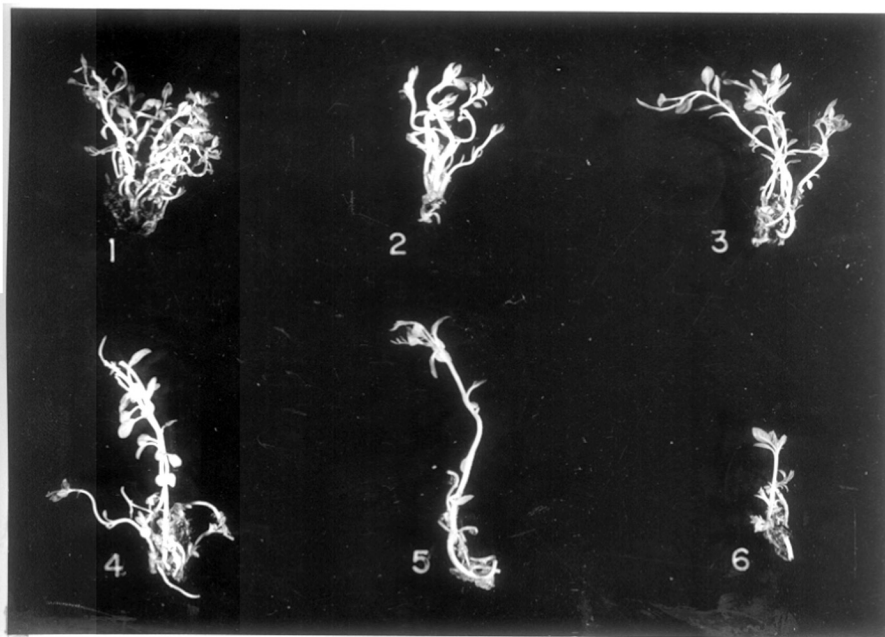


Fig. 11 Effect of Gibberellic acid concentration (mg/l)

- |              |          |        |        |
|--------------|----------|--------|--------|
| 1. No $GA_3$ | 2. 0.1   | 3. 0.5 | 4. 1.0 |
| 5. 5.0       | 6. 10.0. |        |        |

with  $GA_3$  shoots elongated markedly and appeared very tall and etiolated. Maximum elongation was observed with 5 mg/l  $GA_3$ , but at this level only 1-2 shoots developed.

#### Effect of different growth supplements

In this experiment CH, Ed, YE, ME and CM were added individually to MS-3 medium at concentrations given in Table 11. In the presence of all the supplements tested a depression in shoot formation was observed, the maximum inhibition being seen with CM (10%). Both with edamin and CM callus formation was observed (Fig. 12).

#### Effect of different growth retardants and ethrel

The effect of different growth retardants, ABA, morphactin A, B, CCC and also ethrel were tested by addition to MS-3 medium individually at 0.01 mg/l (Table 12). All the growth retardants and ethrel were inhibitory for shoot induction. With CCC 5 - 7 shoots <sup>were</sup> seen accompanied by a little callusing at the base of the explant. The maximum inhibitory effect was obtained with Mor B (Fig. 13).

#### Physical factors

##### Effect of different temperatures

A wide range of temperatures from 5 - 35°C using a series of growth chambers and incubators was tested. All cultures for these explants were supplied with a constant light intensity (1,000 lux) for 16 h (Table 13).

The best result was seen with cultures incubated at  $25 \pm 2^\circ C$  followed by 30°C and 20°C. At 5°C and 30°C bud formation was inhibited (Fig. 14).

Table 11 Effect of different growth supplements

Medium - MS-3

| Organic nitrogen source<br>% |           | Shoot formation |
|------------------------------|-----------|-----------------|
| 1.                           | N11       | +++++           |
| 2.                           | CH (0.05) | +++             |
| 3.                           | Ed (0.1)  | ++ Callusing    |
| 4.                           | YE (0.1)  | ++              |
| 5.                           | ME (0.1)  | ++              |
| 6.                           | CM (10)   | + Callusing     |

## Number of Shoots

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |

Table 12 Effect of different growth retardants and ethrel

Medium - MS-3

| Growth retardant and Ethrel<br>(0.01 mg/l) |        | Shoot formation |
|--------------------------------------------|--------|-----------------|
| 1.                                         | ABA    | ++              |
| 2.                                         | Mor A  | ++              |
| 3.                                         | Mor B  | +               |
| 4.                                         | GCC    | +++ Callusing   |
| 5.                                         | Ethrel | ++              |
| 6.                                         | Nil    | +++++           |

## Number of Shoots

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |

Table 13 Effect of different temperatures

Medium - MS-3

|    | Temperature<br>°C | Shoot formation |
|----|-------------------|-----------------|
| 1. | 5                 | +               |
| 2. | 15                | ++              |
| 3. | 20                | +++             |
| 4. | 25                | +++++           |
| 5. | 30                | ++++            |
| 6. | 35                | +               |

## Number of Shoots

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |



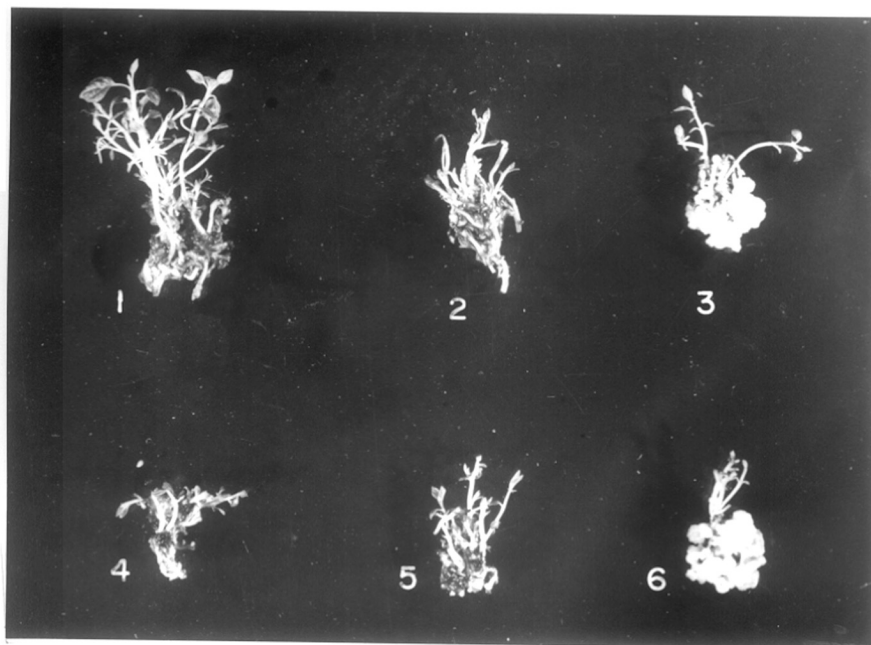


Fig. 12 Effect of different growth supplements

1. Control    2. CH    3. Ed    4. YE  
5. ME        6. CM.

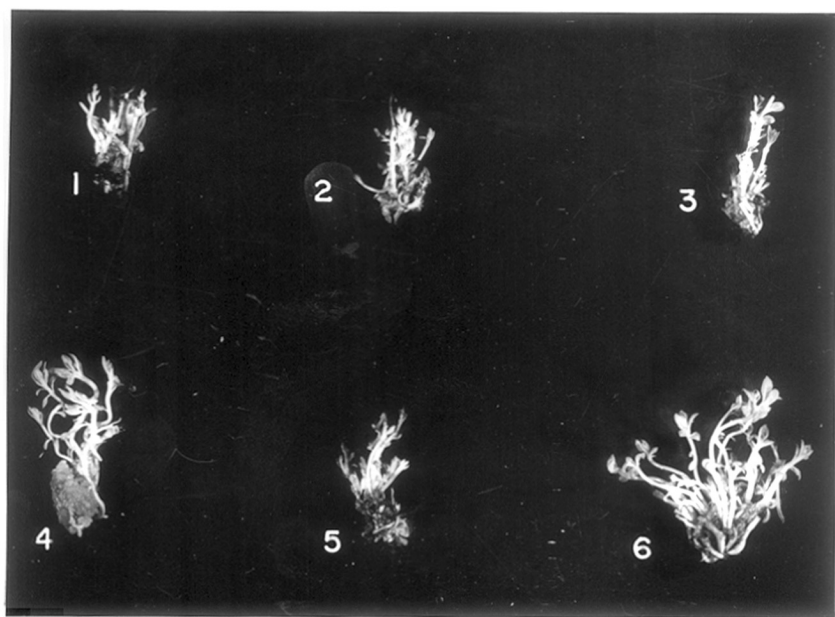


Fig. 13 Effect of different growth retardants and ethrel

1. ABA      2. Mor. A.    3. Mor. B    4. CCC  
5. Ethrel    6. Control.

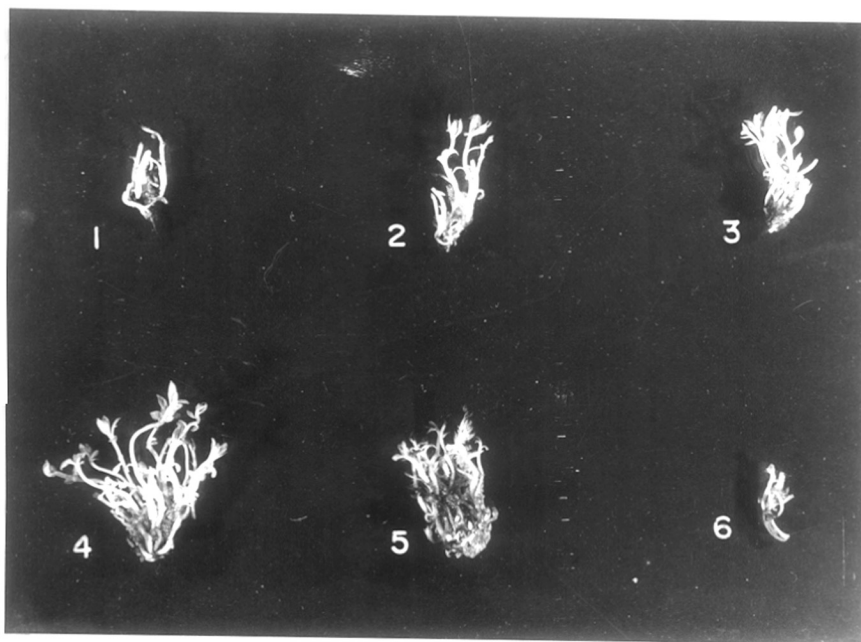


Fig. 14 Effect of different temperatures ( $^{\circ}\text{C}$ )

1. 5    2. 15    3. 20    4. 25

5. 30    6. 35.

### Effect of light intensity

In this experiment cultures were incubated at  $25 \pm 2^{\circ}\text{C}$  but were provided with varying intensities of light (Table 14). The amount of light supplied had a distinct effect on the number of shoots developed. At lower intensities shoot formation was reduced, gradually increasing at 1,000 lux and then remaining constant after 1,500 lux (Fig. 15).

### Effect of subculture

This experiment was conducted from the first subculture onwards to determine whether subculture affected the intensity of shoot formation. At intervals of 25 - 30 days shoots 20 - 30 mm long were excised and subcultured to medium of the same composition (MS-3) (Table 15). On subculture the shoot formation capacity increased becoming fairly uniform after 4 subcultures (Fig. 16). The cultures have now undergone 30 subcultures without any further change in this pattern (Fig. 16).

### Effect of liquid medium

The previous experiments were carried out using medium which were made semisolid with 0.8% agar. In these studies cultures were inoculated into 10 ml MS-3 liquid medium in 100 ml Erlenmeyer flasks. Some flasks were kept stationary and some agitated on a rotary shaker at 120 rpm. In both sets of experiments continuous light (500 lux) was provided. A very large number of shoots were obtained in flasks which were rotated on the shaker.

Table 14 Effect of light intensity

Medium - MS-3

|    | Light intensity<br>(lux) | Shoot formation |
|----|--------------------------|-----------------|
| 1. | 300                      | ++              |
| 2. | 500                      | +++             |
| 3. | 1000                     | ++++            |
| 4. | 1500                     | +++++           |
| 5. | 2000                     | +++++           |
| 6. | 2300                     | +++++           |

## Number of Shoots

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |

Table 15 Effect of subculture

Medium - MS-3

|    | Subculture No.  | Shoot formation |
|----|-----------------|-----------------|
| 1. | S <sub>1</sub>  | ++              |
| 2. | S <sub>2</sub>  | +++             |
| 3. | S <sub>3</sub>  | ++++            |
| 4. | S <sub>4</sub>  | +++++           |
| 5. | S <sub>5</sub>  | +++++           |
| 6. | S <sub>30</sub> | +++++           |

## Number of Shoots

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |

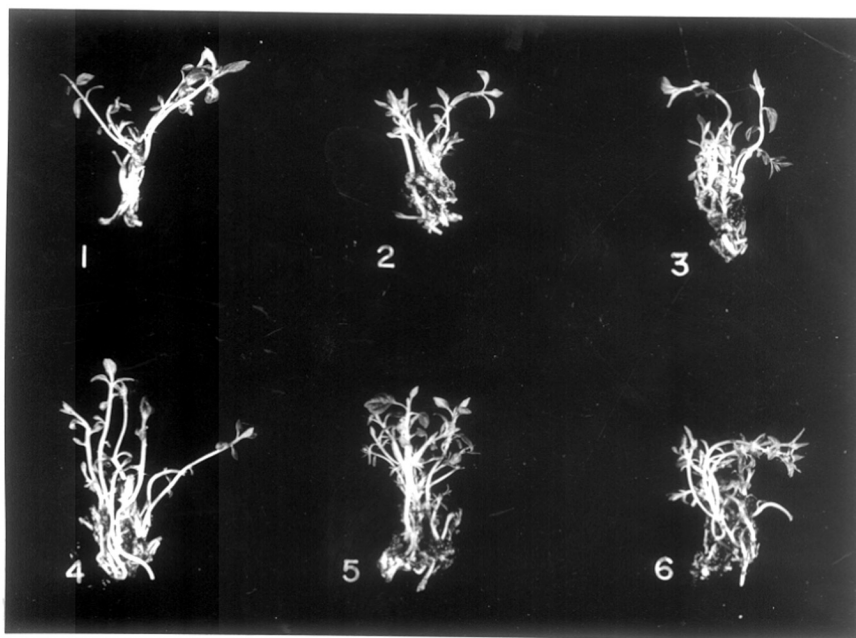


Fig. 15 Effect of light intensity (lux)

1. 300    2. 500    3. 1,000  
4. 1,500    5. 2,000    6. 2,300.

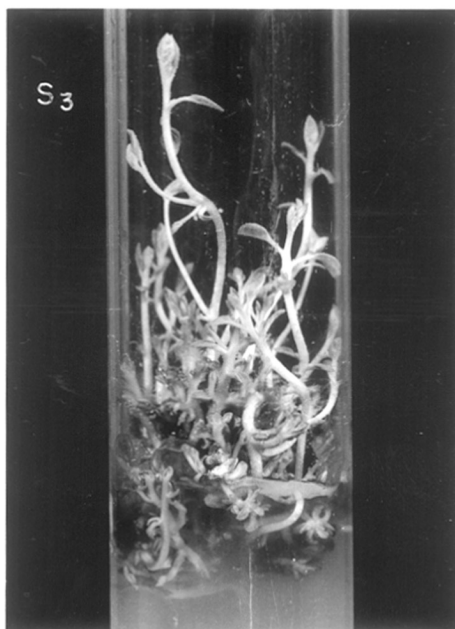
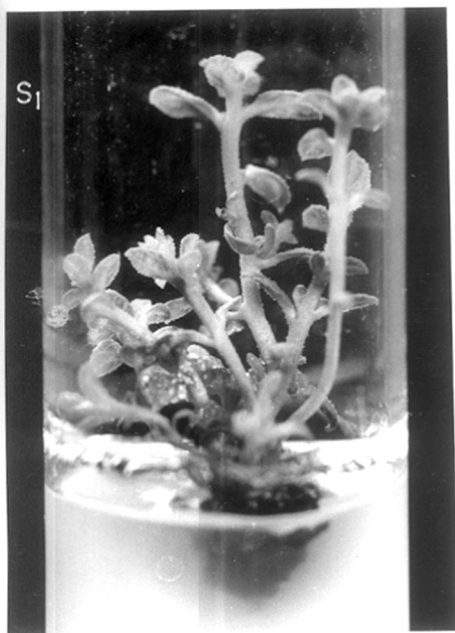


Fig. 16 Effect of subculture -  $S_1$  - first subculture;

$S_2$  - second subculture;  $S_3$  - third subculture.

Note - The magnification for the first three subcultures  $S_1$ ,  $S_2$  and  $S_3$  is higher than those for the subcultures  $S_4$ ,  $S_5$  and  $S_{30}$  in order to focus on the difference in number of shoots.



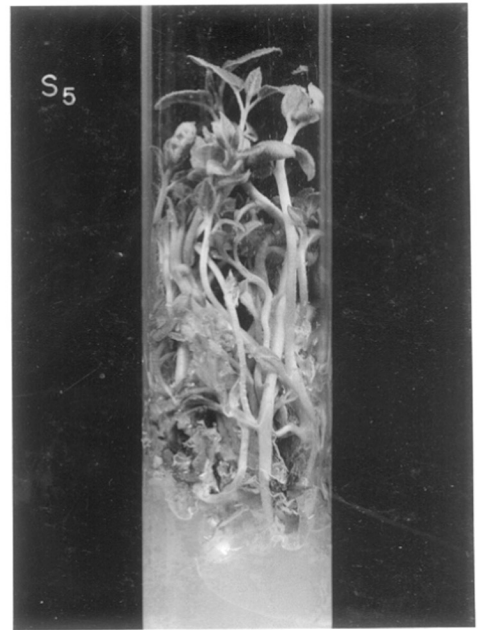


Fig. 16 Effect of subculture -  $S_4$  - fourth subculture;  
 $S_5$  - fifth subculture;  $S_{30}$  - thirtieth subculture.

The number of shoots being over 20 (Fig. 17). In stationary cultures 2 - 3 shoots were seen (Table 16)(Fig.17). This could have been due to the fact that aeration was insufficient.

#### Effect of additional vitamins

MS-3 medium, in addition to containing the vitamins present in Murashige and Skoog medium, was also arbitrarily supplemented with Ca.P. and Bio. (Table 17), each at 0.1 mg/l. This experiment was carried out to determine whether these two vitamins were essential, and whether they were the cause of the healthy shoot formation. Although the number of shoots which developed, were identical in media in which either one of these vitamins or both were omitted, the shoots that grew in the presence of both these vitamins were healthier, had a thicker stem and a higher number of leaves.

Table 16 Effect of liquid medium

Medium - MS-3 (liquid)

|    | Physical condition               | Shoot formation |
|----|----------------------------------|-----------------|
| 1. | Stationary                       | +               |
| 2. | Agitation on shaker<br>(120 rpm) | +++++           |

## Number of Shoots

- (None)

+ (1 - 3)

+++++ (20 and more)



1. Stationary



2. Agitation on shaker (120 rpm)  
Fig. 17 Effect of liquid medium.

Table 17   Effect of additional vitamins  
                   (Calcium Pantothenate and Biotin)

Medium - MS-7

|    | Additional vitamins<br>(mg/l) | Shoot formation |
|----|-------------------------------|-----------------|
| 1. | None                          | +++++           |
| 2. | Ca.P. (0.1)                   | +++++           |
| 3. | Bio. (0.1)                    | +++++           |
| 4. | Ca.P. (0.1) + Bio. (0.1)      | +++++           |

Number of Shoots

|       |               |
|-------|---------------|
| -     | (None)        |
| +     | (1 - 3)       |
| ++    | (3 - 5)       |
| +++   | (5 - 7)       |
| ++++  | (7 - 10)      |
| +++++ | (10 and more) |

## Section 2    Discussion

There are now many reports on differentiation from herbaceous plants (Murashige, 1974; 1978) and also from woody trees (Bonga, 1977; Sommer and Caldas, 1981). Some of these studies on trees have been referred to in Tables (1a - 1g). Organogenesis in vitro is known to be influenced by growth regulators and other physical factors. However reports with potential application in clonal propagation of forest trees is still limited to a very few trees (Gupta et al., 1980; 1981; Smith et al., 1982; Paranjothy and Rohani, 1982). Since most of the literature refers to methods which have been developed mainly with seedlings or juvenile trees (Bonga, 1977; Winton, 1978). In order to develop a method for rapid multiplication of E. citriodora by tissue culture the effect of different growth regulators, physical factors etc. were tested using explants initially from seedlings and later from mature trees of E. citriodora.

