CHAPTER I - INTRODUCTION

CHAPTER I

INTRODUCTION

I-1 ABOUT GUGGUL, COMMIPHORA WIGHTII (Arnott) Bhandari

I-2 PLANT TISSUE AND CELL CULTURE :
REVIEW OF LITERATURE

I-2.a General

I-2.b Clonal propagation of woody species

I-2.c Secondary Metabolites

I-2.d Steroids

I-3 PRESENT INVESTIGATIONS

CHAPTER - I

INTRODUCTION

I-1 ABOUT GUGGUL, COMMIPHORA WIGHTII (Arnott) Bhandari

The genus Commiphora (Family : Burseraceae) comprising of about 165 species of spiny, balsamiferous trees and shrubs is fairly distributed in Africa and tropical Asia. Many of the species yield resins of commercial importance. About five species occur in India of which Commiphora wightii (Arnott) Bhandari and Commiphora roxburghii yield Indian Bdellium (Anonymous, 1950).

Commiphora wightii is a small tree with spinescent branches occurring in the arid, rocky tracts of Rajasthan, North Gujarat, and costal areas of Saurashtra and Kutch. (Fig. I-1).

Commiphora wightii is a source of Indian Bdellium, a gum-resin (guggul) obtained by incision of the bark. Each plant yields about 0.75 to 1 kg of guggul collected during the months of November to June. The gum-resin occurs in the form of conglomerates of tears, pale yellow brown or dull green in colour with a bitter agromatic taste and balsamic odour.

This is the guggul of commerce, which is very largely used in incense industry and as a fixative in perfumery

Fig. I-1 Commiphora wightii (Arnott) Bhandari

Fig. I-1

industries. The classical Ayurvedic literature (Fig. I-2) claims its efficacy in a variety of metabolic disorders especially rheumatoid arthritis and obesity. It is astringent, antiseptic and anti-inflammatory. When taken internally, it acts as a bitter, stomachic and carminative, stimulating the appetite and improving the digestion. The resin is used in the form of a lotion for indolent ulcers and as a gargle in treating caries of the teeth, weak and spongy gums, pyorrhoea, chronic tonsilitis, pharengytis and ulcerated throat (Anonymous, 1950; 1972).

Inhalation of the fumes from burnt guggul is recommended in hay fever, acute and chronic nasal catarrh, chronic laryngitis chronic bronchitis and phthisis (Anonymous, 1950; 1972).

However, the current interest in this plant is on account of anticholesterol activities found in guggul. Modern pharmacological investigations have revealed significant anti-inflammatory, anti-rheumatic (Gujrat et al., 1960; Santha-kumari et al., 1964; and Satyavati et al., 1969 a) and hypocholesterolemic/hypolipaemic activity (Mehta et al., 1968; Satyavati et al., 1969 b; and Malhotra et al., 1970) for the crude drug as well as for certain fractions, thus lending support to the ancient claims.

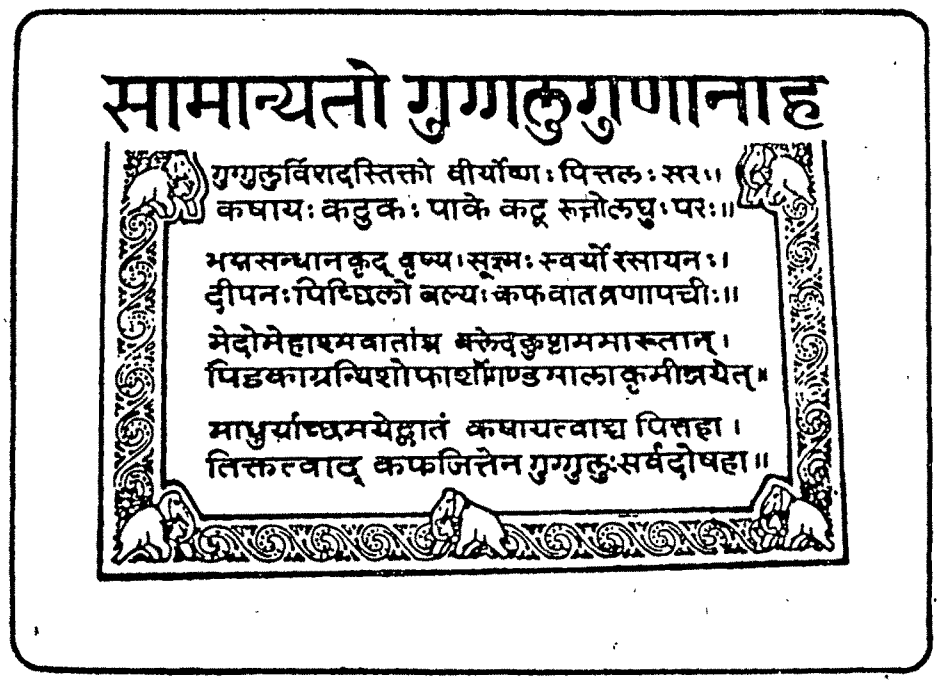


Fig. I-2

Fig. I-2 The classical Ayurvedic literature (Sushruta Samhita)

Further anti-inflammatory, anti-arthritic and anti-rheumatic activity have been confirmed (Arora et al., 1971; Srivastava et al., 1984). Thus, the claims of Ayurveda about its use are well supported.

Satyavati et al. (1969 a) carried out investigations on the effect of the oleo-resin of Commiphora wightii on the serum and tissue lipids, particularly the serum cholesterol, lipid-phosphorus and cholesterol/phospholipid ratio which has been claimed to play a significant role in the pathogenesis of atherosclerosis. The oleo-resin was found not only to lower the serum cholesterol in hypercholesterolemic rabbits, but also to protect the animals against hypercholesterolemia induced by hydrogenated vegetable oil and cholesterol (Satyavati et al., 1969 b).

Anion exchange property detected by means of chloride retention and bile acid sequestrating activity in the oleo-resin fraction suggested that it acts by a mechanism similar to cholestyramine, an anion exchange resin developed by Tennet et al. (1958). Cholestyramine has been reported to act by binding with bile acids and accelerating the excretion of cholesterol and bile acids. Nityanand and Kapoor (1971) have reported that both the alcoholic extract of resin and a steroid fraction (ketonic fraction) have a hypocholesterolemic

effect. Hypocholesterolemic effect of the fraction was found to be more marked than the alcoholic extract. They have further reported that in the presence of resin and steroid, in in vitro experiments, no significant inhibition of acetate-1-^{14}C incorporation into cholesterol was found suggesting that the hypolipaemic effect of the ketonic fraction is unlike to be the effect of Clofibrate (CPIB-P-chlorophenoxyisobutyrate). The latter is known to inhibit cholesterol synthesis, thus causing interference in lipoprotein formation and lipid turnover. Tripathi et al. (1975) reported significant reduction in levels of serum cholesterol, phospholipids and triglyceroids by oral administration of the keto steroid from Commiphora wightii. They further investigated the thyroid stimulating action of z-guggulsterone by administrating z-guggulsterone to albino rats (Tripathi et al., 1984).

Dr. Sukh Dev and co-workers (Maltichem Research Centre, Nandesari, Dist. Baroda) undertook a systematic chemical investigation (Patil et al., 1972, 1973; Prasad and Sukh Dev, 1976; Bajaj et al., 1981; and Bajaj and Sukh Dev, 1982) especially with a view to isolate and characterise compound(s) responsible for hypocholesterolemic/hypolipaemic activity. These investigations were carried out in collaboration with the pharmacological group headed by Dr. S. Nityanand (at the Central Drug Research Institute, Lucknow) who was responsible for the pharmacological screening of different fractions/pure compounds (Sukh Dev, 1983).

The term gum-resin (guggul) indicates that it consists of two components, the gum and the resin. Gum consists of polysaccharide material; while the resin material, mainly lipoidic that can be separated from the gum by solvent extraction. Guggul, is a complex mixture of steroids, diterpenoids, aliphatic esters, carbohydrates and a variety of inorganic ions, besides minor amounts of sesamin and other unidentified constituents (Patil et al., 1972).

The gum-resin was fractionated by successive solvent triturations into a petroleum ether fraction, an Ethyl acetate fraction and an Ethyl acetate insoluble residue. The Ethyl acetate insoluble fraction was a carbohydrate (gum portion of the gum-resin) studied earlier by Bose and Gupta (1964, 1966), and was found to be toxic. The Petroleum ether fraction contained essentially diterpenoids, besides a small quantity of cholesterol. It contained a new cembrenoid hydrocarbon 'Cembrene A', the diterpene alcohol 'mukulol'.

The Ethyl acetate soluble portion 'Guggulipid' carried both the hypocholesterolemic and antiinflammatory activity. It was further separated into bases, acids and the neutral fractions. The neutral fraction carried practically all hypocholesterolemic activity, while the acids displayed significant anti-inflammatory activity, the basic fraction showed none of the two activities (Sukh Dev, 1983).

It was soon found that the neutral fraction contained several ketones, and had significant lipid-lowering activity, and hence this fraction was further segregated into ketonic and non-ketonic fractions with the aid of semicarbazide on silica gel (Singh et al., 1981). The non-ketonic fraction though devoid of any significant lipid-lowering activity, none-the-less appeared to exert a synergistic action on the biological activity of the ketonic fraction.

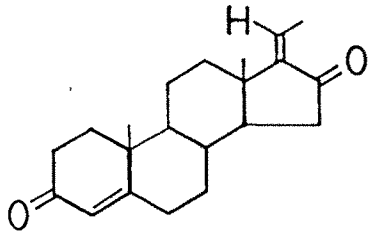
Guggul has proved to be a rich source of steroids. At least ten steroids have been isolated and characterised (Fig. I-3). Besides, cholesterol, guggulsterol-I, - II and -III are the C_{27} steroids, whereas the remaining ones are pregnane derivatives. Z-Guggulsterone and E-Guggulsterone, which together constitute some 2 per cent of gum resin are responsible for the lipid-lowering activity. Moreover, they appear to offer economically viable alternative as raw material for synthesis of important corticosteroidal drugs such as dexamethasone and betamethasone (Sukh Dev, 1983).

Though Z- and E- Guggulsterones exhibited the lipid-lowering activity, since the total ethyl acetate extract of the gum-resin (guggulipid) also had comparable activity, further work with a view to evolve a drug had been carried out on 'Guggulipid'. Fig. I-4 gives some comparative data on 'guggulipid' and another well-known hypocholesterolemic drug, 'clofibrate'

1. Z- Guggulsterone

C_{21} H_{28} O_2

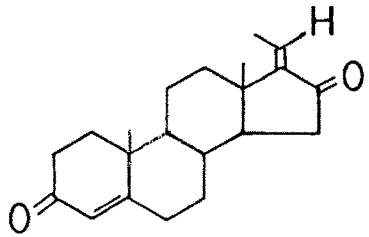
Trans isomer



2 E- Guggulsterone

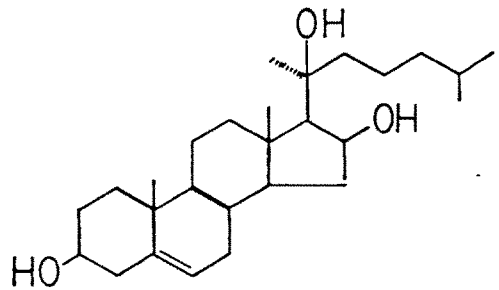
C_{21} H_{28} O_2

Cis isomer



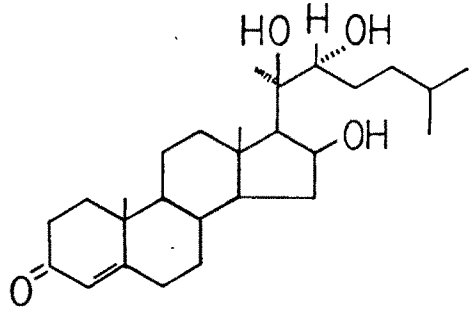
3. Guggulsterol - II

C_{27} H_{46} O_3



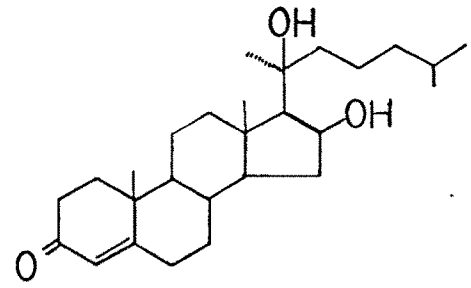
4. Guggulsterol - I

C_{27} H_{44} O_4



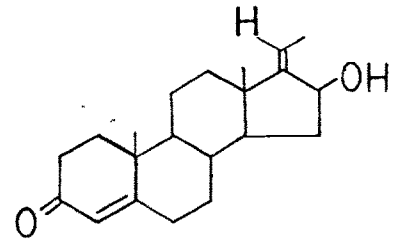
5. Guggulsterol - III

C_{27} H_{44} O_3

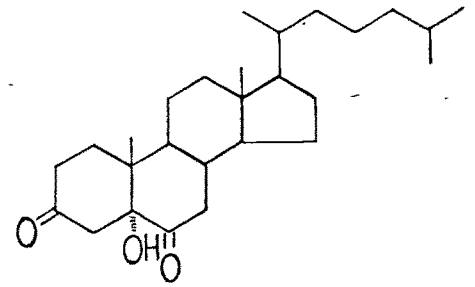


6. Z - Guggulsterol

C_{21} H_{29} O_2



7 Guggulsterol - IV



8 Guggulsterol - V

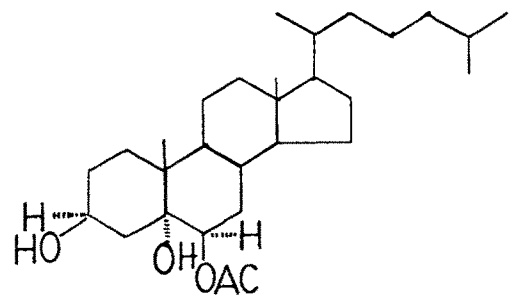


Fig. I-3 STEROIDS

COMPARISON OF EFFICACY OF GUGGULIPID AND CLOFIBRATE (Normal rats; 30-day administration)

Drug	Dose mg./kg.	Lowering of Serum Lipids (%)	
		Cholesterol	Triglycerides
Guggulipid	50	23	21
Clofibrate	50	27	22
Guggulipid	100	34	26
Clofibrate	100	39	30
Guggulipid + Clofibrate	50 + 50	45	33

Data by C.D.R.I. (Dr S Nityanand)

Fig. I-4

Fig. I-4 Comparative data on efficacy of 'guggulipid' and 'clofibrate'.

(ethyl p-chlorophenoxyisobutyrate). As can be seen 'guggulipid' has pronounced activity.

'Cipla' Laboratories, Bombay, which has obtained the technology for manufacture of 'guggulipid' from the Central Drug Research Institute, Lucknow, has established the drug's cholesterol lowering properties. 'Guggulipid' has been widely tested for its efficiency and safety on human volunteers. Recently 'Cipla' has introduced the drug 'Guggulipid' in the Indian market.

Another group of workers (Purushothaman and Chandrasekharan, 1976) had reported the isolation and identification of two more sterols, viz. Guggulsterol IV and Guggulsterol V from the neutral fraction, while Kakrani (1981) identified the major flavonoid compounds of the flowers of C. wightii as quercetin and its derivatives.

Tree exudates are natural products secreted by a tree usually in response to wounding and include true gums, resins, gum-resins, oleo-resins and mucilages. True gums are hydrophilic carbohydrates such as gum-karaya and gum-ghatti. Resins are complex substances containing a variety of compounds mainly lipoidic in nature, and are soluble in many organic solvents (Dell and McComb, 1978). The gum resins are a natural mixture of gum and resin. An oleo-resin is a natural mixture of essential oil and resin.

Gums and gum-resins exude from the plant parts normally through the injured regions. The exudation may be immediate or delayed. In the former condition, the plant organ has a natural tissue system and metabolism for producing gum or gum-resin. In the latter condition, where exudation is delayed, the system is induced due to injury and hence a delayed response (Shah, 1983). It is induced mainly by mechanical injury, physiological perturbation, microorganisms, drought, mineral deficiencies, auxins and ethylene.

Gum-resin canals in C. wightii occur in all the plant parts; however, they are more abundant in the bark of the stem (Setia et al., 1977). The gum-resin canals are lined by a layer of epithelial cells, possibly the principal site of synthesis and secretion of gum-resin. These cells are characterised by dense cytoplasm, conspicuous nuclei and high levels of nucleic acids, proteins, lipids and carbohydrates. The mode of development of a gum-resin duct in C. wightii is schizogenous (Setia et al., 1977). As both gum and resin are produced simultaneously in the ducts, it is obvious that two systems are operating in the epithelial cells for their synthesis (Shah and Setia, 1976).

The epithelial cells showed strong activity of peroxidase and acid phosphatase. Higher levels of amylase as well as

lipase were observed in the epithelial cells indicating the breakdown and conversion of starch and lipid into gum and resin compounds (Shah et al., 1980). Nair et al. (1981 a) observed a mass dissolution of cells at the site of injury. The intracellular spaces show the presence of gum as well as resin droplets. Since plasmodesmata are absent on the inner tangential wall of the epithelial cell in C. wightii (Setia et al., 1977), the only pathway through which the secretary products can be transferred into the canal lumen is through the wall (Nair et al., 1981 b; Bhatt, 1987).

Upon incision of the duct, the gum-resin present in the duct exudes out and gets hardened after exposure to air. Gum-resin is produced by the plant for clogging the wound, for keeping the water balence and preventing pathogen entry.

Gum-resin is exploited commercially by the tribes as well as various organisations for its medicinal application. Between 400-500 quintals of guggul is collected annually from the Saurashtra coast and Kutch areas. It is gathered by local tribes 'Waghirs'.

The plant, therefore, not only provides employment to local tribal population, but also supplies an essential raw material for the industries and is extensively used in the medicines.

I-2 PLANT TISSUE AND CELL CULTURE : REVIEW OF LITERATURE

I-2.a General

Recent advances made in the field of Plant Cell and Tissue Culture have resulted in the utilization of this technology for basic and applied research in a range of disciplines-biochemistry, genetics, developmental physiology, cell biology, agriculture and horticulture.

The term 'Plant Tissue and Cell Culture' broadly refers to the *in vitro* and aseptic cultivation of any plant part, organ, tissue, cell, now even protoplast, wherein their growth and nutrition, metabolism and morphogenetic pattern can be examined under a wide variety of deliberately varied conditions on a defined nutrient medium (Biondi and Thorpe, 1981). Any given tissue composed of living cells is a suitable explant for the initiation of plant tissue culture which permits investigations at many levels organization including cellular and molecular.

Historical background and some Landmarks

Since Schleiden (1838) and Schwann (1839) put forth cell theory, pronouncing the cell as a basic unit of life, extensive data has accumulated regarding the structure of the cell. Today, we know the cell as a very important unit bubbling with metabolic activity, meticulously organised

and consisting of many organelles with specific functions leading to the synthesis of primary and secondary metabolites, differentiation, maturation and reproduction.

Fortunately, plant cells unlike animal cells have an inherent capacity to produce whole plants, a property which led to the principle of totipotency. This idea which was first conceived by Haberlandt (1902) has now become a reality whereby somatic cells given the proper nutritional and environmental conditions can be made to organize, differentiate and ultimately produce whole plantlets under an aseptic atmosphere. In 1922, working independently, Robbins and Kotte reported some success with growing isolated root tips, but the first successful report of continuously growing cultures of tomato root tips was made by White in 1934 (White, 1934). Viable callus culture from procambial tissue of hybrid Nicotiana glauca X N. langsdorffii was reported by White in 1939 (White, 1939). At the same time, Gautheret (1939) and Nobe´Court (1939) separately reported proliferation of carrot explants. Since then a large number of reports has been published on the establishment of callus cultures from a whole range of plants belonging to different species.