### Seedlings

#### Source of explant

The source of the explant cultured is an important factor in determining the organogenetic potential (Narayanswamy, 1977). Plantlets regenerated from herbaceous plants have been successfully accomplished from explants of cotyledons, hypocotyl, stem, leaf, shoot apex, root, young inflorescences, flower petals, petioles, ovular tissues or embryos (Murashige, 1974). There are also several reports on forest trees, where

embryogenic tissues were used for propagation (Bonga, 1977; Winton, 1978).

Isikawa (1974) used hypocotyl segments from 2 week old seedlings of Cryptomeria japonica for adventitious shoot induction on basal medium with a cytokinin and an auxin. Chalupa (1975) excised upper parts of the hypocotyl from embryos of germinating Norway spruce seeds, when cotyledons were beginning to emerge, and placed them on a basal medium containing cytokinin for adventitious bud formation. Campbell and Durzan (1975; 1976a; 1976b) made extensive use of hypocotyl segments in development of a system to obtain plantlets of Picea glauca. When the hypocotyl with the apical end exposed was placed on nutrient medium containing  $10^{-5}M$  BAP and  $10^{-7}M$  NAA, adventitious buds were produced. If the hypocotyl was placed in the nutrient medium with the apical end in a medium containing  $10^{-5}M$  NAA and BAP at  $10^{-7}M$  or with these withheld completely, then roots could be obtained from the basal end of the hypocotyl. David and David (1977) inoculated hypocotyl cultures of Pinus pinaster with apex intact and some times also containing the cotyledons for adventitious shoot formation. In a series of experiments based on the stage of development of 2 - 4 week old Biota orientalis and Cupressus seedling, adventitious bud formation was observed from different regions, from shoots including hypocotyl, cotyledon, primary needles etc. on media containing different BAP

concentrations (Thomas et al., 1977). Proliferation of adventitious buds were obtained from cotyledons or primary needle segments of Pseudotsuga menziesii (Cheng, 1975; 1976; 1977). Mott and coworkers (Mott et al., 1977) inoculated cotyledons directly from the embryos of Pinus taeda and obtained adventitious buds. Smith et al. (1982) reported formation of a large number of shoots from hypocotyl and cotyledon tissues of embryos of Pinus radiata on basal medium supplemented with a cytokinin and auxin.

Multiple shoot bud formation was reported by Rao and Bapat (1978; 1979) from hypocotyl segments of Santalum album on a medium supplemented with a cytokinin.

Adventitious shoot formation has also been reported from Eucalyptus species from diverse regions of the seedlings (Goncalves et al., 1979). From the shoot apex, nodal segments, hypocotyl and cotyledons of E. grandis seedlings (Cresswell and de Fossard, 1974); from hypocotyl and nodal segment of E. ficifolia seedlings (de Fossard and Bourne, 1976; 1977; de Fossard, 1978); from nodal and hypocotyl of E. alba seedlings (Kitahara and Caldas, 1975); from nodal segments of E. camaldulensis, E. curtisii, E. globulus, E. nitens, E. regnans, E. orcadensis, E. obtusiflora (Hartney and Barker, 1980). In earlier studies on E. citriodora multiple shoot formation was reported from different seedling explants, from lignotuber (Aneja and Atal, 1969); shoot apex (Lakshmisita and Vaidyanathan,



1979) and from cotyledons (Lakshmisita, 1980).

In the present studies, maximum number of shoots developed from hypocotyl explants followed by segments from the nodal region and shoot apex. Cotyledon explants formed only callus and the root explants died after 30 days.

#### Basal media

The composition of the medium is an important factor in the successful regeneration of plantlets (Gamborg et al., 1978). At present the most commonly used mineral salts formulation for differentiation is Murashige and Skoog medium (1962). This medium contains a high level of ammonium (20 mM). Different media are now classified based on salt concentration. Among the low salt media White (1963), Heller (1953), Knop (1856) etc. are included whereas Murashige and Skoog (1962), Gamborg B<sub>5</sub> (1968), Smith (1967) media are classified as high salt media.

There are now several reports, where different basal media have been used for adventitious shoot induction from different forest tree species (Sommer and Brown, 1979). The media of Wolter and Skoog (1966) or Gamborg B<sub>5</sub> (1968) with a cytokinin was used for Norway spruce (Chalupa, 1975); for Cryptomeria (Isikawa, 1974); for Populus spp. supplemented with BAP (Winton, 1971). Shoot development occurred <sup>from</sup> explants of Thuja occidentalis, Biota orientalis and Cupressus spp. ~~when~~ cultured on Murashige and Skoog or Heller basal medium with various combinations of cytokinins and auxins (Thomas et al., 1977); from

Pseudotsuga menziesii when embryos were cultured on a modified Murashige and Skoog basal medium with a cytokinin and an auxin (Cheng, 1975); from young needles of Abies balsamea, Picea glauca and Pseudotsuga menziesii on modified Murashige and Skoog basal medium (Brown and Lawrence, 1968) with various levels of growth regulators (Bonga, 1981).

In sandalwood, embryoid formation occurred from stem callus on Murashige and Skoog (1962) basal medium supplemented with GA<sub>3</sub> (Lakshmisita et al., 1979).

Shoot and root formation was obtained from nodal segments of seedling of Eucalyptus camaldulensis, E. robusta, E. grandis on Nash and Davies (1972) basal medium with 1.0 mg/l IBA (Goncalves, 1975); from E. camaldulensis, E. curtisii, E. ficifolia, E. globulus, E. nitens, E. orcadensis and E. regnans on a modified defined medium of Murashige and Skoog (Hartney and Barker, 1980).

In the present study the largest number of shoots were obtained on Murashige and Skoog (1962) basal medium supplemented with cytokinins (MS-3) followed by Smith, Gamborg B<sub>5</sub>, Heller, White and Knop media. Murashige and Skoog basal medium was also earlier used for multiple shoot induction from seedling segments of E. citriodora (Aneja and Atal, 1969; Lakshmisita and Vaidyanathan, 1979; Lakshmisita, 1980). These results indicate the importance of a high salt medium for E. citriodora for multiple shoot formation.

### Mature trees

The literature on organogenesis from seedlings or embryonic tissue is expansive (Bonga, 1977; Abbott, 1978; Sommer and Caldas, 1981), but the methods used with these tissues are often not effective with tissues of mature trees (Gupta et al., 1980; 1981). This is also evident from our studies with E. citriodora. Some of these observations could, therefore, be of interest particularly for the propagation of other mature tree species for which methods have been developed for the embryonic or seedling tissues (Bonga, 1981).

### Pretreatment

The primary explants of 20 year old trees did not respond to the media used for seedling explants either for shoot elongation or multiplication. This indicated that either the media had to be modified or that the tissue had some other requirements. Several unsuccessful experiments were performed mainly by modification of the medium used for seedlings. However a percentage response was obtained by pretreatment of the primary explants on MS-3 medium for 72 h at 15°C and then incubated at 25°C for 14 days followed by transfer of the explants to shake flasks containing MS-3 liquid medium and agitated on a shaker at 27°C with continuous illumination for 15 days before being transferred back to agar media at 25°C. These combined pretreatments resulted in the breakage of the dormancy of the buds.. Recently Von Arnold (1982) also reported that

the pre cold treatment (0 - 5°C) of buds from mature trees of Picea abies is necessary for in vitro adventitious shoot induction.

In order to optimize the media and other factors for maximum shoot formation from buds from mature trees, several experiments were carried out with in vitro grown shoots by addition of different supplements to MS-3 medium for determining the optimum concentrations or essentially of some of the components in this medium.

#### Relative concentration of cytokinins and auxins

Two important groups of plant hormones, cytokinins and auxins and their relative concentrations are very important in obtaining organogenesis and embryogenesis (Thorpe, 1981).

Campbell and Durzan (1975) reported that BAP is essential for adventitious bud induction from hypocotyl segments of Picea glauca. When BAP was present at  $10^{-5}M$ , and NAA at  $10^{-5}M$  (but not at  $10^{-7}M$ ) adventitious shoot induction was inhibited. However, when BAP was added at  $10^{-7}M$ , shoot induction was inhibited by NAA even at  $10^{-7}M$ . This indicated that the ratio of cytokinin to auxin was important for differentiation of Picea glauca, as it was for tobacco (Skoog and Miller, 1957). Juvenile and mature tissues of Thuja plicata were induced to form multiple adventitious buds, when cultured on defined nutrient media containing a high concentration of BAP at  $1 \times 10^{-5}M$  without any auxin (Coleman and Thorpe, 1977). Thomas et al. (1977) studied organogenesis from seedling explants of Biota

orientalis and other species of the Cupressaceae. Among the growth substances tested, only BAP was effective in inducing bud formation at 2.22  $\mu$ M concentration. However, basal media supplemented with NAA and Kin or zeatin were able to induce roots from callus. Sommer et al. (1975) working with long leaf pine, found that 1.0 mg/l BAP and 2.0 mg/l NAA induced adventitious shoots. Cheng (1975) reported organogenesis in Douglas-fir by first treating cells with an 0.5 - 1.0 mM solution of two cytokinins, BAP and 2iP and subsequently transferring them to a cytokinin-free medium. She suggested that this allowed the concentration of cytokinin within the cells to decrease as cellular proliferation continued and eventually the optimal condition for triggering shoot formation was reached. Reilly and Washer (1977) found that a high BAP concentration (10 - 25 mg/l) was required initially for multiple bud induction from cotyledon explants of Pinus radiata, which elongated when transferred to basal medium without cytokinins. Cheng (1976) demonstrated that the morphogenetic response of cotyledon explants of Tsuga heterophylla was strongly influenced by the interaction between exogenously added auxin and cytokinin. Adventitious bud formation occurred only when IAA, IBA and BAP were each present at 2.5  $\mu$ M in the basal medium. Isolated needles of Picea abies were induced to form adventitious bud primordia when cultured on media containing a relatively high cytokinin and auxin ratio

(10  $\mu$ M BAP and 0.55 nM NAA). Shoot primordia could also be induced on media containing 2iP, Kin and zeatin (Von Arnold and Eriksson, 1979). Minocha (1980) reported adventitious bud formation from hypocotyl and cotyledon explants of Pinus strobus on media containing IBA (1 - 5 mg/l) or TIBA (0.5 - 1.0 mg/l), which increased by addition of BAP (0.1 mg/l). Konar and Singh (1980) induced formation of shoot buds from cotyledon callus of Pinus wallichiana on Murashige and Skoog basal medium supplemented with 1.0 mg/l BAP.

Winton (1968) induced multiple shoot formation from Populus tremuloides callus on defined nutrient medium with 0.05 mg/l BAP. Whitehead and Giles (1976) cultured axillary buds of Populus nigra, P. deltoides, P. nigra hybrid and P. yunnanensis on modified Murashige and Skoog basal medium with 0.2 mg/l BAP. If the cultures at the initiation of bud formation were transferred to media with 0.1 mg/l BAP and 0.02 mg/l NAA, 120 - 220 shoots formed from each bud after 6 - 8 weeks. Thompson and Gordon (1977) obtained adventitious shoots from shoot tips of Populus tristis and P. balsamifera cultured on a modified Murashige and Skoog basal medium supplemented with  $1.2 \times 10^{-6}$  M BAP and  $5.7 \times 10^{-7}$  M IAA. Sato (1981) showed the requirement of zeatin ( $10^{-5}$  M) with  $10^{-7}$  M NAA for multiple shoot formation from Populus species. Bapat and Rao (1978) reported that hypocotyl segments of Santalum album formed multiple shoots on a basal medium supplemented with 1 mg/l

BAP. Sommer and Brown (1980) obtained bud differentiation from hypocotyl of sweetgum on medium containing higher cytokinin/auxin ratios 0.8 - 1.6 mg/l BAP to 0.1 - 1.0 mg/l NAA, while root formation occurred at lower cytokinin/auxin ratios (0.2 mg/l BAP to 1.0 - 4.0 mg/l NAA), which was similar to the observation of Skoog and Miller (1957) on tobacco.

A combination of two cytokinins, Kin and BAP each added at 0.1 mg/l, or at 0.2 mg/l Kin and 0.5 mg/l BAP were required for multiple shoot induction from buds and nodal segments of teak (Gupta et al., 1980) and tamarind (Kulkarni et al., 1981). Regeneration of plantlets from callus cultures of Betula pendula were induced on basal medium supplemented with IAA (2 mg/l), Kin (6 mg/l) and adenine (40 mg/l) (Srivastava and Steinhauer, 1981); with 1.0  $\mu$ M BAP and 5  $\mu$ M IBA or nodal segments of seedlings of E. camaldulensis, E. curtisii, E. ficifolia, E. globulus, E. grandis, E. nitens, E. obtusiflora, E. paniculata, E. regnans (Hartney and Barker, 1980); from hypocotyl callus of E. alba on medium containing 1 mg/l IAA (Kitahara and Caldas, 1975); from lignotuber callus of E. citriodora with 1 mg/l NAA alone (Aneja and Atal, 1969); from seedling segments of E. citriodora on basal medium supplemented with 0.5 mg/l BAP (Lakshmisita and Vaidyanathan, 1979). When shoots on this medium were subcultured to media with a higher concentration of BAP (4 mg/l), the number of shoots greatly increased (Lakshmisita and Vaidyanathan, 1979). Cotyledon callus of E. citriodora could also be

induced to produce several shoots on media supplemented with zeatin (1 mg/l) and IAA (0.2 mg/l) (Lakshmisita, 1979).

In the present investigation, BAP induced maximum shoot formation followed by Kin, 2iP and DPU when added singly. Zeatin was less effective. This effect was enhanced with a combination of two cytokinins Kin and BAP, the optimum concentrations being 0.2 mg/l Kin and 0.3 mg/l BAP in MS-3 medium. DPU, 2iP or zeatin in combination with Kin did not enhance the Kin effect.

Addition of auxins, individually at the concentrations tested (NAA, IBA, IAA, 2,4-D, B-NOA, 2,4,5-TCP) along with the cytokinins present in MS-3 medium induced callus formation at the base of the explants and reduced the number of shoots.

These results demonstrate the superiority of the presence of 2 cytokinins Kin and BAP for maximum shoot development.

#### Auxins

An auxin: cytokinin ratio was not necessary for multiple shoot formation as was reported earlier (Aneja and Atal, 1969; Lakshmisita, 1979; Lakshmisita and Vaidyanathan, 1979). This however needs further confirmation by testing the individual auxins over a wide concentration range.

#### Gibberellins

There are several reports that although gibberellins, stimulate elongation of organs, they generally repress the



process of organ initiation (Bigot and Nitsch, 1968) Murashige (1963; 1964; Schraudolf and Reinert, 1959). According to Gautheret (1969), gibberellins could substitute light in stimulating root initiation in Helianthus tuberosus tuber sections. Recently there are many reports on the stimulation of growth of plant cultures by  $GA_3$  which has been attributed mainly or entirely to its promotion of cell expansion on tobacco callus (Murashige, 1964; Lance et al., 1976). Morselli et al. (1974) found that  $GA_3$  could release the dormancy of isolated apical shoots of Acer saccharum. Within 2 weeks four leaves had grown and four weeks later root primordia appeared. A defined medium supplemented with IAA or Kin could not stimulate this effect of  $GA$  in releasing dormancy. Lakshmisita et al. (1979) obtained differentiation of embryoids from stem callus of Santalum album on a medium supplemented with  $GA_3$  (0.5 - 2.0 mg/l). Subsequently in 1980 they obtained embryoids from endosperm callus of sandalwood on basal medium supplemented with  $GA_3$  (1 mg/l).

In the present studies  $GA_3$  was found inhibitory for multiple shoot formation at the different concentrations tested in MS-3 medium. Only shoot elongation was observed at 5 mg/l  $GA_3$  but shoots were unhealthy.

#### Growth retardants and ethrel

There are several reports on the effect of growth retardants on shoot elongation, root development, flower

initiation, sex expression, maturation and crop yield, and metabolic changes in intact plants (Cathey, 1964). The majority of reports indicate that CCC retards plant growth. However, there is also evidence that low doses of CCC significantly stimulate growth and increase the endogenous levels of gibberellins (Halevy and Wittwer, 1965; Reid and Crozier, 1970). Konar and Konar (1974) showed a stimulation of shoot bud formation from hypocotyl segments of Ranunculus sceleratus in presence of CCC (0.001) and GA<sub>3</sub> (1.0). A slight promotive effect of CCC on sprouting of the immature bulbils of 3 species of Dioscorea was reported by Okagami and Tanno (1977). Growth regulators such as morphactins, ethrel, ethephon and CCC are known to effect developmental processes in a number of plants (de Fossard, 1973; Ogura, 1975). Stimulatory effects on branching, retardation of shoot growth, shift in sex expression is reported (Mohan Ram and Jaiswal, 1972; Helena et al., 1976; Balsubramanian and Narayanswamy, 1980). Schneider (1964) reported that morphogenetic changes are brought about by morphactin, a derivative of fluorene-9-carboxylic acid. Ogura (1975) showed that morphactin was effective in inducing shoot regeneration from callus of Nicotiana tabacum (Wisconsin No. 38), which was greatly increased when combined with Kin. Kudo and Koga (1981) showed that morphactin promoted the multiplication of pineapple seedlings, primarily by the induction of buds

from fruit and also by increasing the induction of normal foliar buds such as slips and suckers. Skoog and Tsui (1951), and Miller and Skoog (1953) reported the inhibition of organogenesis from tobacco stem segments by growth retardants such as Amo-1618, CCC, phosphon and B-995.

ABA with a few exceptions inhibits the growth of whole plants or excised parts of plants (Chen and Osborne, 1970; Walton et al., 1970). In addition to the inhibitory effects of ABA on plant growth, some promotive effects of ABA on cell division (Minocha, 1979); shoot initiation (Shepard, 1980) and asexual embryogenesis (Kochba et al., 1978) have recently been reported.

Isikawa (1974) showed that in a basal medium with 500 mg/l malt extract and 1 mg/l ABA, 33% of cultures from Cryptomeria japonica developed buds, whereas without ABA only 6% of the cultures formed buds. In 1978 he reported the requirement of morphactin (chlorflurenol) at a concentration of 0.25 - 0.5 mg/l, with 2 mg/l BAP for bud regeneration from hypocotyl segments of Cryptomeria japonica and Chamaecyparis obtusa.

Our results clearly indicate that the growth retardants ABA, morphactins, CCC and also ethrel are inhibitory for multiple shoot formation at the concentrations tested.

#### Organic compounds

Organic natural products such as CM, YE, ME and CH

(Sharp and Crocomo, 1975) are beneficial for organogenesis and embryogenesis (Murashige and Tucker, 1969). This response is due either to a supply of particular amino acids, or vitamins or nucleotides or of reduced nitrogen (Button and Kochba, 1977). Malt extract has been found to be either beneficial or essential (Bitters et al., 1974). Embryogenesis in nucellar cultures of citrus was stimulated by ME (Kochba and Spägel, 1973; Mitra and Chaturvedi, 1972; Rangan et al., 1969). Steward et al. (1958) showed that a cell suspension derived from carrot phloem explants developed embryo like structures if allowed to continue growth in a medium containing CM. Reinert (1963) found that root development took place when carrot callus was grown on a medium containing 7% CM. Shoot formation was obtained on transfer of embryo callus of Pinus wallichiana to basal medium with 10% CM (Konar and Singh, 1980). Bachelard and Stowe (1963) established root cultures of E. camaldulensis on medium containing 5% CM. Bapat and Rao (1979) reported the initiation of embryo differentiation, when hypocotyl callus of Santalum album was transferred to medium containing CM (10%) or CH (400 mg/l) or YE (400 mg/l). Recently Mascarenhas et al. (1982) reported the requirement of CH 500 mg/l for multiple shoot formation from segments of mature rose wood and rubber trees.

In the present studies, it was observed that the addition of CM, ME, CH and YE to the medium decreased the multiple shoot formation at the level tested.

### Effect of subcultures

Many cultured tissues exhibit a high potential for organogenesis or embryogenesis when first initiated, but this potential gradually declines as subculture proceeds with eventual loss of morphogenetic response (Torrey, 1967; Murashige and Nakano, 1967). Ishihara and Katano (1982) reported an increase in shoot formation from apple shoot tips on repeated subculture. Rugini and Verma (1982) obtained abundant shoot formation from a mature almond cultivar every 20 days for 2 years, without observing culture deterioration. Standardi (1982) showed no change in shoot, root formation and chromosome number on repeated subculture upto 29th from tissue of Actinidia chinensis.

Lakshmisita and Vaidyanathan (1979) also showed that shoot formation increased from shoot tips of E. citriodora on subculture.

Our results showed that multiple shoot formation increased on subculture becoming constant after four subcultures and showing no reduction even after 30 subcultures which the tissues have so far undergone. This observation differs from some of the earlier reports on callus culture (Barba and Nickell, 1969; Narayanswamy, 1977) where the morphogenetic potential decreases on subculture.

### Effect of temperature

Incubation temperature also has a profound influence

on the frequency of multiple shoot production. Skoog (1944) showed that shoot production in tobacco callus was highest at a temperature of 18°C. A temperature of 23°C was too high and 12°C was too low. A high frequency of plantlet formation was observed in Douglas-fir cultures at 19°C (Cheng, 1979). A few plantlets were also formed at 24°C but these were abnormal, exhibiting a discontinuity in their anatomical structures caused by a proliferation of friable callus at the base of the explant. Von Arnold and Eriksson (1977) isolated needles of Picea abies in culture which were capable of forming buds at 15°C and 30°C. The most effective temperature, however, for formation of maximum number of buds was 20°C.

In the present studies, the optimum temperature for shoot formation was observed to be  $25 \pm 2^\circ\text{C}$ . This temperature was very critical since at temperatures just below or above 25°C shoot formation was reduced.

#### Light intensity

The light requirements have clearly been shown to influence organized development in vitro (Thorpe, 1981; Gamborg and Shyluk, 1981). Murashige (1974) showed that the light requirement for differentiation involves a combination of several components including intensity, daily light period and quality for tissues of Gerbera and Saxifraga. Hasegawa et al. (1973) have shown that light intensity of 1,000 lux was optimum for multiple shoot formation from Asparagus at a photoperiod of 16 h.

Gautheret (1969) described a 12 h daily light period of an intensity of 5,000 lux for root initiation in Helianthus tuberosus tuber sections. Bonga (1977) obtained shoot formation from embryonic tissues of Abies balsamea at 540 lux light intensity for 16 h daily. Isikawa (1978) obtained optimum growth of Cryptomeria japonica seedlings in the presence of 6,000 lux light intensity and 5 h photoperiod at 10°C.

We observed that shoot formation was maximum at a light intensity of 1,500 lux for 16 h.

#### Effect of physical condition of medium

The physical state of the medium (whether liquid or semisolid) can influence organogenesis in vitro (Thorpe, 1981). White (1939b) observed that Nicotiana callus differentiated into shoots when transferred from a nutrient agar medium to liquid shake flask medium. Winber (1965) noted that fresh explants of Cymbidium initiated shoot development faster in an agitated liquid medium. Earle and Langhans (1975) obtained high rates of shoot multiplication when explants of carnation, after being grown for 2 - 3 weeks on agar medium were transferred to 500 ml Erlenmeyer flasks containing 30 ml liquid medium and rotated at 10 rpm. 59 clumps of shoots were produced in shake flasks from one explant after 6 weeks as compared to only about 10 shoots on agar medium.

Lakshmisita et al. (1980) reported embryoid formation from sandalwood callus in a liquid nutrient medium and

in flasks rotating at 70 rpm.

In the present studies the number of shoots which developed when the medium was agitated at 120 rpm was over 6 times higher than in stationary liquid media. This clearly demonstrates the importance of aeration for multiple shoot formation.



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## **CHAPTER IV**

### **STUDIES ON ROOT REGENERATION**

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## STUDIES ON ROOT REGENERATION

### Section 1 Experimental

This Section describes studies on root initiation and plantlet formation from in vitro grown shoots obtained from seedling segments and mature trees.

#### Effect of auxins

##### Shoots from seedlings

The experiments for root initiation were initially carried out with seedling raised shoots. In order to induce rooting on seedling shoots, initially, the effect of 4 different auxins; NAA, IAA, IBA and IPA were tested at concentrations ranging from 0.1 - 5.0 mg/l. 30 - 40 mm long shoots which had grown from hypocotyl explants on MS-3 medium, were excised after 25 - 30 days and treated with the different auxins by dipping 5 mm of the cut end of the shoots in White (W-3) liquid medium containing the test auxins. These cultures were then incubated in dark at 25°C for 5 days and later grown in light. Observations were made 15 days after auxin treatment.

A little callusing was observed with 0.2 and 0.5 mg/l NAA and 1.0 mg/l IAA. All the auxins were inhibitory at concentrations above 1.0 mg/l, the shoots turning brown and dying at 5.0 mg/l. Root initiation was not observed in presence of any of the auxins, at the concentration ranges tested. (Table 18).

Table 13 Effect of different auxins on root formation

Inoculum - Seedling shoots

Medium - W-3

|     | Auxin | mg/l | Observation (15 days) |
|-----|-------|------|-----------------------|
| 1.  | NAA   | 0.1  | -                     |
| 2.  | NAA   | 0.2  | Slight callus -       |
| 3.  | NAA   | 0.5  | Slight callus -       |
| 4.  | NAA   | 1.0  | -                     |
| 5.  | NAA   | 5.0  | dead                  |
| 6.  | IAA   | 0.1  | -                     |
| 7.  | IAA   | 0.2  | -                     |
| 8.  | IAA   | 0.5  | -                     |
| 9.  | IAA   | 1.0  | Slight callus -       |
| 10. | IAA   | 5.0  | dead                  |
| 11. | IBA   | 0.1  | -                     |
| 12. | IBA   | 0.2  | -                     |
| 13. | IBA   | 0.5  | -                     |
| 14. | IBA   | 1.0  | -                     |
| 15. | IBA   | 5.0  | dead                  |
| 16. | IPA   | 0.1  | -                     |
| 17. | IPA   | 0.2  | -                     |
| 18. | IPA   | 0.5  | -                     |
| 19. | IPA   | 1.0  | -                     |
| 20. | IPA   | 5.0  | dead                  |

- No root initiation

Effect of treatment with different NAA concentrations for varying periods

In these experiments individual shoots grown as above on MS-3 medium were excised and treated with different NAA concentrations present in W-3 liquid medium (0.1 - 5 mg/l) for varying periods (24, 48 and 72 h) in dark and then transferred to W-3 medium without the auxin and incubated in light (Table 19).

60% of shoots treated with NAA (1 mg/l) for 48 h in dark, rooted on W-3 medium within 12 - 15 days in light. With the other treatments rooting was not observed even if the cultures were incubated for longer periods. The roots appeared healthy, grew long and contained root hairs (Fig. 18).

Shoots from mature trees

Effect of different auxins

Initially the same conditions which were found successful for seedling cultures were tested for shoots from the mature trees viz. treatment with NAA (1.0 mg/l) for 48 h but this was not successful.

Following these studies various other conditions were tested using shoots which had sprouted from the primary explants of the mature tree on MS-3 medium 40 - 50 days after inoculation. Individual shoots (30 - 40 mm long) were dipped, as in the case of seedling shoots, in the auxin solutions prepared in W-3 medium. Four different auxins were tried individually, NAA, IAA, IBA and IPA at five

**Table 19** Effect of treatment with different NAA concentrations for varying periods

Inoculum - Seedling shoots

Medium - W-3

|    | NAA<br>(mg/l) | Treatment |      |      |
|----|---------------|-----------|------|------|
|    |               | 24 h      | 48 h | 72 h |
| 1. | 0.1           | -         | -    | -    |
| 2. | 0.2           | -         | -    | -    |
| 3. | 0.5           | -         | -    | -    |
| 4. | 1.0           | -         | +    | -    |
| 5. | 3.0           | -         | -    | -    |
| 6. | 5.0           | -         | -    | -    |

- No root initiation

+ Root initiation

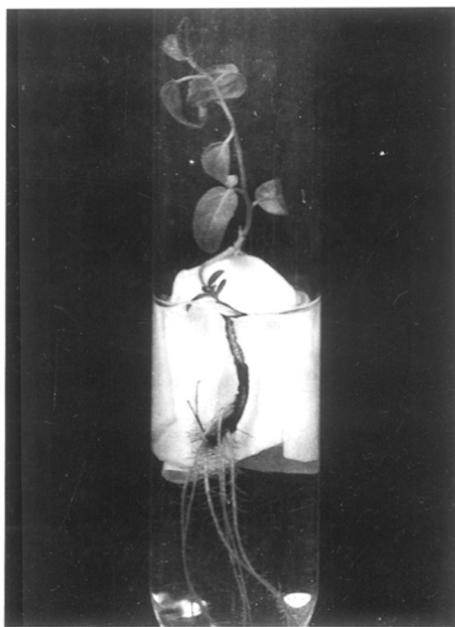


Fig. 18 Rooted plantlet from  
seedling on W-3 medium.



concentrations each ranging from 0.1 - 5.0 mg/l. The shoots were treated with the auxin solution prepared in W-3 medium in the dark for varying periods (24, 48 and 72 h) and later transferred to W-3 liquid medium without the auxins and incubated in light for 30 days. Root initiation was not observed with any of the treatments (Table 20). These results indicated that the requirements for root initiation in mature tree explants differed from seedling explants. The presence of root inhibitory compounds in the shoot explants could also be the cause.

#### Effect of subculture

In these studies individual shoots from mature trees grown and subcultured on MS-3 medium were excised as in the previous experiment, at each subculture, and treated with W-3 medium containing separately 6 different concentrations of NAA (0.1 - 5.0 mg/l) for 24 or 48 h respectively in dark. After this treatment shoots were transferred to W-3 medium without NAA and incubated in light (Table 21).

Root initiation was not observed in any of the treatments upto the 3rd subculture. At the fourth passage 35 - 40% of shoots treated with 2.0 mg/l NAA for 48 h rooted within 8 - 10 days. In the fifth and subsequent passages rooting increased from 40 to 50% (Table 22). The roots were long, healthy and contained secondary roots with root hairs (Fig. 19).

The rooted plantlets on W-3 medium however did not survive as the leaves gradually turned yellow, abscised and

**Table 20** Effect of treatment with different auxins  
concentrations for varying periods

Inoculum - Mature tree shoots

Medium - W-3

Period of - 30 days  
incubation

|     | Auxin | mg/l | Treatment |      |      |
|-----|-------|------|-----------|------|------|
|     |       |      | 24 h      | 48 h | 72 h |
| 1.  | NAA   | 0.1  | -         | -    | -    |
| 2.  | NAA   | 0.2  | -         | -    | -    |
| 3.  | NAA   | 0.5  | -         | -    | -    |
| 4.  | NAA   | 1.0  | -         | -    | -    |
| 5.  | NAA   | 5.0  | -         | -    | -    |
| 6.  | IAA   | 0.1  | -         | -    | -    |
| 7.  | IAA   | 0.2  | -         | -    | -    |
| 8.  | IAA   | 0.5  | -         | -    | -    |
| 9.  | IAA   | 1.0  | -         | -    | -    |
| 10. | IAA   | 5.0  | -         | -    | -    |
| 11. | IBA   | 0.1  | -         | -    | -    |
| 12. | IBA   | 0.2  | -         | -    | -    |
| 13. | IBA   | 0.5  | -         | -    | -    |
| 14. | IBA   | 1.0  | -         | -    | -    |
| 15. | IBA   | 5.0  | -         | -    | -    |
| 16. | IPA   | 0.1  | -         | -    | -    |
| 17. | IPA   | 0.2  | -         | -    | -    |
| 18. | IPA   | 0.5  | -         | -    | -    |
| 19. | IPA   | 1.0  | -         | -    | -    |
| 20. | IPA   | 5.0  | -         | -    | -    |

- No root initiation



Table 21 Effect of subculture from shoots of mature trees

Inoculum - Mature tree shoots

Medium - W-3

| NAA<br>(mg/l) | S <sub>1</sub> |     | S <sub>2</sub> |     | S <sub>3</sub> |     | S <sub>4</sub> |     | S <sub>5</sub> |     |
|---------------|----------------|-----|----------------|-----|----------------|-----|----------------|-----|----------------|-----|
|               | 24h            | 48h | 24h            | 48h | 24h            | 48h | 24h            | 48h | 24h            | 48h |
| 1. 0.1        | -              | -   | -              | -   | -              | -   | -              | -   | -              | -   |
| 2. 0.2        | -              | -   | -              | -   | -              | -   | -              | -   | -              | -   |
| 3. 0.5        | -              | -   | -              | -   | -              | -   | -              | -   | -              | -   |
| 4. 1.0        | -              | -   | -              | -   | -              | -   | -              | -   | -              | -   |
| 5. 2.0        | -              | -   | -              | -   | -              | -   | -              | +   | -              | +   |
| 6. 5.0        | -              | -   | -              | -   | -              | -   | -              | -   | -              | -   |

- No root initiation

+ Root initiation

S Subculture No.

the plants died within 25 days (Fig. 19).

For obtaining healthy plantlets it was found essential to transfer the plants growing on W-3 medium, just after a good root system had developed, (after 8 - 10 days) to MS-9 liquid medium which did not contain cytokinins. On this medium after 7 - 10 days healthy plants could be recovered (Fig. 20).

Effect of activated charcoal on rooting of shoots from primary explants of mature trees

Recently root initiation has been obtained with shoots developed from primary explants of the mature tree at the first subculture. By this procedure excised shoots after treatment with NAA (2.0 mg/l) for 48 h in dark in W-3 medium, were transferred to MS-10 agar medium containing 0.25% activated charcoal and the salts and organic supplements at half the original concentration and were incubated in light. Root initiation was observed within 8 - 10 days (Fig. 21). At this stage plantlets were immediately transferred to MS-11 liquid medium in which charcoal was omitted. By this procedure healthy plantlets with a good root system were obtained (Fig. 22). The percentage of rooting by this improved method increased to 70% as compared to 50% by the method described earlier.

Multiplication of plantlets from a 20 year old tree

When plantlets had attained a height of about 50 - 60 mm either on MS-9 after 3 subcultures or MS-11 medium

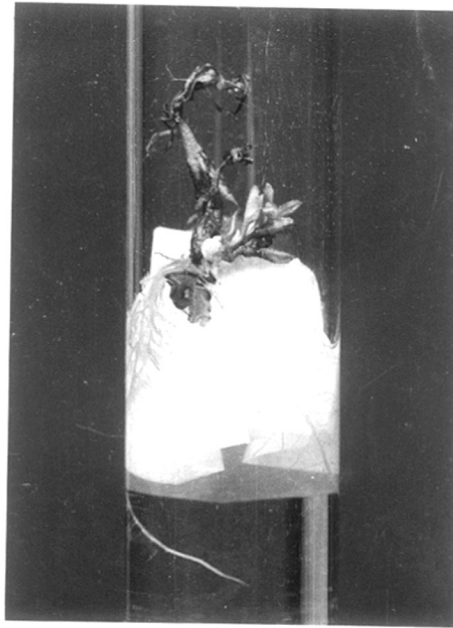


Fig. 19 Plantlet from mature tree showing healthy root system but unhealthy shoot on W-3 medium.

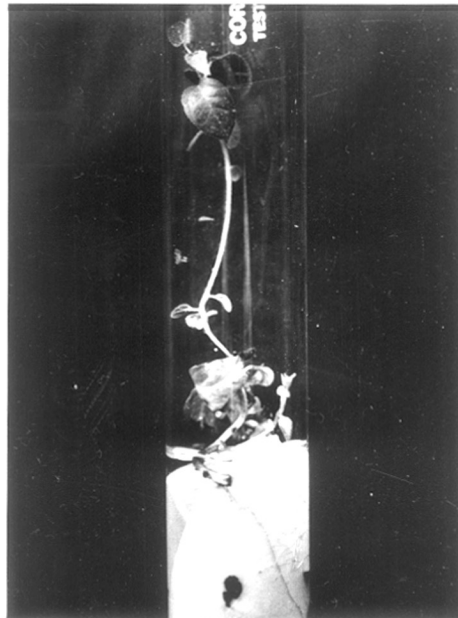


Fig. 20 Healthy plantlet from mature tree on MS-9 medium.



Fig. 21 Rooted plantlet from mature tree on MS-10 medium (activated charcoal medium).

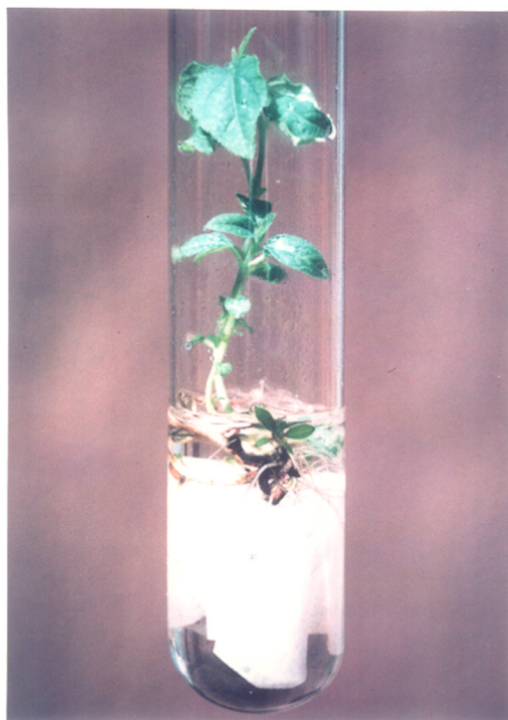


Fig. 22 Healthy plantlet from mature tree on MS-11 medium.

they were transferred to pots containing a soil:sand:compost (3:3:1) mixture, and incubated at  $25 \pm 2^{\circ}\text{C}$  for 10 days for hardening. With the emergence of new leaves the potted plants were transferred to the green house, where they grew into healthy plants. The survival of rooted plantlets in the pots was about 40 - 50% (Table 22). Details on field data are given in the next Chapter.V.

30 subcultures have so far been carried out and healthy viable plantlets obtained at each subculture without any diminution in the capacity for shoot or root formation (Table 22). At this rate of multiplication it is estimated that over 100,000 plants can be obtained when MS-9 medium was used, or 120,000 plants when MS-11 medium was used in a year from a single bud of a mature Eucalyptus citriodora tree by subcultures at intervals of 30 days, and with survival rates of 40 to 50%.

Table 22 Multiplication of plantlets from 20 year old tree of Eucalyptus citriodora on MS-9 and MS-11 medium

| Subculture No.      | No. of shoots per explant | Shoots which form roots in MS-9 medium % | Shoots which form roots in MS-11 medium % | Survival in pots % |
|---------------------|---------------------------|------------------------------------------|-------------------------------------------|--------------------|
| 1. Primary* explant | 5 - 6                     | 0                                        | 65 - 70                                   | 40 - 50            |
| 2. S <sub>1</sub>   | 6 - 8                     | 0                                        | 65 - 70                                   | 40 - 50            |
| 3. S <sub>2</sub>   | 8 - 10                    | 0                                        | 65 - 70                                   | 40 - 50            |
| 4. S <sub>3</sub>   | 8 - 10                    | 0                                        | 65 - 70                                   | 40 - 50            |
| 5. S <sub>4</sub>   | 10 - 12                   | 35 - 40                                  | 65 - 70                                   | 40 - 50            |
| 6. S <sub>5</sub>   | 12 - 15                   | 45 - 50                                  | 65 - 70                                   | 40 - 50            |
| 7. S <sub>6</sub>   | 12 - 15                   | 45 - 50                                  | 65 - 70                                   | 40 - 50            |
| 8. S <sub>30</sub>  | 12 - 15                   | 45 - 50                                  | 65 - 70                                   | 40 - 50            |

\* Primary explants - 30% of buds survived and formed shoots.