Van Overbeek et al. (1941) suggested the importance of coconut milk in culturing the plant tissues which proved a turning point in the field of embryo culture. The growth of

single cells by placing them on a actively growing 'nurse' culture was an important observation made by Muir et al. (1954). An useful biological technique of cloning large numbers of single cells of higher plants was, however, developed in 1960 by Bergmann (Bergmann, 1960).

The classic experiments conducted by Skoog and Miller showed that the differentiation of roots and shoots in tobacco pith cultures was a function of auxin-cytokinin ratio (Skoog and Miller, 1957). Auxins and cytokinins had proved a powerful stimulus to future work on plant tissue culture. The first report of embryogenesis in cultured tissues appeared in 1958 by Steward and his collaborators which aroused considerable interest (Steward et al., 1958).

These findings made tissue culture a tool of great practical application. Soon after, Morel (1960) by meristem culture opened up orchid industry leading to clonal propagation possibilities and production of disease-free plants (Murashige, 1974, 1978). Murashige was instrumental in giving the technique of in vitro culture a status of a viable practical approach (Gautheret, 1985).

The horizon widened with time from horticultural plants via crop plants to forest and fruit trees. This aspect with reference to woody trees has been dealt with in greater detail later, since it is directly connected with the present thesis.

The meristem culture technique is used for virus eradication and has had important consequence at the economic level. This method could however, not only supplement but probably obviate thermotherapy in many cases (Spiegel-Roy and Kochba, 1977) with the possibility of eliminating a greater range of viruses (Navarro et al., 1975 and Wang and Huang, 1975). This has also enabled plants to be freed from other pathogens, including viroids, mycoplasmas, bacteria and fungi (Wakley, 1978).

For geneticists and plant breeders, induction of haploids through anther culture (Guha and Maheshwari, 1964) and isolated microspore culture methods are significant achievements over the conventional methods often laborious and time consuming. Later work of Bourgin and Nitsch (1967) confirmed the totipotency of pollen grains. Androgenesis allows the production of haploid and doubled haploid plants, important in plant breeding from young pollen grains. It also provides a base for mutagenesis and selection in the vegetative phase (Bajaj, 1983).

Plant cell and Tissue Cultures are a source of enormous genetic variability. Plant improvement via somaclonal variation may be exploiting genetic changes that preexist in the whole plant or changes that occur in cultured cells (Barbier and Dulieu, 1980). Further, mutagenesis can be

induced in plant cells efficiently by using physical and chemical mutagenic treatments. The most common mutants isolated *in vitro* that are economically valuable are resistance to phytotoxins (Gengenbach and Green, 1975), herbicides (Chaleff and Parsons, 1978), salinity and chilling resistance (Nabors *et al.*, 1980). Many other mutants, while not of direct economic importance, may be useful as selective markers in somatic hybridization.

New crop varieties with enhanced disease resistance could also be obtained by employing selection pressures to plated cells (Selvapandiyan *et al.*, 1987; Widholm, 1977) and dual cultures. Moreover, this could be used to investigate numerous aspects of host pathogen interactions (Ingram and Robertson, 1965; Ingram, 1980).

Another major break through in the field of plant tissue culture during recent years has been the isolation, culture and fusion of protoplasts, paving the way to one of the most important research field of genetic engineering and somatic hybridization (Cocking, 1972; Bajaj, 1977).

Today, several somatic hybrids have been produced between sexually compatible and sexually incompatible parents. These techniques have great value in studies on plant improvement by cell modification and somatic hybridization and also for fundamental problems in experimental plant biology (Cocking *et al.*, 1981).

The emphasis, however, has shifted from transfer of entire 'genome' by somatic hybridization to isolation of specific 'genes', and tagging them to a suitable vector, for transfusion of desired traits from the wild and incompatible plants to the cultivated species.

Furthermore, the increasing competence to manipulate plant DNA and genes has led to the application of cell culture in genetic engineering of higher plants. All in all, it is now readily apparent that cell culture is the keystone for the progress in plant biotechnology (Evans et al., 1983).

I-2. b Clonal Propagation of Woody Species

Clonal Propagation

Plant population derived from a single individual by asexual reproduction constitutes a clone. In nature clonal propagation occurs by apomixis and/or vegetative reproduction from any plant part like stem, leaf and root. It can also be induced by rooting of cuttings, grafting, layering and air-layering.

Interest in the use of tissue culture techniques as an alternative to traditional asexual multiplication methods is spreading rapidly. The size of the propagule in culture is

so minute, that the *in vitro* asexual propagation technique has been referred to as 'micropropagation'.

The 'micropropagation' techniques offer the following potential advantages over the conventional practices of asexual propagation (Hu and Wang, 1983; Bhojwani and Razdan, 1983; Murashige, 1977; Bonga and Durzan, 1982).

1. In relatively short time and space, large number of plants can be produced starting from a single individual.

2. Many plant species are highly resistant to conventional bulk propagation practices. *In vitro* propagation provides a possible alternative method for these species.

3. To produce, maintain, multiply and transport pathogen-free plants safely and economically.

4. International or regional exchange of genetic stocks is expedited and rendered safer. The sterile status of the culture eliminates the danger of disease introduction.

5. Cryogenic preservation of plant germplasm is distinctly possible.

6. Tissue culture plant multiplication can continue throughout the year irrespective of the season.

7. An enhanced rate of *in vitro* plant multiplication reduces considerably the period between the selection and release of new cultivar.

8. Methods are available to free plants from virus diseases by meristem culture. Employing this technique, virus free plants can be produced in large number.

9. *In vitro* propagation from adult trees gives the same upright growth habit and freedom from virus, with the added advantage of clonal propagation of the 'elite' trees.

10. Micropropagation is extremely important in the case of dioecious species where seed progeny yields 50% males and 50% females; but plants of one of the sexes are more desirable commercially, such as papaya, carob, etc..

11. With *in vitro* techniques greater manipulative control over cells and tissues is available.

There are three possible approaches available for *in vitro* propagule multiplication :

1. Induced proliferation of the shoot apex or the axillary buds to produce multiple shoots that can eventually be rooted.

2. Production of adventitious shoots directly from excised plant parts or from callus; the shoots can then be rooted.

3. Induction of somatic embryogenesis directly from the explant or from callus and/or suspension culture.

Each method of propagation has its merits and shortcomings (Minocha, 1980). Since a clear distinction between forest and nonforest (e.g. fruit and ornamental) trees is difficult and since the techniques and problems of propagation are largely the same for both, tissue culture of both forest and nonforest species will be discussed. However, it is attempted to use literature preferably dealing with forest tree species.

Enhanced axillary branching

Two methods are in use :

(i) shoot tip culture, and

(ii) single node culture.

Both depend on stimulating axillary shoot growth by overcoming the dominance of the shoot apical meristem. In culture lateral buds and stem terminals, containing quiescent or active shoot tips, are stimulated of their axillary branching process by furnishing a suitable nutrient medium (Murashige, 1977).

The method stands out among others because of (George and Sherrington, 1984; Hussey, 1978; Hu and Wang, 1983; Murashige, 1974) :

1. The high frequency,
2. An acceptable level of genetic stability,
3. Its applicability to a large and diverse number of species,
4. Success in propagation of older trees,
5. Its economic feasibility.

Gupta et al. (1980) reported multiple shoot formation from seedling explants and mature trees of Tectona grandis L. Other reports on mature forest trees are Eucalyptus citriodara Hook (Gupta et al., 1981); Eucalyptus torelliana and Eucalyptus camaldulensis (Gupta et al., 1983), Eucalyptus grandis L. (Rao and Venkateswara, 1985; Lakshmi Sita and Shobha Rani, 1985); Dalbergia sissoo (Datta et al., 1983), Quercus robus L. and Tilia cordata Mill. (Chalupa, 1984); Leucaena leucocephala (Goyal et al., 1985; Dhawan and Bhojwani, 1985); Halesia carolina L. (Brand and Lineberger, 1986), Cinnamomum zeylanicum (Rai et al., 1987); Populus alba X P. grandidentala (Chun et al., 1986); Juglans nigra (Heile-Sudholt et al., 1986); Fagraea fragrans (Lee and Rao, 1986); Carob (Thomas and Mehta, 1982).

Apple rootstocks produced by axillary shoot proliferation appeared to be true to the parental material

as assessed by morphological characteristics (Jones *et al.*, 1977). Sutter and Barker (1985) reported that the plants of *Liquidambar styraciflua* derived from micropropagation were phenotypically similar to the parent plants.

Production of adventitious shoots :

Shoots or buds arising from any place other than leaf axil or the shoot apex are termed adventitious shoots or buds. This can proceed either directly on pieces of tissue or organ or indirectly on unorganised callus tissues or in suspension cultures.

Direct adventitious shoot initiation :

Formation of shoots and roots from hypocotyl of *Prosopis cineraria* was studied by Goyal and Arya (1981). Arnold (1982) observed induction of adventitious bud primordia on newly isolated embryos of *Picea abis* when cultured on media containing cytokinin. Ramawat and Arya (1982) reported that with the increase in size of the explant, number of shoots per explant also increased in *Albizia lebbeck*. In *Paulownia*, organs such as hypocotyls, cotyledons and shoot tips regenerated roots and shoots on defined media *in vitro*. (Marcotrigiana and Stimart, 1983).

In vitro organogenesis and multiple plantlet formation directly from fragments of mature cotyledons was reported in

Anacardium occidentale L. by Philip (1984). Another reports of adventitious shoot formation on embryo-free cotyledons are by Kouider et al. (1984) on Red Delicious apple and Kim et al. (1985) on Morus alba L.. Arnold and Eriksson (1985) reported development of adventitious buds on embryos of Picea abies after a pulse treatment with BA. Ishii (1986) found Campbell and Durzan's basic medium optimum for adventitious bud induction on juvenile seedlings of Hinoki cypress (Chamaecyparis obtusa). Maximum shoot proliferation from juvenile and mature tissues of Camellia sasanqua was obtained when GA_3 was incorporated in media with BA and NAA (Torres and Carlisi, 1986).