S Subculture No.

Subculture period - 30 days.

## Section 2 Discussion

In recent years the subject of root development particularly of woody forest trees has received special attention. This has mainly been due to the fact that woody trees are more difficult to root than the herbaceous plants. Also, among the woody trees grown in culture the Gymnosperms have presented more problems for rooting than the Angiosperms (Sommer and Caldas, 1981).

Thimann and Went (1934) reported that auxins exert a primary control over root formation. There are several reports where auxins are used in vivo for root formation from cuttings of different woody tree species such as willow, Populus, pear (Hartman and Kester, 1968). Auxins however are often not the only factors necessary. Several phenolic compounds (Hess, 1964; Girouard, 1969; Gasper et al., 1977; Schmidt, 1978), carbohydrates (Nanda and Jain, 1972), abscisic acid (Basu et al., 1970) in combination with auxins also promote adventitious root formation. Such a synergistic action between an auxin and phloroglucinol on rhizogenesis was reported for in vitro cultured shoots raised from root stock or scions of different Malus cultivars (Jones and Hatfield, 1974; Jones and Hopgood, 1979; James and Thurbon, 1979). Welander and Huntrieser (1981) reported that apple root stock A<sub>2</sub> can be readily rooted in the presence of both 5  $\mu$ M IBA and  $10^{-4}$  M phloroglucinol in the medium. Smith

and Thorpe (1977) obtained root initiation from hypocotyl shoots of Pinus radiata in the presence of phenolic cofactors like aromatic amino acids or simple phenyl propanoids with IBA.

Adventitious root formation of excised shoots is generally induced by a good O<sub>2</sub> supply (liquid medium), and the presence of sugar, macro and micro-elements and an auxin in the culture medium. Shoots from juvenile plants generally root easier than shoots of adult plants (Pierik, 1975).

Sommer et al. (1975) reported root formation from shoots of Pinus palustris cotyledons after treatment with 10 mg/l IBA for four weeks followed by transfer to a hormone free medium. Cheng (1976) obtained root formation of shoots produced from somatic cells of Tsuga heterophylla directly in soil. In this method each individual shoot was placed in a soil mixture "mica-peat". An aqueous solution of NAA at 15 uM (pH 6.2) was applied at two-day intervals for two weeks, after which shoots were watered without any additives. A relatively high humidity was provided during the initial rooting period by placing a plastic cover over the rooting apparatus. After four weeks 90% of shoots rooted. Coleman and Thorpe (1977) obtained rooting when shoots of Thuja plicata were transferred to basal medium containing  $5 \times 10^{-5}$  M IBA.

Reilly and Washer (1977), working with Pinus radiata observed root initiation on a basal medium containing



0.1 mg/l NAA and 2.0 mg/l IBA. This treatment was successful only with shoots which were maintained without hormones for 6 months. Cheng (1979) found that adventitious root formation from Douglas-fir shoots was stimulated by NAA and 2iP. In addition a sucrose concentration, 6-fold lower than that used for bud initiation also favoured root formation. Mehra-Palta et al. (1978) obtained root initiation from shoots of loblolly pine on a medium containing a low concentration of cytokinin (0.01 mg/l BAP) and an auxin (0.1 mg/l NAA).

Chalupa (1974; 1975) reported root formation from shoots of different species of Populus on modified Wolter medium with 0.4 mg/l NAA. Rooting could also be obtained when the shoots are placed aseptically in a sterile perlite and sand mixture containing half strength modified Wolter and Skoog medium with 0.2 mg/l NAA and 0.2% sucrose.

Thompson and Gordon (1977) obtained root initiation from shoots of Santalum album seedlings on basal medium containing NAA (0.5 mg/l) and IBA (0.5 mg/l). Minocha (1980) reported root formation from shoot tips of Betula papyrifera and B. alleghaniensis on a medium containing 6.0 mg/l IBA.

Rooting has also be obtained from shoots of different Eucalyptus species; from E. ficifolia seedlings on a basal medium containing 5  $\mu$ M IBA (de Fossard, 1978)) from E. camaldulensis, E. curtisi, E. grandis, E. obtusiflora seedlings on a low salt, defined medium with 5  $\mu$ M IBA

(Barker and Hartney, 1980). Cresswell and Nitsch (1975) rooted nodal segments of E. grandis after soaking the explants for at least 2 h in sterile distilled water. Soaking evidently leaches out the substances which inhibit root formation.

Recently a mixture of auxins have been found to be more effective than a single auxin for root formation in teak, two Eucalyptus species, rose wood and rubber cultures (Gupta et al., 1980; Mascarenhas et al., 1982). They obtained root initiation from shoots of mature trees using a mixture of IAA, IBA, IPA and NAA. A similar observation was made by Kulkarni et al. (1981) for inducing rooting from shoots of Tamarindus indica after treatment with IAA, IPA and IBA at (1 mg/l) each and of NAA 0.2 mg/l).

Lakshmisita (1979) obtained root formation from shoots of E. citriodora cotyledon callus on a medium containing 1 mg/l NAA or 1 mg/l IBA. The percentage rooting has not been stated.

We have obtained root initiation from shoots of seedlings on treatment with NAA (1 mg/l) in W-3 medium for 48 h in dark following by transfer to W-3 liquid medium without auxin in light. This treatment was not successful for root formation with shoots from a 20 year old tree either from the initial explants or in the first three subcultures. At the fourth subculture 35 - 40% shoots rooted. In the fifth and subsequent passages the percentage of rooting enhanced to 45 - 50% as compared to 60 - 70% rooting from seedling shoots.

After root formation on W-3 medium it was necessary to transfer the plantlets to MS-9 medium for survival and healthy development. Recently McComb and Bennett (1982) also reported a lower percentage of rooting in shoots isolated from mature trees than in seedling shoots of E. marginata.

The reason for the failure of shoots from the 20 year old tree to root after 3 subcultures is not clear. The possibility that inhibiting factors are removed by repeated subculture requires investigation.

Recently Sriskandarajah et al. (1982) reported a similar observation that freshly cultured apple shoots did not root. Root initiation started after four subcultures and after 9 subcultures 95% of shoots formed roots.

Recently activated charcoal has been used in the medium for root initiation. Fridborg and Eriksson (1975) used 1% activated charcoal for root initiation from shoots of Allium cepa. Preil and Engelhardt (1977) obtained root initiation from shoots of Rhododendron simsii on a medium containing 0.25% activated charcoal.

Mascarenhas et al. (1982) reported root initiation from shoots of mature trees of E. terreticornis, E. globulus, Hevea brasiliensis, Dalbergia latifolia on a medium containing 0.25% activated charcoal.

By incorporating activated charcoal into the medium root initiation could be induced from freshly cultured shoots of the 20 year old tree. Addition of 0.25% activated

charcoal stimulated root formation even in primary shoots which had not undergone any subcultures. This effect of activated charcoal on root initiation from freshly cultured shoots of mature trees could be due to the adsorption of the root inhibitory substances which were released from the growing shoots into the medium and which required a series of subculture before their removal (Fridborg and Eriksson, 1975; Schell et al., 1981).

Our results clearly show that some root inhibiting substances are present in shoots of mature trees of E. citriodora which are either diluted on subculture or adsorbed by activated charcoal.

Smith et al. (1982) could produce 16,700 shoots from 100 seeds of Pinus radiata. With a 60% conversion of shoots to plants suitable for plantation establishment, a 100-fold amplification could be obtained within one year from one seedling. We can obtain over 100,000 plants from a single bud of mature tree of Eucalyptus citriodora within a year.



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## **CHAPTER V**

**MONOTERPENES OF FIELD GROWN  
TISSUE CULTURE RAISED PLANTS**

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## MONOTERPENES OF FIELD GROWN TISSUE CULTURE RAISED PLANTS

### Section 1    Experimental

In this Chapter plants raised clonally by tissue culture from a 20 year old Eucalyptus citriodora tree grown at Poona and from an elite tree growing in the Tamil Nadu forests (South of India) were raised in the field, and the total oil citronellal and citronellol contents analyzed and compared with the parent trees.

In the previous Chapter conditions were given for rooting of shoots both from seedlings and from mature tree explants. In these studies healthy plantlets with a good root system were developed on MS-11 liquid medium. When the plantlets on this medium had attained a height of about 60 mm they were carefully removed with a pair of forceps from the culture tubes. The roots were washed thoroughly with tap water to remove traces of nutrient and the plants transferred to small plastic pots (8 cm x 8 cm x 4 cm size), containing a mixture of sterile soil:sand:compost in the ratio 3:3:1. The pots were incubated at  $25 \pm 2^{\circ}\text{C}$  temperature under a 16 h photoperiod and watered regularly at 48 h intervals. To prevent dessication the plants were covered with glass beakers. At this stage only 40 - 50% of the plants survived (Table 22).

#### Transfer to field

Within 20 - 25 days new leaves opened and the plants appeared steady, green and healthy. This indicated that the plants had established in soil.

At this stage potted plants were transferred to a green house where they were kept for 5 - 6 days for further hardening, without the glass beaker covers, before being transplanted to earthen pots (30 mm x 30 mm x 15 mm size), containing soil in which a small quantity of compost and sand was mixed (Fig. 23). These plants were then immediately transferred to the glass house where they were watered daily. After about 5 - 6 weeks when 4 - 5 new sets of leaves had emerged and the plants had attained a height 30 - 40 cm (Fig. 23b), they were transplanted into previously prepared pits (90 cm x 60 mm x 45 cm depth), filled with a mixture of good soil (river silt) containing 4 kg of farm yard manure. Before planting, pits were dranced with 0.1% copper fungicide to avoid fungal attack. The survival in the field at this stage was very high being about 100%. Plants in field were watered at two days intervals. Each plant received 15 g of an N:P:K (15:15:15) fertilizer, "Suphala" manufactured by Fertilizer Corporation of India, initially at 30 days intervals for 6 - 8 months, after that once in a year. Over 150 plants are growing in field, some of which are now 3 - 4 years old (Fig. 24).

#### Analysis of oil, citronellal and citronellol in mature trees

To determine the oil, citronellal and citronellol contents of these plants the procedure described earlier in Chapter II followed. These analyses was conducted with culture raised plants all clonally produced from the same mature parent trees, in order to determine whether





Fig. 23 Plants from mature tree in pot.

A. About 30 days old

B. About 2 1/2 months old.





Fig. 24 Tissue culture raised plants  
growing in field.

there was any variation in the concentrations of these constituents, when compared with the parent trees. The analyses were also performed on plants of different ages. This data could be important in determining the correct <sup>stage</sup> at which propagules raised from mature trees are capable of synthesizing these compounds.

Table 23 shows the pattern of the oil content, citronellal and citronellol of plant of different ages (3, 8, 15, 22, and 36 months old). In this experiment the analyses were carried out on different clonal plants, raised from the same parent tree. The analyses indicated a striking similarity in the oil, citronellal and citronellol concentrations in all plants from 3 - 36 months old, which in turn was similar to the levels of these compounds in the 20 year old parent tree. These results are important, in that the application of tissue culture for clonal multiplication of the high oil containing elite trees can be confirmed when the plants are a few months old without having to wait for many years as in the case with forest trees.

In the second experiment four clonally raised plants each were analyzed in two age groups, 10 and 32 months respectively (Table 24). There was a very close similarity in the oil, citronellal and citronellol contents of the parent tree with each of the plants analyzed, both at 10 and 32 months.

Table 23 Analyses of tissue culture raised plants at different ages

|                                    | Parent tree<br>(20 year old) | Months |        |         |         |         |
|------------------------------------|------------------------------|--------|--------|---------|---------|---------|
|                                    |                              | 3<br>% | 8<br>% | 15<br>% | 22<br>% | 36<br>% |
| Total oil<br>(dry weight<br>basis) | 1.15                         | 1.06   | 1.10   | 1.11    | 1.09    | 1.13    |
| Citronellal                        | 71.50                        | 70.80  | 71.02  | 70.91   | 70.25   | 70.56   |
| Citronellol                        | 17.30                        | 15.90  | 16.28  | 16.86   | 16.90   | 17.00   |

Table 24 Analyses of clonally raised plants

| Months                       |    | Total oil<br>(dry weight<br>basis)<br>% | Citronellal<br>% | Citronellol<br>% |
|------------------------------|----|-----------------------------------------|------------------|------------------|
| 1.                           | 10 | 1.10                                    | 71.00            | 16.00            |
| 2.                           | 10 | 1.08                                    | 70.90            | 15.90            |
| 3.                           | 10 | 1.12                                    | 70.60            | 17.00            |
| 4.                           | 10 | 1.06                                    | 70.10            | 16.50            |
| 5.                           | 32 | 1.12                                    | 71.10            | 17.00            |
| 6.                           | 32 | 1.10                                    | 70.90            | 17.10            |
| 7.                           | 32 | 1.13                                    | 70.50            | 16.80            |
| 8.                           | 32 | 1.00                                    | 70.20            | 17.20            |
| Parent tree<br>(20 year old) |    | 1.15                                    | 71.50            | 17.30            |

### Analysis of elite plants

The success of any tissue culture method developed for clonal propagation depends on its application for reproducing certain plus characters in the progeny. In this experiment buds were collected from the high oil and aldehyde containing E. citriodora trees grown in the forest at Dodabetta, Ooty (South of India). These buds were inoculated under the conditions described in Chapter III and then rooted as in the procedure given in Chapter IV. The rooted plantlets were then transferred to pots and the field. Analyses were conducted on the tissue culture plants raised from these elite trees, and the results are the average of three plants in each case.

Table 25 gives the data of 2 and 4 month old plants raised from a 5 year old 'elite' E. citriodora tree, having 3.5% oil, 92% citronellal, and 7% citronellol (dry weight basis). The concentration of the total oil, citronellal and citronellol in tissue culture plant was more or less identical to that of the parent elite tree.

Tissue culture raised plants flowered and fruited within 2 1/2 years (Fig. 25), whereas seed raised plants take a longer period.

**Table 25** Analyses of tissue culture raised plant from  
a mature elite tree

| Tree                                                             | Total oil<br>% | Citronellal<br>% | Citronellol<br>% |
|------------------------------------------------------------------|----------------|------------------|------------------|
| 1. Elite parent tree<br>(5 year old)<br>(on dry weight<br>basis) | 3.50           | 92.00            | 7.00             |
| 2. 2 months old<br>tissue culture                                | 3.00           | 89.80            | 6.00             |
| 3. 4 months old<br>tissue culture                                | 3.10           | 90.20            | 6.30             |



Fig. 25 Tissue culture raised plant  
(2 1/2 years old) showing  
flowers and fruits.

## Section 2 Discussion

It is generally a difficult procedure, where a high mortality is observed when plantlets are transferred from the sterile culture environment to the free living condition (Bonga, 1977). There are also some reports that this transfer can be made without much difficulty, as with Populus tremuloides and P. tremula (Winton, 1970; 1971) and Betula pendula (Huhtinen and Yahyaoglu, 1974). A gradual adaptation to the soil environment is usually required, involving a gradual simplification of the nutrient medium and increased light intensities (Murashige, 1974). Water stress is often a problem and high atmospheric humidities have to be provided (Winton, 1970; Bitter et al. , 1972). It has been noted that roots penetrating the nutrient medium often lack root hairs. For example, the roots of plantlets of Populus spp. had to be kept above the nutrient surface for root hairs to develop and for the roots to become functional (Winton, 1970; Chalupa, 1974), which is necessary for a high percentage of survival in the field.

Under the conditions followed for obtaining a healthy root system with roots having root hairs on MS-11 medium (Chapter IV), a 50% survival of plants was obtained when transferred to a free living environment.

A number of papers covering the procedure for producing plants by tissue culture of a whole range of forest trees belonging to the Angiosperms (Rao and Bapat,



1978; Lakshmisita et al., 1979; 1980a; 1980b) and Gymnosperms (Bonga, 1977; Sommer and Brown, 1979; Winton, 1978; Sommer and Caldas, 1981) have been recently reported. However very little data is available regarding transfer and growth of these plants in field. This is particularly the case with mature woody trees with which there are only a few reports e.g. teak (*Mascarenhas et al.*, 1981); oil palm (Choo et al., 1981), *Pinus radiata* (Smith et al., 1981; 1982). Mascarenhas et al. (1981) reported that the growth rates of 18 month old tissue culture plants of teak raised from 100 year old elite trees were nearly 20% taller and grew faster than seed raised plants of the same age.

Some of the field data on tissue culture raised plants of oil palm are described by Choo et al. (1981). A more detailed statistical analysis of the field data is given by Corley et al. (1981). The variation in petiole width x depth (widely used as an index of vegetative vigour in oil palm) is much less in clone 931 than among seedlings in the same trial. The variation in fruit composition, in terms of both percentage mesocarp and percentage kernel to fruit, is greater among seedling trees than in clone 931. The important yield components of the oil in clone 931 is less variable than seedlings. Corley et al. (1977) indicated that variation between oil palm within clones is appreciably less than variation between plants grown from seeds.

Our results clearly indicate that the concentration of the total oil, citronellal and citronellol is more or less identical in different clonally produced plants, being similar to the mature parent trees. No differences in the oil contents were found in the clonally raised plants from the elite trees even though they were grown in two different agroclimatic zones.

The results immediately demonstrate the utility of tissue culture as a complementary vegetative method for propagation of elite high oil containing E. citriodora trees. These results could also indicate that this character is genetic and not due to environmental or agroclimatic differences, since in the latter experiment, plants with a high oil, high aldehyde, content were obtained from the southern states of India and grown in Poona on the west of India. These two regions having different agroclimatic conditions. Tissue culture thus has a potential for verification of genotype versus environment interaction by planting the same clones in different environments.

In all our field trials which were conducted with plants of E. citriodora raised by tissue culture from mature trees a general observation made was that they flowered and set fruit within two years unlike in the normal methods where it takes a longer period. This finding increases the usefulness of tissue culture for inclusion in breeding programmes.

A similar result was also obtained with pomegranate plants raised by tissue culture where flowering and fruit setting was observed in six months (Mascarenhas et al., 1981). At AFOCEL (France) tissue culture is the method used to establish clonal orchards from 10 - 500 year old elite trees of Sequoia sempervirens. In vitro techniques are regularly applied for the production of 30,000 plantlets from approximately 200 selected clones (Poissonnier et al., 1980). Similarly a small production laboratory has been built at the Forest Research Institute of Rotorua (Newzealand) where micropopagation techniques are applied to produce 100,000 radiata pine plantlets annually (Smith et al., 1981; 1982).

These results indicate an immediate application of tissue culture for growing elite tree orchards. If the observation that tissue culture raised plants flower earlier than seed raised plants then breeding programmes can be undertaken after fairly short periods for obtaining elite seed orchards.

Tissue culture can thus have a tremendous potential and can be introduced into forestry breeding and improvement programmes.

As far as we are aware this is the first report giving field data of clonally propagated plants from elite trees of Eucalyptus citriodora Hook.

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## **CHAPTER VI**

### **INITIATION OF CALLUS AND STUDIES ON NUTRITION**

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## INITIATION OF CALLUS AND STUDIES ON NUTRITION

### Section 1    Experimental

This Section of Chapter VI describes the studies on the isolation of callus and nutritional requirements for the growth of E. citriodora callus. These experiments were performed with the objective of determining the optimum conditions for obtaining maximum growth of healthy friable callus.

#### Initiation of callus

Ten mm segments excised from the stem or leaf regions, collected from a one year old tree, after surface sterilization as described in Chapter II, in preliminary experiments were inoculated into different media. In these preliminary experiments, good healthy callus growth was obtained from leaf explant on MS-1 medium (Fig. 26), which could be subcultured every 25 days.