Indirect organogenesis :

It has been increasingly clear in recent years that through plant regeneration from in vitro cultures a vast reservoir of genetic variability is available termed as 'Somaclonal variation' by Larkin and Scowcroft (1981). The diverse variation characteristic of the plants obtained from differentiated cell cultures might be of great use to plant breeders (Reisch, 1983).

Callus establishment from Pinus strobus and P. echinata and a limited amount of differentiation and shoot regeneration from Pinus strobus was reported by Kaul and Kochhar (1985). Geier (1986) found regeneration from

Anthurium scherzerianum callus to be highly dependent on genotype and leaf age. He also observed that of the various medium factors tested, the NH_4NO_3 level had the most significant effect on callus and shoot formation. Fink et al. (1986) reported shoot organogenesis when the callus derived from internodes of actively elongating shoots of the hybrid cultivar 'Pioneer' elm was transferred from a primary medium containing various cytokinins to a secondary medium with reduced level of BA. Plantlets, isolated roots, leaves and shoots were differentiated in various callus cultures derived from root, hypocotyl, cotyledon and leaf of seedlings of Punica granatum (Jaidka and Mehra, 1986). High frequency shoot regeneration from callus and suspension cultures of quaking aspen (Populus tremaloides) was obtained by Noh and Minocha (1986).

However, reports on the organogenesis in callus cultures obtained from mature trees are very few. Jaiswal and Narayan (1985) reported regeneration of shoots in the callus cultures derived from stem segments of adult plants of Ficus religiosa. Regeneration of shoots and their subsequent rooting from callus cultures of Dalbergia latifolia Roxb. was reported by Lakshmi Sita et al. (1986) and Rao (1986).

Somatic embryogenesis

Somatic embryogenesis offers tremendous potential for the large-scale production of plant material (Ammirato and Styer, 1985). It can proceed in two ways. Either directly from sporophytic or gametophytic cells of a cultured explant or indirectly from an unorganized callus or cell suspension.

A shoot bud or an embryo are distinguishable on the basis of recognizable morphological differences between the two. The formal is a monopolar structure. It develops procambial strands which establish connection with the pre-existing vascular tissue dispersed within the callus or the cultured explant. On the other hand, an embryo is a bipolar structure with a closed radicular end. It has no vascular connection with the maternal callus tissue or the cultured explant (Haccius, 1978; Bhojwani and Razdan, 1983).

Direct embryogenesis

Embryo formation frequently occurs in certain explants, preferably juvenile tissues. Occurrence appears to be limited to the tissues that were pre-embryogenetically determined (George and Sherrington, 1984).

Hypocotyl explants of seedling of the leguminous tree Albizzia lebbek showed signs of cracking after 2 weeks in

culture and young embryoids subsequently emerged (Gharyal and Maheshwari, 1981). Somatic embryos have also been observed on the needles of cultured shoots of various gymnosperm trees (Bonga, 1977a).

Somatic embryos are frequently formed on the nucellus tissue of cultured ovules. Instances have been especially recorded in Citrus species (Rangaswamy, 1958; Mitra and Chaturvedi, 1972). Conditions for induction and growth of nucellar callus and efficient somatic embryogenesis was reported by Litz et al. (1984) in Mangifera indica.

Indirect embryogenesis

Indirect formation of somatic embryos from callus or suspension culture is observed more frequently. Although primary callus arising from an explant may show no morphogenetic capacity, it can sometimes be induced to give rise to new embryogenic tissue by transferring to inductive media, e.g. in Santalum album L. when the established callus cultured on 2,4-D and kinetin was transferred to a medium supplemented with gibberellic acid (GA_3), embryoids developed in the callus (Lakshmi Sita et al., 1979). In Sapindus trifoliatus L. reduction of 2,4-D conc during subsequent subcultures resulted in formation of embryoids; which developed further when transferred to a medium containing BAP and Kn and then to a hormone free medium. Unless 5-methyl tryptophan was

added and the level of sucrose raised, the embryoids began to recallus (Desai et al., 1986). Somatic embryos derived from callus of Christmas palm, developed and matured on a hormone-free, glutamine-containing medium (Srinivasan et al., 1985). In Dendrocalamus strictus callus culture was obtained on B5 medium supplemented with 2,4-D. On transfer to a germination medium (B5 liquid, high sucrose, IBA and ∝-NAA) embryoids developed into plantlets (Rao et al.,1985).

High frequency somatic embryogenesis offers the opportunity to develop an artificial seed production, using the fluid drilling technique

Clonal propagation of woody species

The gradual commercial application of tissue culture to herbaceous ornamental plants brought serious attention to tissue culture as a possible means of propagating tree species (Mott, 1981). In recent years the micropropagation methods have been applied successfully to many fruit, ornamental and some of the forest trees as already stated.

Initially, these methods met with little success with trees, particularly in adult form; upto some years ago, reports mostly related to seed or seedling material (Winton, 1978). These pioneering studies greatly encouraged increased efforts. Methods of culture in vitro have now been applied successfully to a wide range of species including many adult

trees (Mascarenhas et al., 1981; Sankara Rao and Venkateswara, 1985; Datta et al., 1983; Marcotrigiano and Stimart, 1983; Desai et al., 1986; Pally and D'souza, 1986). Even though considerable progress has been made in developing micro-methods for many tree species, they are still not available for some important species (George and Sherrington, 1984) and there remains much scope for improving these methods on the commercial scale.

The potential usefulness of these techniques for the production of tree species has been discussed in a number of reviews (Bonga, 1977b; Tsai-Ying Cheng, 1978; Abbott, 1978; Minocha, 1980; Sommer and Caldas, 1981; Rao and Lee 1982; Jones, 1983).

Although there could be dangers form susceptibility to pests and diseases if monoculture of a long-lived tree crop were to be practiced, they can be minimised by the planting of more than one clone at any one time.

Even though, the knowledge obtained from the successful propagation of scores of herbaceous plant species by cell and tissue culture, provides basis for the research on tree species, tissue culture of woody species has lagged behind that of herbaceous species. The main reasons for that are as follows :

1. Inherent difficulties with trees :

a. Most tree populations are highly heterogenous resulting in a high variability in culture response (Sommer and Caldas, 1981). Each species presents somewhat unique problems for determining its need for propagation by tissue culture.

b. Getting adventitious shoot primordia or buds in culture is not so difficult as getting them to elongate and became plants ready to soil (Mott, 1981).

c. A tree does not express its full genetic potential till it reaches the mature age (David, 1982). So micro-propagation of mature trees selected on the basis of superior phenotypes is desirable.

d. Long reproductive cycles of trees place severe limitations in the field study.

e. Greenhouse material is even hardly available from mature trees and explants have to be taken from field grown trees. Consequently considerable physiological variation in explants can be expected.

f. Endogenous microbial contaminants are often present, especially in tissues of field grown material. Removal of these contaminants is often difficult or impossible and high contamination rates are common (Banga and Durzan,1982).

2. Problems associated with transplanting and hardening of plantlets :

Plantlets produced *in vitro* must be acclimatised to withstand the less humid, warmer temperatures and higher light intensities existing outside. Plantlets removed from cultures are usually susceptible to desiccation and wilting because of their highly succulent nature, inadequate covering etc. and excessive water loss before roots can become physiologically functional after transplanting and adaptation to stressful environment.

3. Production costs :

The use of tissue culture should be compatible with or offer advantages over conventional systems of propagation (Brown and Sommer, 1982). Mass generation of plantlets transplanting and hardening off operations prior to establishment in the field are highly labour intensive, and require constant supervision.

Thus many problems of propagation are peculiar to woody species and attempts to solve them using tissue culture methods have been often disappointing. But the nature of the problems dectates that tissue culture should find its place in practical nursary stock production (Abbott, 1978).

Forest trees :

Forest trees represent a renewable resource of fiber, chemicals and energy. The steadily increasing use of forest products worldwide, forces the view of forests as a crop to be managed and harvested efficiently. The long period of growth of trees and the pronounced juvenile-mature phases of growth typical of forest species present some formidable obstacles to rapid genetic improvement. During the maturation process, several physiological changes take place in the tree that affect the *in vitro* behaviour of the tree explants.

Present research has shown that treatment of the plants before excision of the explant (heavy and repeated pruning of the tree and *in vitro* culture, as well as manipulation of the environment after regeneration will occasionally result in the reappearance of juvenile behaviour (Bonga, 1982). But the knowledge of the physiological and biochemical basis of rejuvenation is incomplete (Kestor, 1976). Study of biochemical markers of morphogenesis such as IAA-peroxidase relations (Johnson and Carlson, 1978) and the biochemical basis for rejuvenation phenomena would appear to require some priority as research topics (Jones, 1983).

Current Trends and Prospects :

Recent progress has been excellent and already the success of *in vitro* propagation of adult trees is bringing important practical benefit (Jones, 1983). In the near future, it is conceivable that some of the limitations in hybridization of tree species will be overcome by the production of mutants and polyploid types through regeneration of the cells. In the longer term application of pollen culture and protoplast culture techniques would be expected to transform possibilities for the breeding of trees as for other crops. Already there have been some significant achievements with trees in respect of such methods.

With *Coffea arabica* haploid callus and subsequent formation of embryoids were observed (Sondahl and Sharp, 1977). Nair *et al.* (1983) reported induction of haploid plants from anther callus of *Annona squamosa*. Gautam *et al.* (1987) observed green nodules and root formation in callus derived from anthers of *Azadirachta indica*. Culture of endosperm, has so far been reported in more than forty species in angiosperms. Plantlets via embryoid formation in endosperm derived callus has been obtained in some species viz. *Citrus* sp. (Wang and Chang, 1978). Morphogenesis and plant regeneration from cultured endosperm of *Emblica officinalis* was reported by Sehgal

and Khurana (1985). Nair *et al.* (1986) reported the differentiation of callus and formation of triploid roots and shoots from endosperm of *Annona squamosa*. Prakash Kumar *et al.* (1985) observed high degree of aneuploidy in the callus cells from the cellular endosperm of *Cocus nicifera*.

Gabr and Tisserat (1984) reported a short-lived embryogenic callus produced from protoplasts derived from date palm callus. *Citrus sinensis* L. was the first arboreous angiosperm species for which a reproducible procedure for the regeneration of functional plants from isolated protoplasts was reported (Vardi *et al.*, 1975). In subsequent studies the protoplast to plant system was extended to several other *Citrus* species and cultivars from which nucellar-callus could be derived. Furthermore, *Citrus* protoplasts were utilized to obtain somatic-hybrid plants. Vardi *et al.* (1986) developed the protoplast to tree system in *Microcitrus* to render this genus an efficient protoplast-fusion partner to produce somatic hybrids and cybrids between *Microcitrus* and *Citrus* cultivars. Gupta and Durzan (1986) described a method of isolation and culture of protoplasts from callus, cell suspension, cotyledons and needles from mature trees of sugar pine (*Pinus lambertiana*).

Sequential Consideration :

Normally, the process of plant multiplication *in vitro* must proceed through a series of steps, each with a distinct objective and sometimes specific set of requirements (Murashige, 1977; de Fossard, 1985). The following stages are recognised :

Stage '0' : Mother plant selection and preparation

Before micropropagation commences, careful attention should be given to the selection of a mother plant which is typical of a variety and is disease free. It may be advantageous to treat the plant in some way to make *in vitro* culture successful.

Stage 'I' : Culture Establishment

The concern in 'Stage I' is the freshly excised tissue. The aim is to achieve prolonged survival of an infection-free plant explant *in vitro*. Completion of stage 'I' is determined variously, such as growth of shoot tip, multiple shoot formation or formation of callus. Frequently, difficulties may be encountered in achieving disinfection.

Many plants are rich in polyphenolic compounds. After tissue injury, such compounds get oxidized and the tissue turns brown or black. The oxidation products are known to inhibit enzyme activity, reduce growth and darken the tissue

and culture media, imposing a serious block in the establishment of primary cultures.

Some of the procedures used by various workers to combat this problem are :

1. Adding antioxidants to culture media
2. Presoaking explants in antioxidant before inoculations such as ascorbic acid, PVP, etc.
3. Incubation during the initial period of primary cultures in reduced light or darkness
4. Frequent transfer of explants into fresh medium (Hu and Wang, 1983).

Stage 'II' : Multiplication of Propagule :

The main objective of this stage is to produce the maximum number of useful propagule units. According to the procedure of multiplication, this is followed by the newly derived axillary or adventitious shoots or embryoids. The multiplication rate is mainly governed by composition of medium, growth supplements and physical factors such as light, state of medium, temperature.

Stage 'III' : Preparation for growth in the natural environment

Stage 'III a': The elongation of buds formed during 'Stage II'

Stage 'III b' : Rooting of Stage 'III a' shoots *in vitro* or *extra vitrum* (Debergh and Maene, 1985).

Stage 'IV' : Transfer to the Natural Environment

The method whereby plantlets are transferred from the *in vitro* to the *extra vitrum* external environment is extremely important. Factors that should be considered in transplantation are infections and desiccation. Sterilizing the soil mixture eliminates serious infection problems. Excessively high water loss was recorded from the leaves of plants immediately after transplanting (Brainerd and Fuchigami, 1981). Such a high rate of water loss is related to :

a. Shoots developed in culture have often been produced in high humidity and low light intensity. This results in the reduced quantities of epicuticular wax (Grout and Aston, 1977; Sutter and Langhans, 1979).

b. The high volume of mesophyll intercellular spaces (Brainerd *et al.*, 1981), and

c. The slowness of stomatal response to water stress (Brainerd and Fuchigami, 1981). To compound the problem, the xylem tissue in the regenerated plants formed a closed system across the base of the shoot prior to root formation.

The de novo formed roots, arising from callus, have poor connections to the main vascular system of the shoot (Grout and Aston, 1977). Such a structuring is of no consequence in culture but it severely restricts acropetal water transport after transplantation.

A period of humidity acclimation is required for the newly transferred plantlets to adapt to the outside environment. The application of an anti-transparent film to the leaves has been recommended in this stage (Selvapandiyan et al., 1988).

The details of the stages are not described here, since they have been covered in the sections where the present results have been discussed.

I-2.c Secondary Metabolites

Plants have been for a long time of great importance, not only as food sources but also as a rich source of supply of a wide range of chemicals. These are secondary metabolites which include pharmaceuticals, insecticides, flavours, fragrance, colours and enzymes.

The term secondary metabolites or secondary products include those compounds which are not thought to be essential to all plant cells or compounds without any clear function in the vital primary processes of plant cells. This definition also includes compounds such as lignins and flower pigments which have known functions (Butcher, 1977; Staba, 1980). Primary and secondary metabolites have the following characters (Campbell, 1984).

Primary metabolites :

1. Relatively simple chemical composition,
2. Produced by biosynthetic pathway that is neither usually lengthy nor involved,
3. Attributed readily to a role in the physiological make-up,
4. Turn over actively through interactions of these biosynthetic pathways with catabolic processes,
5. Present throughout the life cycle,
6. Necessary for growth.

Secondary metabolites :

1. Complex chemical compounds,
2. Produced by biosynthetic pathways that are involved and often lengthy,
3. Relatively free of turn over,
4. Difficult to integrate meaningfully into the immediate general physiology of the producer,
5. Not necessary for the growth of producer,
6. Normally produced only after the rapid growth phase of the organism has passed.

It is not always possible to assign a natural product unequivocally to either of these categories. The difficulty is, both these metabolites are derived biosynthetically from the same precursor and are essential for survival. While primary metabolites essentially provide the basis for growth and reproduction, the secondary metabolites provide basis for adaptation and interaction with the environments (Crocomo et al., 1981). These are not only sources of reserve materials but also contribute to specific functions to plants as follows (Jalal, 1981).

1. Secondary metabolites like pectin give mechanical strength to plants,
2. Some of the poisonous alkaloids and non-protein amino acids repel insects and herbivorces, e.g.

alkaloids present in the seeds of legume species are lethal to the larvae of seed eating bettle,

3. Some compounds have adverse effect on other plant species growing nearby and thus discourage competing plant species e.g. The compound juglene produced by walnut tree,

4. Compounds like isoflavones help the plant in resisting microbial infections,

5. Coloured pigments such as anthocynins and some carotenes attract pollinators and symbionts.

While the demands of these drug and economically important compounds yielding plants are increasing, the plant resources are fast diminishing, mainly due to human disturbance of the natural environment and ruthless exploitation. On the other hand, inspite of advances in the knowledge of microbial and chemical production methods, plants are still a source of compounds which are too complex or expensive to produce in any other way (Mehta, 1984; Dixon, 1985).

The demonstration of biochemical potential following the morphological totipotency of plant cells grown in culture, has led researchers to resort to plant cell and tissue culture systems for the production of useful compounds (Mehta and Staba, 1970). The feeling of using

plant tissue cultures as an alternative to whole plants as a source of these compounds came from the demonstrations that plant cells can be grown in suspension in the same way as microorganisms.

The major advantages cell culture systems confer over the conventional cultivation of wholeplants are as follows : (Tabata, 1977; Zenk and Deus, 1982; Fowler, 1983; Crocomo et al., 1981).

1. Useful compounds could be produced under controlled nutritional and environmental conditions, independent of climatic changes and soil conditions. Further, it would also provide freedom from political interference.
2. Cultured cells would be free of contamination by microbes and insects.
3. Once the procedure is established, the desired compounds can be produced round the year.
4. The biotransformation and biosynthetic pathway can be easily manipulated.
5. Possibility for automation of cell culture system will reduce labour cost.
6. By rational regulation of growth and metabolism, the production of desired compounds could be enhanced.
7. Technology is now available for the relatively large scale production of plant cell suspensions from batch

cultures, chemostats and turbudostats, to industrial bioreactors. This permits to enhance the yield of desired compounds.

8. Cells in culture can be genetically modified and those that accumulate specific intermediates and metabolites can be isolated.

During the last three decades, a wide variety of plants have been cultured on chemically defined media. The list of compounds already shown to be produced in static or submerged culture is quite impressive and includes :

1. Flavonoids : flavones, flavonols, chalcones, isoflavones, anthocynins and tannins,
2. Anthraquinones and Naphthoquinones,
3. Coumarins and lignins,
4. Isoprenoids : latex, monoterpenes, sesquiterpenes, diterpenes, sterols, steroidal alkaloids and carotenoids,
5. Fatty acids and related compounds,
6. Flavones and sweetners (Fowler, 1983; Staba, 1980; Butcher, 1977).

In many cases, the desired products are either not produced in culture or when they are, the yields are extremely low. Various techniques and methods have been

tested and described to improve the synthesis of valuable metabolites in cell cultures. These include : Optimization of media and growth conditions, type and concentration of hormones, physical environment, feeding of precursors and manipulation of biosynthetic pathways, as well as clone selection and genetic improvement (Rokem et al., 1985). Optimization of the nutrient regime is obviously the key factor in enhancing product yield (Fowler, 1983).

The step-wise strategy developed now includes the following steps :

1. Selection of high yielding plant,
2. Establishment of cell cultures from the selected plant,
3. Development of an optimum growth medium (without consideration of secondary natural product formation),
4. Development of methods to induce secondary product formation,
5. Clonal selection of stable and high yielding cell lines and
6. Development of optimum production medium.

With adaptation of this approach, it has now become possible to produce desired compounds from many cultures in amounts as high as and in some cases even higher than in the parent plant.

Factors affecting products produced :

Genetic, morphological and biochemical factors do affect the ability of plant tissue cultures to produce significant amount of product. The expression of these factors is interrelated (Staba, 1977).

Genetic Factors : The variation in yield of desired compound include the species, the individual plant and perhaps the specific part of the plant from which the culture was initiated (Dougall, 1985). Further, there is evidence for differences in yield between cells in a population (which can be exploited as somaclonal variations).

Tissue origin : Generally high yielding parent plant is chosen to start the culture with. This was a major point of controversy for some years. From the Kinnersley and Dougall's work (1980), it was evident that the cultures derived from a high yielding N. tabaccum plant gave high yield of nicotine. Zenk et al. (1977) also concluded that high yielding Catharanthus roseus plants tend to give high yielding cultures. However, the converse is also true in a few cases (Dougall, 1985).