This callus grown for 25 days was used in all these studies as inoculum, the wet weight of the inoculum being 100 - 150 mg per tube. The studies were performed with cultures which had undergone over 5 passages, at which stage uniformity in the growth was observed. The results were taken after 25 days.

#### Effect of different basal media

Six different basal media were tested for the growth of callus (Tables 1a and 1b). All the media contained the vitamins and glycine according to Murashige and Skoog medium

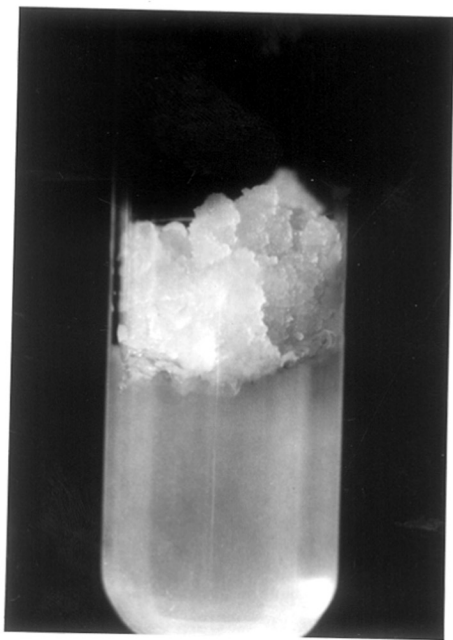


Fig. 26 Callus from leaf  
explant of seedling.

(1962) (Table 1c). Profuse and healthy growth was obtained on Murashige and Skoog basal medium (MS-1) followed by GB<sub>5</sub>-1 and S-1, W-1, H-1, and K-1 gave poor growth being less than half the growth obtained on MS-1 medium (Table 26). Callus was whitish and friable on Murashige and Skoog medium whereas in the other media callus was dark brown. Callus grown on MS-1 medium was used in the subsequent studies.

#### Effect of nitrogen sources

To study the effect of different nitrogen sources, the media was prepared by omission of the inorganic and organic nitrogen sources. The potassium content was kept constant in all media by addition of KCl. The inorganic compounds tested were added to the medium at 0.84 g of nitrogen/litre, equivalent to the total nitrogen present in MS medium (Table 27). Organic nitrogen sources were added arbitrarily at 0.05% except for CM and urea which were added at 5% and 0.018% respectively (Table 28). It was observed (Tables 27 and 28) that neither the inorganic salts nor organic sources of nitrogen were adequate when added singly. It will be seen from Table 27 that  $\text{KNO}_3$  and  $\text{NH}_4\text{NO}_3$  added combined was better than if these compounds were added singly. Similarly when edamin and CM were added combined to a medium containing  $\text{KNO}_3$  and  $\text{NH}_4\text{NO}_3$ , growth of the tissue was over 40% higher, than if edamin was added alone (Table 28), and was 66% higher than on  $\text{KNO}_3$  and  $\text{NH}_4\text{NO}_3$  (Table 27 No. 8). This was the best medium for growth and was used in subsequent studies. Poor growth was observed

Table 26 Effect of different basal mediaMedia - MS-1, S-1, W-1, H-1, K-1, GB<sub>5</sub>-1

|    | Basal medium       | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|--------------------|-------------------------|-------------------------|
| 1. | MS-1               | 5299 $\pm$ 13           | 160                     |
| 2. | S-1                | 3240 $\pm$ 40           | 110                     |
| 3. | W-1                | 2035 $\pm$ 10           | 52                      |
| 4. | H-1                | 2035 $\pm$ 36           | 77                      |
| 5. | K-1                | 1935 $\pm$ 37           | 72                      |
| 6. | GB <sub>5</sub> -1 | 3812 $\pm$ 46           | 126                     |



Table 27 Effect of inorganic nitrogen sources

Medium - MS-2

|    | Nitrogen sources                                          | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|-----------------------------------------------------------|-------------------------|-------------------------|
| 1. | None                                                      | 365 $\pm$ 28            | 18                      |
| 2. | $\text{NH}_4\text{NO}_3$                                  | 1207 $\pm$ 26           | 50                      |
| 3. | $\text{KNO}_3$                                            | 974 $\pm$ 37            | 41                      |
| 4. | $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$      | 813 $\pm$ 12            | 34                      |
| 5. | $\text{NaNO}_3$                                           | 694 $\pm$ 49            | 27                      |
| 6. | $(\text{NH}_4)_2\text{SO}_4$                              | 878 $\pm$ 31            | 38                      |
| 7. | $\text{NH}_4\text{Cl}$                                    | 584 $\pm$ 11            | 25                      |
| 8. | $\text{NH}_4\text{NO}_3 + \text{KNO}_3$<br>(as MS medium) | 1685 $\pm$ 32           | 65                      |

Table 28 Effect of organic nitrogen sources

Medium - MS-2

|    | Nitrogen sources<br>%                                                                        | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|----------------------------------------------------------------------------------------------|-------------------------|-------------------------|
| 1. | None                                                                                         | 365 $\pm$ 28            | 18                      |
| 2. | YE (0.05)                                                                                    | 766 $\pm$ 26            | 34                      |
| 3. | ME (0.05)                                                                                    | 589 $\pm$ 29            | 27                      |
| 4. | CH (0.05)                                                                                    | 1007 $\pm$ 32           | 45                      |
| 5. | Ed (0.05)                                                                                    | 1428 $\pm$ 15           | 53                      |
| 6. | CM (5)                                                                                       | 1751 $\pm$ 54           | 68                      |
| 7. | Urea (0.018)                                                                                 | 655 $\pm$ 22            | 29                      |
| 8. | NH <sub>4</sub> NO <sub>3</sub> + KNO <sub>3</sub><br>(as MS medium)<br>+ Ed (0.05)          | 3245 $\pm$ 53           | 109                     |
| 9. | NH <sub>4</sub> NO <sub>3</sub> + KNO <sub>3</sub><br>(as MS medium)<br>+ Ed (0.05) + CM (5) | 5078 $\pm$ 29           | 158                     |

with either  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{Ca}(\text{NO}_3)_2$  or  $\text{NaNO}_3$  or  $\text{NH}_4\text{Cl}$  as inorganic nitrogen source and yeast extract or malt extract or urea as organic nitrogen source. Slight growth was obtained in the presence of casein hydrolysate.

#### Effect of different carbon sources

Several carbohydrates were tested for their suitability as carbon sources (Table 29). Sucrose was omitted from the basal medium and the different carbon sources were added at a concentration of 2%. Growth was maximum on sucrose, glucose and slightly less on fructose. Lactose gave moderate growth. Galactose, xylose, starch and maltose showed poor growth. Glycerol was inhibitory. Callus growth on media containing sucrose, glucose and fructose, remained whitish and was friable.

#### Effect of sucrose concentrations

The optimum sucrose concentration was found to be between 2 and 3%. At higher and lower concentrations, there was a drop in growth (Table 30).

#### Effect of coconut milk concentrations

Maximum growth was obtained between 5 and 10% coconut milk. With increase in CM concentration a very gradual reduction in growth was observed, even upto 30% concentration (Table 31).

The callus remained soft, friable and was white in colour at 5% coconut milk, whereas callus was dark brown and compact at 10% coconut milk.

Table 29 Effect of different carbon sources

Medium - MS-1 (without sucrose)

|     | Carbon source<br>2% | Wet weight<br>mg/tube | Dry weight<br>mg/tube |
|-----|---------------------|-----------------------|-----------------------|
| 1.  | None                | 266 $\pm$ 11          | 16                    |
| 2.  | Sucrose             | 5202 $\pm$ 52         | 162                   |
| 3.  | Glucose             | 5106 $\pm$ 31         | 156                   |
| 4.  | Fructose            | 4821 $\pm$ 39         | 141                   |
| 5.  | Lactose             | 2238 $\pm$ 56         | 87                    |
| 6.  | Galactose           | 1213 $\pm$ 44         | 52                    |
| 7.  | Xylose              | 838 $\pm$ 59          | 33                    |
| 8.  | Starch              | 1142 $\pm$ 30         | 49                    |
| 9.  | Glycerol            | 361 $\pm$ 25          | 20                    |
| 10. | Maltose             | 919 $\pm$ 42          | 46                    |

Table 30 Effect of sucrose concentration

Medium - MS-1 (without sucrose)

|    | Sucrose<br>% | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|--------------|-------------------------|-------------------------|
| 1. | 0            | 520 $\pm$ 23            | 23                      |
| 2. | 1            | 3026 $\pm$ 51           | 102                     |
| 3. | 2            | 5288 $\pm$ 46           | 160                     |
| 4. | 3            | 4845 $\pm$ 48           | 150                     |
| 5. | 4            | 2435 $\pm$ 35           | 90                      |
| 6. | 5            | 1050 $\pm$ 37           | 53                      |
| 7. | 6            | 822 $\pm$ 16            | 36                      |

Table 31 Effect of coconut milk concentration

Medium - MS-1 (without CM)

|    | CM<br>% | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|---------|-------------------------|-------------------------|
| 1. | 0       | 1915 $\pm$ 34           | 75                      |
| 2. | 5       | 5296 $\pm$ 97           | 162                     |
| 3. | 10      | 5937 $\pm$ 53           | 176                     |
| 4. | 15      | 4943 $\pm$ 61           | 151                     |
| 5. | 20      | 4144 $\pm$ 39           | 136                     |
| 6. | 25      | 2105 $\pm$ 53           | 81                      |
| 7. | 30      | 2009 $\pm$ 5            | 79                      |

### Effect of edamin concentration

Optimal growth was obtained at 0.05% edamin (Table 32). Moderate growth was observed at 0.01, 0.1 and 0.2% edamin concentration. Edamin added at concentrations above 0.2% were inhibitory.

### Effect of different auxins

Of several auxins tested each at 3.0 mg/l (Table 33), best growth was obtained with 2,4-D followed by B-NOA, 2,4,5-TCP, NAA, 2-BTOA, IBA, IAA and IPA.

### Effect of 2,4-D concentration

Different concentrations of 2,4-D (1.0 mg/l to 10 mg/l) were tested. 2,4-D enhanced growth over a wide range of concentrations (1 - 7 mg/l) (Table 34). Maximum growth was obtained at 3 mg/l (Table 10). 10 mg/l 2,4-D was inhibitory.

The callus was whitish, soft and friable at lower concentrations (1 - 3 mg/l), whereas it turned hard and darker at higher concentrations of 2,4-D (5 - 7 mg/l).

### Effect of different cytokinins

The effect of various cytokinins was tested for optimum growth (Table 35). Kin, BAP, 2iP and DPU were added at 0.5 mg/l concentration to MS-1 medium (lacking cytokinin), while zeatin was added at 1 mg/l to the medium. All the cytokinins showed inhibitory effect as compared to control on growth of callus.

### Effect of different growth retardants and ethrel

Different growth retardants and ethrel were tested

Table 32 Effect of Edamin concentration

Medium - MS-1 (without Edamin)

|    | Edamin<br>% | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|-------------|-------------------------|-------------------------|
| 1. | 0           | 2044 $\pm$ 41           | 80                      |
| 2. | 0.01        | 3032 $\pm$ 34           | 98                      |
| 3. | 0.05        | 5239 $\pm$ 31           | 163                     |
| 4. | 0.1         | 4555 $\pm$ 52           | 146                     |
| 5. | 0.2         | 3226 $\pm$ 40           | 108                     |
| 6. | 0.5         | 1464 $\pm$ 26           | 54                      |
| 7. | 1.0         | 752 $\pm$ 33            | 37                      |
| 8. | 2.0         | 300 $\pm$ 10            | 16                      |



Table 33 Effect of different auxins

Medium - MS-1 (without 2,4-D)

|    | Auxin<br>(3.0 mg/l) | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|---------------------|-------------------------|-------------------------|
| 1. | None                | 743 $\pm$ 26            | 26                      |
| 2. | -NAA                | 2965 $\pm$ 46           | 105                     |
| 3. | B-NOA               | 3747 $\pm$ 34           | 125                     |
| 4. | IAA                 | 2066 $\pm$ 47           | 80                      |
| 5. | 2,4-D               | 5371 $\pm$ 36           | 162                     |
| 6. | IBA                 | 2173 $\pm$ 39           | 86                      |
| 7. | IPA                 | 1937 $\pm$ 45           | 77                      |
| 8. | 2-BTOA              | 2966 $\pm$ 33           | 106                     |
| 9. | 2,4,5-TGP           | 3341 $\pm$ 61           | 112                     |

Table 34 Effect of 2,4-D concentration

Medium - MS-1 (without 2,4-D)

|    | 2,4-D<br>(mg/l) | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|-----------------|-------------------------|-------------------------|
| 1. | 0.0             | 650 $\pm$ 21            | 27                      |
| 2. | 1.0             | 3202 $\pm$ 58           | 105                     |
| 3. | 2.0             | 4712 $\pm$ 72           | 142                     |
| 4. | 3.0             | 5399 $\pm$ 65           | 163                     |
| 5. | 4.0             | 4625 $\pm$ 84           | 130                     |
| 6. | 5.0             | 3092 $\pm$ 43           | 104                     |
| 7. | 7.0             | 2057 $\pm$ 25           | 81                      |
| 8. | 10.0            | 6601 $\pm$ 05           | 24                      |

Table 35   Effect of different cytokinins

Medium - MS-1

|    | Cytokinin<br>(mg/l)    | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|------------------------|-------------------------|-------------------------|
| 1. | None                   | 5288 $\pm$ 36           | 164                     |
| 2. | Kin (0.5)              | 2966 $\pm$ 29           | 98                      |
| 3. | BAP (0.5)              | 3726 $\pm$ 34           | 124                     |
| 4. | 2iP (0.5)              | 2374 $\pm$ 24           | 85                      |
| 5. | DPU (0.5)              | 2361 $\pm$ 62           | 86                      |
| 6. | Zeatin (1.0 $\mu$ g/l) | 3105 $\pm$ 98           | 109                     |

each at 4 different concentrations (Table 36). At 0.001 mg/l all the retardants showed either no inhibition or very little inhibition. As the concentration was increased there was a gradual decrease in growth with all the compounds tested, maximum inhibition being observed with Mor B.

#### Effect of different vitamins

The effect of the B vitamins, B<sub>1</sub>, B<sub>2</sub> and B<sub>6</sub> were tested individually and in combination. Optimum growth was obtained with a combination of all three vitamins as contained in the standard medium of Murashige and Skoog (1962) (Table 37). Individually thiamine was the more effective followed by niacin and pyridoxine.

#### Effect of inositol concentration

Different concentrations of inositol were tested (Table 38). Poor growth was obtained in absence of inositol and at inositol concentration of 1,000 mg/l. Callus growth was good over a wide range of inositol concentrations from 100 - 200 mg/l, the optimum being 120 mg/l. Callus remained soft, friable and whitish at 120 mg/l inositol concentration but appeared brown at higher concentrations.

#### Physical factors

##### Effect of different temperatures

For studies on the effect of temperature cultures were incubated at different temperatures ranging from 5 - 37°C in dark. Optimum growth was at 25 - 30°C (Table 39).

Table 36 Effect of different growth retardants and Ethrel  
Medium - MS-1

|     | Growth retardants<br>(mg/l) | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|-----|-----------------------------|-------------------------|-------------------------|
| 1.  | None                        | 5263 $\pm$ 43           | 106                     |
| 2.  | Ethrel (0.001)              | 4910 $\pm$ 39           | 156                     |
| 3.  | Ethrel (0.01)               | 4028 $\pm$ 37           | 138                     |
| 4.  | Ethrel (0.1)                | 2052 $\pm$ 47           | 86                      |
| 5.  | Ethrel (1.0)                | 1595 $\pm$ 13           | 57                      |
| 6.  | CCC (0.001)                 | 5669 $\pm$ 25           | 168                     |
| 7.  | CCC (0.01)                  | 4542 $\pm$ 47           | 143                     |
| 8.  | CCC (0.1)                   | 3964 $\pm$ 39           | 134                     |
| 9.  | CCC (1.0)                   | 3301 $\pm$ 08           | 108                     |
| 10. | ABA (0.001)                 | 4827 $\pm$ 24           | 151                     |
| 11. | ABA (0.01)                  | 3803 $\pm$ 41           | 130                     |
| 12. | ABA (0.1)                   | 2559 $\pm$ 32           | 97                      |
| 13. | ABA (1.0)                   | 1867 $\pm$ 21           | 76                      |
| 14. | <del>Mor</del> A (0.001)    | 5652 $\pm$ 62           | 159                     |
| 15. | Mor A (0.01)                | 3426 $\pm$ 105          | 111                     |
| 16. | Mor A (0.1)                 | 2065 $\pm$ 32           | 85                      |
| 17. | Mor A (1.0)                 | 1762 $\pm$ 39           | 74                      |
| 18. | Mor B (0.001)               | 4095 $\pm$ 97           | 136                     |
| 19. | Mor B (0.01)                | 3169 $\pm$ 59           | 105                     |
| 20. | Mor B (0.1)                 | 2076 $\pm$ 37           | 86                      |
| 21. | Mor B (1.0)                 | 987 $\pm$ 15            | 40                      |

Table 37 Effect of different vitamins

Medium - MS-4 (without vitamins)

| Vitamins<br>(mg/l)                                        | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|-----------------------------------------------------------|-------------------------|-------------------------|
| 1. None                                                   | 3100 $\pm$ 61           | 100                     |
| 2. Thiamine (0.1)                                         | 4107 $\pm$ 52           | 141                     |
| 3. Niacin (0.5)                                           | 3660 $\pm$ 49           | 115                     |
| 4. Pyridoxine (0.5)                                       | 3196 $\pm$ 15           | 102                     |
| 5. Pyridoxine (0.5) +<br>Thiamine (0.1)                   | 4926 $\pm$ 37           | 156                     |
| 6. Pyridoxine (0.5) +<br>Niacin (0.5)                     | 4373 $\pm$ 24           | 148                     |
| 7. Thiamine (0.1) +<br>Niacin (0.5)                       | 4686 $\pm$ 67           | 151                     |
| 8. Thiamine (0.1) +<br>Pyridoxine (0.5) +<br>Niacin (0.5) | 5115 $\pm$ 58           | 160                     |

Table 38 Effect of Inositol concentration

Medium - MS-1 (without inositol)

|    | Inositol<br>(mg/l) | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|--------------------|-------------------------|-------------------------|
| 1. | None               | 1298 $\pm$ 34           | 50                      |
| 2. | 50                 | 2165 $\pm$ 43           | 85                      |
| 3. | 100                | 4634 $\pm$ 36           | 152                     |
| 4. | 120                | 5078 $\pm$ 37           | 160                     |
| 5. | 150                | 4586 $\pm$ 52           | 156                     |
| 6. | 200                | 4699 $\pm$ 51           | 158                     |
| 7. | 500                | 2818 $\pm$ 15           | 98                      |
| 8. | 1000               | 1152 $\pm$ 50           | 45                      |

Table 39 Effect of temperature

Medium - MS-1

|    | Temperature<br>°C | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|-------------------|-------------------------|-------------------------|
| 1. | 5                 | 165 $\pm$ 07            | 15                      |
| 2. | 15                | 857 $\pm$ 35            | 36                      |
| 3. | 25                | 5360 $\pm$ 67           | 166                     |
| 4. | 30                | 5486 $\pm$ 33           | 169                     |
| 5. | 37                | 987 $\pm$ 10            | 44                      |



Callus grown at 25°C was whitish and soft whereas at 30°C was brown and compact. Poor growth was observed at 37°C, and at 5°C there was no growth.

#### Effect of hydrogen-ion concentration

The growth of callus was determined at various pH values (Table 40). Good growth was obtained at pH 6.0 and 7.0. With increase in pH above 7.0 there was a marked inhibition of growth. Growth at pH 5.0 was about 60% that obtained at pH 6.0, whereas at pH 9.0 and 10.0 growth was poor.

#### Effect of agar concentration

Different concentrations of agar were tested. The best growth was observed at 0.8 and 0.9% (Table 41). The tissue showed growth even at the highest concentration of agar tested at 1.4%.

#### Effect of inoculum size

Growth of the callus was determined using various amounts of inoculum (Table 42). It was found that the ratio of the final fresh and dry weight, to the inoculum weight was maximum at an inoculum size of 100 - 200 mg/tube, followed by an inoculum of 50 mg fresh weight.

#### Growth pattern of callus

In this experiment callus growth was determined at different time intervals as shown in Fig. 27. Several tubes were inoculated with about 200 mg fresh tissue in each tube. At specified periods the wet<sup>3</sup> weight and dry weight of tubes respectively were taken. The results given in the

Table 40   Effect of hydrogen-ion concentration

Medium - MS-1

|    | pH   | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|------|-------------------------|-------------------------|
| 1. | 5.0  | 3157 $\pm$ 50           | 104                     |
| 2. | 6.0  | 5030 $\pm$ 25           | 158                     |
| 3. | 7.0  | 4941 $\pm$ 61           | 155                     |
| 4. | 8.0  | 2601 $\pm$ 18           | 94                      |
| 5. | 9.0  | 5598 $\pm$ 06           | 30                      |
| 6. | 10.0 | 455 $\pm$ 25            | 21                      |

Table 41 Effect of agar concentration

Medium - MS-1

|    | Agar<br>% | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----|-----------|-------------------------|-------------------------|
| 1. | 0.6       | 3444 $\pm$ 115          | 116                     |
| 2. | 0.7       | 4656 $\pm$ 62           | 151                     |
| 3. | 0.8       | 5110 $\pm$ 56           | 160                     |
| 4. | 0.9       | 5294 $\pm$ 43           | 162                     |
| 5. | 1.0       | 4022 $\pm$ 41           | 142                     |
| 6. | 1.4       | 2077 $\pm$ 36           | 90                      |

Table 42 Effect of inoculum size

Medium - MS-1

| Fresh weight       |       |               | Ratio<br>final and<br>initial<br>fresh<br>weight | Dry weight         |       | Ratio<br>final and<br>initial<br>dry<br>weight |
|--------------------|-------|---------------|--------------------------------------------------|--------------------|-------|------------------------------------------------|
| Initial            | Final |               |                                                  | Initial            | Final |                                                |
| (mg/tube)(mg/tube) |       |               |                                                  | (mg/tube)(mg/tube) |       |                                                |
| 1.                 | 50    | 1296 $\pm$ 08 | 25.3                                             | 5                  | 52    | 10.4                                           |
| 2.                 | 100   | 2637 $\pm$ 37 | 26.3                                             | 9                  | 100   | 11.1                                           |
| 3.                 | 200   | 5242 $\pm$ 29 | 26.2                                             | 14                 | 160   | 11.5                                           |
| 4.                 | 300   | 5781 $\pm$ 47 | 19.2                                             | 18                 | 170   | 9.4                                            |
| 5.                 | 400   | 6053 $\pm$ 58 | 15.1                                             | 24                 | 179   | 7.4                                            |

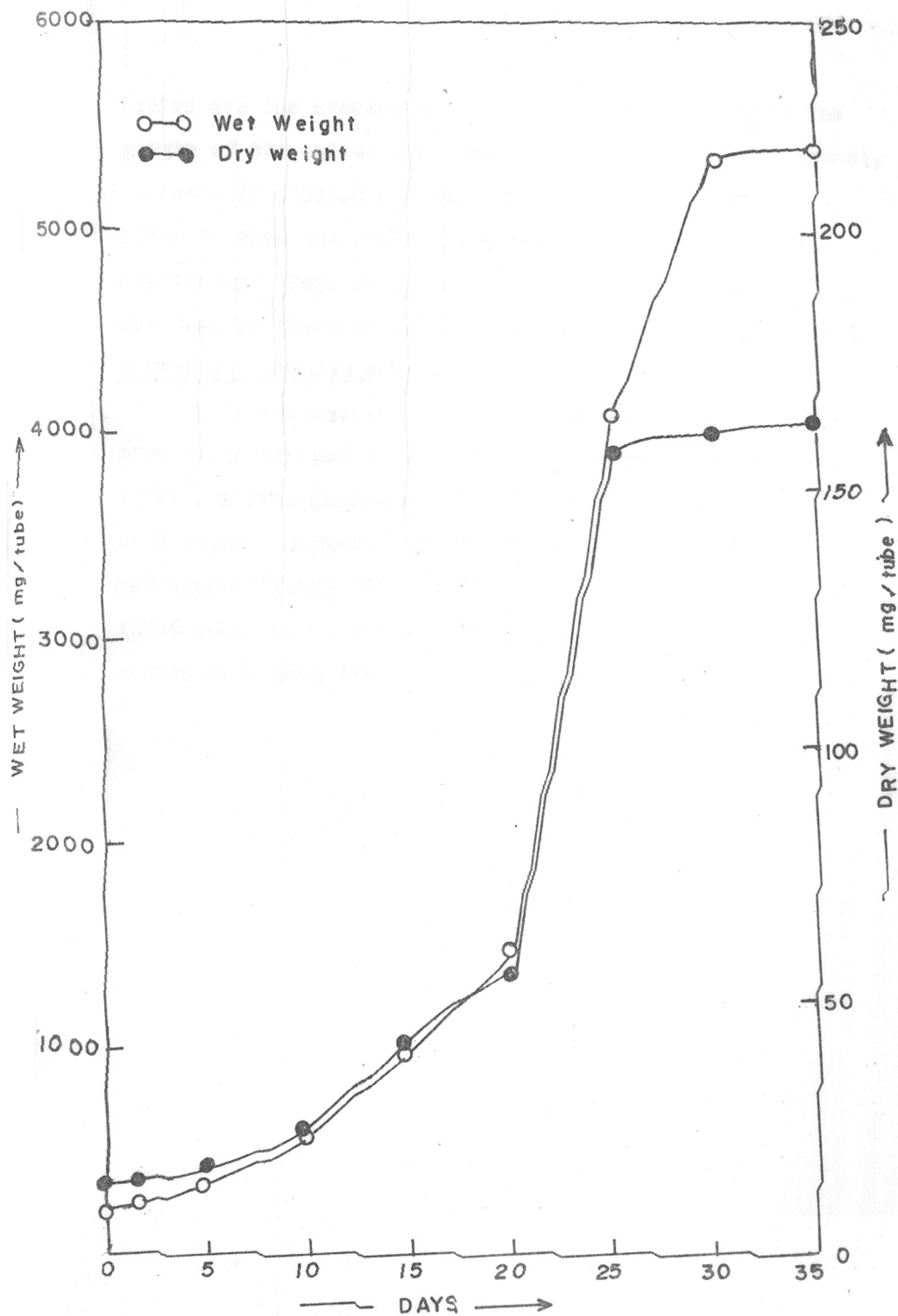


FIG. 27. GROWTH RATE OF CALLUS CULTURE

tables are the average values. It was observed that the growth of the callus was very slow upto 5 days (lag phase), followed by a phase of rapid growth till the 30th day. After 30 days the growth of the callus remained almost stationary. This pattern in the growth curve was obtained for both the fresh and dry weight of the tissue.

#### Growth on repeated subcultures

There are several reports of callus cultures which grow initially and then stop growth, whereas growth in other cultures increases on subculture. In this experiment the growth of the callus was observed at every subculture (Table 43). Poor growth was obtained in the first three subcultures. Callus maintained a steady growth rate from the 5th subculture onwards.

Table 43 Growth of callus on repeated subcultures

Medium - MS-1

| Subculture No. | Wet weight<br>(mg/tube) | Dry weight<br>(mg/tube) |
|----------------|-------------------------|-------------------------|
| 1              | 912                     | 40                      |
| 2              | 1256                    | 53                      |
| 3              | 2566                    | 97                      |
| 4              | 3946                    | 139                     |
| 5              | 5016                    | 159                     |
| 6              | 5215                    | 162                     |
| 7              | 5426                    | 167                     |
| 8              | 5315                    | 165                     |
| 9              | 5526                    | 168                     |
| 10             | 5410                    | 166                     |
| 11             | 5325                    | 164                     |
| 12             | 5126                    | 162                     |
| 13             | 5201                    | 161                     |
| 14             | 5126                    | 162                     |
| 15             | 5025                    | 160                     |
| 16             | 5100                    | 161                     |
| 17             | 5016                    | 160                     |
| 18             | 5007                    | 159                     |
| 19             | 5010                    | 166                     |
| 20             | 5009                    | 160                     |
| 21             | 5098                    | 161                     |
| 22             | 5112                    | 161                     |
| 23             | 5007                    | 160                     |
| 24             | 5126                    | 161                     |
| 25             | 5126                    | 161                     |

## Section 2 Discussion

The nutritional requirements of plant tissues in culture vary from species to species and often with the variety. The composition of the medium has a marked effect on the growth of callus.

### Effect of different basal media

Formulae for the many different mineral salts medium for growth of plant tissue and cell cultures have now been reported. The composition of some of these are given in Tables 1a, 1b and 1c in Chapter II and some of the basal media are discussed in Chapter I.

Recently Beuwens (1976) studied the nutritional requirements of coconut explants and formulated a medium with high concentrations of potassium, ammonium chloride and iodine.

The media developed for Picea glauca by Reinert and White (1956) consisted of White salts, 11 vitamins and 16 amino acids. Later, Risser and White (1964) refined this medium, to contain modified White salts with 4 vitamins, and glutamine. Similarly the medium used by Brown and Lawrence (1968) was based on Murashige and Skoog (1962), salts containing in addition 4 vitamins, asparagine and 2,4-D. This medium supported growth of callus cultures of Pinus elliottii for 5 years.

Harvey and Grasham (1969) formulated a common defined low salts basal medium for callus cultures of Conifer species of Abies grandis, Larix occidentalis, Picea abies,



Picea engelmannii, Picea pungens, Pinus albicaulis, Pinus contorta, Pinus flexilis, Pinus nigra, Pinus ponderosa, Pseudotsuga menziesii and Thuja plicata.

Minocha (1980) grew embryo callus of Pinus strobus on Murashige and Skoog (1962) medium supplemented with NAA. McCowen and Lloyd (1981) formulated a new defined medium for the growth of callus for woody plants and named it as woody plant medium (WPM). They compared the growth of callus for Rosa, Rhododendron, Betula and Kalmia on three mineral formulations viz. Murashige and Skoog (1962), Gresshoff and Doy (1972) and WPM media. Best growth was obtained from all the tissues on WPM. Mathes (1964) isolated callus from cambium tissue of Populus tremuloides on modified White medium (1963) supplemented with coconut milk. Chalupa (1974; 1975) obtained callus from Populus nigra, P. tremula, P. canescens on both modified Wolter (Wolter and Skoog, 1966), and modified Linsmaier and Skoog (1965) basal medium. Riou et al. (1975) obtained friable callus on a modified Murashige and Skoog (1962) medium containing Nitsch and Nitsch vitamins.

Sommer and Brown (1980) isolated callus from germinated seeds of sweet gum on medium containing the major salts of Risser and White (1964), Iron as FeEDTA as in Murashige and Skoog medium (1962), Brown trace elements (Brown and Lawrence, 1968) and vitamins of Gresshoff and Doy (1972).

Lakshmisita et al. (1979) obtained good callus growth

from shoots of Santalum album on MS-basal medium (1962).

A series of nutrient media were tested by de Fossard (1974), Lee and de Fossard (1974) for callus growth of Eucalyptus grandis. Varying degrees of success were obtained with the different media of Murashige and Skoog (1962), White (1963), Linsmaier and Skoog (1965), Tulecke et al. (1965), Nash and Davies (1972), Sommer et al. (1975), with varying concentrations of the different compounds. The best callus developed from leaf blades of E. grandis on the medium of Nash and Davies (1972) and from anthers callus on White (1963) medium. It may be significant that both these media have relatively low salt concentrations.

Eucalyptus callus grew well on chemically defined media (de Fossard, 1974; Goncalves, 1975). Callus growth was obtained from lignotuber on modified MS basal medium (Aneja and Atal, 1969); from hypocotyl and cotyledon of Eucalyptus citriodora on Murashige and Skoog (1962) inorganic salts and Gamborg (1968) vitamins with m-inositol and ferric citrate (16 mg/l).

In the present studies, the best growth of callus was obtained on Murashige and Skoog basal medium followed by Gamborg and Smith media. White, Heller and Knop basal media gave poor growth. This result indicates that to grow viable callus a high salt concentration is possibly necessary.

#### Inorganic and organic nitrogen sources

Nitrogen has been supplied in different forms such as

nitrite, nitrate, ammonium and also in organic form to different plant species grown in vitro. Riker and Gutsche (1948), Burkholder and Nickell (1949) and Heller (1953) were among the first to study the nitrogen nutrition of plant tissues. They found that nitrate was the best source of inorganic nitrogen. Among the different nitrogen sources tested for the growth of tobacco and Populus callus, nitrate was found best as inorganic nitrogen source: (Matsumoto et al., 1971).

The media of Murashige and Skoog (1962) and Linsmaier and Skoog (1965) are characterized by having a high level of potassium and supply of nitrogen is by a mixture of nitrate and ammonium salts separately. These media have been widely used for growth and morphogenesis of plant tissues and cell cultures. Caldas and Caldas (1976) found a similar stimulating effect of nitrate and ammonia for growth and morphogenesis of Paul's Scarlet Rose Callus; for Haworthia tissue (Paul and Sabharwal, 1972); for papaya tissue (Mendora et al., 1979); for teak callus (Dharva, 1974); Nadgir, 1980). Eeuwens (1976) obtained good callus growth from coconut tissue, when nitrogen was supplied as ammonium ( $\text{NH}_4\text{Cl}$ ) and nitrate ( $\text{KNO}_3$ ). Ahmed and Mandour (1977) reported a new medium containing  $\text{NH}_4\text{NO}_3$ ,  $\text{KNO}_3$  and  $\text{Ca}(\text{NO}_3)_2$  as nitrogen source for tissue culture of medicinal plants.

Konar and Singh (1980) showed faster callus growth of Pinus wallichiana, when Murashige and Skoog (1962) medium

further supplemented with higher concentration of ammonium nitrate (200 mg/l) was used.

Goncalves et al. (1979) reported that the concentration and form of nitrogen added also greatly affects callus growth of E. grandis. 2.5 mM  $\text{NO}_3^-$  was very effective for growth of leaf blade callus on Nash and Davies (1972) medium. Ammonium alone as the nitrogen source gave consistently lower fresh weight values than those encountered at comparable nitrate concentrations, and no stimulating effect of ammonium on dry weight were noted. Effect of nitrate concentration on anther callus of E. grandis indicated that growth was optimal on White medium (1963), when further supplemented with 12 times higher concentration of  $\text{KNO}_3$  (10 mM).

Nitrogen incorporated in the medium in the form of organic compounds has often been found to be stimulatory to growth and morphogenesis when added as a mixture of amino acids or vitamins as in CH, YE, ME, CM etc. Glycine was inhibitory for growth of Pinus callus, whereas asparagine and aspartic acid were stimulatory (Brown and Lawrence, 1968). Arginine and tyrosine stimulated callus growth of conifers (Harvey and Grasham, 1969).

Callus growth of Acer pseudoplatanus cells was obtained in medium with urea and nitrate as nitrogen sources (King, 1976); with urea and arginine for callus tissue of Populus spp. (Sato, 1978a). Malt extract was shown to support callus growth of conifer tissues (Geisbuhler, and Skoog, 1957); edamin for tobacco tissue (Murashige and

Skoog (1962); Casein hydrolyzate for callus growth of Populus spp. (Riou et al., 1975) and Betula pendula (Srivastava and Steinhauer, 1981).

Callus growth has also been reported from different trees on media supplemented with coconut milk at different concentrations, with 10% for Populus tremuloides (Mathes, 1964), White spruce and pine (Durzan and Steward, 1970); Eucalyptus camaldulensis (Bachelard and Stowe, 1963; Sussex, 1965); with Eucalyptus grandis hypocotyl segments (Goncalves et al., 1979). Coconut milk added at 15% was optimum for lignotuber callus (Aneja and Atal, 1969) and for cotyledon callus of E. citriodora (Lakshmisita, 1979).

In our studies callus grew well on media containing a combination of  $\text{KNO}_3 + \text{NH}_4\text{NO}_3$  at concentrations present in Murashige and Skoog (1962) basal medium. Callus growth increased in the presence of edamin (0.05%) or CM (5%) added individually, but the optimum growth was obtained with Murashige and Skoog (1962) mineral salt medium supplemented both with edamin at 0.05% and CM at 5% (MS-1).

#### Carbon sources

Sugars are essentially the main energy source used for plant tissue cultures. Sucrose at 1 - 4% concentration is the most commonly used carbohydrate, although in a number of cases it has been shown that other sugars such as glucose and fructose are also equally effective (Maretzki et al., 1974). The utilizable carbohydrates not only act

as source of carbon and energy but can effect cyto-differentiation (Geffs and Northcote, 1967), including chloroplast differentiation and function (Jaspers, 1965).

Risser and White (1964) found sucrose at 5% concentration as the best carbohydrate source for spruce tumor cells. No other sugar except dextrose approached sucrose in effectiveness. With fructose, mannose and raffinose, 50% growth was obtained as compared to sucrose. Sucrose was found optimum at 4% concentration for Populus and tobacco callus (Matsumoto et al., 1971); glucose for Paul's scarlet Rose Callus (Nash and Davies, 1972); mannose and galactose for Sequoia tissue (Ball, 1953). In general pentoses are not effective in supporting growth (Street, 1966).

Button (1978) reported that sucrose, glucose and fructose at 0.15 M were the best carbohydrate sources for growth of callus while lactose and maltose were effective in organogenesis of citrus ovular callus. Sucrose was found optimum at 7% for date palm and coconut explants (Eeuwens, 1978). With increasing sucrose concentration from 0.6 - 4%, dry weight of E. grandis leaf blade callus correspondingly increased (Goncalves, 1979). 5% sucrose concentration was inhibitory. 2% and 3% sucrose concentration was optimum for Eucalyptus bancroftii stem callus and for E. laevopinea and E. melliodora stem callus respectively (Lee and de Fossard, 1974; de Fossard, 1974). Higher sucrose concentration (10%) was

found inhibitory.

In the present studies sucrose and glucose gave best callus growth followed by fructose and lactose. Poor growth was obtained with galactose, xylose, maltose and starch. Glycerol was inhibitory. The optimum sucrose level was 2% to 3%.

### Auxins

Auxins are normally used for the initiation and maintenance of callus cultures. The chief auxins used in tissue culture are IAA, NAA and 2,4-D. Only IAA is a natural auxin. It is very sensitive to biological degradation unlike NAA and 2,4-D which are both stable in solution. Differences exist in the effectiveness of these auxins in callus induction for different species. Species specificity for NAA, IAA or 2,4-D was seen in 12 species of conifers (Harvey and Grasham, 1969). In Pinus 2,4-D was better than IAA (Brown and Lawrence, 1968).

Risser and White (1964) studied the effect of 3 auxins, IAA, NAA and 2,4-D for growth of Picea glauca tumor cells and found that 2,4-D was the most effective at 0.1 mg/l. Durzan and Steward (1970) found NAA at 5.0 mg/l best for White spruce callus; NAA at 5 mg/l for Douglas fir cotyledon callus (Winton and Verhagen, 1977); NAA at 1.0 - 2.0 mg/l or 2,4-D 0.1 - 1.0 mg/l for Pinus strobus embryo callus (Minocha, 1980); NAA 0.1 mg/l for hypocotyl callus of Pinus wallichiana (Konar and Singh, 1980); with 2,4-D 0.5 mg/l for stem

callus of Populus nigra (Venverloo, 1973; Brand and Venverloo, 1973); with IAA (2 mg/l) for Betula pendula plants (Srivastava and Steinhauer, 1981).