Plant tissue part : The plant tissue part selected for culture may be critical for product formation. It has been reported that, the root-synthesized tropane (West and Mika, 1957) and reserpine (Mitra and Kaul, 1964) alkaloids are

best produced by root derived callus cultures. The artemisinin content was observed to be consistently below 1 mg% for shoot cultures when the variability in artemisinin content was determined in cultures of different parts of Artemisia annua seedling (Martinez et al., 1986).

'Varient' cell lines : Because of the heterogenity associated with cultured plant cells, the selection of cells producing high amounts of secondary metabolites has been made possible (Yamada and Fujita, 1983). There are two possibilities for the small amounts of the desired compounds produced by the cultures. First, all cultured cells produce only insignificant amount of the desired product, or second, most of the cultured cells do not have the biochemical potential to produce and accumulate the products, but there are a few 'variant' cells present which produce the compounds at high concentration. By means of radioimmunoassay techniques, it was possible to show that the second case is valid.

By determining the heterogenity within the culture by cloning and measuring the yield given by the various sublines, a high yielding clone or subline can be selected (Dougall, 1985). In selecting specific cells, the two methods generally used are cell-aggregate cloning and single-cell cloning.

The cell-aggregate cloning method has been successfully used to obtain high anthocyanin producing cells from Euphorbia milli by Yamamoto et al. (1981). Other reports are by Sato and Yamada (1984), Watanabe and Yamada (1982). Mizukami et al. (1978) reported the isolation of cell line of Lithospermum erythrorhizum with a high pigment content of shikonin by repeated clonal selection. Sareen and Khanna (1986) analysed the different coloured strains of Papaver somniferum showing maximum yield of codeine, the baine and narceine in dark grey coloured strain, whereas the amount of papaverine and oxalate was found to be maximum in light grey coloured strain. These methods are based on the visual selection of coloured cell aggregates and no analytical methods were used for the selection.

Through the use of semi-automated Radioimmunoassay (RIA) technique, Zenk et al. (1977) carried out extensive studies on the selection of Catharanthus roseus cell strains capable of producing the useful alkaloids serpentine and ajamalicine. Suspension cultures of Anchusa officinalis established by clonal propagation of microspectrophotometrically analyzed single cells were found to produce rosmarinic acid at different levels (Ellis, 1985). Matsumoto et al. (1980) succeeded in producing highly producing strain (UQ-10) of tobacco cultured cells by cell cloning technique, producing Uniquinone-10.

The biosynthetic capacity of cultured cells may be improved by the artificial induction of genetic mutation (Nishi et al., 1974). It is possible that the frequency of obtaining mutant cells may be markedly increased by mutation treatment of haploid cells (Widholm, 1977).

Morphological Differentiation

In higher plants, there are certain compounds which are synthesized or accumulated only in particular organs or tissues. The failure of unorganized cells or cultures to produce these compounds suggest that morphological differentiation of specific organs or tissues are required for their formation. Induction of differentiation in unorganized cultures induces production of desired compounds, e.g. the differentiated tissues of Solanum nigrum produced significantly more solasodine than the callus (Bhatt et al., 1983). In Solanum dulcamara cultures also the alkaloid accumulation could be enhanced by the induction of organogenesis (Emke and Eilert, 1986). Manoharan et al. (1987) observed an association between greening and shoot differentiation with an increase in the content of total lipid and the galactolipid fraction in Datura innoxia cultures. On the other hand, cases are now known in which unorganized tissue will produce compounds found exclusively in specific organs/tissues of intact plant. This is chemical differentiation without

morphological differentiation; e.g. Sugisawa and Ohnishi (1976) showed that cells of Perilla frutescens, leaves in culture contained as much essential oil as the intact leaves.

However, since some of the organized tissues have better ability to produce the desired compounds and since selection can be done from organized tissues to increase product yields e.g. Scopolamine from Hyoscyamus niger root suspension cultures, a need has arisen to grow even the organized tissues economically on large scale (Staba, 1985). Hamill et al.(1986) established 'Hairy root' cultures of Beta vulgaris and Nicotiana rustica which synthesised their characteristic secondary products, the betalain pigments and nicotine alkaloids respectively, at levels comparable with those of in vivo roots from the same variety. Heble and Staba (1980) established the shoot cultures of Dioscorea camposita and demonstrated their ability to synthesize diosgenin. The amounts of atropine and scopolamine was observed to be same or ever higher than plants in hairy root culture of Atropa belladona (Kamada et al., 1986). Ushiyama et al. (1986) studied large scale production of Panax ginseng root by 30l, 2 kl and 20 kl fermentors. Essential oil production from in vitro - grown shoots and callus of Rosmarinus officinalis was studied by Jain et al. (1987).

Biological Controls :

The biosynthetic activity of cultured cells usually varies with cell growth and substrate utilization. Kinetic studies of the rates of cell growth and product formation are essential to obtain a basic understanding of secondary metabolite production.

Experimental data on the time-course of product formation indicates that product-growth patterns may be classified into three major types :

1. Product formation proceeds almost in parallel with cell growth,
2. Product formation is delayed until cell growth declines or ceases,
3. Production curve is diphasic and lags behind the growth curve (Crocomo et al., 1981).

Generally, a primary metabolite is synthesized as a direct result of metabolic processes and correlates with increase in dry weight. In contrast, a secondary metabolite is generally not formed as a direct result of metabolism and accumulation of these metabolites tends to lag behind the cell growth. It is characteristic of many plant cultures that, after a phase of rapid cell division on a batch culture system, the growth rate slows down and secondary

product formation and other features of cell specialisation begin (Luckner et al., 1977).

At any one stage of culture, the accumulation of secondary metabolites is the result of a dynamic balance between biosynthetic, biotransformational and biodegradative processes.

Environmental Control :

Factors in the external cultural environment

1. Light, photoperiodicity, light-quality and light intensity influence the behaviour of cultures (Seibert and Kadkade, 1980). It has been shown in a number of cultures that light generally stimulates the formation of compounds including conteroids, flavonoids, polyphenols and plasto-quinones (Tabata, 1977). Ruiz and Valadez (1985) reported that in Gomphrena globosa callus cultures blue light is essential for obtaining good growth and pigmented callus cultures containing betacyanins. Morris (1986) observed serpentine as major alkaloid in the light and ajmalicine in dark in the callus cultures of Catharanthus roseus. In contrast to these triggering effects of light, several reports indicate that light can have inhibitory effect, e.g. suppressed alkaloid production in Scopolia parviflora cultures (Tabata et al., 1972). Photodegradation of certain metabolites and/or enzymes may be responsible for the

higher levels of accumulation of secondary metabolites under dark rather than light conditions, although there is no direct evidence to substantiate this (Mantell and Smith, 1983).

Temperature :

Little information is available on temperature optima for secondary metabolite production. Nettleship and Slaytor (1974) showed that optimum growth of Peganum callus cultures occurred at 30°C, while maximum alkaloid production was attained at 25°C.

Factors in the internal cultural environment :

The factors associated with culture itself i.e. the growth medium, the metabolic stage of cells and the gaseous environment above the culture surface form complex multi-component entities.

Growth regulators :

Nature and concentration of auxins and kinins have a marked effect on the primary and secondary metabolism. This aspect has been discussed in many reviews e.g. Staba (1980), Mantell and Smith (1983).

In most studies, auxins have been supplied to cultures in combination with cytokinins. Ravishankar and Mehta (1979)

reported that a combination of 2,4-D with kinetin enhanced growth and ecdysterone in Trianthema portulacastrum callus cultures. Callus cultures of Saffron grown on MS medium supplemented with 2,4-D and kinetin, when transferred onto indolebutyric acid-kinetin containing media, produced pigmentation and showed metabolites comparable to authentic saffron (Venkataraman et al., 1987).

Not much study has been devoted to the effect of other growth regulators such as gibberellic acid (GA_3), abscisic acid (ABA) ethylene and 2-chloroethyltrimethyl ammonium chloride (CCC). A favourable effect of GA_3 was observed in quinine production in leaf shoot organ cultures of Cinchona ledgeriana (Chung and Staba, 1987). Ohlsson et al. (1986) reported increases in growth and cardenolide accumulation when the cultures of Digitalis lanata were grown with GA_3.

Macro and Micronutrients :

This aspect has been reviewed in detail by Dougall (1980).

Generally, increased levels of nitrate, potassium and phosphate tend to support rapid cell growth, while depletion or deficiency of some of these is associated with growth limitation and concomitant secondary metabolism.

The nature and concentration of sugar generally affects

the yield of secondary product. In general, raising the initial sucrose levels leads to an increase in the secondary metabolite yields in culture. Perhaps the effect of high initial levels of sucrose is to raise the osmotic potential of media. In Dioscorea suspension cultures the initial sucrose concentration influenced the rate of synthesis and final concentration of diosgenin (Rokem et al., 1985).

Precursors :

Among attempts to increase the potential of plant cell cultures to produce particular metabolites, have been those involving the feeding of cultures with known precursors and/or intermediates with the intention of stimulating particular enzyme pathways. However, administration of a direct precursor does not always produce the desired effect, and finding the most efficient precursor compound may sometimes prove to be difficult.

Khanna and Khanna (1976) observed considerable increase in growth, free ascorbic acid and pyrethrins, when tissue cultures of Tagetes erecta L. were fed with various concentrations of exogenous ascorbic acid. The accumulation of betacyanins could be increased by feeding tyrosine to the cell culture of Chenopodium rubrum (Berlin et al., 1986).

The internal cultural environmental factors will be discussed in more details in Chapter VI.

Elicitors :

Of late elicitors find applications in studies on the biosynthesis of diverse secondary metabolites in vitro. Looking at the literature of phytopathogen-host-interaction, it becomes clear that a large number of secondary natural products are phytoalexins, i.e. secondary metabolites synthesized in plants due to physical, chemical, microbiological and fungal damage of the plant.

Recently a review has been published discussing the stimulation of alkaloid accumulation in Papaver somniferum and Catharanthus roseus cell cultures by elicitors (Eilert et al., 1987).