Lakshmisita et al. (1979) studied the effects of three auxins, IAA, NAA and 2,4-D (0.2 - 5 mg/l) for callus from shoot tips of Santalum album. Maximum callus growth was obtained on medium supplemented with NAA.

Lee and de Fossard (1974) found that callus isolated from stem and lignotuber of Eucalyptus bancroftii had different auxin requirements. For stem callus optimum growth occurred with 4 mg/l NOA whereas for lignotuber 2,4-D at 4 mg/l was best.

Goncalves et al. (1979) reported growth of Eucalyptus grandis leaf blade callus in the presence of 20 mg/l IAA and 8 mg/l NAA when added separately. Aneja and Atal (1969) obtained callus from lignotuber of E. citriodora with 1 mg/l NAA. For callus from leaf and shoot explants of mature trees of E. citriodora 5 mg/l each of NAA and IAA was found necessary (Lakshmisita, 1979).

In the present studies, maximum callus growth was observed with 2,4-D followed by B-NOA, 2,4,5-TCP, 2-B-TOA and NAA. A growth reduction of 50% was obtained with IAA, IBA and IPA. The optimum concentration of 2,4-D was 3 mg/l. Higher concentrations were inhibitory.

#### Cytokinins

Several tissues fail to respond to an auxin alone for growth of callus unless a cytokinin is also added to the



medium (Sharp and Crocorno, 1975). The most commonly used cytokinins are Kin, BAP, 2iP and zeatin. Kinetin has been widely used to initiate and maintain callus cultures (Sharp and Crocorno, 1975), and is particularly active as an interactant with IAA or 2,4-D in the cultures of tobacco pith (Miller et al., 1955).

Winton and Verhagen (1976) showed the requirement of 0.1 mg/l BAP with NOA for good growth of Douglas fir cotyledon callus. Callus from buds of Pinus abies required BAP or 2iP and IAA or IBA at  $5 \times 10^{-6}$  M and  $5 \times 10^{-5}$  M (Von Arnold and Eriksson, 1979). Bornman and Jansson (1980) obtained best callus growth of Pinus sylvestris on medium containing 5  $\mu$ M BAP and 30  $\mu$ M IAA. Minocha (1980) showed enhanced callus formation from embryos of Pinus strobus with BAP or zeatin (0.01 - 5.0 mg/l) and an auxin. However very little growth occurred in the absence of an auxin. Good callus growth from stem of Santalum album was obtained in the presence of 0.5 - 1.0 mg/l Kin and 1.0 mg/l 2,4-D (Lakshmisita et al., 1979). Maximal growth of callus from shoots of Drimys winteri was observed in the presence of Kin 0.1 mg/l and NAA 1.0 mg/l (Jordon and Cortes, 1981); for Eucalyptus bancroftii lignotuber callus  $2 \times 10^{-6}$  M BAP and  $2 \times 10^{-5}$  M 2,4-D (Lee and de Fossard, 1974); for Eucalyptus grandis leaf blade callus in the presence of 1.0 mg/l Kin with 8.0 mg/l NAA (Goncalves et al., 1979).

The results of Aneja and Atal (1968) and Lakshmisita

(1979) indicated that callus cultures of E. citriodora did not respond to cytokinin. Auxins alone are sufficient for good callus growth.

In these studies maximum callus growth occurred in the absence of cytokinins, and was similar to the earlier reports of Aneja and Atal (1969) and Lakshmisita (1979). Kinetin when present in the medium inhibited callus growth.

### Vitamins

The most commonly used vitamins, thiamine, inositol, nicotinic acid and pyridoxine are now generally incorporated into all media (Gamborg et al., 1976). Thiamine, however, is critical and it is usually used at 0.1 - 0.5 mg/l. Matsumoto et al. (1971) showed an absolute requirement of thiamine for Populus callus. Jacquot (1964) showed that m-inositol added at 50 - 100 mg/l significantly stimulated callus growth of Ulmus campestris. Similar effects were found from Pelargonium hortorum (Narayana, 1963); tobacco (Linsmaier and Skoog, 1962); Catharanthus roseus (Wood and Braun, 1961) and Fraxinus pennsylvanica callus (Wolter and Skoog, 1966). Other vitamins such as nicotinic acid, pyridoxine, biotin, calcium pantothenate and folic acid have also been used in media and found beneficial for the growth of some plant cells (Gamborg and Shyluk, 1981). For callus growth of spruce tumor callus thiamine (1 mg/l) niacin (10 mg/l), ascorbic acid (10 mg/l) and inositol (100 mg/l) were found

necessary. Vitamin B<sub>12</sub>, calcium panthothenate, thiamine, pyridoxine and inositol ( $2.5 \times 10^{-4}M$ ) were required for optimal callus growth of E. bancroftii (Lee and de Fossard, 1974).

In the present studies callus growth was not enhanced when vitamin, niacin, thiamin<sup>e</sup> or pyridoxine were added singly. However, maximum callus growth was obtained with a combination of all the 3 vitamins. This was further enhanced with inositol over a wide range from 100 - 200 mg/l. The callus was soft and friable at lower inositol concentrations and turned dark and compact at higher levels.

#### Growth retardants and ethrel

Several compounds have now been isolated which have a growth retardāng effect on plants. These include CCC, morphactins, ABA etc. Although some of these compounds are known as growth retardants, in some cases in tissue culture, callus growth is often enhanced in their presence, especially if added at lower concentrations. ABA was reported to influence phenol production and was found to inhibit callus growth of tobacco (Li et al., 1970), whereas in citrus ABA enhanced callus growth (Altman and Goren, 1971). Similarly ABA was found to enhance growth of carrot callus but to retard development of embryoids (Norreel and Nitsch, 1968). ABA promoted growth of soybean callus in presence of higher levels of kinetin (Blumenfeld and Gazit, 1970). Increased fresh and dry weight of Spinacia oleracea callus was obtained at lower concentrations

of ABA (0.01 mg/l) whereas higher concentration 1.0 mg/l were inhibitory (Neskovic et al. , 1977).

Good growth of tobacco callus was obtained in the absence of cytokinins <sup>when</sup> treated with morphactine (Bedner and Linsmaier, 1971). Inhibition of citrus callus growth was reported in the presence of CCC, ethephon and Alar (0.01 - 1.0 mg/l) but embryogenesis was stimulated (Kochba et al., 1978). Promotion of sweet potato callus was demonstrated by Chalatz and de Vay (1969) in the presence of ethrel. Nadgir (1979) reported inhibition of teak callus growth in the presence of ABA, CCC, morphactins (0.01 mg/l) and total inhibition at higher concentrations (1.0 mg/l).

In the present studies, all the compounds tested showed either no inhibition or very little inhibition at 0.001 mg/l. As the concentrations were increased there was a gradual decrease in growth.

#### Physical factors

There are many physical factors that can influence the growth of plant cells in vitro (Thorpe, 1981) such as pH, temperature, agar concentrations and inoculum size.

#### pH

The pH of the medium plays an important role in success on obtaining good callus growth (Thorpe, 1981). Optimum growth was obtained at pH 5.5 to 5.9 for tobacco callus and for sunflower at pH 6.0 to 6.4 (Hildebrandt et al. , 1945); for elm, Populus, Willow and grape tissue

the optimum pH was 4 to 4.5 (Pelet et al., 1960; Sommer et al., 1962); for peach stem callus the optimum was pH 7.0 (Sommer et al., 1962); for Picea glauca callus at pH 5.5 to 6.5 (Risser and White, 1964). Fraxinus callus (Wolter and Skoog, 1966) grew well over a wide pH range from 4.0 to 7.0. Nadgir (1979) obtained good teak callus growth at pH 6.0.

The optimum pH for good callus growth was found to be between pH 6.0 to 7.0 in the present investigation. Total inhibition was obtained at higher pH values above 9.0.

#### Incubation temperature

Growth rate is closely related to the prevailing temperature. A temperature between 26 - 28°C is considered optimum for achieving maximum growth of many tissue cultures (Gamborg and Shyluk, 1981). Maximum growth of citrus callus was obtained at 30°C in dark (Murashige and Tucker, 1969); at 27 - 29°C in continuous light with apple callus (Chong and Taper, 1974a; 1974b); at 25°C in dark for teak callus (Nadgir, 1979); at 20°C in dark for spruce tumor tissue (White and Risser, 1964).

In the present work, best callus growth was observed at temperatures between 25 - 30°C in dark. At 25°C the callus was whitish brown and soft, whereas at 30°C it turned brown and hard.

#### Agar concentration

In general agar is added to media at concentrations between 0.6 - 1.0% (Narayanaswamy, 1977) as a support for

the growing tissue. The optimum agar concentration was found to be 0.5% for White spruce (White and Risser, 1964); 1% for Fraxinus (Wolter and Skoog, 1966); 0.6 - 0.8% for teak (Nadgir, 1979) and 0.4% for Eucalyptus bancroftii (Lee and de Fossard, 1974) callus cultures respectively.

We obtained best callus growth at 0.8 or 0.9% agar concentration.

#### Inoculum size

Different weights of inoculum have been used. Gautheret (1959) used an inoculum of 80 - 150 mg/tube for different studies on carrot tissues, whereas Murashige and Skoog (1962) found an inoculum of 40 - 50 mg/tube good for their experiments on tobacco callus. The results of Caplin (1962) indicated that more uniform growth was obtained with smaller inoculum of the order of 5 - 10 mg/tube for tobacco and carrot tissue. Similar observations were made by White and Risser (1964) who obtained good results of Picea glauca callus with 20 mg/tube inoculum weight.

In the present studies 100 - 200 mg/tube inoculum weight was optimum for maximum growth of callus.



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## **CHAPTER VII**

### **STUDIES ON SYNTHESIS OF ESSENTIAL OIL, CITRONELLAL AND CITRONELLOL IN TISSUE CULTURE**

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STUDIES ON SYNTHESIS OF ESSENTIAL OIL, CITRONELLAL ANDCITRONELLOL IN TISSUE CULTURESSection 1 Experimental

This Chapter describes studies on the synthesis of the essential oil, citronellal and citronello~~l~~ using three different tissue culture systems - (i) Callus, (ii) Differentiating tissues and (iii) Organized shoot cultures. The purpose of the experiments was to determine the exact stage at which these secondary metabolites are produced.

Callus was isolated from leaves and stem segments of four month old and one year old seedlings of E.citriodora respectively on MS-3 medium as described earlier in Chapter VI. Leaf callus was soft, friable and whitish brown (Fig. 28), whereas stem callus was hard and dark brown in colour (Fig. 29). Tissues were subcultured at intervals of 30 days.

Shoot regeneration was obtained from 4 month old seedling stem callus on MS-3 medium which was used earlier for shoot multiplication. The conditions for further development of shoots and roots and for transfer of plantlets to pots and the field were similar to the procedures followed for clonal multiplication from seedlings and have been described in Chapters III, IV and V.

The method used for extraction of oil from leaves and estimation of total oil, citronellal and citronello~~l~~ have been described earlier in Chapter II.





Fig. 28 Leaf callus



Fig. 29 Stem callus

The concentration of total oil has been calculated on the basis of the dry weight of the leaves.

The typical GLC pattern of E. citriodora oil is shown in Fig. 30. This pattern is identical to the patterns shown by tissue cultures and normal plants. Comparisons made in the present studies have been only with respect to total oil and the main components, citronellal and citronellol. Structures of these components have been shown in Fig. 31.

### Callus

#### Effect of subculture

The total oil, citronellal and citronellol contents were estimated from callus isolated from leaf tissue at the initial stage and on subculture. This data was compared with the content of these components in its parent plants. Primary callus contained the essential oil and its components at concentrations lower than the parent plant (Table 44). These concentrations gradually diminished<sup>h</sup> further on subculture and were not detected by the tenth passage.

#### Effect of organogenesis

Murashige and Skoog medium (MS-4) was tested as given in Table 45, to which different growth regulators were added in order to obtain shoot differentiation from leaf and stem callus. Shoot differentiation was obtained from stem callus only on MS-3 medium (Fig. 32). Leaf callus did not differentiate under these conditions.

The further procedure followed to the stage of plantlet

Model — SHIMADZU (6-AMPF)  
Column used - 10% Carbowax 20M on  
Chromosorb P. 60-80 mesh 2mm X 2m  
Column Temp. - 175  
T.C.D. Temp. - 200  
Flow rate - 40 ml/min  
Carrier gas - Hydrogen

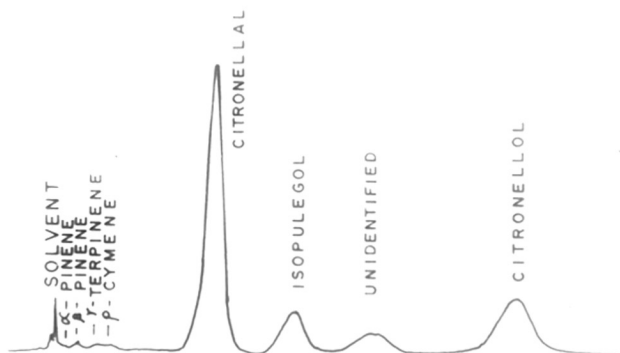


Fig. 30 Standard GLC pattern of E. citriodora oil.

CITRONELLAL

$C_{10}H_{18}O$

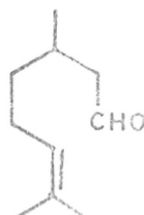
(3,7-Dimethyl-6-octen-1-al)

MOLECULAR WEIGHT -- 154.25

BOILING POINT -- 89-90°/14 mm

SPECIFIC GRAVITY  $d_4^{20}$  -- 0.8554

REFRACTIVE INDEX  $n_D^{20}$  -- 1.4485



CITRONELLOL

$C_{10}H_{20}O$

(3,7-Dimethyl-6-octen-1-ol)

MOLECULAR WEIGHT -- 156.26

BOILING POINT -- 116°/15 mm

SPECIFIC GRAVITY  $d_4^{17.5}$  -- 0.8545

REFRACTIVE INDEX  $n_D^{20}$  1.4563

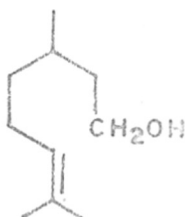


FIG. 31. MAIN COMPONENTS OF E. CITRIODORA OIL  
BEDOUKIOSN (1967)

Table 44 Effect of subculture on oil, citronellal and citronellolcontent in callus

|                              | Parent plant<br>% | Primary<br>% | S <sub>1</sub><br>% | S <sub>2</sub><br>% | S <sub>3</sub><br>% | S <sub>4</sub><br>% | S <sub>10</sub><br>% |
|------------------------------|-------------------|--------------|---------------------|---------------------|---------------------|---------------------|----------------------|
| Total oil<br>(dry wt. basis) | 2.25              | 0.92         | 0.56                | 0.30                | 0.10                | 0.10                | N.D.                 |
| Citronellal                  | 70.20             | 42.50        | 25.00               | 12.10               | 8.20                | 7.00                | N.D.                 |
| Citronellol                  | 16.80             | 8.00         | 5.10                | 2.20                | N.D.                | N.D.                | N.D.                 |

Initial inoculum - leaf callus from plant (1 year old)

N.D. - not detectable

Subculture period- 30 days

Medium - MS-8

**Table 45** Effect of different media on shoot regeneration from leaf and stem callus

Medium - MS-4

|                                        | Medium<br>(mg/l) | Observation after 30 days<br>shoot regeneration from<br>callus |      |
|----------------------------------------|------------------|----------------------------------------------------------------|------|
|                                        |                  | Leaf                                                           | Stem |
| 1. MS-4 (without growth regulators)    |                  | -                                                              | -    |
| 2. Kin (0.1 - 5.0)                     |                  | -                                                              | -    |
| 3. BAP (0.1 - 5.0)                     |                  | -                                                              | -    |
| 4. 2iP (0.1 - 5.0)                     |                  | -                                                              | -    |
| 5. Zeatin (10 ug - 100 ug)             |                  | -                                                              | -    |
| 6. Kin (0.2) + IAA (0.1)               |                  | -                                                              | -    |
| 7. Kin (0.2) + NAA (0.1)               |                  | -                                                              | -    |
| 8. BAP (0.2) + IAA (0.1)               |                  | -                                                              | -    |
| 9. BAP (0.2) + NAA (0.1)               |                  | -                                                              | -    |
| 10. CM (10%)                           |                  | -                                                              | -    |
| 11. CM (10%) + Kin (0.2)               |                  | -                                                              | -    |
| 12. CM (10%) + Kin (0.2) + IAA (0.1)   |                  | -                                                              | -    |
| 13. CM (10%) + BAP (0.2)               |                  | -                                                              | -    |
| 14. CM (10%) + BAP (0.2) + IAA (0.1)   |                  | -                                                              | -    |
| 15. CH (0.05%)                         |                  | -                                                              | -    |
| 16. CH (0.05%) + Kin (0.2)             |                  | -                                                              | -    |
| 17. CH (0.05%) + Kin (0.2) + IAA (0.1) |                  | -                                                              | -    |
| 18. CH (0.05%) + Kin (0.2) + NAA (0.1) |                  | -                                                              | -    |
| 19. CH (0.05%) + BAP (0.2)             |                  | -                                                              | -    |
| 20. CH (0.05%) + BAP (0.2) + IAA (0.1) |                  | -                                                              | -    |
| 21. Kin (0.2) + BAP (0.3) (MS-3)       |                  | -                                                              | +    |
| 22. Kin (0.2) + 2iP (0.3)              |                  | -                                                              | -    |
| 23. Kin (0.2) + Zeatin (10 µg/l)       |                  | -                                                              | -    |

- No Shoot regeneration

+ Shoot regeneration

was as  
formation/described in Chapters III and IV. Fig. 33 shows  
a rooted plant growing on MS-11 liquid medium regenerated  
from stem callus.

These plantlets were successfully grown in pots and  
then in field by similar methods as described in Chapter V.

Total oil, citronellal and citronellol were estimated  
from primary stem callus and at different stages during  
shoot and root development and in the regenerated plants.

In primary stem callus and also just after shoot  
initiation no oil was detected. With shoot initiation and  
elongation after 45 days traces of oil and citronellal  
appeared. This amount increased with further elongation  
of the shoots. With the initiation of roots there was a  
sudden increase in the total oil and citronellal concentration  
and also the appearance of citronellol. The concentration  
of these compounds increased as the root system was  
developed, and reached a higher level in plants which were  
grown in pots for 4 months (Table 46).

#### Organized shoots

Shoots both from seedlings and mature trees could be  
subcultured repeatedly without any reduction in their  
organizing ability. To determine the effect of subculture  
on the presence of the oil, citronellal and citronellol,  
these components were analyzed both in seedling and mature  
tree subcultured shoots at different passages.

#### Effect of subculture on shoots of seedlings

Table 47 illustrates the effect of subculture on oil



Fig. 32 Shoot differentiation  
from stem callus.



Fig. 33 Rooted plant from stem  
callus on MS-11 medium.



Table 46 Effect of organogenesis on oil, citronellal, and citronellol content

|                                    | Callus | Shoot<br>initiation<br>(0.5 - 0.8<br>cm length) | Shoot<br>(2 - 3<br>cm length) | Shoot<br>(4 - 6<br>cm length) | After<br>initiation<br>of root | Rooted<br>plant<br>in<br>tube | Plant<br>in pot<br>(4 months) |
|------------------------------------|--------|-------------------------------------------------|-------------------------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------|
|                                    | %      | %                                               | %                             | %                             | %                              | %                             | %                             |
| Total oil<br>(dry weight<br>basis) | N.D.   | N.D.                                            | 0.06                          | 0.07                          | 0.30                           | 0.48                          | 0.75                          |
| Citronellal                        | N.D.   | N.D.                                            | 5.00                          | 5.20                          | 23.10                          | 40.20                         | 50.90                         |
| Citronellol                        | N.D.   | N.D.                                            | N.D.                          | N.D.                          | 2.00                           | 5.00                          | 10.10                         |

Initial callus from seedling (4 month old) stem

N.D. - not detectable

Period for shoot development - 60 days

Period for root development - 30 days

Table 47 Effect of subculture on oil, citronellal and citronellol content of shoots from seedlings

|                                    | Parent<br>explant<br>% | Primary<br>% | S <sub>1</sub><br>% | S <sub>2</sub><br>% | S <sub>3</sub><br>% | S <sub>4</sub><br>% | S <sub>10</sub><br>% |
|------------------------------------|------------------------|--------------|---------------------|---------------------|---------------------|---------------------|----------------------|
| Total oil<br>(dry weight<br>basis) | 1.06                   | 0.80         | 0.38                | 0.15                | 0.12                | 0.12                | 0.10                 |
| Citronellal                        | 62.60                  | 43.80        | 20.20               | 11.00               | 10.80               | 10.30               | 10.20                |
| Citronellol                        | 13.50                  | 6.20         | 2.40                | 1.00                | N.D.                | N.D.                | N.D.                 |

Initial inoculum - Seedling (4 month old)

Subculture period - 30 days

N.D. - not detectable

content and its main components from shoots grown on MS-3 medium from explants of 4 month old seedlings. There was a gradual reduction in the concentration of the oil and citronellal upto third subculture and later maintained a fairly constant level after the 10th passage. On the other hand citronellol was not detected after the 3rd passage.