Industrialization :

Certain pharmaceutically important chemicals such as shikonins, digoxin, vinblastine, berberine and rosmarinic acid have been successfully produced by cell cultures in bioreactors under precise growth conditions (Heble and Chadha, 1987). The industrial production of useful secondary products by cell cultures has been realised through the development of adequate culture media, elucidation of regulatory factors of the secondary metabolism, selection of specific cells and the establishment of two-stage culture media. Since 1984, Shikonin produced by the large-scale culture of Lithospermum cells has commercially been used for cosmetics.

In order to be useful as an alternative industrial source of secondary compounds, cell cultures must satisfy several requirements.

1. The rates of cell growth and biosynthesis should be high enough to give a good yield of final product,

2. Its accumulation in the cell or release into the medium should be rapid in comparison to its degradation,

3. To get a constant yield of product, the cells must be genetically stable,

4. The production must be profitable including the inherent cost of culture medium and the extraction and purification procedures (Tabata, 1977),

When grown in bioreactors, the differences between plant cell suspensions and microbial culture become significant.

1. Growth rate of plant cells is very slow,
2. Requirement of high inoculum density of plant cells in order to obtain growth,
3. The large size of plant cells in culture associated with a rigid cell wall and large vacuole makes them sensitive to shear,
4. Problems in mixing, sampling and aeration are caused due to the formation of cell aggregates. Production of extracellular polysaccharides also poses problems,

5. Requirement of oxygen is very low and high airation rates may have a deleterious effect on growth and product formation.

In spite of all these difficulties, still there are some examples of large scale production of secondary products by using techniques such as phase partition and air-lift bioreactors. Hegarty et al. (1986) carried out investigations to examine the effect of aeration on the growth of Catharanthus roseus suspension cultures in airlift bioreactors. Smith et al. (1986) selected cell lines which naturally over-produce catharanthine (important in the biosynthesis of vinblastine and vincristine) and also illustrated the scaling up from 50 ml cultures, through 1 l, 10 l, and 30 l to full pilot scale of 200 l.

Immobilization of cells :

Plant cells have been successfully immobilized in a number of matrices either by entrapment in a polymeric network or by growth into preformed structures. Cells are commonly immobilized by entrapment in calcium alginate, potassium currageenan or in agarose beeds. For plant cells attachment to solid supports by adsorption or covalent coupling has proved to be unsuitable.

The main reasons for the use of immobilized plant cells as an alternative to mass cell culture for production

of secondary metabolites are :

1. To reuse biomass which is expensive to produce,

2. To separate physically cells from the medium and hence from products,

3. To utilize efficiently expensive fermentors by using high biomass/low volume vessels,

4. The cells being trapped in a rigid base, the problem of disintegration etc. are overcomed,

5. To operate a continuous process.

(Brodelius and Nilsson, 1982).

Immobilization may provide a method of exploiting the improved biosynthetic capacity of slow growing, aggregated or partially differentiated tissue cultures (Lindsey and Yeoman, 1983). Immobilization of plant cells is seen as an intermediate state between homogenous suspension culture and the highly differentiated tissue matrix of the whole plant (Morris et al., 1985; Brodelius and Nilsson, 1980).

The possibility of immobilizing partially differentiated celery cultures was studied by Watts and Collin (1985). The immobilized cells of Coffea arabica produced considerably more alkaloids than the freely

suspended cells (Haldimann and Brodelius, 1986).

It is essential that, in the operation of an immobilized cell system, at least some of the product is released from the cells into the medium, where it can be recovered. Since the products being preferentially accumulated in the vacuole, liberation of products is difficult. In such cases two stage culture system consisting of repeated product accumulation and product release phase have been used. However, treatments used in the product release phase must retain membrane integrity, or ensure rapid repair of membrane function. From the industrial point of view, the most successful type of reactor is the expanded or fluidised bed reactor.

The use of microbes as carriers of plant genetic information is an attractive in terms of large scale fermentation. But before such a leap forward in technology could occur, a thorough understanding must be gained of the enzymes involved in the synthesis of the compounds together with the genetic control (Misawa and Samejima, 1978).

I-2.d Steroids :

Steroids belong to a large group of compounds known as terpenoids or isoprenoids. Terpenes are formed by the polymerization of isoprene units and steroids are triterpenes or triterpenoids. They have the perhydro-1,2-cyclopentanophenanthrene ring structure (Grunwald, 1980; Lalitha and Ramasarma, 1987).

Sterols : Sterols are secondary alcohols. The hydrocarbon skeleton has a secondary hydroxy group at C-3 with few exceptions, angular methyl groups at C-10 and C-13 and generally an 8 to 10 carbon chain at C-17. The hydroxy, the two methyl groups and the C-17 side chain all have the β-orientation. The various sterols found in plants differ in the number of carbon atoms in the C-17 side chain.

Distribution :

Sterols have been isolated from a large number of species and probably occur in all angiosperms and gymnosperms. The phytosterols are ubiquitous in higher plants and probably also in plant tissue cultures (Grunwald, 1980; Butcher, 1977).

Biosynthesis :

The formation of squalene is through the familiar pathway i.e. through acetate, acetoacetate, hydroxymethyl-

glutaric acid, mevalonic acid, isopentyl pyrophosphate, 3,3-dimethylallyl pyrophosphate, geranyl pyrophosphate, farnesyl pyrophosphate and squalene (Fig. I-5) (Jacobsohn, 1970; Goad and Goodwin, 1972; Grunwald, 1975).

The cyclization of squalene to form the cyclopentanophenanthrene ring system is via squalene-2,3-oxide (Benveniste and Massy Westropp, 1967; Reid 1968). However, the major difference is the formation of cycloartenol (Rees et al., 1968) instead of lanosterol as in animals. Experiments with radioactive sterol precursors suggest that cycloartenol is a branch point, not only for cholesterol synthesis, but also for the synthesis of other major phytosterols. (Benveniste et al., 1966; Ehrhardt et al., 1967; Knapp and Nicholas, 1971).

Cultures of N. tabacum and Rubus fruticosus have provided excellent experimental systems for studying phytosterol biosynthesis. When 14C labelled acetate, mevalonate and other sterol precursors are incubated with callus tissues they are efficiently incorporated into the intermediates of the sterol biosynthesis (Benveniste, 1968; Heintz et al., 1972). After a detailed series of studies the scheme shown in Fig. I-6 was proposed as the pathway, for sterol biosynthesis.

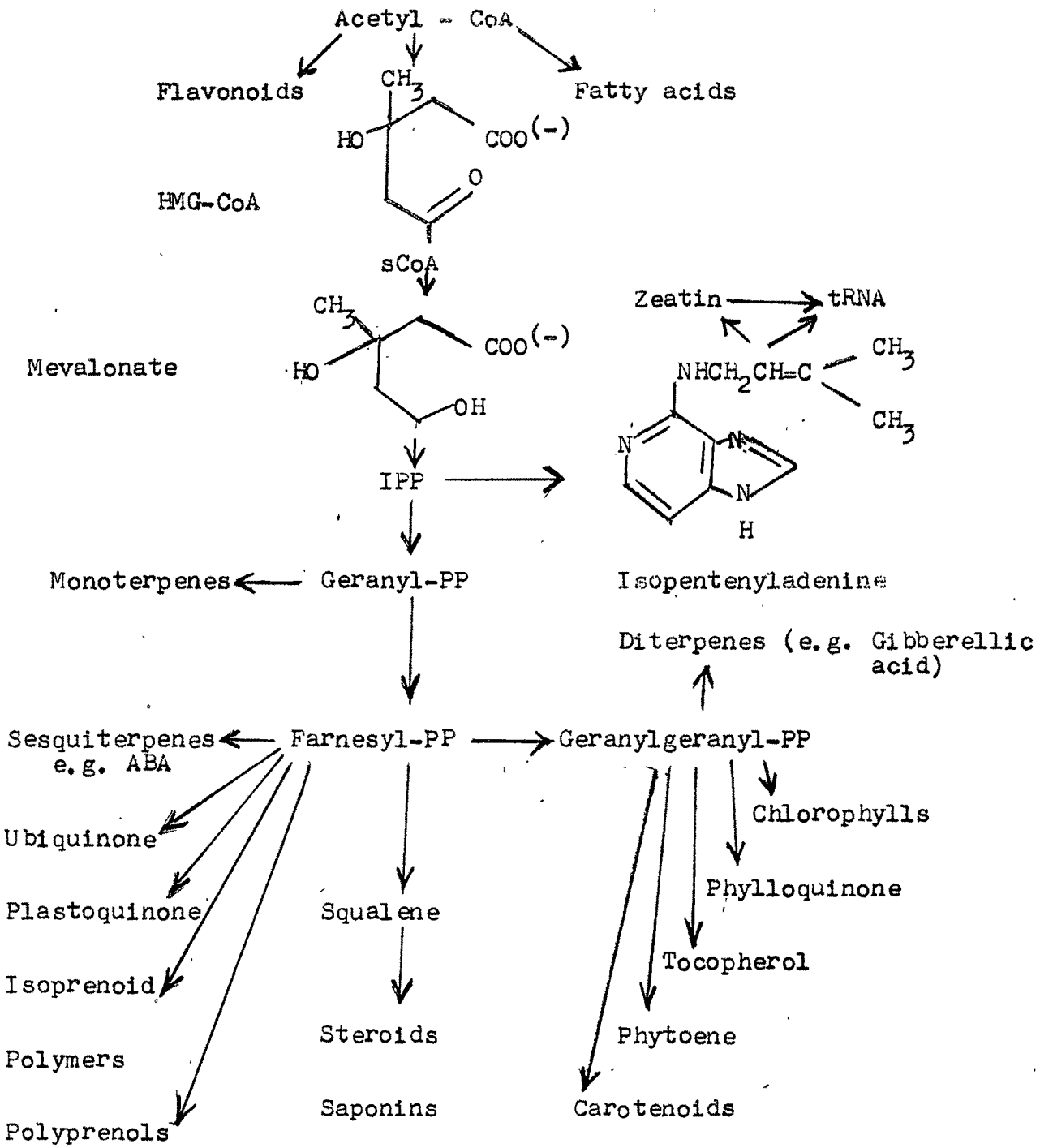


Fig I-5.

Fig. I-5 General pathway for isoprenoid biosynthesis in plants.

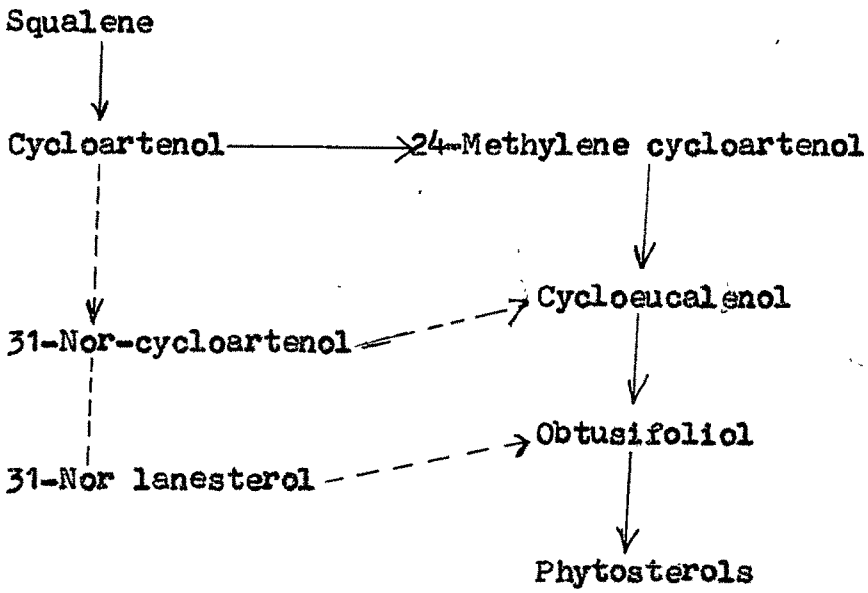


Fig. I-6 (Butcher, 1977)

Physiological function :

It is probable that they are essential components of many cellular membranes. Thus in a strict sense they are not secondary products, however, their role has not been unequivocally established and the kinds and proportions of the sterols vary considerably from one plant species to another. The most widespread sterols are β-sitosterol, Stigmasterol, campesterol and cholesterol (Butcher, 1977). Sterols have at least two functions :

1. As precursors in the formation of other steroids
2. As components of membrane.

It has been suggested that the interaction of sterols with phospholipids stabilizes membranes and thereby controls permeability (Grunwald, 1971). For effective phospholipid interaction, the C-3 hydroxyl group of sterol molecule must be free and cannot be substituted.

The importance of sterols in the function of membranes in several biological systems has been demonstrated. Filipin, a polyene antibiotic, is known to complex with sterols and causes cellular leakage which can be reversed by addition of sterols (Mudd and Kleinschmidt, 1970). The amount of leakage induced with filipin in wheat endosperm of different sterol phenotypes is directly related to its free sterol conc (Carbonero et al., 1975). Sterols also restored the K^+ and NO_3^- uptake capability in pea stem sections treated with filipin (Hendrix and Higinbotham, 1973). The saponin, digitonin, forms equimolar insoluble complexes with free sterols, and when added to chloroplasts inhibits oxygen evolution (Robinson and Wiskich, 1975). Cholesterol as well as $CaCl_2$, the classical membrane stabilizer, reversed phytochrome-induced anthocyanin production in red cabbage, probably by acting at the membrane level (Bassim and Pecket, 1975).

Exposure of plants to ozone increases cell permeability and it is interesting to note that ozone-treated plants show a decrease in free sterols and an increase in steryl

glycosides (Tomlinson and Rich, 1971). Antisenescent compounds prevent or minimize ozone damage and plants treated with N-6-benzyladenine and kinetin did not show the decrease in free sterols (Tomlinson and Rich, 1973).

Garg and Paleg (1986) observed the sterol levels (Sitosterol, cholesterol, campesterol and stigmasterol) much higher in the shoot apex than those in the leaf in Lolium temulentum plants during floral development. A much greater proportion of cholesterol was found in the shoot apex reflecting a specific association of cholesterol with meristematic and/or reproductive tissues. Quenther and Quenther (1985) investigated diosgenin and sterols from Costus spiralis rhizomes. The amounts of free and esterified sterols increased towards the heartwood in Pinus sylvestris, but, in contrast, steryl glycosides decreased simultaneously (Saranpää, 1987).

Plant cell and tissue culture studies :

The widespread sterols, β-sitosterol, stigmasterol and campesterol have been isolated from cultures of Nicotiana tabacum (Benveniste et al., 1966), Dioscorea tokoro (Tomita et al., 1970), Withania somnifera (Yu et al., 1974), Paul's scarlet Rose (Williams and Goodwin, 1965), Tylophora indica (Benjamin and Mulchandani, 1973), Helianthus annuus (Butcher et al., 1974); Morus alba (Kulkarni et al., 1970),

Datura tissue cultures (Nag, 1975), Lycium barbarum L. (Nag and Harsh, 1982a), Dioscorea floribunda (Khanna et al., 1980), Solanum mammosum (Gunawan and Isnaeni, 1986), Solanum aviculare (Galanes et al., 1984), Solanum nigrum (Bhatt and Bhatt, 1984a), arid zone plants (Nag and Harsh 1982b).

Brain and Lockwood (1954) studied the effect of hormonal control on steroid level in tissue cultures from Trigonella foenumgraecum. Heble et al. (1976) isolated several sterols from the callus cultures derived from the hypocotyl of germinated seedlings of the plant, Holarrhena antidysenterica, of which the predominant ones were identified as cholesterol, 24-methylene cholesterol, 28-isofacosterol, sitosterol and stigmasterol. Khanna et al. (1975) reported effects of various hormones on production of sapogenins and sterols in Trigonella foenumgraecum L. suspension cultures. Bhatt and Bhatt (1984 a) observed that sterols (Cholesterol, campesterol, stigmasterol and sitosterol) were about three times higher in the differentiated cultures (roots and shoots) of Solanum nigrum than in the undifferentiated cultures (callus). They further studied the regulation of sterol biosynthesis in Solanum species and reported that cholesterol inhibited conversion of acetate to mevalonate, which is taken as evidence of a negative feedback control on sterol biosynthesis (Bhatt and Bhatt, 1984 b). Cholesterol was found to be a predominant sterol (42% of

total sterol) in the leaves of intact plant of Solanum dulcamara L. but its level reduced to 1 to 7% in cell suspension culture (Bhatt et al., 1986). Khanna and Purohit (1983) carried out studies on steroidal sapogenins from Yucca aloefolia L. in vivo and in vitro tissue cultures.

Metabolism of steroids in plant tissue cultures :

As an experimental tool, plant tissue cultures offer great convenience in the addition of precursors and steroidal substrates when investigating biotransformations. However, in using tissue cultures one may encounter a situation where one or none of the steroid metabolizing enzymes normally found in intact plants are absent in the corresponding tissue systems (Stohs, 1977). Basically three approaches have been used to investigate the biosynthetic and metabolic potential of plant tissue cultures with respect to steroids :

1. The isolation and identification of the steroids produced by callus and suspension cultures,
2. The use of tissue cultures to study the biosynthetic pathways of steroids, frequently employing radioactive precursors,
3. The incubation of tissue cultures with steroidal substrates in order to develop potentially useful modifications, and to investigate the general steroid metabolizing enzyme systems present.

Graves and Smith (1967) studied the transformation of pregnenolone and progesterone by cultured plant cells from a range of dicotyledonous plants. Steroidal metabolism in plant tissue cultures has been reviewed in details by Stohs and Rosenberg (1975) and Stohs (1977).

The use of some inhibitors of plant sterol biosynthesis proved evidence that active sterol synthesis is essential for plant cell growth and division. Sterols are apparently required for membrane structures but there is also a specific need for 24-alkylsterol before cell division can proceed (Goad, 1987). Inhibition of plant sterol biosynthesis by Paclobutrazol and its effect on cell growth in suspension culture of Celary was studied by Haughan et al. (1987).

I-3 PRESENT INVESTIGATIONS

As mentioned in Section I-1, guggul, the oleoresin-gum complex of Commiphora wightii has numerous applications in pharmaceutical and perfumery industries. It is also an important source of foreign exchange (Farooqi, 1979).

Most of the tree populations inhabit inhospitable areas where conventional agriculture is not feasible and there are no methods available for its cultivation. The available literature on this plant reveals that even though substantial work has been carried out on the chemical and pharmaceutical investigations of the gum-resin and on the histochemical aspects on the development of resin-ducts (Section - I), there are no reports available about the studies on tissue culture aspects of this plant. The present work was carried out with two main objectives.

1. To screen the 'guggul' tree population from different arid and semiarid zones and identify elite plants. To work out in vivo and in vitro methods for propagating them.
2. To raise the content of active principle - Guggulsterone Z and E in the cell and tissue cultures.

The active principle was observed to be present at a very low concentration in the cell cultures. Initial

experiments designed to increase the content by cultural manipulations were not successful. Since β-C-3 sterols act as precursors of this compound, experiments were planned to raise the content of β-C-3 sterols in the culture.

With the above mentioned objectives in mind, various experiments were conducted and the results obtained are presented in the Chapters III, IV, and V as outlined below.

Chapter III

III-1 Studies on identification of Elite plants.

III-2 In vivo propagation by stem cuttings and by germinating seeds

III-3 In vitro propagation from seedling material.

III-4 Clonal propagation of Elite clones.

III-5 Studies on morphogenesis in callus cultures.

Chapter IV

IV-1 Studies on growth of callus cultures

IV-2 Studies on nutrition of callus cultures.

IV-3 Studies with suspension cultures.

Chapter V

V-1 Studies with different parts of a plant and callus derived from these parts regarding β-C-3 sterol production.

V-II Effect of Cultural parameters on growth and β-C-3 sterol production in suspension cultures.

V-III Effect of ethephon on guggulsterone production in cell cultures.

* * * * *