#### Mature trees

In shoots from mature trees the effect of subculture on total oil synthesis was more or less similar to that of the effect from seedling shoots, except that the concentration of the oil and citronellal at the end of 10th subculture was nearly double that of the seedling shoots (Table 48).

#### Rooting

##### Seedlings and mature trees

The effect of rooting on the concentration of oil, citronellal and citronellol was studied both with tissues raised from seedlings and mature trees. This study was conducted on cultures which were in the fourth passage. In studies both on seedling and mature tree cultures, the initiation of roots resulted in a sharp increase in the concentration of the oil and citronellal and was found to be nearly double the concentration in shoots alone (Tables 49 and 50). Citronellol which was not detected at the fourth passage, was also present at this stage. The levels of all the components increased with root elongation. Plants grown for 4 months in pots

Table 48 Effect of subculture on oil, citronellal and citronellol content of shoots from mature tree

|                                    | Parent tree | Primary | S <sub>1</sub> | S <sub>2</sub> | S <sub>3</sub> | S <sub>4</sub> | S <sub>10</sub> |
|------------------------------------|-------------|---------|----------------|----------------|----------------|----------------|-----------------|
|                                    | %           | %       | %              | %              | %              | %              | %               |
| Total oil<br>(dry weight<br>basis) | 1.15        | 0.80    | 0.50           | 0.30           | 0.27           | 0.27           | 0.25            |
| Citronellal                        | 71.00       | 50.15   | 40.00          | 28.29          | 25.50          | 24.00          | 24.50           |
| Citronellol                        | 17.50       | 8.50    | 6.00           | 3.50           | 1.00           | N.D.           | N.D.            |

Initial inoculum - Apical buds from mature tree (20 year old)

Subculture period - 30 days

N.D. - not detectable.

Table 49 Effect of root formation on oil, citronellal and citronellol content  
from seedlings

|                                    | Parent<br>seedlings<br>Ø 4 months)<br>% | Shoot<br>% | After root<br>initiation<br>% | Rooted plant<br>in tube<br>% | Plant<br>in pot<br>Ø 4 months)<br>% |
|------------------------------------|-----------------------------------------|------------|-------------------------------|------------------------------|-------------------------------------|
| Total oil<br>(dry weight<br>basis) | 1.06                                    | 0.12       | 0.32                          | 0.65                         | 0.92                                |
| Citronellal                        | 62.60                                   | 10.70      | 25.60                         | 49.60                        | 58.70                               |
| Citronellol                        | 13.50                                   | N.D.       | 3.00                          | 7.50                         | 11.30                               |

Shoot rooted at fourth subculture

Initial inoculum from seedling (4 months)

N.D. - not detectable.

Table 50 Effect of root formation on oil, citronellal and citronellol content  
from mature tree

|                                    | Potest tree<br>(20 years) | Shoot | After<br>initiation<br>of root | Rooted<br>plant<br>in tube | Plant in pot<br>(4 months) |
|------------------------------------|---------------------------|-------|--------------------------------|----------------------------|----------------------------|
|                                    | %                         | %     | %                              | %                          | %                          |
| Total oil<br>(dry weight<br>basis) | 1.12                      | 0.25  | 0.52                           | 0.93                       | 1.04                       |
| Citronellal                        | 71.50                     | 24.00 | 40.00                          | 67.50                      | 70.30                      |
| Citronellol                        | 17.00                     | N.D.  | 4.38                           | 11.25                      | 15.50                      |

N.D. - not detectable

Period for root development - 30 days.

contained more or less identical levels of all the components as that present in the parent plants (Tables 49 and 50).

Table 51 summarizes the data on the oil, citronellal and citronellol contents in plants regenerated from callus, seedlings and mature trees. The percentage of oil present in shoots obtained from callus, seedlings and mature trees was approximately 6, 9 and 19% respectively, when compared to the parent plants/trees. These levels increased in rooted plants from callus, seedlings and mature trees being 40, 65 and 90% respectively as that present in the parent. More or less similar differences in the concentration of citronellal was also observed in shoots and rooted plants from callus, seedlings and mature trees. The content of citronellal of rooted plants from mature trees was slightly higher than from callus and seedling plants. Rooting however had a striking effect on the synthesis of citronellol, which was not traceable in shoots but which was detected with the onset of roots.

**Table 51** Comparative data on oil content in shoot and rooted plant regenerated from callus, seedling and mature tree

|                                    | Parent<br>plant<br>(4 month<br>seedling) | Parent<br>tree<br>(20<br>year<br>old) | Shoot<br>(stem<br>seedling<br>callus) | Shoot<br>(seedling) | Shoot<br>(mature<br>tree) | Rooted<br>plant<br>in tube<br>(callus) | Rooted<br>plant<br>in tube<br>(seedling) | Rooted<br>plant<br>in tube<br>(mature<br>tree) |
|------------------------------------|------------------------------------------|---------------------------------------|---------------------------------------|---------------------|---------------------------|----------------------------------------|------------------------------------------|------------------------------------------------|
|                                    | %                                        | %                                     | %                                     | %                   | %                         | %                                      | %                                        | %                                              |
| Total oil<br>(dry weight<br>basis) | 1.06                                     | 1.15                                  | 0.07                                  | 0.12                | 0.25                      | 0.48                                   | 0.65                                     | 0.93                                           |
| Citronellal                        | 62.60                                    | 71.50                                 | 5.00                                  | 10.70               | 24.00                     | 40.20                                  | 49.80                                    | 67.50                                          |
| Citronellol                        | 13.50                                    | 17.30                                 | N.D.                                  | N.D.                | N.D.                      | 5.00                                   | 7.50                                     | 11.20                                          |

N.D. - not detectable



## Section 2 Discussion

A large variety of secondary products have now been shown to be present in plant callus or tissue cultures (Krikorian and Steward, 1969; Constabel et al., 1974). This observation has stimulated considerable interest in the possibility of using plant tissue cultures for the biosynthesis of commercially important products (Shuler, 1981). These compounds are generally produced at concentrations less than normally present in the parent plants (Krikorian and Steward, 1969; Staba, 1969). Although in a few cases there are reports that the products produced in tissue culture exceed the in vivo rate of production (Constabel et al., 1974; Zenk et al., 1977; Doller, 1978), in others disappearance of the compounds are reported where as in some cases, a replacement with a related compound or even completely novel compounds are observed (Furuya et al., 1972; Butcher, 1971). It has been suggested that organization in tissue culture plays an important part in the induction of some secondary products. RajBhandary et al. (1969), for example, showed that synthesis of major alkaloids in Atropa belladonna was achieved only with the organization of root structures. Nicotiana tissue cultures studies by Tabata et al. (1970) have also indicated an intimate relationship between alkaloid synthesis and bud formation. Similar results have been reported for tissue cultures producing volatile oils, such as Mentha reported by Krikorian and Steward (1969)

and onion (Mackenzie et al., 1974). They showed that none of the calli which lacked organized structure produced volatile oil as comparable to organized structures. Ozehra and Komamine (1981) also showed the induction of anthocyanin synthesis in relation to embryogenesis in carrot suspension cultures. Waltrand et al. (1981) reported an increase in glycoside formation during organ differentiation in tissues of Digitalis purpurea cultured in vitro. There are also some reports in which secondary product synthesis is not decreased in callus cultures. Furuya et al. (1966), for example found both nicotine and anatabine even in five year old callus cultures of N. tabacum. There are other reports where secondary products, such as certain steroids and furanochromes, are produced in higher amounts in undifferentiated cells compared to differentiated tissues (Kaul and Staba, 1967).

Our results clearly demonstrate that:

- (1) The capacity for synthesis of significant quantities of monoterpenes, normally produced within or in association with specialised cells is lost during callus growth. This confirms earlier reports. These reports substantiate earlier reports that calli derived from Mentha piperita, Mentha spicata and other species do not produce detectable quantities of the monoterpenes characteristic of the whole plant (Krikorian and Steward, 1969). Becker (1970) has surveyed cultures from Pimpinella anisum, Foeniculum vulgare, Levisticum officinalis, Mentha piperita, Mentha pulegium, Origanum vulgare, Salvia officinalis, Rosmarinus

officinalis for their volatile oil contents. None of the calli which lacked organised structures gave steam distillates with component spectra comparable to those of the intact plants. Similarly it has been shown that callus cultures of Nepeta cataria do not synthesise the methyleyclopentane monoterpenes e.g. nepetactone, found in the intact plants (Downing and Mitchell, 1975).

Cultures derived from a number of rose varieties also do not produce monoterpenes (Macrae and How, via Jones, 1974).

Recently Takayuki et al. (1980) reported that none of the monoterpenoids characteristic of an essential oil of the intact plants of M. spicata were found from leaf callus tissues. Very recently Ishii et al. (1982) have also shown that callus cultures of Eucalyptus dives do not produce monoterpenes which are abundant in the mother plant.

There are only a few reports concerning monoterpene biosynthesis in tissue cultures. Callus cultures of Tanacetum vulgare produced the same monoterpenes as the parent plant (Banthorpe and Wirz-Justice, 1972). Tissue culture of Ruta graveolens are reported to reproduce the monoterpene patterns of the parent and it is claimed that monoterpene biosynthesis in the organized culture system is controlled by both wavelength and the intensity of light (Negel and Reinhard, (1975) Corduan and Reinhard, 1972). The monoterpenes of a Perilla species are reported to be synthesized by callus cultures (Hiroshi and Yoshiyuki,

1976). Very recently Purohit and Khanna (1981) reported essential oil production from callus cultures of Ocimum basilicum.

In all these reports the yield of monoterpene biosynthesis in callus cultures is very low as compared to intact plants.

Apart from these isolated reports, it is clear from all these studies that callus cultures do not usually synthesize significant quantities of monoterpenes, which are normally produced within or in association with specialized cells.

The lack of the formation of such monoterpenes may be attributed to the defect of an oil gland in these callus tissues, as Becker (1970) has described that no callus tissues form oil glands even when the tissues are induced from essential oil producing plants. In addition, it is well known that the redifferentiation ability of callus tissues decreases on subculture of the tissue over a long period. This could also be responsible for the change in the ability to produce monoterpenes (Suga et al., 1980).

Another explanation that can be given as to why monoterpenes rarely accumulate in callus culture is that although such compounds are formed, they are rapidly degraded, and hence are never present at sufficiently high concentrations to be detected (Charlwood and Banthrope, 1978). On the other hand some or all of the enzymes

required for monoterpene biosynthesis may be completely missing from the cultured cells (Charlwood and Banthrope, 1978). Further investigations are however necessary to confirm these points.

(2) A second conclusion one could draw from our results is that organogenesis is necessary, and root development is required for high essential oil production. This is evident from the fact that there is a marked increase in the oil and citronellal content and appearance of citronellol after root initiation. Tabata et al. (1970) in studies on Nicotiana tabacum have shown that the average nicotine content of undifferentiated callus was only half that of calli with buds produced by growth on an auxin free medium.

Dhoot and Henshaw (1977) showed that alkaloid production in Hyoscyamus was slightly increased by an increase in the degree of organization and after the appearance of roots. Garve et al. (1980) obtained 10 - 100 fold increase in cardenolide levels in tissue cultures of Digitalis lanata in medium which favours the formation of green embryoid structures with roots.

Recently Tabata et al. (1981) have shown that essential oil content increased with increase in degree of organization in tissue cultures of lemon grass. These plants induced from callus cultures of lemon grass, when grown in soil contained identical contents of the essential oil as the parent plants.

The results cited above substantiate our results that

organization plays an important part in biosynthesis of secondary products.

(3) A third important observation from our data is that plants regenerated from mature tree explants contain higher concentrations of oil, citronellal and citronellol than plants regenerated from seedlings or callus cultures. There have been no reports where this type of comparison has been made on the essential oil production from tree species.

As far as we are aware this is the first report on the production of essential oil during organogenesis from callus and organized cultures of forest trees from seedlings and mature trees of Eucalyptus citriodora.

## CHAPTER

MARY AND SON



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## **CHAPTER VIII**

### **SUMMARY AND CONCLUSIONS**

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## SUMMARY AND CONCLUSIONS

The present studies were carried out on plant tissue cultures of Eucalyptus citriodora hook with two main objectives: (1) to develop a tissue culture method for clonal propagation of the 'elite' trees, and (2) to determine the stage at which the essential oil and its main components citronellal and citronellol are synthesised in culture.

These studies have been divided into five main Chapters. The results obtained in Chapters III, IV, V, VI and VII are summarized as follows:

### III. Studies on shoot regeneration

In this Chapter studies were carried out to obtain a high rate of shoot multiplication from seedling segments and from buds of a 20 year old tree of E. citriodora.

#### Seedlings

- a) The hypocotyl segment of the seedling had the highest potential for shoot formation followed by nodal and apical regions respectively.
- b) A modified Murashige and Skoog (1962) basal medium supplemented with two cytokinins, kin and BAP and two vitamins Ca.P. and Biotin (MS-3) gave optimum shoot formation.

#### Mature trees

The medium used for obtaining shoot multiplication from seedling segments was not applicable to explants from mature trees which required additional treatments viz.



a short cold treatment of the primary explants for 72 h at 15°C on MS-3 and then incubation at 25°C for 14 days followed by growth in shake flasks at 27°C with continuous illumination, before occurrence of shoot development on MS-3 medium at 25°C. For subculture, cold or shake flask pretreatments were not necessary.

Some of the results with different growth regulators and physical factors on multiple shoot formation carried out on in vitro grown shoots are as follows:

- (a) Two cytokinins kin at 0.2 mg/l and BAP at 0.3 mg/l supplemented to a modified Murashige and Skoog (1962) basal medium containing Ca.P. (0.1 mg/l) and biotin (0.1 mg/l) was best.
- (b) The auxins, IAA, IBA, IPA, NAA and 2,4-D tested at varying concentrations in the medium were inhibitory.
- (c) CM, Ed, YE, ME and CH were also found inhibitory.
- (d) A similar inhibition was also found by ABA, ethrel, Morphactin, CCC and GA<sub>3</sub> at the concentrations tested.
- (e) Healthy shoot development was observed by incubation at 25 ± 2°C with a light intensity of 1,500 lux.
- (f) Shoot formation greatly increased on subculture reaching a constant level after 4 passages.
- (g) Agitation of cultures in liquid medium resulted in a marked increase in the number of shoots as compared to stationary cultures grown on agar or liquid medium of the same composition.

From these results the best medium for maximum healthy

shoot formation was, Murashige and Skoog (1962) basal medium supplemented with Kin (0.2 mg/l), BAP (0.3 mg/l), GA<sub>3</sub>P. (0.1 mg/l) and biotin (0.1 mg/l), on incubation at  $25 \pm 2^{\circ}\text{C}$  with a light intensity of 1,500 lux and a 16 h photoperiod.

#### IV. Studies on root regeneration

In this Section studies were carried out on root initiation from shoots obtained from seedlings and a 20 year old tree.

Seedling raised shoots rooted after treatment with NAA (1 mg/l) in W-3 medium for 48 h followed by transfer to W-3 liquid medium without auxins. Shoots from the 20 year old tree did not respond to this treatment. Rooting however occurred after shoots had undergone 3 subcultures, by treatment of shoots with double the auxin concentration (2 mg/l) NAA in W-3 medium for 48 h, following by transfer to W-3 medium lacking the auxin. For further healthy development of plants transfer to Murashige and Skoog basal liquid medium (MS-9) was found necessary. Only 45 - 50% rooting was obtained by this method.

A second method was also developed whereby primary shoots, (before any subculture) regenerated from mature trees could be induced to root after treatment with NAA (2 mg/l) for 48 h by sequential transfer from Murashige and Skoog half strength mineral salts solid medium containing 0.25% activated charcoal (MS-10) to Murashige and Skoog half strength salts liquid medium without charcoal (MS-11) for

healthy plant development. By this method 70% rooting was obtained.

Rooting was found to be undiminished<sup>b</sup> even after shoot cultures had undergone over 30 passages.

#### V. Monoterpenes of field grown tissue culture-raised plants

The concentration of total oil, citronellal and citronellol in clonally propagated plants from the 20 year old trees and from elite high oil containing trees were identical to the parent trees. This observation could be detected even in plants at a very young age (3 months old).

#### VI. Initiation of callus and studies on nutrition

Callus isolated from leaves of a one year old

E. citriodora plant on Murashige and Skoog basal medium supplemented with coconut milk, edamin, inositol and 2,4-D (MS-1).

(a) The study with different basal media showed that Murashige and Skoog medium (1962) was the best for callus growth.

(b) Different inorganic and organic nitrogen sources were also tested. Growth was best with a mixture of  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  at the concentration present in Murashige and Skoog basal medium (1962). When coconut milk and edamin were also present in the medium 5 - 10% coconut milk and 0.05% edamin were found optimum.

(c) Of various carbon sources tested, sucrose at a concentration of 2 - 3% gave maximum growth of callus.

(d) Auxins were observed to play an important role in callus

growth. 2,4-D at the level of 3 mg/l gave maximum callus growth followed by B-NOA, 2,4,5-TCP, 2-BTOA, NAA, IAA, IBA and IPA.

(e) All the cytokinins tested were slightly inhibitory for callus growth.

(f) None of the growth retardants or ethrel tested at 0.001 mg/l inhibited growth although at concentrations above this, a slight growth retardation was observed.

(g) Maximum growth was obtained with a combination of pyridoxine, thiamine, nicotinic acid and inositol. These vitamins when added singly did not markedly stimulate callus growth.

(h) The optimum temperature for incubation of cultures was 25 - 30°C in the dark with media adjusted to a pH, ranging from 6 - 7, and an agar concentration of 0.8%. The best inoculum size was found to be 100 - 200 mg/tube, attaining maximum growth within 25 - 30 days.

From these studies, the best conditions that emerged for growth of E. citriodora callus were Murashige and Skoog (1962) medium supplemented with coconut milk (5%), edamin (0.05%), inositol (120 mg/l) and 2,4-D (3 mg/l) with sucrose (2%), by incubation in dark at 25°C.

#### VII. Studies on synthesis of essential oil, citronellal and citronellol in tissue cultures

Studies carried out using callus and organized cultures from seedlings and mature trees to determine the stage for synthesis of the essential oil, citronellal and citronellol indicated:

(a) The absence of these secondary products in

undifferentiated callus cultures.

- (b) The first appearance of oil and of citronellal with the commencement of shoot differentiation, the concentration increasing gradually with root initiation.
- (c) The shoots from buds of mature trees having a higher oil content than those regenerated from seedling segments.
- (d) The concentration of the oil and citronellal in shoot culture decreasing on subculture.
- (e) A sharp increase in oil content which coincided with initiation of roots, demonstrating the importance of a root system for oil synthesis.

From these results it can be concluded that organization and root formation in particular is necessary for secondary product synthesis in E. citriodora trees.

These studies demonstrate the applicability of tissue culture for clonal propagation of high oil containing 'elite' trees of Eucalyptus citriodora.



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